SEX, SIGNALLING, AND STEROIDS:

REGULATION of NUCLEAR SIZE
and INTRACELLULAR TRAFFICKING in SECONDARY CELLS
in the *D. MELANOGASTER* MALE ACCESSORY GLAND



Josephine E. E. U. Hellberg

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DECLARATION

I, Josephine E. E. U. Hellberg, confirm that the contents of this Dissertation are the result of my own work, except where otherwise acknowledged.

The work described in this Dissertation was undertaken in the laboratory of Professor Clive Wilson, at the Department of Physiology, Anatomy and Genetics, at the University of Oxford between Michaelmas 2014 and Trinity 2017, and completed in Hilary 2018.

No portion of the work described in this Dissertation have been submitted for any other degree at this University or any other institute of learning.



Josephine E. E. U. Hellberg

Edinburgh, Hilary 2018

A little learning is a dang'rous thing;

Drink deep, or taste not the Pierian spring:

There shallow draughts intoxicate the brain,

And drinking largely sobers us again.

Fir'd at first sight with what the Muse imparts,

In fearless youth we tempt the heights of Arts,

While from the bounded level of our mind

Short views we take, nor see the lengths behind;

But more advanc'd, behold with strange surprise

New distant scenes of endless science rise!

- Alexander Pope
(in POPE, 1711: *p.* 14)

SUMMARY

REGULATION of NUCLEAR SIZE and INTRACELLULAR TRAFFICKING in SECONDARY CELLS in the *D. MELANOGASTER* MALE ACCESSORY GLAND

by
Josephine E. E. U. Hellberg
of
Magdalen College, University of Oxford

Submitted for the degree of

Doctor of Philosophy in Physiology, Anatomy and Genetics

The fruit fly, *Drosophila melanogaster*, can be a useful *in vivo* model for understanding the biology of cells and the signalling pathways by which this biology is regulated. My Dissertation focuses on the accessory glands of male *D. melanogaster*, where two types of cells—main cells and secondary cells—are responsible for producing many seminal fluid proteins. The work presented in this Dissertation describes some of the aspects of secondary cell biology that are involved in controlling the growth and secretory activity of these cells.

Secondary cells are secretory cells, and they contain large intracellular compartments—some of which accumulate proteins destined for secretion. The BMP ligand, Decapentaplegic (Dpp), is one such protein, which can regulate secondary cell biology via an autocrine signalling mechanism. Previous work has shown that BMP signalling regulates a *D. melanogaster* steroid receptor—the ecdysone receptor (EcR)—to form a BMP/EcR signalling axis that controls secondary cell nuclear growth.

In this Dissertation, I show that the BMP/EcR signalling axis regulates the expression of the *D. melanogaster* initiator caspase, DRONC, and that this caspase and other related regulators control intracellular trafficking in secondary cells. I propose that this process might be orchestrated via non-apoptotic mobilisation of the cytoskeleton. In addition, I show that secondary cells produce the lipoprotein, Hedgehog, and that it is released into the seminal fluid. Like Dpp, Hedgehog seems to signal back into secondary cells to regulate their biology and nuclear growth. This growth is reduced when *hedgehog* (and its downstream signalling components) is knocked-down in secondary cells. Conversely, activating the Hedgehog-dependent transcription factor, Cubitus interruptus (Ci), promotes this growth. Like BMP signalling, autocrine Hedgehog signalling might play an important role in co-ordinating growth and secretion in secondary cells.

Previous studies have shown that secondary cell nuclei grow after mating. In an effort to understand how this growth is regulated, I identified the BMP/EcR signalling axis as a key regulator—alongside cell cycle regulators, like Cyclin D and Cyclin E. My work shows that secondary cell nuclei seem to grow after mating, partly as the result of BMP/EcR-dependent increases of the secondary cell DNA content—perhaps to increase the cells' biosynthetic potential. This suggests that secondary cell nuclei can grow via two distinct mechanisms: one that is mating-dependent, and one that is not. Altogether, the work presented in this Dissertation describes important roles for steroid signalling and sexual activity in the regulation of secondary cell biology. This complex co-ordination of BMP- and steroid signalling might also be relevant to other organisms.



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PREFACE





PREVIOUS PAGE:

A winter view of the University of Oxford University Church, St. Mary the Virgin, from the High Street, with pigeons overhead.

PREFACE

In the elder days of Art,
Builders wrought with greatest care
Each minute and unseen part;
For the Gods are everywhere.

– Henry Wadsworth Longfellow
(in LONGFELLOW, 1860, *p.* 250)

This Dissertation details results obtained during my *D. Phil.* studies in the laboratory of Professor Clive Wilson, at the Department of Physiology, Anatomy and Genetics, at the University of Oxford. The aim of my work was to better understand the genetic regulation of the cell biology of the secondary cells in the male accessory gland in the fruit fly, *Drosophila melanogaster*. A particular emphasis of this work has been on understanding how the nuclear growth of these cells is regulated, and to explain the changes that occur after mating. In a world full of more pressing matters, one might wonder: is there a point to such (seemingly) esoteric research? For me, there are plenty. I am a great believer in the merits of basic science—of 'doing science for science's sake'—and therefore, I wanted to spend my doctorate working

on something that was properly *esoteric*. This kind of exploratory, 'blue-sky' science is full of risk, but with the potential to uncover something truly novel, while at the same time avoiding to become mired with concerns of human application and clinical benefit. Those come down the line—sometimes decades later.

Reading this Dissertation, you will notice that I have drawn on a large body of work done by those who have come before me, and, wherever possible, I have attempted to track down the original studies and publications where genetic stocks or scientific findings were described for the first time. In addition, I have made a point of identifying papers written by joint-first authors, to give both their due. Science proceeds by increment; of countless curious minds each chipping away at one aspect of a much greater whole; the accomplishments of an individual lifetime being crucial for science to inch forward, yet becoming dwarfed by the findings of the next generation. Sir Isaac Newton explained this beautifully in the (now cliché) phrase that: "If I have seen further, it is by standing on the shoulders of giants" (NEWTON, 1675). In this sense, science is the most human of all pursuits: of individual scientists seeking to understand the universe, yet being fated to contribute only one miniscule aspect of an unfathomable whole. Such sentiment perfectly encapsulates the contributions of a single doctoral dissertation; representing but an incremental advance of human knowledge, yet one begotten through several years of hard work. To cite the work of others properly is the least one can do to acknowledge the efforts of those from whose work this increment has been derived.

Traditionally, a 'masterpiece' was the final piece of work produced by an apprentice aspiring to become a master craftsman; serving as an example of the work that resulted from efforts to the best of their abilities, and providing an example of the kind of work they would produce after embarking on their professional lives.

Considering that a *D. Phil.* is (by any definition) an *apprenticeship* in science, the dissertation—the final culmination of the doctoral work—would represent the *masterpiece* of the academic training process. Therefore, the dissertation ought to be representative of the doctoral candidate's best work; showcasing their skill as a researcher and communicator to the already-qualified academics who ultimately assess the merits of the work through the final examination. It is my belief that this kind of work, the dissertation and doctoral monograph, is the most important aspect of the doctoral training process; representing not only the final word on the work that has been performed, but also standing testament to the skills acquired through this process. To compile a monograph resulting from several years' academic work requires important skills in project management, data interpretation and analysis—as well as communication skills; analytical skills; and an understanding of the historical context of the work. All these skills being important to the doctoral training process, the monograph must be considered a crucial component of the *D. Phil.*

I consider this Dissertation my masterpiece; it representing the kind of work that results once I have been employed to the best of my abilities. As always, there are extenuating circumstances associated with every project—without which one would have been able to work with more dedication, and with more passion. For example, I admit to never having been very fond of laboratory work. Rather, I have a tendency to see experiments as something to be performed in order to support my genuine passions of tabulating and interpreting data; turning results into a coherent story and argument; considering the wider scientific context and implications of my work; designing diagrams, figures, and illustrations; and producing the final report itself.

*

Structuring this Dissertation, I have tried to be conservative and keep to the orthodox, six-chapter format—while also making reasoned choices about deviating from this structure whenever required to increase the communicative value of the work. The title of the Dissertation itself, '*Sex, Signalling, and Steroids'*, draws on its contents: describing roles for sexual reproduction and steroid-dependent intracellular signalling in the regulation of secondary cell biology.

In the first Chapter of this Dissertation, CHAPTER 1, I introduce concepts that I believe are the most relevant to the Dissertation as a whole: the fruit fly, *Drosophila melanogaster*; its use as a genetic model to study signalling; and the tissue on which most of the work presented in this Dissertation has been performed: the male accessory gland. In addition, this first Chapter also provides an overview of the cell type that forms the focus of most of the work in this Dissertation: the secondary cell—providing an overview of the cell type itself; previous work on this cell type; and questions relating to this cell type and its functions that remain to be answered. I also provide a list of the general aims of the work. Because each of the results chapters is concerned with a separate aspect of secondary cell biology, I have made the decision to keep the initial introduction to this Dissertation brief, and instead introduce key concepts in more detail at the beginning of each individual results chapter. I feel that this improves the structure of the Dissertation itself, as each chapter then is sufficient to accurately describe one aspect of my doctoral work—complete with background and context. Although this format is not orthodox, I am hopeful that the examiners will understand my reasons for having made this decision, and instead focus their efforts and criticism on the scientific content of the work.

The second chapter, CHAPTER 2, is very brief, and details the materials and methods used and employed for the work presented in this Dissertation. This

Chapter also includes a discussion on the analysis and interpretation of data in this Dissertation, drawing on the contemporary research literature on this topic.

The first results chapter, CHAPTER 3, provides a brief introduction to a class of proteolytic enzymes, known as 'caspases'. The Chapter begins with an overview of caspase biology and function in *D. melanogaster*, before listing the aims of the Chapter itself. Briefly, the Chapter shows that caspases are present in secondary cells, and that they might be regulated by a BMP signalling and steroid receptor (EcR) axis. Crucially, I provide data suggesting that caspases might function non-apoptotically to regulate intracellular trafficking in secondary cells. I conclude the Chapter by presenting a model based on my data; illustrating how I believe this regulation might be orchestrated and translate to cell secretion in other tissues and organisms.

The second results chapter, CHAPTER 4, provides a brief introduction to Hedgehog signalling—drawing on parallels between the development of the *D. melanogaster* and mammalian male reproductive systems. In this Chapter, I provide a brief description of the changes that the accessory gland undergoes as the male fly approaches sexual maturity, as well as presenting evidence that suggests that Hedgehog signalling becomes active during this time. I also show that Hedgehog signalling has a role in regulating secondary cell nuclear size, and I conclude the Chapter with a discussion of the potential implications of Hedgehog signalling on the reproductive biology of male (and female) *D. melanogaster*.

The final results chapter, CHAPTER 5, concerns itself with the mechanisms governing secondary cell nuclear growth—with an emphasis on the growth that results after mating. I begin the Chapter with an introduction to the cell cycle and the molecular regulation thereof, before I show how the secondary cell nuclei behave over the first ten days of the male's adult life. The main purpose of the Chapter is to

show that the BMP/EcR signalling axis might be activated by mating in secondary cells, and that it might interact with the cell cycle to promote DNA synthesis—to drive mating-dependent nuclear growth. I conclude the Chapter with a discussion of the potential implications of this mating-dependent DNA synthesis on male reproductive biology, and propose a model where mating helps secondary cells to coordinate their secretory output with the reproductive demand. This is potentially relevant for the regulation of secretion in other tissues and organisms.

In the final chapter, CHAPTER 6, I provide a summary of the findings presented in this Dissertation, followed by a brief discussion of how the results fit together into a greater whole, and how my work has furthered our understanding of the male *D. melanogaster* accessory gland and male-specific secretory cell biology. Importantly, I also discuss some of the caveats of my results and the conclusions that can be drawn from these.

Following the Dissertation proper, I have also provided a selection of Appendices; containing tables with the numerical data generated from my work—as well as more detailed discussions of results that, although relevant to the work discussed in this Dissertation, did not merit inclusion in the Dissertation itself.

*

This Dissertation is not perfect. There are always more experiments to be done; further references to cite; and more insightful connections to be made. In particular, the work forming the basis of this Dissertation came to a somewhat premature end when I was given the opportunity to embark on a new career, doing something very different—making the quality of this Dissertation suffer as a result. Nevertheless, I hope that you, as a Reader, will enjoy exploring this work, and come away from it

having learnt something new. I have the same hope for myself: to keep learning new things, although I no longer consider myself to be a student. Yet, I feel that one has a duty to oneself to never stagnate, but to keep learning and to improve oneself. As Oscar Wilde (alumni of my Oxford college, Magdalen) wrote in his book, *The Picture of Dorian Gray* (1891[2008]):

> The aim of life is self-development. To realize one's nature perfectly—that is what each of us is here for. People are afraid one themselves, nowadays. They have forgotten the highest of all duties, the duty that one owes to one's self.

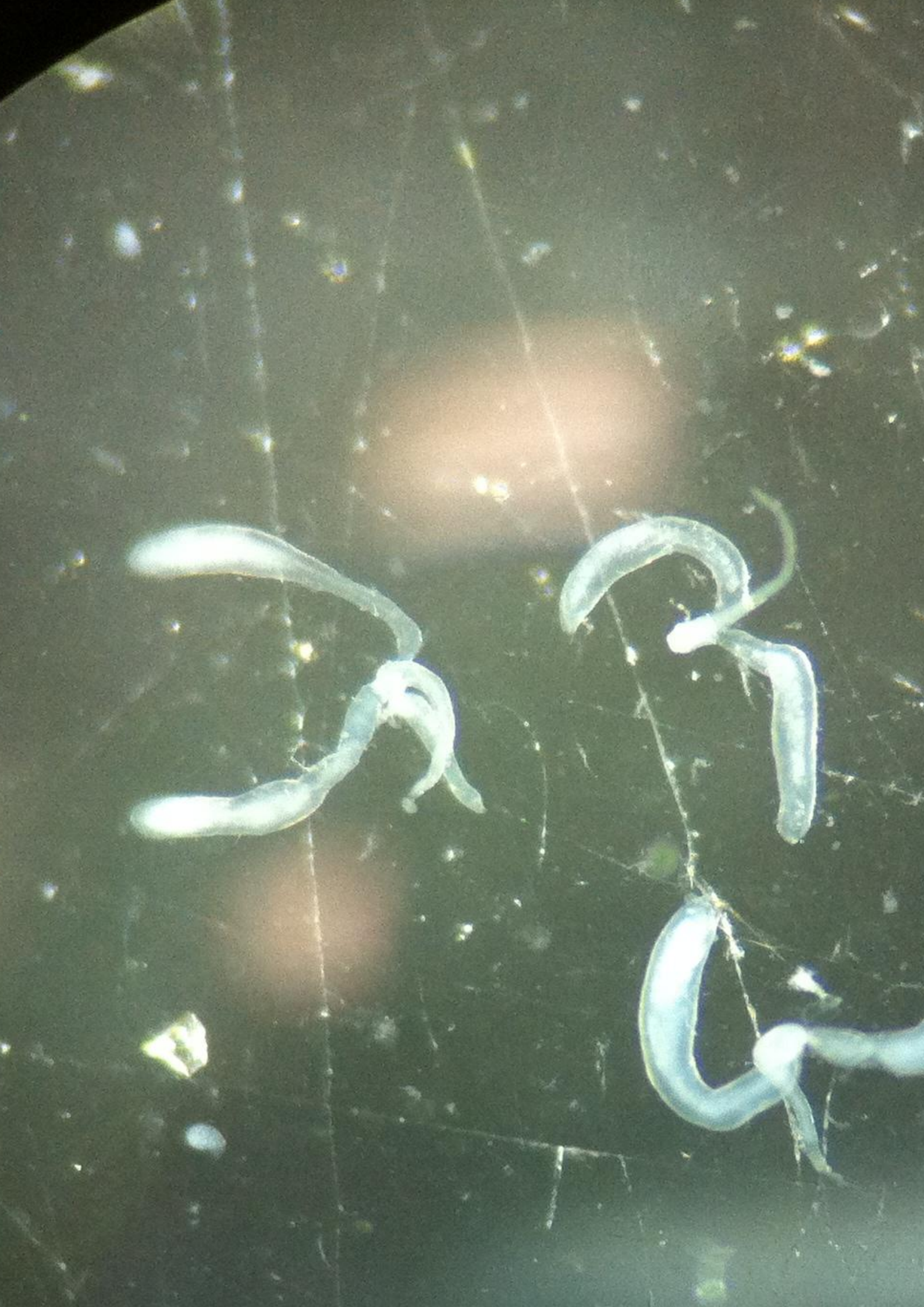
I think of this Dissertation as the culmination of one chapter of my life—with other, equally insightful, already having followed. I have done my best to make the Dissertation approachable and clear, but I also know that it is by no means perfect. To allow me to keep improving, even as my student days are over, please read and criticise this Dissertation constructively—to allow me to learn from my mistakes and to keep improving as a thinker and researcher; as a writer and communicator; as a student and professional.

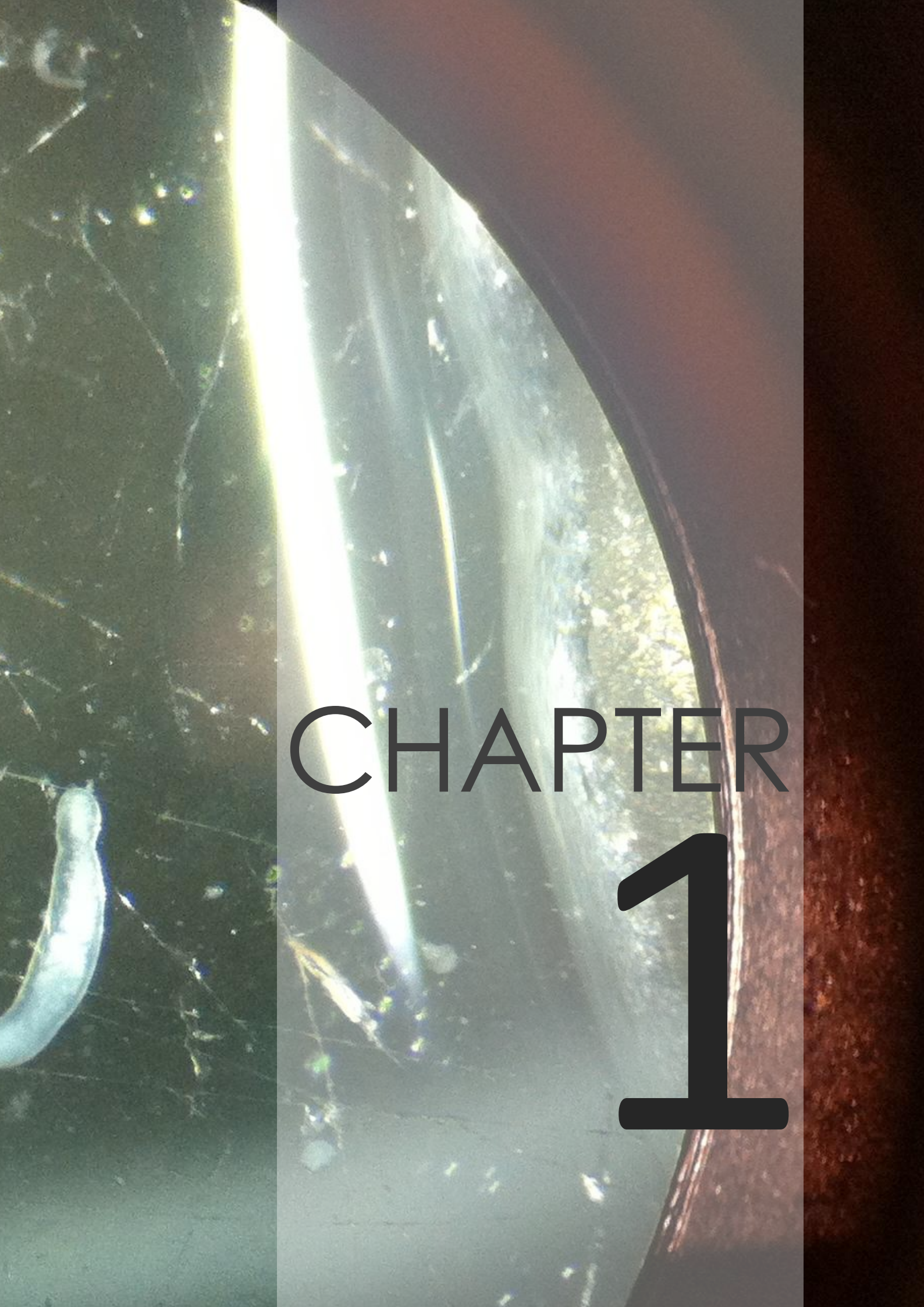
Finally, *thank you* for taking the time to read this Dissertation.



Josephine E.E.U. Hellberg

Edinburgh, Hilary 2018





CHAPTER 1

PREVIOUS PAGE:

Photograph of the male accessory glands and ejaculatory ducts of three different males, dissected in PBS, as seen down a stereo dissection microscope. Note that the leftmost dissection shows a very rare conformation where the ejaculatory duct bears three accessory gland arms instead of the usual two.



CHAPTER 1

INTRODUCTION

The chief advantage of Drosophila initially was one that historians have overlooked: it was an excellent organism for student projects.

– Robert E. Kohler
(in KOHLER, 1994: *p.* 33)

The fruit fly, Drosophila melanogaster

The fruit fly, *Drosophila melanogaster*, has its roots in the African tropics, but is currently distributed worldwide (MARKOW & O'GRADY, 2008). Whereas the genus name '*Drosophila'* means 'dew-loving' (referring to the species' habit of being active in the early morning), the species name '*melanogaster*' ('black-bellied') refers to the darkened abdomen of the male fruit fly (**FIGURE 1. 1. B**). Although terms like 'fruit fly' or, simply, '*Drosophila',* are arguably the most common names used to refer to this species, it is more formally (and correctly) referred to as 'pomace[1] fly', or 'vinegar fly'.[2] As suggested by these names, the species is commonly associated with fermenting fruit and decaying vegetable and plant matter, where it feeds on the associated microbes (MARKOW, 2015). As a microbivore, the fly is an ecological

[1] 'Pomace' are the solid remnants of fruits pressed for juice or oil.
[2] Although the *Drosophila* flies are referred to as 'common fruit flies', they are technically 'vinegar flies' and not true fruit flies. The true fruit flies belong to the family *Tephritidae.*

generalist in the wild and is capable of persisting on a variety of food-sources—a habit that might have enabled *D. melanogaster* to become a commensal species of human activity (MARKOW, 2015; MARKOW & O'GRADY, 2008).

Like many insects, *D. melanogaster* goes through several separate life stages, including a complete (*holometabolous*) metamorphosis that transforms the crawling larva into a free-flying adult fly (**FIGURE 1. 2.**). The *D. melanogaster* life cycle begins with a female fruit fly finding a deposit of decomposing plant material (often a fruit or other sugar-rich material) (MARKOW, 2015). Following courtship by males



FIGURE 1. 1. | THE FRUIT FLY, *D. MELANOGASTER*.

Photographs of male *D. melanogaster*, as seen through the stereo dissection microscope.

A: Dorsal view of a young male which has recently eclosed from his puparium and is in the process of inflating his adult tissues before the cuticle hardening occurs. Notice the pale colour and bloated abdomen of the fly. The *meconium* would be visible in the ventral view.

B: Lateral view of a young male (< 24 hours) who has undergone the initial process of cuticle hardening and pigmentation. Notice the darkened distal abdomen, which is characteristic of the males of this species.

The scale bar in both panels represents 2 mm (approx.).

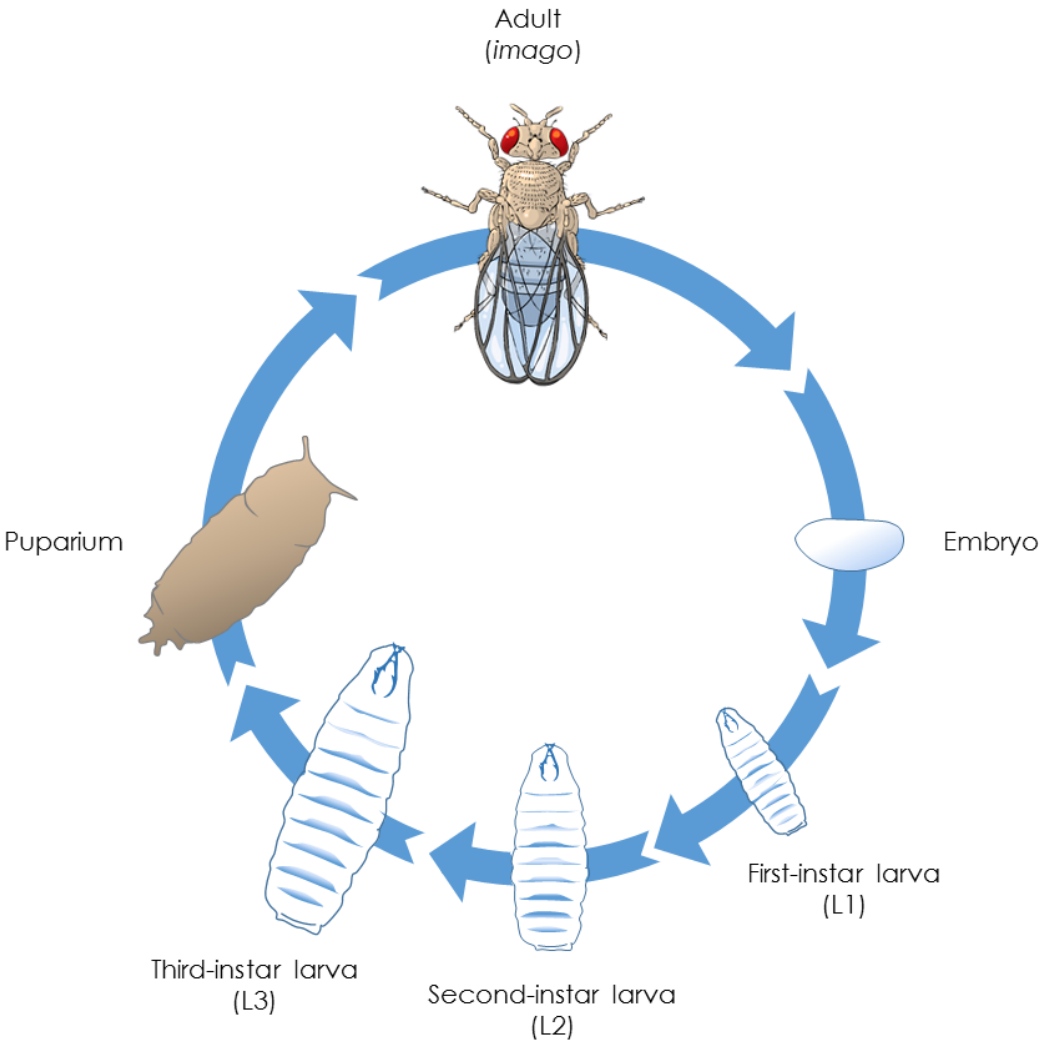


FIGURE 1. 2.| THE LIFE CYCLE OF *D. MELANOGASTER*.

D. melanogaster is a *holometabolous* insect and goes through a complete metamorphosis as part of its life cycle. After the fertilised oocyte (embryo) has been deposited into a suitable substrate of fermenting vegetable matter, and embryogenesis is complete, a larva emerges. The larva goes through three successive larval 'instars' (L1–L3), during which the adult tissues develop within the larva as 'imaginal discs'. The larva grows throughout its development, and the late third-instar larva leaves the food substrate that has supported its development to find a suitable place where to metamorphose. Having located such a place, the larva attaches itself to its puparation substrate and forms a puparium. The puparium protects the larva as the imaginal tissues reorganise to form those of the adult fly. The adult fly emerges from the puparium through a process known as '*eclosion*'. The adult fly matures fully during the first days after eclosion, after which the life cycle is complete and ready to begin anew. The illustration of the male fruit fly was adapted from FIGURE 6 in MORGAN (1925: *p.* 20).

competing for her attention, the female is fertilised and deposits her fertilised eggs (embryos) into the fermenting fruit. The yeasts that have colonised the food matter sustain the developing fly larvae as they pass through three successive larval stages.

The adult life stage of a metamorphic insect is called the '*imago'*, and the adult tissues that are developing as discs of material within the larva are therefore called 'imaginal discs'. When the larva reaches the end of its third and final larval stage, a steroid hormone pulse compels it to leave its food source and find a suitable place to metamorphose. Having located such a place, the larva's salivary glands empty their contents, coating the ventral side of the larva with proteinaceous secretions that quickly harden, attaching the larva to its chosen puparation substrate (FRAENKEL & BROOKES, 1953). Meanwhile, the larva contracts and its cuticle hardens and darkens, forming a protective case (*puparium*). The puparium protects the metamorphosing larva as its body and larval tissues reorganise and the imaginal tissues mature into their adult form. The developmental timing of *D. melanogaster* development is temperature-dependent: taking from 7 days at 28 °C up to 20 days at 18 °C, to include 10 or more generations per year in the wild (MARKOW & O'GRADY, 2008). Once the metamorphosis is complete, the adult fly emerges from its puparium through a process known as '*eclosion'*.

At eclosion, the fly is translucent and pliable, but assumes its adult form as the soft adult structures are inflated, and allowed to harden (**FIGURE 1. 1. A**). During this process, remnants of the fly's last meal as a larva (*meconium*) are easily observed through the transparent, extended abdominal wall.[3] Compared to other species of *Drosophila*, sexual maturation is rapid in *D. melanogaster*; with the female being able to

[3] Since the presence of the *meconium* is a reliable indicator that the fly has not yet mated, it is sometimes referred to as a 'virgin spot'.

oviposit within two days of emerging from her *puparium* (MARKOW & O'GRADY, 2008). Yet, this process of sexual maturation is not instantaneous (RUHMANN *et al.,* 2016).[4] For example, although the newly eclosed male fly emerges with the testes containing mature sperm (MARKOW & O'GRADY, 2008), he is reluctant to mate during the first day or two after eclosion (STRØMNAES, 1959).

Using *D. melanogaster* as a model for genetic research

Historical significance of *D. melanogaster* in the study of genetics[5]

The genetics of the fruit fly are among the best understood of any multicellular organism (GILBERT, 2000), and much of this is due to the work of one pioneering laboratory and research group: that of Thomas Hunt Morgan. Although the foundation of modern genetics was laid by Gregor Mendel, who used the garden pea (*Pisum sativum*) to work out some of the fundamentals of genetic inheritance (*e.g.* the law of segregation and the law of independent assortment) (MENDEL, 1925), it was much because of the work in the Morgan laboratory that genetics matured into a modern scientific discipline.

The pea was Mendel's organism of choice because many phenotypes are easy to score and female flowers are easy to fertilise manually (MENDEL, 1925). Similar characteristics made the fruit fly an agreeable animal model for early geneticists: being hardy, breeding rapidly, and being easy to sustain on human food products (*e.g.* bananas and other over-ripe fruits) (LEWIS, 1998). In addition, techniques for

[4] There are however reports of *D. melanogaster* males in the wild successfully mating with females only just emerged from their puparia (*e.g.* MARKOW, 2000).

[5] Few references in the scientific literature seem to be available (to my knowledge) that satisfactorily describe the story of how *D. melanogaster* came to be used as a genetic model and its significance for contemporary work. For this reason, I feel it is justified to include a brief summary (drawing from both LEWIS (1998) and MORSE (2007)) of this event in the Dissertation, hoping it will be of general interest to the reader.

identifying mutants were easy to learn, making the fruit fly ideal for students who, then (as now) acted as "eager, renewable student labor" (KOHLER, 1994: *p.* 34).

Reputably, the first person to realise the potential of the fruit fly as a model organism was the American entomologist, Charles Woodworth. Woodworth was an economic entomologist, and would have been familiar with the *Drosophila* flies (HOLDEN, 2015), since several members of the genus are important agricultural pests (*e.g. D. suzukii* (ATALLAH *et al.,* 2014)). Having learned to breed *D. melanogaster* in large numbers, Woodworth recommended the fruit fly to one of his colleagues, William Castle (MORSE, 2007), who used the fruit fly to study the effects of several generations of inbreeding (CASTLE, 1906). Morgan himself was introduced to the fruit fly by Castle and a colleague, both of whom had been impressed with the usefulness of the fruit fly as a laboratory animal (MORSE, 2007). At the time, Morgan himself was sceptical of the budding field of genetics where hereditary 'factors' were postulated, but with nobody having a firm idea of what actually constituted those factors (MORGAN, 1910a; LEWIS, 1998; GREEN, 2010). The end result of Morgan's work to determine the physical nature of heredity was that the mysterious 'factors' that governed inheritance were recast as physical entities in the form of genes occupying discrete *loci* on the chromosomes (GREEN, 2010).

The Morgan laboratory hosted many students and researchers, who made fundamental contributions to the field of *D. melanogaster* research. Particularly notable among these are Alfred Sturtevant, Calvin Bridges, Hermann Muller, Theodosius Dobzhansky, and Clara Lynch. Work under Morgan came to define *D. melanogaster* genetics—as well as the discipline of genetics in general. For example, Sturtevant and Bridges studied the giant chromosomes of the *D. melanogaster* larval salivary

gland, and showed that the genes—the hereditary materials—reside on (material, tangible) chromosomes ('coloured bodies') (STURTEVANT, 1913; BRIDGES, 1914); thus establishing the 'Chromosome Theory of Heredity' (MORGAN, 1910a; MORGAN *et al.,* 1915). The Morgan group reconciled these new findings with those of Mendel, finding that the segregation of genes depends on their positions along the chromosome (STURTEVANT, 1913); effectively laying the foundation for genetic linkage mapping and allowing the spatial distance of genes (measured in honorific 'Morgans') on the chromosome to be determined (**FIGURE 1. 3.**).

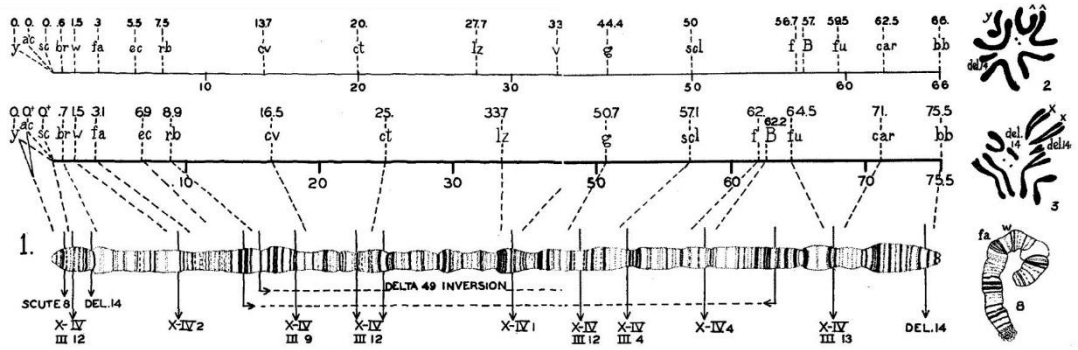


FIGURE 1. 3.| REFERENCE MAP OF THE *D. MELANOGASTER* LARVAL SALIVARY X-CHROMOSOME.

Composite schematic drawing of the X chromosome in the salivary gland of a female larva, drawn in segments using a *camera lucida* (1). The characteristic, distinctive banding pattern on the chromosome is clearly visible, and the two rows above show the location of individual genes. The giant salivary gland X chromosome is shown, to scale, alongside the entire cellular chromosome complement (2) of an oocyte in metaphase. The figure is taken from PAINTER (1934b): FIGURE 1 (detail).

In addition to the scientific contributions of the Morgan laboratory, the student-based research group also made contributions to the fruit fly research culture that persist to this day—with the open sharing of fly stocks, techniques and information (KOHLER, 1994). Notable examples—aside from the multitude of research papers that constitute the ever-growing literature on *D. melanogaster*—are initiatives such as FlyBase (GRAMATES *et al.*, 2016) and the *D. melanogaster* stock centres, such as the Bloomington *Drosophila* Stock Centre ("**BDSC**") and the Vienna *Drosophila* Resource Centre ("**VDRC**"); resources which have proven crucial for the work presented in this Dissertation.

Following the Morgan era of fruit fly genetics, the study of genetics shifted its focus onto bacteria and their phage (ASHBURNER & BERGMAN, 2005). *D. melanogaster* genetics did not come to the fore again until the 1970's, when it proved an essential tool for the study of developmental biology. Previously, the study of development in the fruit fly was hindered by the complexity of the fruit fly embryo, and the fact that it was not large or translucent enough for its development to be easily followed using light microscopy. As a result, the use of *D. melanogaster* as a model in the study of developmental biology could only be realised through improvements in molecular biology techniques that, effectively, made the invisible visible (GILBERT, 2000; ASHBURNER & BERGMAN, 2005). Notable among developmental biology studies making use of advances in molecular biology to better understand development in *D. melanogaster*, is the Nobel prize-winning screen of NÜSSLEIN-VOLHARD and WIESCHAUS (1980). The *D. melanogaster* embryo is intricately patterned, and this pattern is set up through increasingly fine sub-division of the embryo. Trying to better understand how the protein gradients that govern this patterning are set up and maintained, Nüsslein-Volhard and Wieschaus screened

for genes that, when mutated, altered the embryonic segmentation pattern. The screen succeeded in isolating 15 genes; representing several genetic pathways with roles in embryonic development—including molecules such as Hedgehog and Patched (NÜSSLEIN-VOLHARD & WIESCHAUS, 1980).

More recent work has shown that a large proportion of genes involved in human disease processes are conserved between humans and *D. melanogaster* (YAMAMOTO & JAISWAL *et al.*, 2014). Many of the major molecular signalling pathways are also conserved between the two species (PERRIMON *et al.,* 2012). These relationships allow the use of *D. melanogaster* as a model in the study of molecular processes implicated in human disease—for example through the functional analysis of genes, and facilitating the identification of gene expression patterns (both spatially and temporally) (BELLEN & YAMAMOTO, 2015). Through the publication of the complete fly genome (ADAMS *et al.*, 2000), research on the fruit fly has entered a 'genomic era', ushering the way for post-genomic approaches to better understand and apply the genetics of *D. melanogaster* to contemporary research questions (ASHBURNER & BERGMAN, 2005).

Using *D. melanogaster* to study molecular signalling

Following the successful use of *D. melanogaster* as a model of developmental biology in the 1970's, the fruit fly became an important part of the geneticists' toolkit once again. Commencing with the view that: "the genes controlling pattern organization are expected to have their primary expression within the cell itself" (GARCIA-BELLIDO & SANTAMARIA, 1972: *p.* 87), work on *D. melanogaster* helped create a more nuanced understanding of how large-scale biological patterns emerge. Far from autonomous, cells are in constant communication with their environment; with

signals reaching the cell to orientate it within the tissue, and to co-ordinate its functional output with the needs of the organism (KHOLODENKO, 2006).

Separate components are required for molecular signalling to proceed, and these components exist both outside and within the signalling cells, acting together in 'pathways' of variable logic and complexity. Stereotypically, these pathways are formed from the interaction of an extracellular component (*e.g.* a 'ligand') with a membrane-bound receptor, which is capable of modulating the behaviour of intracellular transducers—with the effect of activating a transcription factor to alter gene expression patterns (**FIGURE 1. 4. A**) (PIRES-DASILVA & SOMMER, 2002). Examples of well-known signalling pathways are those of the transforming growth factor-β ("**TGF-β**") superfamily (which includes the bone morphogenetic proteins; "**BMPs**") (SCHMIERER & HILL, 2007), and small molecules like steroids (KING-JONES & THUMMEL, 2005) and the lipoprotein, Hedgehog (BRISCOE & THÉROND, 2013). In animals, only a handful of signalling pathways are required for the development of diverse body plans (PIRES-DASILVA & SOMMER, 2002), and many of these pathways have similar roles—governing processes such as cell proliferation and differentiation; functions that are important for the proper development and patterning of an organism. Many of these pathways are conserved between *D. melanogaster* and mammals (PERRIMON *et al.*, 2012).

One tissue that was crucial for elucidating the relationship between signalling and pattern formation is the 'wing disc'; the imaginal disc in the *D. melanogaster* larva that gives rise to the tissues of the adult wing (**FIGURE 1. 5. A, B**). The wing disc is formed from a folded, bi-layered epithelium, where the apical surfaces of the epithelia face a lumen at the interior of the disc (**FIGURE 1. 5. B'**) (MORATA, 2001). The disc itself is divided into anterior ("**A**") and posterior ("**P**") compartments

(**FIGURE 1. 5. A**)—whose component cells are derived from separate founder lineages (GARCIA-BELLIDO *et al.,* 1973). Initially, the separation of the anterior and posterior lineages was believed to be the result of differential patterns of 'selector' gene expression—whose identification, it was hoped, would provide the genetic basis for pattern formation in development (LAWRENCE, 1985). The first selector gene to be proposed was the gene, '*engrailed*' ("***en***"), whose expression pattern helps define the posterior compartment (GARCIA-BELLIDO & SANTAMARIA, 1972). Subsequently, Engrailed was shown to be a transcription factor, with a role regulating the expression of other genes (JAYNES & O'FARRELL, 1991). This finding was quickly followed by the discovery that Engrailed regulates the expression of the gene, '*hedgehog*' ("***hh***"), in the posterior compartment (TABATA *et al.,* 1992).

The *hedgehog* gene had been discovered already in the 1950's, based on an eye phenotype (allele: *bar-3*) (INGHAM, 2016). However, the gene earned its name because of its effect on disrupting the *D. melanogaster* embryonic segmentation pattern; causing the embryo to grow a patch of bristles on its back that makes it resemble a little hedgehog (**FIGURE 1. 6.**) (NÜSSLEIN-VOLHARD & WIESCHAUS, 1980). The Hedgehog molecule itself is a lipoprotein, containing lipid modifications that affect its spread through tissue (BRISCOE & THÉROND, 2013). This allows Hedgehog to act as a 'morphogen'; a molecule with a role in tissue patterning (BRISCOE & THÉROND, 2013; TURING, 1952). In the wing disc, Hedgehog spreads through the tissue in an anterior direction, forming a gradient across a narrow stripe of tissue (**FIGURE 1. 5. A**) (BASLER & STRUHL, 1994). As a result of this gradient forming, cells close to the source of Hedgehog

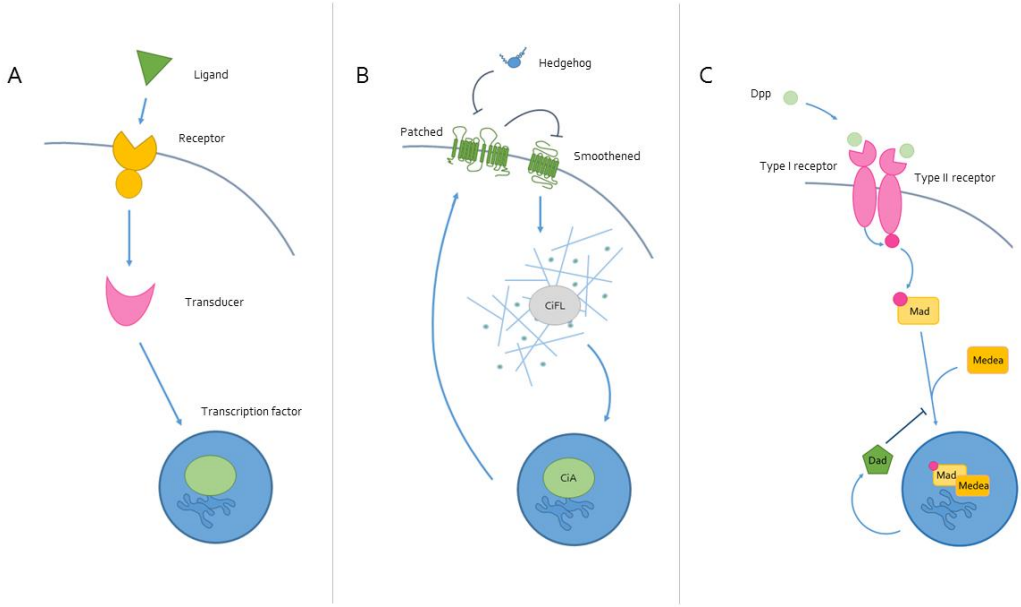


FIGURE 1. 4. | SIGNALLING PATHWAYS ARE FORMED FROM THE INTERACTION OF MULTIPLE COMPONENTS.

A: Diagrammatic representation of a stereotypical signalling pathway, where the binding of a ligand to its receptor permits the receptor to interact with signal transducers, culminating with the regulation of gene expression by a transcription factor.

B: Diagrammatic representation of the *D. melanogaster* Hedgehog signalling pathway, to illustrate a signalling pathway with a negative logic, where the Hedgehog ligand inhibits its receptor, Patched, allowing the signal transducer, Smoothened, to become active. Smoothened activity causes the transcription factor, Cubitus interruptus (Ci), to be processed into an activator form, allowing Ci to translocate to the nucleus and promote Hedgehog-dependent gene expression. One of the Hedgehog target genes is *patched*, whose expressions completes the pathway by forming a negative feedback loop.

C: Diagrammatic representation of the *D. melanogaster* BMP signalling pathway, to illustrate a signalling pathway with a positive logic, where the Dpp ligand activates its receptor, allowing it to dimerise, and activate the signal transducer, Mothers against Dpp (Mad). Activated Mad complexes with its binding partner, Medea (Med), allowing the complex to translocate to the nucleus to promote BMP-dependent gene expression. One of the BMP target genes is the BMP signalling inhibitor, *Daughters against dpp* (*Dad*), whose expression completes the pathway by forming a negative feedback loop.

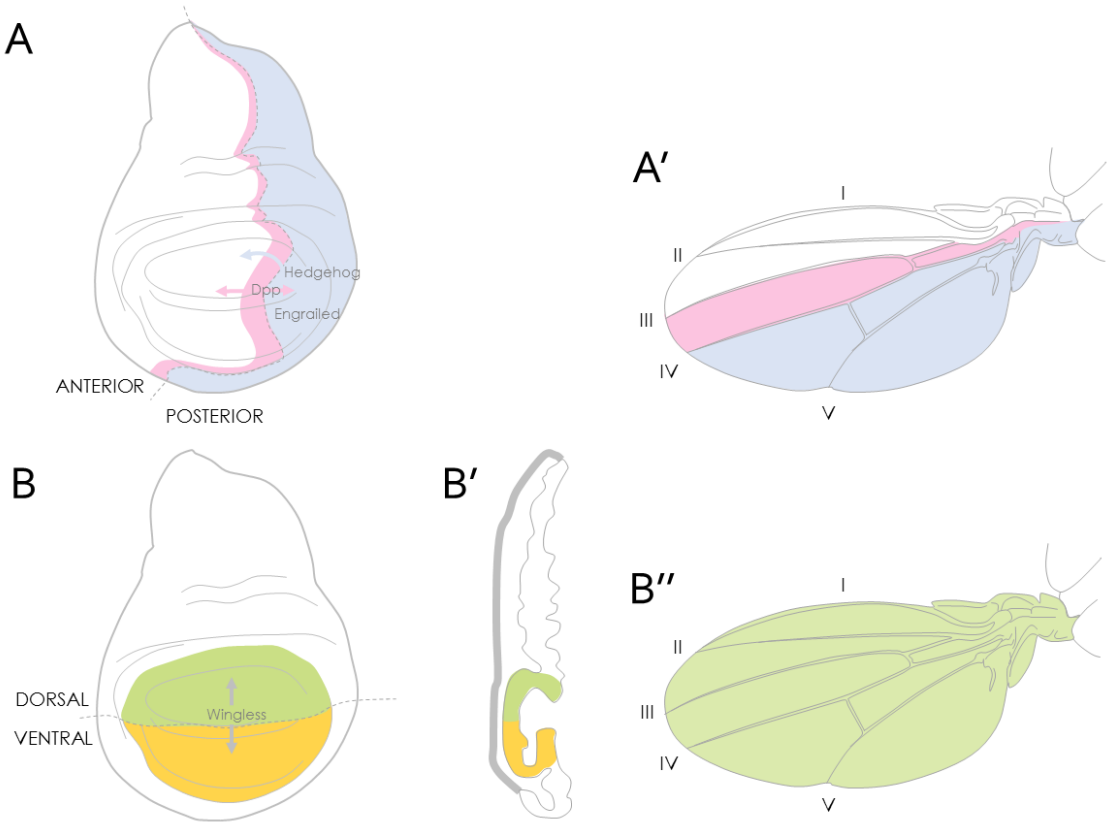


FIGURE 1. 5. | MOLECULAR SIGNALLING EVENTS PATTERN THE DEVELOPING ORGANISM.

A: Semi-diagrammatic representation of a wing imaginal disc in dorsal view, showing how the disc is divided into anterior (A) and posterior (P) compartments. The transcription factor, Engrailed, is expressed in the posterior compartment, where it regulates the expression of *hedgehog*. Hedgehog diffuses in an anterior direction in a thin stripe of tissue, where it induces the expression of the BMP signalling ligand, *decapentaplegic* (*dpp*).

A': Semi-diagrammatic representation of the adult *D. melanogaster* wing in dorsal view, showing how the organisation of the wing disc translates into the adult structure, and how the Dpp gradient helps set up the distance between the third (III) and fourth (IV) wing veins.

B: Semi-diagrammatic representation of the wing disc in dorsal view, showing how the wing is divided into dorsal (D) and ventral (V) compartments. The morphogen, Wingless (Wg), helps define the dorso-ventral boundary.

B': Semi-diagrammatic representation of the wing disc in the posterior view, showing how the disc is composed of a sac-like epithelium.

B": Semi-diagrammatic representation of the adult wing in dorsal view, showing how the organisation of the wing disc translates into the adult structure.

The wing disc diagrams in this figure have been adapted from FIGURE 3 in DAHMANN & BASLER (1999: *p.* 323), whereas the wing diagram has been adapted from FIGURE 2 in GARCIA-BELLIDO and SANTAMARIA (1972: *p.* 90).

receive high levels of the molecule, whereas cells farther from the source receive smaller amounts; allowing the receiving cells to effectively orient themselves within the tissue along the anterior-posterior axis. Appropriate patterns of gene expression are activated by different levels of morphogen signalling. For example, high levels of Hedgehog activate one set of genes (such as the Hedgehog receptor, *patched* ("***ptc***")), and low levels of Hedgehog activate another (including the BMP ligand, *decapentaplegic* ("***dpp***")) (STRINGINI & COHEN, 1997).

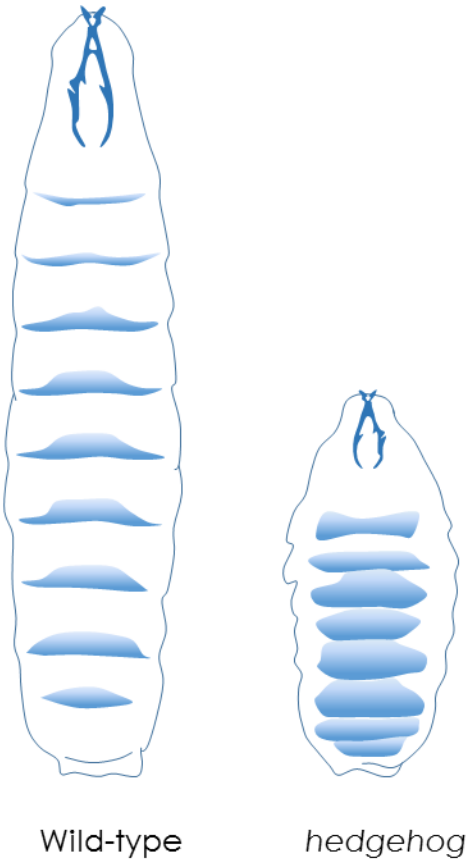


FIGURE 1. 6. | *hedgehog* GENE MUTATION DISRUPTS THE EMBRYONIC SEGMENTATION PATTERN

Semi-diagrammatic representation of a wild-type larva and a larva mutant for the gene, *hedgehog*. The body of the wild-type larva is divided into segments, each with its anterior half covered is a type of bristles called 'denticles', and with the posterior part of the segment remaining smooth. Mutation of *hedgehog* disrupts the embryonic segmentation pattern, causing a defect in the formation of the posterior half of the segments. This makes the embryo grow an abnormal patch of denticle bristles on its dorsal side—causing it to resemble a hedgehog.

The larvae in this figure have been adapted from FIGURE 2 in NÜSSLEIN-VOLHARD & WIESCHAUS (1980).

Similar to *hedgehog*, *patched* had been discovered prior to the Nüsslein-Volhard and Wieschaus screen that gave the gene its (current) name (INGHAM, 2016). As the Hedgehog receptor, Patched is a crucial component of the Hedgehog signalling pathway (**FIGURE 1. 4. B**). Other components of the pathway include the Hedgehog signal activator, Smoothened ("**Smo**"), and the Hedgehog-dependent transcription factor, Cubitus interruptus ("**Ci**"). Like *hedgehog* and *patched*, *ci* is also expressed in the wing disc—but its expression is negatively regulated by Engrailed, and therefore restricted to the anterior compartment (EATON & KORNBERG, 1990). As a result, only cells in the anterior compartment are sensitive to Hedgehog signalling—making the signalling 'paracrine'. Aside from *patched*, Ci also regulates the expression of *dpp* in the stripe of tissue receiving low levels of Hedgehog protein, which helps set up the distance between the third and fourth wing veins (**FIGURE 1. 5. A'**) (STRINGINI & COHEN, 1997).

Dpp is a growth factor and ligand of the BMP signalling pathway (**FIGURE 1. 4. C**), and it performs diverse roles during the development of *D. melanogaster*. Like Hedgehog, Dpp can be released from cells to regulate development and tissue patterning in a concentration-dependent manner (WHARTON *et al.,* 1993; AFFOLTER & BASLER, 2007). Dpp is required for the normal development of all the major imaginal discs, including the wing disc (SPENCER *et al.*, 1982).[6] In mammals, members of the TGF-β superfamily (which includes the BMP ligands) interact with two receptor sub-types: each with different ligand-binding activities, and with a 'type I' receptor requiring a 'type II' interaction partner (WRANA *et al.,* 1994). In an effort to identify similar receptors in *D. melanogaster*, the gene '*thickveins*'

[6] There are 15 major imaginal discs in *D. melanogaster*. Mutation of *dpp* affects the development of these, and this phenotype gave the gene its name: 'decapentaplegic' meaning '15 defects' (SPENCER *et al.,* 1982).

("***tkv***"), a serine-threonine kinase, was identified and shown to be a type II TGF-β–like receptor capable of binding Dpp (NELLEN *et al.,* 1994). However, whereas the Hedgehog ligand *inhibits* its receptor, Dpp binding a type II receptor *activates* the receptor; permitting it to interact with a type I dimerisation partner to form a functional type I·type II receptor complex (WRANA *et al.,* 1994). The functional receptor complex is competent of activating the downstream BMP signalling pathway, which proceeds through phosphorylating the intracellular signalling component, Mothers against Dpp ("**Mad**") (SEKELSKY *et al.,* 1995). Phosphorylated Mad ("**pMad**") proceeds by dimerising with an interaction partner, Medea ("**Med**"); forming a functional pMad·Med transcription factor complex. This complex is capable of translocating to the nucleus, where it regulates the transcription of BMP-dependent genes (DAS *et al.,* 1998; KIM *et al.,* 1997).

Experimentally manipulating the genetics of *D. melanogaster*

The first *D. melanogaster* mutant to be isolated was *white*. This first mutant appeared spontaneously, and Morgan wrote of its discovery in a 1910 article in *Science*:

> In a pedigree culture of *Drosophila*, which had been running for a considerable number of generations, a male appeared with white eyes. The normal flies have brilliant red eyes. [MORGAN, 1910b: *p.* 120.]

In the years that followed, further genetic mutants were isolated—having arisen both spontaneously or as the result of mutagenesis (GREEN, 2010), using mutagens such as X-rays (MULLER, 1930). Despite their low efficiency, early molecular biological techniques were also used to generate mutations and to introduce new sequence information. The discovery of transposable elements (*P*-elements)[7] in wild

[7] The P-elements are so-named because of the tendency of natural P-elements to cause 'P-M hybrid dysgenesis' after crossing certain *D. melanogaster* strains; with the hybrid dysgenesis occurring when a

populations of *D. melanogaster* (HIRAZUMI, 1971) paved the way for technological breakthroughs and increased transformation efficiency through the development of P-element–mediated transformation techniques that allow the introduction of stable transgenes into the fruit fly genome (SPRADLING & RUBIN, 1982; RUBIN & SPRADLING, 1982). In parallel, molecular biology helped elucidate the mechanisms of gene regulation in other model organisms—such as how the metabolism of different carbon sources is regulated genetically in the yeast, *Saccharomyces cerevisiae* (*e.g.* DOUGLAS & HAWTHORNE, 1966). One of the genes identified through this work was the transcription factor, GAL4, which regulates the expression of genes involved in galactose metabolism ("***GAL***" genes); which has proven pivotal for the *D. melanogaster* research community (DUFFY, 2002).

GAL4 is constitutively expressed in yeast cells (LAUGHON & GESTELAND, 1982), but is normally kept inactive through interaction with its repressor, GAL80 (DOUGLAS & HAWTHORNE, 1966). The presence of galactose (in the absence of the preferred carbon source, glucose) in the growth medium causes GAL80 to become inactive; allowing GAL4 to regulate the genes required for galactose catabolism through binding to specific sites within the *GAL*-gene promoters; the 'upstream activating sequences' ("**UAS**") (JOHNSTON & HOPPER, 1982; GINIGER *et al.*, 1985) (**FIGURE 1. 7.**). Understanding the molecular basis of the GAL4-*UAS* interaction allowed the regulatory circuit to be successfully introduced into *D. melanogaster* using P-element–mediated transformation; permitting GAL4 to drive the expression of a *UAS*-reporter gene construct (FISCHER *et al.*, 1988). Building on this conceptual advance, a bimodal system of GAL4/*UAS*-targeted gene expression in *D. melanogaster* was designed (BRAND & PERRIMON, 1993). In

male from a P-strain (paternal) is mated to a female from an M-strain (maternal), but not the other way around (SPRADLING & RUBIN, 1982).

this system, targeted gene expression is kept inactive by maintaining the GAL4 transcription factor and the *UAS* enhancer separately, with the system only becoming active in the F_1 progeny resulting from a cross between separate GAL4 and a *UAS* parent flies (**FIGURE 1. 8.**). Generating stock lines with the *GAL4* gene sequence fused to endogenous or transgenic *D. melanogaster* promoters allows the

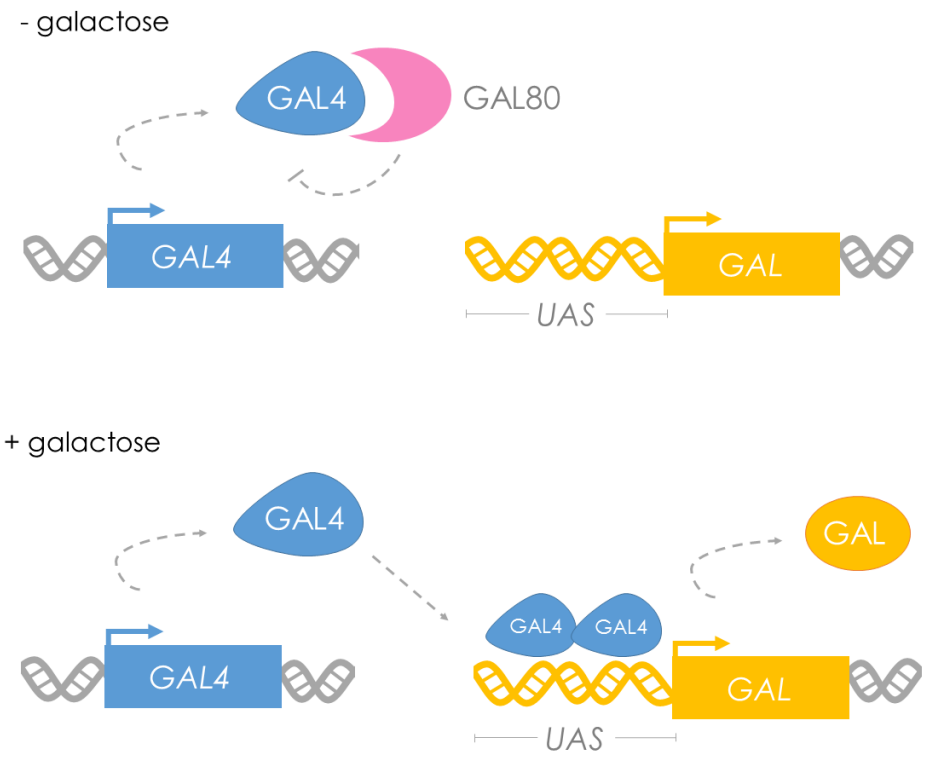


FIGURE 1. 7.| THE GAL4/*UAS* SYSTEM IN *S. CEREVISIAE*.

Schematic diagram showing the logic of galactose-dependent gene expression in *S. cerevisiae*. The transcription factor, GAL4, is constitutively expressed in the cell. In the absence of galactose, GAL4 activity is inhibited by its interaction with GAL80. In the presence of galactose, GAL80 is inactive, allowing GAL4 to bind upstream-activating sequences (*UAS*) in the promoter of galactose-inducible genes—and drive the expression of genes required for galactose metabolism (*GAL*-genes).

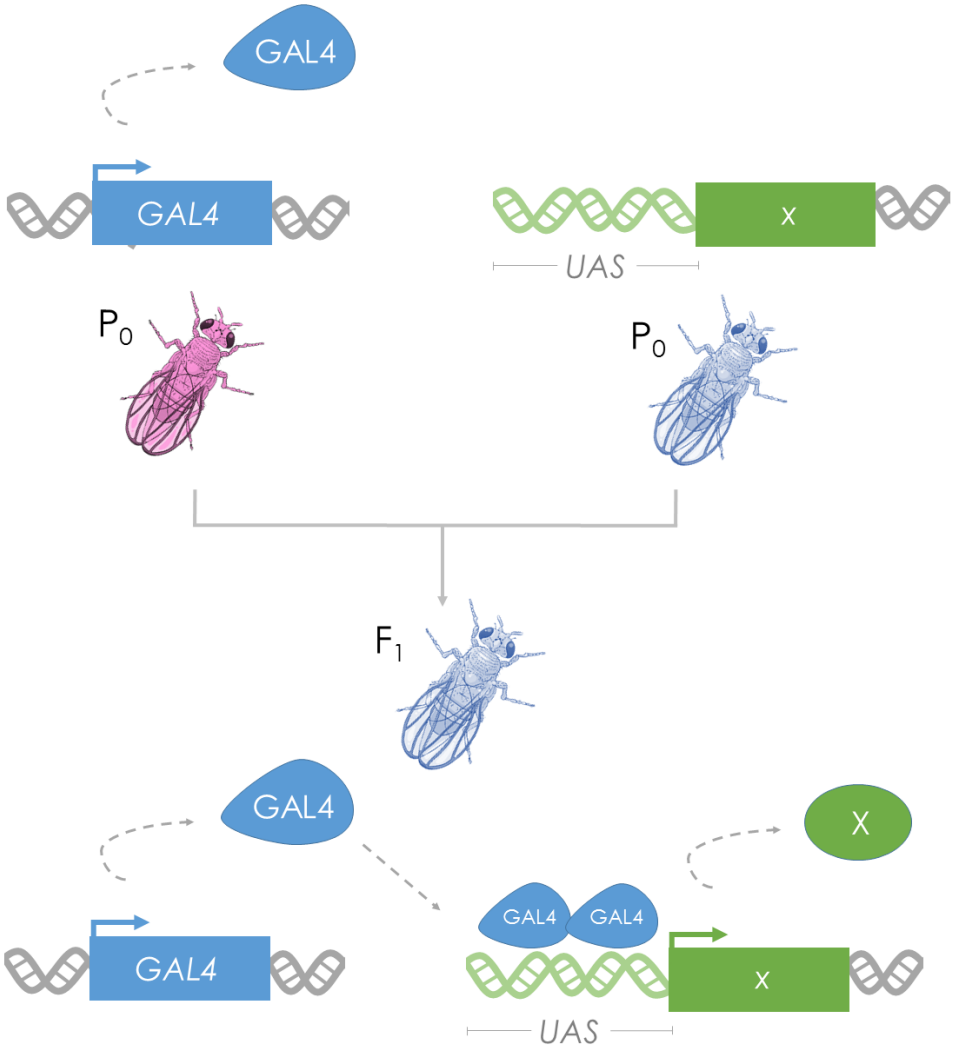


FIGURE 1. 8.| THE GAL4/*UAS* SYSTEM IN *D. MELANOGASTER*.

Schematic diagram showing the logic of GAL4/*UAS*-dependent gene expression in *D. melanogaster*. The system is made flexible and also kept inactive by separation of the GAL4 and *UAS* component in different genetic lines. This separation allows the construction of stock libraries where the *GAL4* gene sequence has been fused to different genetic regions—like enhancers and/or promoters, that then come to regulate the cell- and tissue-specificity of the GAL4 expression—and where the *UAS* sequence has been fused to different genes of interest (*x*). The system is made active by genetically combining the GAL4 and *UAS* components in the F_1 offspring of a cross between GAL4 and *UAS* parent flies: allowing the enhancer/promoter-*GAL4* fusion to drive expression of the *UAS*-dependent gene of interest in a cell- and tissue-specific manner.

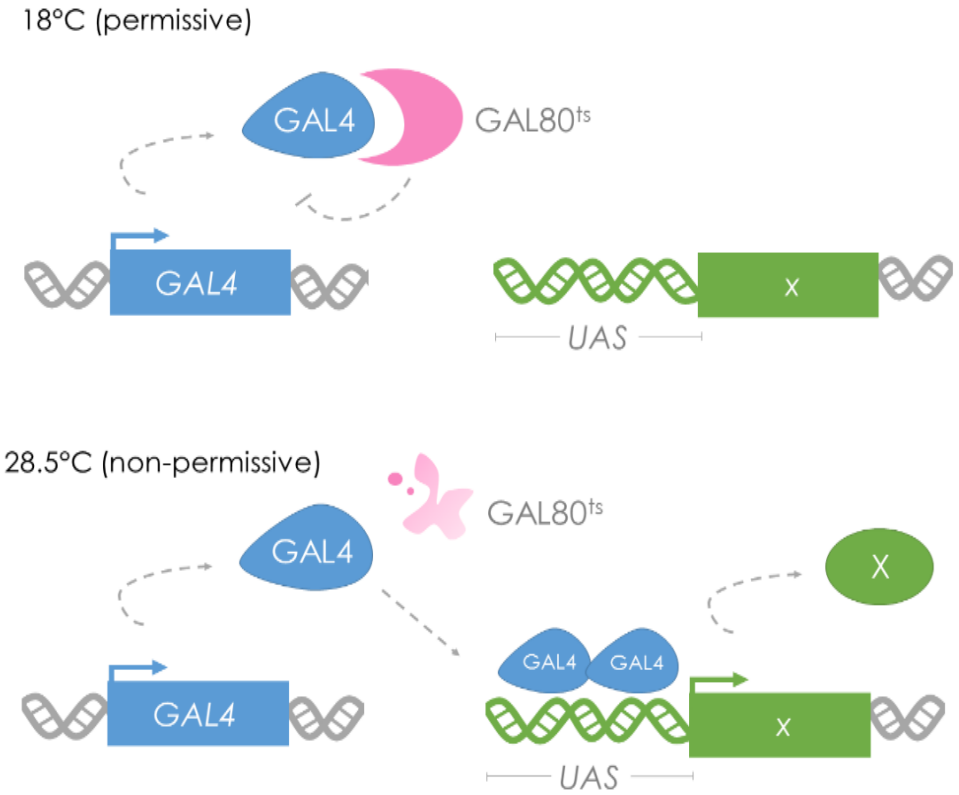


FIGURE 1. 9.| THE CONDITIONAL/INDUCIBLE GAL4/*UAS* SYSTEM.

Schematic diagram showing the logic of the temperature-dependent GAL4/*UAS* system in *D. melanogaster*. At the permissive temperature, the temperature-sensitive form of GAL80 (GAL80^{ts}) is active and able to inhibit the activity of GAL4, rendering *UAS*-dependent gene expression inactive. At the non-permissive temperature, GAL80^{ts} is no longer active, and GAL4 is able to drive *UAS*-dependent gene expression.

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enhancer-specific expression of GAL4; effectively making the transcription factor age-, tissue-, or cell-type–specific, depending on the nature of the enhancer. Combining these lines with genes of interest fused to the *UAS*, allows the very flexible (tempo-spatial) expression of desired transgenes (BRAND & PERRIMON, 1993).

Using the GAL4/*UAS* system, fly stock collections were created and maintained for the benefit of the *Drosophila* community (*e.g.* by the BDSC and VDRC), and in the years since, a temperature-sensitive form of GAL80 ($GAL80^{ts}$) has been added to the fly genetic toolkit to allow the temperature-dependent regulation of GAL4 activity (MCGUIRE *et al.,* 2003) (**FIGURE 1. 9.**); further increasing the flexibility of the GAL4/*UAS* system. The GAL4/*UAS* system also simplified the introduction of other techniques to alter gene expression in *D. melanogaster*—such as RNA-interference ("**RNAi**") (FIRE *et al.*, 1998; PICCIN *et al.,* 2001); the FLP/FRT system of inducible nucleotide excision (THEODOSIOU & XU, 1988; DUFFY *et al.,* 1998); and the CRISPR/Cas9 system of conditional mutagenesis (JINEK *et al.*, 2013; XUE *et al.,* 2014).

The *D. melanogaster* male accessory gland

The reproductive system of adult male *D. melanogaster* is bilaterally symmetrical, and can be divided into three main parts: the testes and seminal vesicles; the ejaculatory duct; and the accessory glands (**FIGURE 1. 10. A; FIGURE 1. 11. A**). In *D. melanogaster*, the testes occur as a looped pair and are responsible for the production of sperm—which is stored in the seminal vesicles (BAIRATI, 1968). The testes and seminal vesicles drain into the ejaculatory duct ('*ductus ejucularis*'), which consists of a secretory epithelium in the shape of a long, narrowing tube, connecting the internal male reproductive system with the ejaculatory bulb ('sperm pump') and the external genitalia. The ejaculatory duct is dilated at its distal end (forming the '*ampullar*' portion)—and the rest of the male reproductive system (seminal vesicles and accessory glands) connects to this *ampulla* (BAIRATI, 1968).

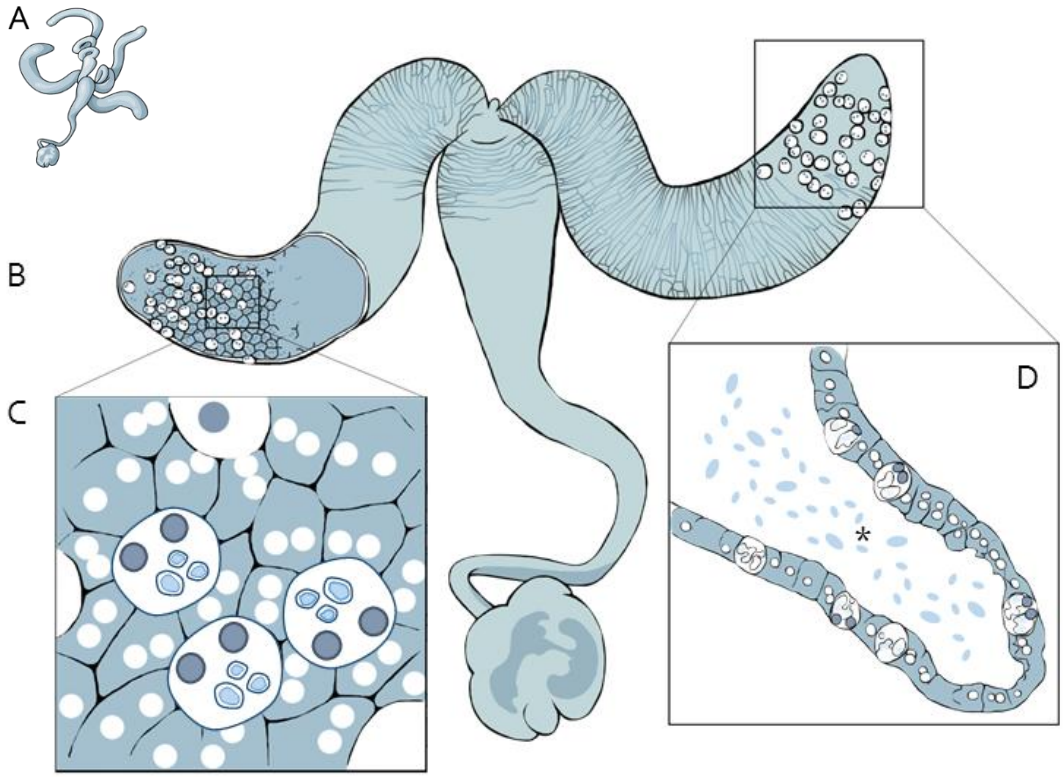


FIGURE 1. 10.| THE *D. MELANOGASTER* MALE REPRODUCTIVE SYSTEM, WITH AN EMPHASIS ON THE MALE ACCESSORY GLANDS.

A: The male reproductive system, complete with looped testes, accessory glands, and the ejaculatory duct.

B: The male reproductive system, *sans* testes. The paired accessory glands are sheathed in a striated muscle layer, and connect to a common ejaculatory duct. During mating, accessory gland contents are transferred to the female through muscle contractions. The ejaculatory duct terminates proximally in an ejaculatory bulb ('sperm pump').

C: The accessory glands consist of a monolayered, secretory epithelium. The epithelium is made up of two cell types: main cells (blue) and secondary cells (white). Main cells are typically smaller and roughly hexagonal in shape, whereas secondary cells are larger and more rounded. Both cell types are binucleated. Whereas main cells make up the main part of the accessory gland epithelium, secondary cells are less numerous and restricted to the distal tips of the glands.

D: The secretory epithelium encloses the accessory gland luminal space (*), that contains the accessory gland secretions (*i.e.* a major component of the seminal fluid).

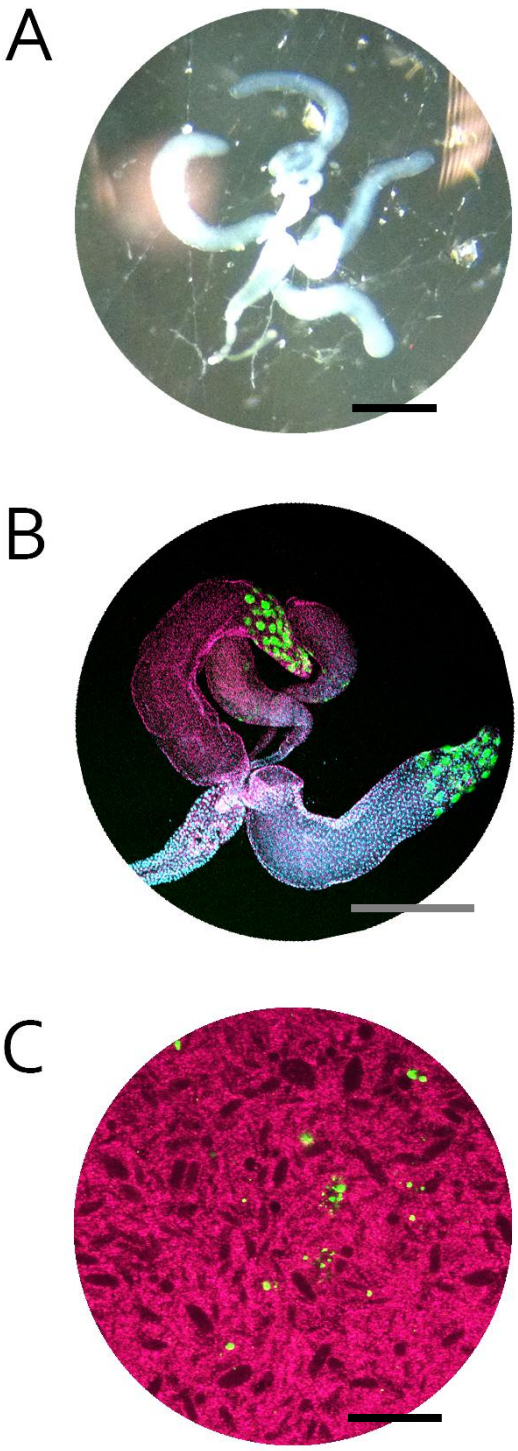


FIGURE 1. 11. | THE *D. MELANOGASTER* MALE ACCESSORY GLANDS ARE PART OF THE MALE REPRODUCTIVE SYSTEM AND CONTAIN PROTEINS IMPORTANT FOR REPRODUCTION.

A: The male reproductive system as seen down a light microscope, dissected in PBS. The paired, arm-like accessory glands budding off horizontally from the ejaculatory duct are clearly visible, as are the vertically looped testes and seminal vesicles.

B: The male reproductive system, as seen down a confocal microscope—with epithelial cell nuclei shown in cyan; cell junctions shown in magenta; and secondary cells shown in green. The seminal vesicles are visible in-between the arms of the paired accessory glands.

C: The accessory gland lumen is filled with epithelial secretions organised into different fractions: an aqueous phase that (among other things) contains the protease, ANCE (magenta); and an immiscible phase that contains both membrane-associated proteins (green) and non-membrane–associated lipophilic proteins that form cigar-shaped (ovoid) flocculates (visible as dark, negative spaces). In this image the labelled membrane-associated proteins were released from secondary cells. The protein flocculates are believed to form from the coalescence of lipophilic products derived from both main cells and secondary cells.

In panels **A–B**, the scale bars represent 400 μm (approx.). In panel **C**, the scale bar represents 20 μm (approx..).

Insect epithelia are typically simple (HAPP, 1984), and, like the ejaculatory duct, the paired accessory glands (*paragonia*) each consist of a monolayered epithelium. A contractile muscle sheath encloses the accessory glands, and during mating, peristaltic contractions of circular muscle fibres cause the glands to contract and push its contents into the ejaculatory duct, to be delivered to the female (BAIRATI, 1968). The epithelium is the secretory portion of the accessory gland and in *D. melanogaster* it is composed of two cell types: 'main cells' and 'secondary cells'.[8] The main cells are the most numerous—numbering around 1,000 per gland—and make up the main bulk of the epithelium (BAIRATI, 1968; BERTRAM *et al.*, 1992). Main cells are hexagonal or near-hexagonal in shape, and rest on the gland basement membrane that separates the epithelium from the underlying muscle cells (BAIRATI, 1968). The secondary cells are located at the distal ends of each gland, where around 40–50 of them are scattered among the surrounding main cells (BAIRATI, 1968; BERTRAM *et al.*, 1992) (**FIGURE 1. 10. B, C; FIGURE 1. 11. B**). Secondary cells are larger and more rounded than main cells, and being mildly pear-shaped, they wedge between main cells to reach from the basement membrane to protrude slightly into the gland lumen (BAIRATI, 1968) (**FIGURE 1. 10. D**). On this apical face, from where secretion happens (HAPP, 1984), the secondary cell is mostly covered by neighbouring main cells, exposing only a small portion of the secondary cell to the lumen—a conformation similar to that seen in mammalian neuroendocrine cells and their surrounding tissues (B. KROEGER & K. CARR, personal comments).

The accessory gland lumen is filled with secretions from both main cells and secondary cells. Just as the two cell types are morphologically distinct, the protein

[8] The cells are so named since the main cell is the main cell type in the accessory gland, whereas the secondary cell is a less numerous, second (secondary) cell type.

content of their secretions are also different (DIBENEDETTO *et al.,* 1990; BERTRAM *et al.,* 1992; KALB *et al.,* 1993; GLIGOROV & SITNIK *et al.*, 2013). In the gland lumen, the joint epithelial secretions are highly heterogeneous; forming a viscous, proteinaceous fluid that contains dispersed filaments and flocculent lipophilic proteins (BAIRATI, 1968; FEDERER & CHEN, 1982; SCHMIDT *et al.*, 1985; CORRIGAN, 2014) (**FIGURE 1. 11. C**). These accessory gland secretions form the bulk of the proteins that go into the seminal fluid; the non-cellular part of the ejaculate.

Role of accessory gland secretions in male manipulation of female physiology

The males of many insect species use their seminal fluid to manipulate female reproductive physiology[9] (reviewed by AVILA *et al.,* 2011). Some of these manipulations are subtle, such as strengthening the female immune system (MUELLER *et al.,* 2007; INNOCENTI & MORROW, 2009). Other changes are more pro-active, such as facilitating sperm activation and storage within the female, or boosting female fertility by increasing her ovulation and oviposition rates (AVILA *et al.,* 2011). Yet other changes run contrary to the reproductive interests of the female—some even being downright detrimental—such as making the recipient female less attractive to, and receptive of, other courting males, or reducing her lifespan through the delivery of toxic proteins (PENG *et al.,* 2005; WIGBY & CHAPMAN, 2005; MUELLER *et al.,* 2007). Altogether, these stereotypic changes that mating has on the behaviour and physiology of female insects are referred to as the female 'post-mating response' ("**PMR**"). Although part of this response can be induced by the receipt of sperm—such as increased oviposition—the majority of the

[9] Which is not to say that the female cannot respond in kind, although the subject of such sexual conflict and intra-species sexual arms-races is beyond the scope of this Dissertation.

female post-mating response is induced by the receipt of seminal fluid (XUE & NOLL, 2000). Similar to males that produce no sperm, male *D. melanogaster* that lack accessory glands, or that are otherwise unable to produce seminal fluid, are sterile (XUE & NOLL, 2000; CHOW *et al.*, 2015). The seminal fluid is, therefore, crucial to male reproductive success.

The seminal fluid in *D. melanogaster* is a complex cocktail of proteins belonging to several different functional classes (FINDLAY *et al.,* 2008), and at least some of the seminal fluid components that are responsible for inducing the female post-mating response are known in some detail. Although the majority of the top-50 most highly expressed proteins in the accessory gland are known only by their computed gene ("**CG**")-numbers (indicating that they have not yet been the subject of formal description) (CHINTAPALLI *et al.*, 2007), a number of accessory gland-derived proteins ("**Acps**") and their physiological effects have been described (FINDLAY *et al.,* 2008; AVILA *et al.*, 2011) (see **TABLE 1. 1.** for a selection). Arguably, the most well-known accessory gland-derived protein is Sex Peptide ("**SP**"; Acp70A[10]) (CHEN *et al.,* 1988; reviewed by KUBLI & BOPP, 2012), which is produced by the accessory gland main cells (XUE & NOLL, 2002). The Sex Peptide itself is short, a mere 36 amino acids, but is involved in the regulation of many aspects of the female post-mating response (KUBLI & BOPP, 2012). Part of this activity is by Sex Peptide associating with sperms and binding to the Sex Peptide-receptor ("**SPR**") that is expressed, for example, by neurons in the female sperm storage organs (YAPICI & KIM *et al.*, 2000). A gradual dissociation of Sex Peptide from the receptor permits its gradual release from stored sperm, to affect female post-mating physiology for as long as sperm remains available (PENG *et al.,* 2005). These dynamics allow the

[10] In a gene name like 'Acp70A', 'Acp' represents 'accessory gland protein', whereas the alpha-numerical '70A' represents the gene *locus* on the salivary gland chromosome (CHEN *et al.,* 1988).

female post-mating response to be divided into two parts: an early, short-term response ("**STR**") that occurs within the first 24 hours post-mating, and a long-term response ("**LTR**") that alters female behaviour and physiology for up to 10 days post-mating (KALB *et al.*, 1993; XUE & NOLL, 2000; KUBLI, 2003; PENG *et al.*, 2005).

The long-term activity of Sex Peptide in the female reproductive tract depends on its interaction with a network of several other seminal fluid proteins (LAFLAMME & WOLFNER, 2013). Some of these—like Ovulin (ovulation-inducing pro-hormone; Acp26Aa) and the glycoprotein, Acp36DE—are produced by main cells (HERNDON & WOLFNER, 1995; NEUBAUM & WOLFNER, 1999). Yet, the function of these main cell products has been shown to require the activity of secondary cells in the gland; with secondary cells (or secondary cell-derived products) being responsible for glycosylation of proteins involved in the Sex Peptide regulatory network (GLIGOROV & SITNIK *et al.*, 2013; SITNIK & GLIGOROV *et al.,* 2016). Additionally, many accessory gland proteins function as regulators of proteolysis (proteases and protease inhibitors) that regulate the function of other proteins (reviewed by LAFLAMME & WOLFNER, 2013). Notable among these are the secondary cell-derived angiotensin-converting enzyme ("**ANCE**"; a thermolysin-like protease) (RYLETT & WALKER *et al.*, 2007), and the main cell-derived seminase (a trypsin-like serine protease) (LAFLAMME *et al.,* 2012). Seminase is involved in the correct localisation of Sex Peptide to the female sperm storage organs and also participates in a proteolytic cascade that culminates with the processing of Ovulin (Acp26Aa) and Acp36DE in the female reproductive tract (RAVI RAM *et al.*, 2006).

Effect of mating on the male accessory gland

Mating depletes accessory gland contents and reduces the male's ability to manipulate female post-mating physiology (BAUMANN, 1974a; ROGERS *et al.,* 2005; RADHAKRISHNAN & TAYLOR, 2008; AVILA *et al.,* 2011). Since the seminal fluid is crucial for male fertility (XUE & NOLL, 2000), there is a need for

TABLE 1. 1. | EXAMPLES OF MALE ACCESSORY GLAND PROTEINS IN *D. MELANOGASTER* AND THEIR FUNCTIONS.

Protein	Source	Type	Effect (+/-)	Reference
Acp26Aa (Ovulin)	MC	Prohormone	Ovulation (+); oviposition (+)	HERNDON & WOLFNER (1995)
Acp29AB	MC	Lectin (predicted)	Sperm storage (+)	WONG & ALBRIGHT *et al*. (2008)
Acp36DE	MC	Glycoprotein	Sperm storage (+)	NEUBAUM & WOLFNER (1999)
Acp62F	MC	Protease inhibitor	Sperm competition (+); ovulin processing (+)	MUELLER *et al.* (2008)
Acp70A (Sex Peptide; SP)	MC	Ligand	Sexual receptivity (-);	LIU & KUBLI (2003)
ANCE	SC	Protease	Not determined	RYLETT & WALKER *et al.* (2007)
CG10284	MC	Protease inhibitor	Immunity (+)	MUELLER *et al*. (2007)
CG17575	SC	Cysteine-rich secretory protein	SP-sperm binding (+)	SITNIK & GLIGOROV *et al.* (2016)
Lectin-46CA	SC	C-type lectin	SP-sperm binding (+)	SITNIK & GLIGOROV *et al.* (2016)
Seminase	MC	Trypsin-like serine protease (predicted)	Oviposition (+); Sexual receptivity (+)	LAFLAMME *et al.* (2012)
Spn38F	MC	Protease inhibitor	Immunity (+)	MUELLER *et al*. (2007)

the gland to recover its contents between copulations to ensure continued reproductive success. In both the stalk-eyed fly (*Cyrtodiopsis dalmanni*), and the Queensland fruit fly (*Dacus tryoni*), it has been demonstrated that the size of the male accessory gland is reduced upon mating but that accessory gland contents are restored within 24 hours (ROGERS *et al.*, 2005; RADHAKRISHNAN & TAYLOR, 2008). In *D. melanogaster*, mating depletes the accessory gland contents by about one-third (BAUMANN, 1974a). However, mating also stimulates the production of accessory gland proteins (BAUMANN, 1974b; DIBENEDETTO *et al.,* 1990)—an effect that can be mimicked by the topical application of two *D. melanogaster* hormones, juvenile hormone ("**JH**") or 20-hydroxyecdysone ("**20-HE**", or "**ecdysone**"), onto the abdominal cuticle (YAMAMOTO *et al.*, 1988; HERNDON *et al.,* 1997). Indeed, in main cells, *Acp* gene promoters contain JH binding elements (HERNDON *et al.,* 1997), which is suggestive of a direct regulatory relationship between JH and accessory gland protein production. In addition, it has been noted that the hormone-induced stimulation of protein production in the male accessory gland involves *de novo* synthesis of ribosomes; increasing the translation of stable mRNA transcripts already present in the secretory cells (SCHMIDT *et al.*, 1985). Altogether, it seems as if mating increases accessory gland protein production by increasing both the transcriptional and translational activity of the tissue.

In other cell types, such as the *D. melanogaster* larval salivary gland, ecdysone also stimulates the synthesis and secretion of secreted proteins (*i.e.* salivary gland proteins; "**Sgps**") (POELS, 1972; BIYASHEVA & DO *et al.*, 2001). Interestingly, it has been shown that the tubular accessory glands of the red flour beetle, *Tribolium castaneum*, are a site of ecdysone synthesis—and there is evidence that this may also

occur in the secondary cells of the *D. melanogaster* male accessory gland (HENTZE *et al.,* 2013). Indeed, the secondary cells of *D. melanogaster* contain many of the enzymes required for converting a cholesterol precursor into ecdysone (HENTZE *et al.,* 2013), suggesting a role for secondary cells in the production of the main insect steroid hormone; allowing them to potentially regulate, not only accessory gland function, but also systemic steroid hormone signalling in the adult male.

Secondary cell biology

Despite being secondary in number to the main cells, secondary cells are important for the function of the *D. melanogaster* male accessory gland. Although main cells produce a large proportion of the proteins that go into seminal fluid, secondary cells are biochemically distinct with their own expression and secretion profile (DIBENEDETTO *et al.*, 1990; BERTRAM *et al.*, 1992; KALB *et al.*, 1993). Indeed, secondary cells are important for the female post-mating response. As mentioned, secondary cells are involved in the glycosylation of main cell protein products (GLIGOROV & SITNIK *et al.*, 2013). Without secondary cells the female post-mating response is reduced; with males being unable to induce the long-term response in their mates (LEIBLICH *et al.*, 2012; MINAMI *et al.*, 2012; GLIGOROV & SITNIK *et al.,* 2013; CORRIGAN *et al.*, 2014). That main cells and secondary cells co-exist in the gland as distinct cell types suggest that their inter- and co-dependence might be important for accessory gland function—similar to how other secretory organs (*e.g.* the mammalian prostate and pancreas) are composed of multiple cell types that co-ordinate their functions to, together, regulate complex physiological processes (GLIGOROV & SITNIK *et al.,* 2013; SITNIK & GLIGOROV *et al.,* 2016). Indeed, RNA sequencing of accessory glands from males with defective

secondary cells has identified a set of 192 genes whose expression seemingly depends on the presence of secondary cells in the accessory gland (SITNIK & GLIGOROV *et al.*, 2016). Of these genes, 77 were down-regulated, and knock-down of some of these down-regulated genes not only affected the female post-mating response but also produced intracellular phenotypes, such as the loss of, or altered morphology of, secondary cell intracellular compartments (SITNIK & GLIGOROV *et al.*, 2016). Therefore, secondary cell-specific genes and intracellular structures seem to be required for optimal accessory gland function.

Morphologically, the most characteristic feature of the secondary cell is the presence of atypically large intracellular compartments (BAIRATI, 1968; FEDERER & CHEN, 1982; RYLETT & WALKER *et al.,* 2007; CORRIGAN *et al.,* 2014) (**FIGURE 1. 12.**). Cells typically use vesicles to transport, store, and release protein cargo. In the *D. melanogaster* larval salivary gland, the intracellular secretory compartments are very large (3–7 μm) (RUOSSO *et al.*, 2016), and similar in size to those of the accessory gland secondary cells (2–5 μm) (BAIRATI, 1968; CORRIGAN *et al.*, 2014).[11] In addition to these secretory compartments (both endolysosomal and exocytic) (CORRIGAN *et al.*, 2014), secondary cells contain well-developed endoplasmic reticuli (ER) and Golgi apparatuses (BAIRATI, 1968; CORRIGAN *et al.*, 2014; REDHAI, 2016; B. KROEGER, personal comments).

Different members of the RAS-in-the-brain ("**RAB**") family of GTPases (which are involved in vesicle recognition and docking events) associate with different types of intracellular compartments; allowing intracellular trafficking to be targeted to distinct compartments (STENMARK, 2009; BARR, 2013). Each RAB GTPase thus

[11] Compare the size of these compartments to the size of the secretory compartments in the typical pancreatic β-cell in the rat, where they measure around 200–300 nm (0.2–0.3 μm) (FAVA & DEHGHANY *et al.*, 2012). Similar compartments have also been identified in the human prostate, where they measure around 1 μm (COHEN *et al.*, 2000).

has its own (approximate) subcellular localisation, and the combination of different RAB GTPases on different compartments allow for the identification of intracellular compartment identity. For example, the RAB GTPase, RAB7, is a marker of the endolysosomal system; being present on late endosomes and lysosomes (STENMARK, 2009). In secondary cells, RAB7-positive compartments are acidic and undergo multiple fusion events as the secondary cell matures; leading to the

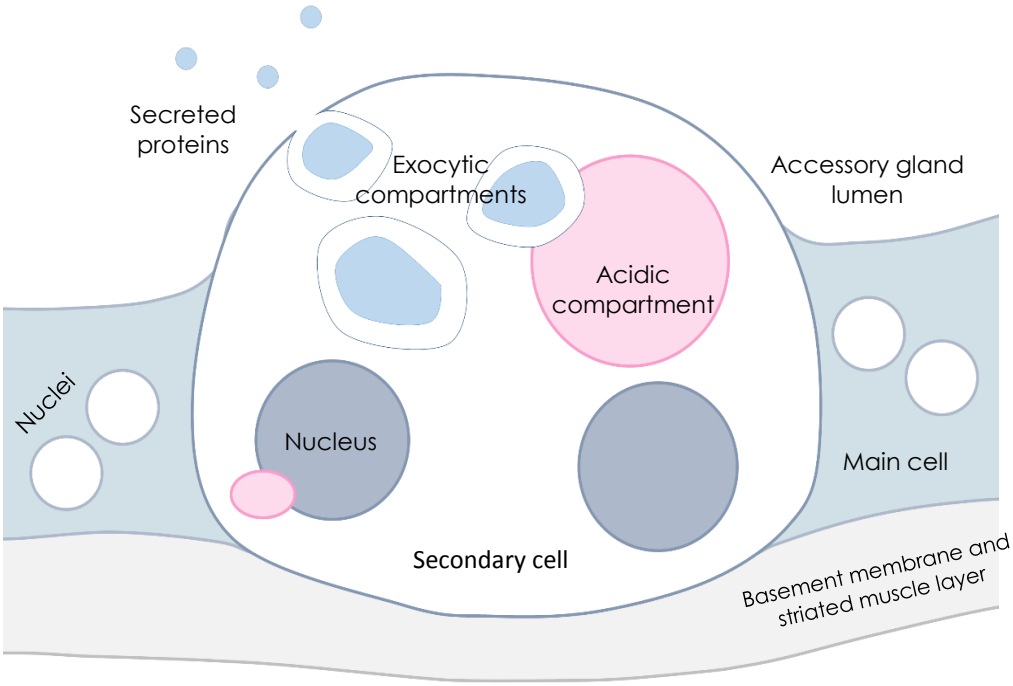


FIGURE 1. 12. | THE SECONDARY CELL.

A schematic of a secondary cell, showing its characteristic cytology. The secondary cell (white) is larger than the surrounding main cells (light blue), and protrudes slightly from the epithelium. Both main cells and secondary cells are binucleate—although main cell and secondary cell nuclei are of a similar size, they are not shown to scale in this diagram. Unlike the main cells, secondary cells contain large internal compartments: exocytic compartments and endolysosomal compartments.

Exocytic compartments contain a secretory granule, whose contents are released into the accessory gland lumen, to mingle with the other components of the seminal fluid. The epithelial cells rest on a basement membrane, whereas the gland itself is surrounded by a striated muscle layer.

growth and formation of large, endolysosomal compartments (CORRIGAN *et al.*, 2014). Similarly, exocytic compartments and recycling endosomes can be identified through their association with RAB11 (STENMARK, 2009). In secondary cells, RAB11 is present on non-acidic internal compartments (CORRIGAN *et al.*, 2014; REDHAI, 2016) that can be labelled with antisera against accessory gland lumen contents (FEDERER & CHEN, 1982; RYLETT & WALKER *et al.*, 2007); indicating an exocytic identity of RAB11-positive compartments in secondary cells.

A significant volume of the secondary cell body is occupied by the exocytic compartments. These compartments contain granulaceous protein cores ('secretory granules'), surrounded by bundles of filaments (BAIRATI, 1968; FEDERER & CHEN, 1982; RYLETT & WALKER *et al.,* 2007). These granules contain protein products that form part of the secondary cell contribution to both the aqueous and immiscible (flocculent) portions of the seminal fluid, such as: ANCE (aqueous; RYLETT & WALKER *et al.,* 2007); lipid-anchored proteins (immiscible; CORRIGAN, 2014); and the BMP ligand, Dpp (immiscible) (REDHAI *et al.,* 2016).

The size and composition of the secondary cell intracellular compartments changes as the accessory gland itself matures. At early ages, (days one to three), the secondary cell already contains exocytic compartments—complete with mature secretory granules (CORRIGAN *et al.,* 2014; REDHAI, 2016). As the accessory gland (and the secondary cell) matures, these compartments experience high turnover rates—presumably as their contents are delivered into the gland lumen (B. KROEGER & F. CASTELLANOS, personal comments). In addition, immature secondary cells contain small and numerous endolysosomal compartments that grow over time—as the result of homotypic compartment fusion events (CORRIGAN *et al.,* 2014; REDHAI, 2016). The secondary cell nuclei have also been shown to grow

during the time between eclosion and full sexual maturity (LEIBLICH *et al.,* 2012). Altogether, it seems as if the maturation of secondary cells follows that of the accessory gland itself; implying that the secondary cells, like the accessory gland, undergo a process of maturation over the time when the male matures sexually.

Regulation of secondary cell biology: questions answered and outstanding

Secondary cells were first described in the late 1960's, as BAIRATI (1968) performed the first electron microscopy on the *D. melanogaster* male accessory gland. In the years following, secondary cells were shown only cursory interest—mainly differentiated as being distinct from the main cells (*e.g.* DIBENEDETTO *et al.,* 1990). Secondary cell-specific gene expression patterns were however described in the decade leading up to the new millennium (*e.g.* BERTRAM *et al.,* 1992; HRDLICKA *et al.,* 2002)—making the first cell type-specific manipulations of secondary cells (theoretically) possible. However, a (perceived) lack of drivers and markers made forays into secondary cell biology remain largely dormant (C. WILSON & A. LEIBLICH, personal comments), with much of the contemporary work focussing on the composition of the accessory gland-derived components of the seminal fluid and its physiological and reproductive functions (reviewed by WOLFNER, 1997; WOLFNER, 2002; RAVI RAM & WOLFNER, 2007).

Our laboratory first became interested in the secondary cells as part of a pioneering effort to better understand human prostate biology through the use of a genetically amenable and analogous model: the *D. melanogaster* male accessory gland (WILSON *et al.*, 2017). Further cell type-specific drivers were identified in secondary cells as a result of these efforts (LEIBLICH, 2015), paving the way for the discovery

that BMP signalling is an important regulator of secondary cell biology; having a role regulating secondary cell nuclear growth (LEIBLICH *et al.,* 2012). Building on this initial cytological work, our laboratory subsequently showed that BMP signalling also regulates intracellular trafficking in secondary cells (*e.g.* CORRIGAN *et al.,* 2014). In tandem, other research groups have added further genes to the secondary cell-specific genetic toolkit (*e.g.* GLIGOROV & SITNIK *et al.,* 2013), and provided further demonstration that secondary cells contribute to male reproductive function (*e.g.* SITNIK & GLIGOROV *et al.,* 2016). Yet, despite the rapid progress that has been made on our understanding of secondary cell function in the past decade, several interesting and important questions remain unanswered. For example, although LEIBLICH *et al.* (2012) showed that secondary cell nuclei grow over time and after mating, the exact mechanisms underlying this growth remain unknown. Furthermore, whereas CORRIGAN *et al.* (2014) showed that secondary cells make an excellent model for a more detailed understanding of intracellular trafficking dynamics, many details concerning the regulation of these events remain unexplored. In addition, although good progress has been made identifying secondary cell-specific drivers (*e.g.* LEIBLICH, 2015; GLIGOROV & SITNIK *et al.,* 2013), our understanding of what signalling pathways are active in secondary cells, and how they affect secondary cell biology, remains to be fully elucidated.

Cell biology of secretion and its regulation

Musing on the interconnectedness of things, the English poet John Donne wrote: "No man is an island, entire of itself; every man is a piece of the continent, a part of the main" (DONNE, 1624[2011]: *p.* 108). In the spirit of the famous quip by French biochemist, Jacques Monod: "what is true of *E. coli* must also be true of elephants"

(FRIEDMANN, 2004); the individual cells of an organism (like people in a society) rely on one another for their function and survival. Individual cells are therefore constantly engaged with releasing products and receiving signals that modify their surroundings and influence the behaviour of other cells—either neighbouring or distant. These processes occur via secretion, the process of which can be either *constitutive* or *regulated* (BURGESS & KELLY, 1987; BURGOYNE & MORGAN, 2003). Constitutive secretion occurs in all cell types: promoting the anterograde (forward) transport and retrograde (backward) flux of receptors and other molecules between the cytoplasm to the plasma membrane; allowing cells to both receive and respond to external signals (FARHAN & RABOUILLE, 2011). Constitutive secretion also allows cells to secrete the products that form and maintain the extracellular matrix (*e.g.* collagen, elastin, and various glycoproteins)—to keep individual cells securely embedded in their host tissue to support their normal function (HAY, 2013). Conversely, regulated secretion is typically a feature of specialised secretory cell types, whereby secretory products (*e.g.* neurotransmitters, hormones, or enzymes) are retained within the cell and only released in response to a signal (BURGESS & KELLY, 1987).

Proteins need to be both modified and sorted in order to function and localise properly within the cell, and many of these processes occur within specialised membrane systems: the endoplasmic reticulum ("**ER**") and the Golgi apparatus. The ER is a continuous membrane system with an internal microenvironment that is permissive for proper protein folding. Proteins that are destined for the ER contain an 'ER signal sequence' that target them to the ER during translation—as their ribosomes associate with a portion of the ER (the rough ER) to feed the growing polypeptides into either the ER membrane (transmembrane proteins) or ER lumen

(soluble proteins) (ALBERTS *et al.*, 2002; LEE & MILLER *et al.,* 2004). Proteins that are destined for secretion via the Golgi are transported to a region of the ER that is free of ribosomes (the smooth ER). Although the smooth ER has a role synthesising both cholesterol and cholesterol-derivatives like steroids, it also contains specialised ER 'exit sites', where proteins are concentrated and packaged into vesicles to bud off into the cytoplasm (ALBERTS *et al.,* 2002). In the cytoplasm, ER-derived vesicles fuse with one another to form 'vesicular tubular clusters' that migrate on microtubules towards the Golgi apparatus, maturing as they are depleted by proteins normally resident in the ER (LEE & MILLER *et al.,* 2004).

The Golgi apparatus is formed from the association of several *cisternae* (flattened membrane-associated compartments), and it is particularly well-developed and prominent in specialised secretory cells (ALBERTS *et al.,* 2002). Protein-containing vesicles enter the Golgi via the *cis*-(entry) face, and are modified as they migrate throughout the Golgi: from the *cis*-Golgi to the *medial* Golgi, to finally be fully modified and ready to be released on vesicles from the *trans*-(exit) face of the apparatus (DE MATTEIS & LUINI, 2008). Vesicles derived from the *trans*-Golgi target proteins to the lysosome, or to the cell surface to be secreted. All proteins destined to be secreted from the cell are all packed onto secretory vesicles, but proteins specifically destined for the regulated secretion pathway are packed onto secretory vesicles specialised for protein storage (DE MATTEIS & LUINI, 2008). The content of these storage vesicles is compacted as the vesicle matures (ARVAN & CASTLE, 1992), and the resultant 'granule' of protein cargo is commonly referred to as a *secretory granule*.[12]

[12] When viewed using electron microscopy, some secretory granules can appear electron-dense, being thus referred to as 'dense-core granules'. However, unless a secretory granule appears appropriately electron-dense in an electronmicrograph, it would be inappropriate to refer to it as a

Vesicles containing secretory granules only fuse with the plasma membrane in the presence of the appropriate signals. These signals ('secretagogues') often take the form of hormones; signalling into the cell for secretory granule-containing vesicles to be mobilised (BURGOYNE & MORGAN, 2003). For example, in pancreatic β-cells, insulin is packed on dense-core granules, and although their release is regulated by elevated blood glucose-levels (CERASI & LUFT, 1967; CURRY *et al.,* 1968), this process can be further stimulated by BMP signalling (GOULLEY *et al.,* 2007). However, although secretory granules are easy to see down even a simple light microscope, their complete contents and the detailed mechanisms underlying their production and release are still not fully understood. Because secretory granule release is altered in various disease states, like the type-II diabetic pancreas (KAHN *et al.*, 2001) and the malignant prostate (COHEN *et al.,* 2000), how secretory granule biogenesis and release is regulated represents an important area of contemporary cell biology research (*e.g.* DELESTRE-DELACOUR & CARMON *et al.*, 2017; PEJLER, FRISK & SJÖSTRÖM *et al.,* 2017; STEPHENS *et al.,* 2017). As described previosuly, secretory granules are also found in the secondary cells of the *D. melanogaster* male accessory gland, where they occupy a significant proportion of the cell volume and carry seminal fluid proteins to be released into the gland lumen. In our laboratory, we believe that the secondary cells represent an important cytological tool for better understanding the mechanisms underlying the biogenesis and regulated secretion of cored compartments, and the work presented in this Dissertation contribute to our understanding of these processes.

'dense-core granule' (K. CARR, personal comments). Notably, the secretory granules imaged using electron microscopy in secondary cells by members of our laboratory do not seem to appear particularly electron-dense (B. KROEGER, personal comments), and are therefore referred to as 'secretory granules' for the purposes of this Dissertation.

Aims of this project

The main aim of my *D. Phil.* work was to build on previous work in the field—to help expand on our understanding of accessory gland biology in general, and secondary cell biology in particular. My hope was that increased understanding of accessory gland function and secondary cell biology would help us to better understand the function of male reproductive glands, in insects, as well as in other organisms. The overall aim of my project was therefore to *better understand the genetic regulation of intracellular signalling in secondary cells*; with a strong emphasis on *how this signalling regulates secondary cell nuclear growth*. More explicitly, the starting aims of my project were:

- To better understand how the *D. melanogaster* male accessory gland develops in the adult, as the male approaches sexual maturity;
- To identify and characterise novel regulators of secondary cell biology, with an emphasis on the identification and characterisation of novel signalling pathways and inputs;
- To better understand the mechanisms underlying secondary cell nuclear growth, with an emphasis on the mating-dependent regulation thereof.

The findings presented in this Dissertation have increased our understanding of the molecular processes that regulate the maturity and function of the *D. melanogaster* male accessory gland, with particular emphasis on the mating-dependent regulation of secondary cell biology. These findings have interesting implications for our understanding of male reproductive glands and male reproductive biology more generally, not only by providing a description of how the male *D. melanogaster*

accessory gland epithelium changes as the male matures, but also by proposing a potential mechanism by which the mating-dependent nuclear growth is regulated. In addition, I also provide a potential model of how a positive feedback mechanism might regulate the dynamics of glandular secretion and replenishment in response to sexual activity and autocrine cell signalling. However, I balance these interpretations with a more nuanced discussion of their implications in the final Chapter of this Dissertation (CHAPTER 6), beginning on *p.* 329.



CHAPTER 2



PREVIOUS PAGE:

A display of antique microscopes at the Norwich Castle Museum & Art Gallery, in Norwich, Norfolk.



CHAPTER 2

MATERIALS AND METHODS

If I could, I would always work in silence and obscurity,
and let my efforts be known by their results.

— Charlotte Brontë
(in GASKELL, 1857: *p.* 195)

Media, buffers, and solutions

Media, buffers and solutions used in this Dissertation are listed in **TABLE 2. 1**.

Fly stocks and culture

Flies were kept on standard yeast-cornmeal agar medium (**TABLE 2. 1.**), and maintained at a 12 hour light–dark cycle. Fly stocks were maintained at 18 °C. Crosses were maintained at 18 °C or 25 °C, with the temperature being kept consistent within individual experiments (for both control and experimental crosses). Crosses maintained at 25 °C were kept at this temperature for 8–10 days before transfer to 18 °C. For crosses, 5–8 female virgins of the driver line were put together with 2–3 males of the line to be crossed to the driver. For crosses where the transgene of interest resided on the X chromosome, the arrangement was reversed and all other experimental crosses belonging to the same experiment was treated equally. For crosses maintained at 18 °C, vials were flipped every 5–7 days, whereas

crosses maintained at 25°C were flipped every 3–4 days. The flipping frequency was kept consistent within individual experiments. A control cross was set up together with each individual experiment and treated the same as the experimental crosses thorough. Flies used for experiments were collected at eclosion and maintained at 28.5 °C, unless stated otherwise. All the fly strains used in this study are listed in **TABLES 2. 2.–2. 7**. Strain w^{1118} is considered wild-type, since many of the transgenic lines used in this Dissertation were generated in that background.

TABLE 2.1. | MEDIA, BUFFERS, AND SOLUTIONS

Media	Components per litre
Yeast-cornmeal agar (YCA)	12.56 g bacteriological agar (F. Gutkind); 0.7 g calcium (Sigma Aldrich); 8.6 g potassium sodium tartrate ($KNaC_4H_4O_6{\cdot}4H_2O$; Sigma Aldrich); 93 g D(+) glucose (Sigma-Aldrich); 75 g organic maize flour (cornmeal; Brian Dewitt); 31.48 g dried baker's yeast (*Saccharomyces cerevisiae*; Fermipan); 2.5 g methyl-4-hydroxybenzoate ('nipagen') (Sigma Aldrich), diluted in 12mL EtOH (Sigma Aldrich).

Buffers	Components
Phosphate-buffered saline (PBS)	0.01M phosphate buffer; 0.0027M KCl; 0.137 M NaCl (pH 7.4) (Sigma Aldrich).
PBS-Triton X-100 (PBST)	PBS, 0.3% (v/v) Triton X-100 (Sigma Aldrich).
PBST-goat serum (PBSTG)	PBST, 10% (v/v) goat serum (Gibco; Life Technologies).

Solutions	Components
PBS-paraformaldehyde (PFA)	PBS, 4% (w/v) paraformaldehyde (Sigma Aldrich).

TABLE 2. 2. | GENETIC ALLELES

Shorthand	Genotype	Description	Source/Reference	FlyBaseID
ecd^{1}	*ecd*^{1} *st*^{1} *ca*^{1}	Heat-sensitive loss-of-function allele of *ecd* (P656 amino acid replacement by ethyl methanesulfonate (EMS) mutagenesis).	BDSC (Bl 218) / GAREN *et al.* (1977)	FBst0000218
w^{1118}	*w*^{1118}; ;	Partial deletion of *w*; considered wild-type (control).	L. PARTRIDGE / HAZELRIGG *et al.* (1984)	FBal0018186
yw	*y*^{1}*w*^{j}; ;	Combination allele for the *y* and *w* loci containing an A to C transversion in the *y* gene ATG translation initiation codon, and a duplication of 2.96kb within the *w* gene.	UMEÅ *DROSOPHILA* STOCK CENTRE[1]	FBst0300266

[1] Now deposited in the Kyoto Stock Center for *Drosophila* Genomics and Genetics Resources (Kyoto DGGR).

TABLE 2. 3. | GAL4-DRIVER LINES

Shorthand	Genotype	Description	Source/Reference	FlyBaseID
Acp26Aa > GFP	*w acp26Aa-GAL4; UAS-GFPnls tubGAL80ts (II)*	Main cell driver with a cytoplasmic/nuclear GFP marker.	L. CORRIGAN	n/a
dronc^{ts} > RFP^{nls}	*w; IF/CyO; Dronc-GAL4 tubGAL80ts UAS-histone-RFP/TM6B*	*dronc* expression reporter.	A. BAENA-LOPEZ	n/a
dsx^{ts}	*w; tub-GAL80ts; dsx-GAL4/SM5.TM6*	Temperature-sensitive *dsx* GAL4 driver.	L. CORRIGAN / CORRIGAN *et al.* (2014)	FBti0168641 (derived from)
dsx^{ts} > CD63::GFP	*w*; *tub-Gal80*^{ts} *UAS-CD63-GFP*; *dsx-Gal4*/SM5.TM6	Secondary cell driver with a CD63::GFP membrane marker.	L. CORRIGAN / CORRIGAN *et al.* (2014)	n/a
esg^{ts} *F/O* > GFP	w; *esg-Gal4 tub-Gal80*^{ts}, *UAS-GFP*$_{nls}$/CyO; *UAS-flp* act>CD2>GAL4/TM6	Cytoplasmic/nuclear GFP marker and secondary cell driver.	A. LEIBLICH / LEIBLICH *et al.* (2012)	n/a
hh-GAL4	*+/Y-hs-hid; hh-GAL4 UAS-diap/TM6B*;	*hh* enhancer-trapped GAL4 driver.	N. TAPON / SZUPLEWSKI *et al.* (2009)	FBti0017278 (derived from)
ptc-GAL4	*w*; P{w[+mW.hs]=GawB} ptc[559.1]*	*ptc* enhance-trapped GAL4 driver.	M. MLODZIK / HINZ *et al.* (1994)	FBst0002017
spi^{ts} > GPI::GFP	*w; spi-GAL4 tubGAL80*^{ts} *UAS-GPI::GFP/CyO*	Secondary cell driver with a GPI::GFP membrane marker.	L. CORRIGAN / REDHAI *et al.* (2016)	n/a

TABLE 2. 4. | REPORTER CONSTRUCTS AND FLUORESCENT TRANSGENIC LINES

Shorthand	Genotype	Description	Source/Reference	FlyBaseID
UAS-GFP^{nls}	*w[1118]; P{w$^{[+mC]}$= UAS-GFP.nls}14;*	*UAS* sequence drives expression of GFP tagged at the N-terminus with the SV40 nuclear localisation signal.	B. EDGAR / NEUFELD *et al.* (1998)	FBst0004775
UAS-HhC85SN::GFP	*yw f; Sp/CyO; UAS-HhC85sNGFP/TM6B*	GFP-tagged Hh, lacking both its cholesterol and palmitic acid modifications.	I. GUERRERO	n/a
UAS-HhC85S::GFP	*yw; UAS-HhC85s::GFP/CyO; MKRS/TM6B*	GFP-tagged Hh, lacking its palmitic acid modification.	I. GUERRERO / GORFINKIEL & SIERRA *et al*. (2004)	n/a
UAS-HhN::GFP	*yw f; UAS-HhN::GFP/CyO; MKRS/TM6B*	GFP-tagged Hh′ lacking its cholesterol modification.	I. GUERRERO / TORROJA *et al*. (2004)	n/a
UAS-Hh::GFP	*yw; UAS-Hh::GFP/CyO;*	GFP-tagged, wild-type Hh.	I. GUERRERO / TORROJA *et al*. (2004)	n/a
UAS-Ptc^{DN}::GFP	*w[*]; P{w[+mC]=UAS-GFP-ptc.DN}2*	*UAS* sequence drives expression of a dominant-negative Ptc protein tagged with GFP at the N-terminus.	BDSC (Bl 52212) / LU *et al*. (2006)	FBst0052212
UAS-Ptc::GFP	*w[*]; ; P{w[+mC]=UAS-GFP-ptc.WT}3*	*UAS* sequence drives expression of a Ptc protein tagged with GFP at the N-terminus.	BDSC (Bl 52213) / LU *et al*. (2006)	FBst0052213
UAS-Smo::GFP	*y[1] w[*]; P{w[+mC]=UAS-smo.GFP}2*	*UAS* sequence drives expression of Smo protein, tagged with GFP at the N-terminus at amino acid 35.	BDSC (Bl 44624) / ZHU *et al*. (2003)	FBst0044624

TABLE 2. 5. | NON-FLUORESCENT OVEREXPRESSION CONSTRUCTS AND NON-RNAi TRANSGENICS

Shorthand	Genotype	Description	Source/Reference	FlyBaseID
UAS-Ci	*w[1118]; P[w+ UAS-Ci];*	*UAS* sequence drives expression of a *Ci* cDNA.	M. VAN DEN HEUVEL / DOMINUEZ *et al.* (1996)	FBtp0006293
UAS-Ci::VP16	*w[1118]; ; P[w+ UAS-CiVP16]*	*UAS* sequence drives expression of the Ci zinc-finger DNA-binding domain fused to the herpes simplex virus VP16 activation domain.	M. VAN DEN HEUVEL / ALEXANDRE *et al.* (1996)	FBtp0006298
UAS-cycD UAS-CDK4	*w; UAS-cycD UAS-cdk4/CyO (J49)*	*UAS* sequences drive expression of both *cyclin D* and its kinase, *CDK4.*	B. EDGAR / DATAR *et al.* (2008)	n/a
UAS-cycE I	*w[*]; ; P{w[+mC]=UAS-CycE.L}ML1*	*UAS* binding sites are fused upstream of *cycE* sequences for CycE overexpression.	BDSC (Bl 4781) / JOHNSTON & EDGAR (1998)	FBst0004781
UAS-cycE II	*w[*]; P{w[+mC]=UAS-CycE.R}2;*	*UAS* sequence drives expression of full-length type I CycE.	BDSC (Bl 30725) / RICHARDSON *et al.* (1995)	FBst0030725

Shorthand	Genotype	Description	Source/Reference	FlyBaseID
UAS-Dad	*w; P[w+ UAS-Dad];*	*UAS* sequence drives expression of a sequence containing the entire coding region of *Dad.*	D. BENNETT / TSUNEIZUMI *et al.* (1997)	FBtp0008763
UAS-dIAP1	*w[*]; P{w[+mC]=UAS-DIAP1.H}3*	*UAS* sequence drives expression of *dIAP1*.	BDSC (Bl 6657) / GUO *et al*. (2004)	FBti0038641
UAS-dronc	*w; GMR-GAL4; UAS-Dronc-wt/SM6A<TM6B*	*UAS* sequence drives expression of wild-type *dronc.*	A. BAENA-LOPEZ / QUINN *et al*. (2000)	n/a
UAS-dronc $^{\Delta N}$	*w; UAS-ΔN-Dronc (activated);*	*UAS* sequence drives expression of a truncated coding sequence of *dronc*; from nucleotides 706 to 1721, missing its inhibitory prodomain.	A. BAENA-LOPEZ / MAIER *et al*. (2000)	FBal0104749
UAS-dronc^{CA}	*w; ; UAS-dronc C → A*	*UAS* sequence drives expression of a catalytically inactive coding sequence of *dronc*; includes nucleotides 369 to 1721, with the catalytic Cys mutated to Ala.	A. BAENA-LOPEZ / MAIER *et al.* (2000)	FBal0104746
UAS-E2F1 UAS-Dp	*w[1118]; P{w[+mC]=UAS-E2f.N}3B P{w[+mC]=UAS-Dp.D}1-4b/TM6B, Tb[1]*	*UAS* sequence drives the expression of *E2F1* and *Dp*.	BDSC (Bl 4770) / BUTTITTA *et al.* (2010)	FBst0004770
UAS-EcR-C UAS-tkv^{Q253D}	*w; UAS-EcRC UAS-Tkv^{QD} /TM6*	*UAS* sequence drives expression of an N-terminal truncated form of EcR containing only the region common to all EcR isoforms and a constituently active form of Tkv.	C. WILSON / LEIBLICH *et al*. (in progress)	n/a
UAS-EcR-C	*w^{1118}; P{UAS-EcR.C}TP1-4;*	*UAS* sequence drives expression of an N-terminal truncated form of EcR containing only the region common to all EcR isoforms.	BDSC (Bl 6868) / CHERBAS *et al.* (2003)	FBst0006868
UAS-RBF^{CA}	*w[*]; P{w[+mC]=UAS-Rbf.280}3/TM3, Sb[1]*	*UAS*-sequence drives expression of a form of RBF with mutation to four of its regulatory phosphorylation sites.	BDSC (Bl 50748) / XIN & WENG *et al.* (2002)	FBst0050748
UAS-smo8	*w[1118]; ; P[w+ UAS-smo8-1-3HA]*	*UAS* sequence drives expression of a full length *smo* cDNA tagged at the 5' end with a triple T:Zzzz\FLAG tag.	M. VAN DEN HEUVEL / INGHAM *et al.* (2000)	FBtp0013297
UAS-tkv^{Q253D}	*w[*]; P{w[+mC]=UAS-tkv.Q253D.Nb}3/TM3, Sb[1] Ser[1];*	*UAS* sequence drives expression of a constitutively active Tkv protein.	BDSC (Bl 36536) / WIERSDORFF *et al.* (1996)	FBst0036536

TABLE 2. 6. | RNAi LINES

Shorthand	Genotype	Description	Source/Reference	FlyBaseID
UAS-smo^{RNAi}	*y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF02363}attP2;*	*UAS* sequence drives expression of a dsRNA for RNAi of *smo* (TRiP).	BDSC (Bl 27037) / NI *et al*. (2009)	FBst0027037
UAS-ci^{RNAi} I	*y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF01715}attP2 - ci*	*UAS* sequence drives expression of an inverted repeat of a fragment of *ci* (TRiP).	BDSC (Bl 28984) / NI *et al*. (2009)	FBst0028984
UAS-ci^{RNAi} II	*y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF01744}attP2 - ci*	*UAS* sequence drives expression of an inverted repeat of a fragment of *ci* (TRiP).	BDSC (Bl 31236) / NI *et al*. (2008)	Bst0031236
UAS-ci^{RNAi} III	*y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF01272} attP2*	*UAS* sequence drives expression of an inverted repeat of a fragment of *ci* (TRiP).	BDSC (Bl 31321) / NI *et al*. (2008)	FBst0031321
UAS-cycD^{RNAi}	*y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00059 }attP2*	*UAS* sequence drives expression of a dsRNA for RNAi of *cycD* (TRiP).	BDSC (Bl 33653) / NI *et al*. (2011)	FBst0033653
UAS-cycE^{RNAi} *I*	*y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00060 }attP2*	*UAS* sequence drives expression of a dsRNA for RNAi of cycE (TRiP).	BDSC (Bl 33654) / NI *et al*. (2011)	FBst0033654
UAS-cycE^{RNAi} *II*	*y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.GL00511} attP40*	*UAS* sequence drives expression of a dsRNA for RNAi of cycE (TRiP).	BDSC (Bl 36092) / NI *et al*. (2011)	FBst0036092
UAS-dark^{RNAi}	*w; UAS-dark-RNAi (TRiP line);*	*UAS* sequence drives expression of a dsRNA for RNAi of *dark* (TRiP).	BDSC (Bl 33924) / NI *et al*. (2011)	FBst0033924
UAS-disp^{RNAi} I	*y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS02877 }attP2/TM3, Sb[1]*	*UAS* sequence drives expression of an inverted repeat of a fragment of *disp.*	BDSC (Bl 44633) / NI *et al*. (2011)	FBst0044633
UAS-disp^{RNAi} II	*y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF02550}attP2 - disp*	*UAS* sequence drives expression of an inverted repeat of a fragment of *disp.*	BDSC (Bl 27247) / NI *et al*. (2009)	FBst0027247
UAS-dpp^{RNAi} I	*y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF01371}attP2*	*UAS* sequence drives expression of an inverted repeat of a fragment of *dpp.*	BDSC (Bl 25782) / NI *et al*. (2009)	FBti0114533
UAS-dpp^{RNAi} II	*y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF01090}attP2*	*UAS* sequence drives expression of an inverted repeat of a fragment of *dpp.*	BDSC (Bl 31530) / NI *et al*. (2008)	FBst0031530
UAS-drice^{RNAi}	*w; UAS-drice-RNAi;*	*UAS* sequence drives expression of an inverted repeat of a fragment of *drice.*	A. BAENA-LOPEZ / VRDC (v28065) / DIETZL *et al.* (2007)	FBst0457273
UAS-dronc^{RNAi}	*w; P{UAS-Dronc.IR.L};*	*UAS* sequence drives expression of a dsRNA for RNAi of *dronc*	A. BAENA-LOPEZ / LEULIER *et al.* (2006)	FBAL0244087
UAS-E2F1^{RNAi}	*y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF01741}attP2 E2f*	*UAS* sequence drives expression of a dsRNA for RNAi of *E2F1* (TRiP).	BDSC (Bl 31214) / NI *et al*. (2008)	*FBst0031214*
UAS-EcR^{RNAi}	*w[1118]; P{w[+mC]=UAS-EcR-RNAi}97;*	*UAS* sequence drives expression of a dsRNA of the region of *EcR* common to all isoforms.	BDSC (Bl 9326) / COLUMBANI *et al.* (2005)	FBst0009326
UAS-EcR^{RNAi} *UAS-Tkv*^{QD}	*w; UAS-EcR-RNAi (Bl 9327); UAS-Tkv*^{QD}*/SM5.TM6*	*UAS* sequence drives expression of a dsRNA for RNAi of *EcR,* combined with *UAS* sequence driving expression of a constitutively active Tkv protein.	C. WILSON / LEIBLICH *et al*. (in progress)	n/a

Shorthand	Genotype	Description	Source/Reference	FlyBaseID
UAS-hh^{RNAi} I	*y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF01488}attP2;*	*UAS* sequence drives expression of an inverted repeat of a fragment of *hh.*	BDSC (Bl 31042)/ NI *et al*. (2008)	FBst0031042
UAS-hh^{RNAi} II	*y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF01270}attP2;*	*UAS* sequence drives expression of an inverted repeat of a fragment of *hh.*	BDSC (Bl 31475) / NI *et al*. (2008)	FBst0031475
UAS-hh^{RNAi} III	*y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00492}attP2/TM3, Sb[1];*	*UAS* sequence drives expression of an inverted repeat of a fragment of *hh.*	BDSC (Bl 32489) / NI *et al*. (2010)	FBst0032489
UAS-ptc^{RNAi}	*y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF03223}attP2;*	*UAS* sequence drives expression of an inverted repeat of a fragment of *ptc.*	BDSC (Bl 28795) / NI *et al*. (2009)	FBst0028795
UAS-ptc^{RNAi}	*y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMC03872}attP40;*	*UAS* sequence drives expression of an inverted repeat of a fragment of *ptc.*	BDSC (Bl 55686)/ NI *et al*. (2010)	FBst0055686
UAS-Rbf^{RNAi}	*y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS03004}attP2/TM3, Sb[1] Rbf*	*UAS* sequence drives expression of a dsRNA for RNAi of *RBF* (TRiP).	BDSC (Bl 36744) / NI *et al.* (2011)	*FBst0036744*
UAS-trr^{RNAi} I	w^{1118} P{GD4501}v10749	*UAS* sequence drives expression of a dsRNA for RNAi of *trr.*	VRDC (v10749) / DIETZL *et al.* (2007)	FBst0450152
UAS-trr^{RNAi} II	P{KK100280}VIE-260B	*UAS* sequence drives expression of a dsRNA for RNAi of *trr.*	VRDC (v110276) / DIETZL *et al.* (2007)	FBst0481854

TABLE 2. 7. | GENE AND PROTEIN TRAPS

Shorthand	Genotype	Description	Source/Reference	FlyBaseID
GCE::GFP	*y^{1} w* Mi{PT-GFSTF.1}gceMI02742-GFSTF.1*	Recombination-Mediated Cassette Exchange (RMCE) of a Mi{MIC} insertion, resulting in expression of GCE tagged with EGFP-FlAsH-StrepII-TEV-3xFlag.	BDSC (Bl 60189) / NAGARKAR-JAISWAL *et al.* (2015)	FBst0060189
N::GFP	*w N^{55e11}; M[p3xP3-RFP, Dp(1;2)Notch-iGFP2388]51D/(+)*	A BAC-encoded, GFP-tagged version of Notch where GFP is inserted in the intra-cellular domain at position 5 (NiGFP5; residue 2388). Rescues the *Notch* null-allele, *N^{55e11}*.	F. SCHWEISGUTH / COUTURIER *et al.* (2012)	FBtp0072075
TANGO7:: GFP	*Tango7 2XTY1-SGFP-V5-preTEV-BLRP-3XFLAG*	A GFP-tagged form of TANGO7.	VDRC / SAROV et al. (2016)	n/a

Fly genetics and experimental manipulations

Expression of UAS-transgenes

Transgene expression was regulated using the *UAS*-GAL4 system (BRAND & PERRIMON, 1993). To induce expression of transgenes in fly strains containing the temperature-sensitive GAL4 inhibitor, $GAL80^{ts}$, flies were shifted from the permissive temperature (18 °C) to the non-permissive temperature (28.5 °C) at the desired time-point. Flies were typically collected at eclosion and immediately transferred to the non-permissive temperature (28.5 °C) to activate GAL4 expression. Controls for each experiment were generated by crossing the relevant driver line with either w^{1118} or *yw*, depending on the genetic background of the experimental lines.

TABLE 2. 8. | LABELLING SECONDARY CELL INTERNAL COMPARTMENTS

Label	Description	Labelling pattern in secondary cells
CD63::GFP	A GFP-tagged form of the transmembrane protein, CD63.	Internal membrane-bound compartments; *e.g*. acidic and exocytic compartments. Traffics into the acidic compartments, and also labels the cell apical surface. Cell debris released into the gland lumen.
GPI::GFP	A GPI-anchored form of GFP.	Cell membrane and exocytic compartments. Traffics into the acidic compartments. Packaged into the exocytic compartment secretory granules.
GFP^{nls}	A form of GFP with a nuclear translocation signal.	Nuclei, but is expressed at sufficiently high levels to also be retained in the cell cytoplasm—acting as a nucleocytoplasmic marker.
α-ANCE	An antibody that recognises the protease, ANCE.	Exocytic secretory granules, but also the secondary cell cytoplasm.

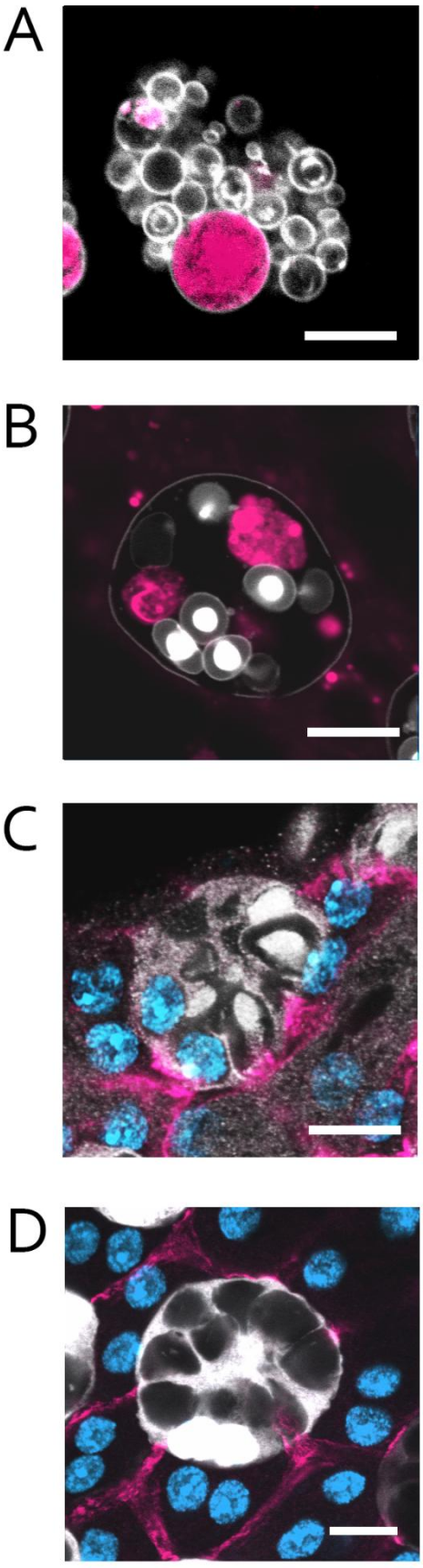


FIGURE 2. 1. | FLUORESCENT MOLECULES LABEL DIFFERENT SECONDARY CELL COMPARTMENTS.

Different fluorescent markers label different secondary cell compartments.

A: A GFP-tagged form of the transmembrane protein, CD63 (white), labels secondary cell internal compartments; *e.g.* acidic compartments (magenta; part of the endolysosomal system) and exocytic compartments. Some CD63::GFP is also trafficked into the endolysosomal system.

B: A GPI-anchored form of GFP (white) labels the secondary cell plasma membrane and secondary cell exocytic compartments. GFP is also packaged into the secretory granules contained within the exocytic compartments. Occasionally, GPI::GFP traffics into the endolysosomal system (magenta).

C: An antibody against the protease, ANCE (white), labels the secondary cell cytoplasm and the secretory granules. ANCE is also released into the accessory gland lumen, and stains the seminal fluid.

D: A form of GFP with a nuclear localisation signal (GFP^{nls}) labels the secondary cell nuclei strongly. The high GFP^{nls} expression achieved with the *UAS*/GAL4-system is sufficient to cause nuclear GFP to leak into the cell cytoplasm, allowing the internal compartments to be resolved as negative spaces.

The scale bar in all panels represents 10 μm. Panels **A** and **B** show secondary cells that were imaged under live conditions, whereas panels **C** and **D** show fixed material. In the latter two panels DNA is shown in cyan and cell junctions are shown in magenta.

Labelling secondary cells and their internal compartments

For ease of imaging and identifying secondary cells within the accessory gland epithelium, secondary cells were labelled using a combination of fluorescent markers (using the *UAS*/GAL4-system) and antibody stainings (**TABLE 2. 8.**; **FIGURE 2. 1.**).

Multiple mating protocol

Multiple mated males for staining experiments were generated as described in LEIBLICH *et al.* (2012), with slight modification. Briefly, upon eclosion, virgin males were placed in individual vials together with seven virgin females to each vial. Multiply mated males used for experiments to detect the synthesis of new DNA were obtained by putting 2 virgin males in a vial together with 15 females, in the interest of reducing the number of vials used. Vials were maintained at 28.5 °C for 6 days under both protocols, after which the males were dissected and the females were discarded. Females were occasionally retained in their individual vials at 25 °C to assess their mating status (mated/not mated) by scoring vials for the presence of progeny (yes/no), in order to estimate the proportion of females mated by each male.

Histochemistry and sample preparation

Acid histochemistry

To visualise acidic compartments within secondary cells, accessory gland samples were prepared as described in CORRIGAN *et al.* (2014). Briefly, flies were anesthetised using CO_2 and dissected with fine forceps in ice-cold phosphate-buffered saline ("**PBS**") under a Motic SMZ-161 stereo-dissection microscope (Leica) with an MFO-90 fibre-optic light source (Microtec Fibreoptics). Male

accessory glands were transferred to 1.5 mL Eppendorf tubes, washed once with ice-cold PBS and incubated with ice-cold Lysotracker-Red DND-99 (1 μM; **TABLE 2. 9.**) for 5 minutes, before being washed again, twice, in ice-cold PBS.

Immunohistochemistry

Material for immunostaining was obtained as by described in LEIBLICH *et al.* (2012). Briefly, flies were anesthetised using CO_2 and dissected using fine forceps in 4 % weight-by-volume ("**w/v**") PBS-formaldehyde ("**PFA**") (**TABLE 2. 1.**). Dissected glands were transferred to Eppendorf tubes and fixed for about 15–20 minutes at 20 °C. Glands were washed and then re-suspended in 0.3 % volume-by-volume ("**v/v**") PBS-Triton X ("**PBST**"). For immunostaining, glands were blocked in PBST with 10 % (v/v) goat serum ("**PBSTG**") and re-suspended in 50–100 μL of PBSTG containing primary antibody at an appropriate dilution (**TABLE 2. 9.**). Preparations were left to incubate at 4 °C overnight. The following day, glands were washed 4 × 15 minutes in PBST before being re-suspended in 100 μL of PBST containing secondary antibody at an appropriate dilution (**TABLE 2. 9.**). Preparations were left to incubate at 20 °C for at least 2 hours (typically overnight), away from light. Glands were washed 4 × 15 minutes in PBST before being washed and re-suspended in PBS. All washes were performed at 20 °C.

TABLE 2. 9. | ANTIBODIES AND STAINS USED IN THIS PROJECT

Protocol	Antibody	Antigen	Dilution	Species	Source	Reference
Immuno-fluorescence	Primary	ANCE	1:2000	Rabbit	E. ISAAC	RYLETT & WALKER *et al*. (2007)
		FASIII[1]	1:10	Mouse	DSHB	PATEL *et al*. (1987)
		DRONC	1:2000	Guinea pig	H. STELLER	ARAMA *et al.* (2003)
		pSMAD 1/5	1:40	Rabbit	Cell Signalling Technology	n/a
	Antibody	**Antigen**	**Dilution**	**Species**	**Fluorophore**	**Source**
	Secondary	Guinea pig	1:400	Goat	Cy3	Merck Millipore
		Mouse	1:400	Goat	Cy3	Merck Millipore
		Mouse	1:400	Donkey	Cy5	Merck Millipore
		Rabbit	1:400	Goat	Cy3	Merck Millipore
		Rabbit	1:400	Donkey	Cy5)	Merck Millipore
		Rabbit	1:400	Goat	ALEXA 488	Jackson Laboratories
		Rat	1:400	Donkey	Cy3	Jackson Laboratories

Protocol	Stain	Concentration		Dilution	Source	Reference
		Stock	Target			
Acid histochemistry	Lysotracker-Red DND-99	1 mM	1 μM	1:1000	Invitrogen	n/a
EdU labelling	Azide-fluor 545	2 mM	5 μM	1:400	Sigma Aldrich	DAUL *et al.* (2010)

[1] Fasciclin III antibody was deposited to the DSHB by C. Goodman (DSHB Hybridoma Product: 7G10 anti-Fasciclin III).

Detection of DNA replication using EdU

To detect DNA replication using the thymidine analogue, 5-ethynyl-2'-deoxyuridine ("**EdU**"), a Click-iT® EdU imaging kit (Invitrogen) was used. Reagents were prepared following the manufacturer's instructions. EdU-labelled tissue was obtained and processed using a modified Cold Spring Harbour protocol (DAUL *et al.*, 2010), where flies were maintained on medium containing 0.2 mM EdU. Medium was prepared by mixing standard yeast-cornmeal agar medium (at a temperature of around 50–60 °C) with a 10 mM EdU stock solution (diluted in PBS, per the manufacturer's instructions); 2 mL of the 10 mM EdU stock solution was used for every 100 mL yeast-cornmeal agar (1:50 dilution). The prepared medium was decanted into fly culture vials (around 3 mL per vial) while still hot, and allowed to solidify and dry (covered with linen cheesecloth) at room temperature overnight. Leftover vials were kept at 4 °C until required.

To label cells undergoing DNA replication, male flies of the desired genotype were collected at eclosion and kept on the EdU-containing medium at the non-permissive temperature for three days. To detect DNA with EdU incorporation, flies were anaesthetised and dissected in PFA as described for immunohistochemistry, above (*p.* 57). Once suspended in PBST, tissues were transferred to 200 μL PBSTG and incubated for 45 minutes at 20 °C to block, before being washed with 200 μL PBST. The Click-iT® reaction mix was prepared following the manufacturer's instructions, using a 1:400 dilution of 2 mM azide-fluor (Sigma Aldrich) in dimethyl sulphoxide ("**DMSO**"). To label DNA in the sample, 200 μL of the reaction mix was added to the vials and left to incubate for 30 minutes

at 20 °C, away from light. Glands were washed three times with 200 μL PBST and then re-suspended in 200 μL PBS.

Imaging and image analysis

Sample preparation

For live imaging, glands in PBS were transferred onto glass microscope slides before blotting excess fluid with tissue paper, and mounting the tissue in Voltalef 10S oil (VWR). For imaging of fixed samples, fixed tissue in PBS were transferred to glass slides and blotted with tissue paper, prior to mounting in Vectashield containing the DNA-stain, 4',6-diamidino-2-phenylindole ("**DAPI**") (Vector Laboratories). Glands prepared for live imaging were kept at 18–20 °C and considered viable for up to 60 minutes, whereas fixed tissue mounted in Vectashield was stored at 4 °C and imaged within 1 month.

Confocal microscopy and image analysis

Samples were imaged using a laser scanning confocal microscope (Axioplan 2, LSM 510 Meta; Zeiss) using LSM 510 Meta software. Images were captured using Zeiss Plan Apochromat 10×/0.45 and 63×/1.4 (oil differential interference contrast) objectives, with RI 1.514 immersion oil (Zeiss). *Z*-sections and images for quantification of intracellular compartments and nuclear size were captured using scanning confocal microscopy according to previously published protocols (LEIBLICH *et al.,* 2012; CORRIGAN *et al.,* 2014).

Widefield microscopy and image analysis

Widefield microscopy was undertaken using a DeltaVision Elite wide-field fluorescence deconvolution microscope (GE Healthcare Life Sciences) at the

Department of Biochemistry Micron Facility (University of Oxford), with the help of Benjamin Kroeger (a *D. Phil.* student in our laboratory). Images were obtained using 10×/0.40 UPlanSApo (Olympus) and 20×/0.75 UPlanSApo (Olympus) objectives, with RI 1.514 immersion oil (Cargill Labs).

Data quantification

For all accessory gland-specific quantifications, both accessory gland arms were quantified, where available. For all secondary cell-specific quantifications, three cells per gland were quantified to give an average per gland, with the aim quantify at least 10 glands (= 10 flies) to produce a genotype average.[1] For nuclear size and intra-cellular compartment numbers, data were scored blind to avoid scoring bias. Data were blinded by giving arbitrary identifiers to the relevant system folders.

Secondary cell numbers

Quantification of secondary cell number to determine total cell numbers or what proportion of secondary cells ("**SCs**") were expressing the reporter of interest was performed by labelling secondary cells with an appropriate marker (*e.g.* dsx^{fs} > CD63::GFP) or antibody stain (*e.g.* α-ANCE), to make them easily identifiable in the accessory gland epithelium. The total number of secondary cells (SC_{total}) and the number of secondary cells positive for the marker or phenotype of interest (SC) was then determined from a confocal *z*-series. The final proportion of secondary cells ($SC_{\%}$) was then calculated using the formula:

$$\frac{SC}{SC_{total}} = SC_{\%}$$

[1] Averages were never calculated from averages, and for the purposes of the Dissertation, 'n_{gland}' will be used a short-hand for the number of individual flies used in the experiments.

Intracellular compartment numbers and sizes

To establish intracellular compartment numbers in live samples, secondary cell intracellular compartments were labelled with CD63::GFP as described in CORRIGAN *et al.* (2014). Secondary cell acidic and non-acidic intracellular compartment numbers were determined from confocal *z*-series of secondary cells expressing the CD63::GFP membrane marker and stained with LysoTracker-Red. The shape of the largest acidic compartment was assumed ellipsoid and calculated using *x, y,* and *z* measurements (m^x, m^y and m^z, respectively) obtained from confocal *z*-series, using the formula:

$$V = \frac{4\pi}{3} \times \frac{m^x \times m^y \times m^z}{2}$$

For secondary cell size measurements, secondary cells were assumed ellipsoid and calculated using the same formula, using *x, y,* and *z* measurements obtained from confocal *z*-series

Nuclear size

For nuclear size measurements, secondary cells were labelled with nuclear GFP (*esg* F/O^{ts} > GFP) as described in LEIBLICH *et al.* (2012). The maximum nuclear area (as determined through a selection of *z*-slices) was measured using the LSM Image Browser (Zeiss). The sum of the areas ("**sum area**") of both nuclei in individual cells was calculated to give a final sum nuclear area for each cell, with the two nuclear measurements for each secondary cell being paired with the two nuclear measurements from a neighbouring main cell (**FIGURE 2. 2.**).

Nuclear DNA content

To measure nuclear DNA content, confocal image pixel intensity was measured using the Image J Fiji package (SCHINDELIN *et al.*, 2012), as described in MCCOY *et al.* (2014). Briefly, measurement parameters were set using *Analyze* > *Set measurements*, selecting: area, mean gray value, and integrated density ("**ID**"; the product of the 'mean gray value' and the area of the region of interest). Confocal channels were split, and a 'Sum Slices' z-projection was created from the DAPI channel. The freehand selection tool was used to outline three pairs of secondary cell nuclei and three pairs of main cell nuclei (regions of interest; "**ROIs**"). Background fluorescence intensity ("**BFI**") measurements were also obtained (using the nuclear selection outline to sample the nearby background). Two nuclear measurements for each secondary cell (with background) were paired with two neighbouring main cell measurements (with background) (**FIGURE 2. 2.**). If background pixel intensity ≠ 0, the corrected total nuclear fluorescence ("**CTNF**") was calculated using the formula:

$$CTNF = ID - (ROI\ area \times BFI)$$

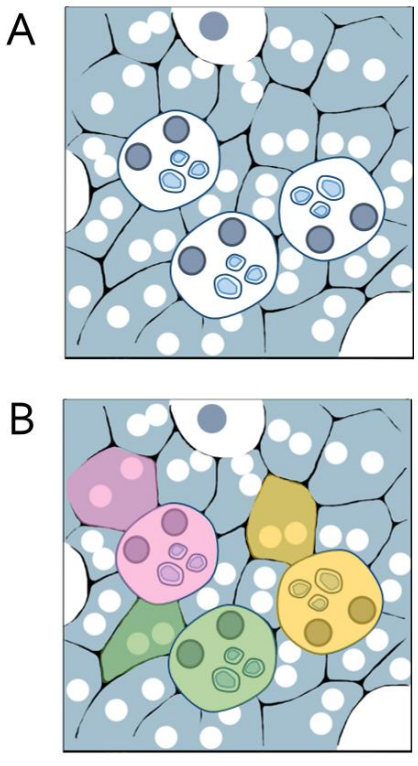


FIGURE 2. 2. | PAIRING MAIN CELLS AND SECONDARY CELLS IN THE ACCESSORY GLAND EPITHELIUM FOR QUANTIFICATION PURPOSES.

To quantify aspects of the accessory gland epithelium (**A**), three secondary cells in the epithelium were paired with an individual neighbouring main cell (**B**).

Once obtained, corrected total nuclear fluorescence was treated as integrated density, and the DAPI fluorescence values for individual nuclei were summed in the appropriate pairs. The pairwise secondary cell nuclear fluorescence values were then compared to their partnered pairwise main cell ("**MC**") measurement, to yield a ID_{SC}:ID_{MC} ratio showing the DAPI fluorescence differential between a summed pair of secondary cell nuclei and those of its partner summed main cell nuclei. According to TANIGUCHI *et al.* (2012), individual main cell nuclei are tetraploid. To approximate secondary cell nuclear ploidy ("**C**"; C_{SC}), corrected total nuclear fluorescence was treated as integrated density, and the following formula was used:

$$C_{SC} = \frac{ID_{SC}}{\left(\frac{ID_{MC}}{4}\right)}$$

Statistical analysis

Data were collated using Microsoft Excel (Microsoft Corporation) and analysed using GraphPad Prism 7.03 (GraphPad Software) and SPSS Statistics 17 (IBM). Statistical analysis was supported by *Intuitive Biostatistics* (MOTULSKY, 2010). The number of males (n_{gland}) was used as the unit of replication in all tests.

Assumptions regarding the normal distribution of data

Biological data is seldom sampled from ideal Gaussian distributions[2], but statistical tests that assume a Gaussian distribution remain robust even when the data only approximates the assumed ideal (MOTULSKY, 2010). Normality tests can be used to determine if the distribution of data is different from the expected distribution of

[2] Ideal Gaussian distributions typically include both very high and very low values, which are normally not present in sampling of biological data (MOTULSKY, 2010).

sampling from a true Gaussian distribution (*i.e.* if the distribution of the data sampled deviates from the expected distribution if the data were sampled from a larger population that is normally distributed) (MOTULSKY, 2010). A Shapiro-Wilk test can be used to test for normality (SHAPIRO & WILK, 1965). Yet, data collected as part of an experimental series should be subjected to the same analysis, and it is not recommended to perform normality tests on individual datasets to decide on what test to use for analysing the data (MOTULSKY, 2010).

Choice of parametric and non-parametric testing methods

Nuclear size data has previously been shown to be normally distributed and analysed using parametric methods (*e.g.* Student's *t*-test; or one-way analysis of variance ("**ANOVA**"), with the appropriate post-test corrections for multiple comparisons) (LEIBLICH, 2015). Using the Shapiro-Wilk test included in the statistical software package, SPSS, I have found that the nuclear size data of control samples is indeed normally distributed. Therefore, parametric analysis was preferred for the nuclear size data also in this Dissertation. No similar trend has been reliably demonstrated for secondary cell intracellular compartment data (CORRIGAN, 2014), so Shapiro-Wilk tests were performed as part of the intracellular compartment measurements in this Dissertation. Typically, neither intracellular compartment numbers (non-acidic and acidic) nor lysosomal volume measurements were found to be normally distributed (see CHAPTER 3: *p.* 90), and this type of data was therefore analysed using non-parametric methods (*e.g.* Mann-Whitney U test; or Kruskal-Wallis one-way ANOVA, with the appropriate post-test to correct for multiple comparisons). For the type of analysis used for other types of data, the reader is referred to **TABLE 2. 10**.

Statistical testing and selecting thresholds of statistical significance

The parametric testing methods that were used to analyse the data in this Dissertation were Student's *t*-test (STUDENT, 1908), and one-way ANOVA testing (VAJDA, 1947). The non-parametric testing methods that were used to analyse the data in this Dissertation were the Mann-Witney U-test (MANN & WITNEY, 1947), and Kruskal-Wallis one- way ANOVA testing (KRUSKAL & WALLIS, 1952). To compare the difference between the mean of two samples of parametric data, a two-tailed, unpaired, Student's *t*-test was used. As part of this test, GraphPad Prism normally performs an *F*-test to compare the sample variance of each group groups, which was found to be similar for the data in this Dissertation that were tested using this method. To compare the difference between the mean of more than two

TABLE 2. 10. | NORMAL DISTRIBUTION OF DATA TYPES IN THIS DISSERTATION, AND THEIR METHOD OF ANALYSIS.

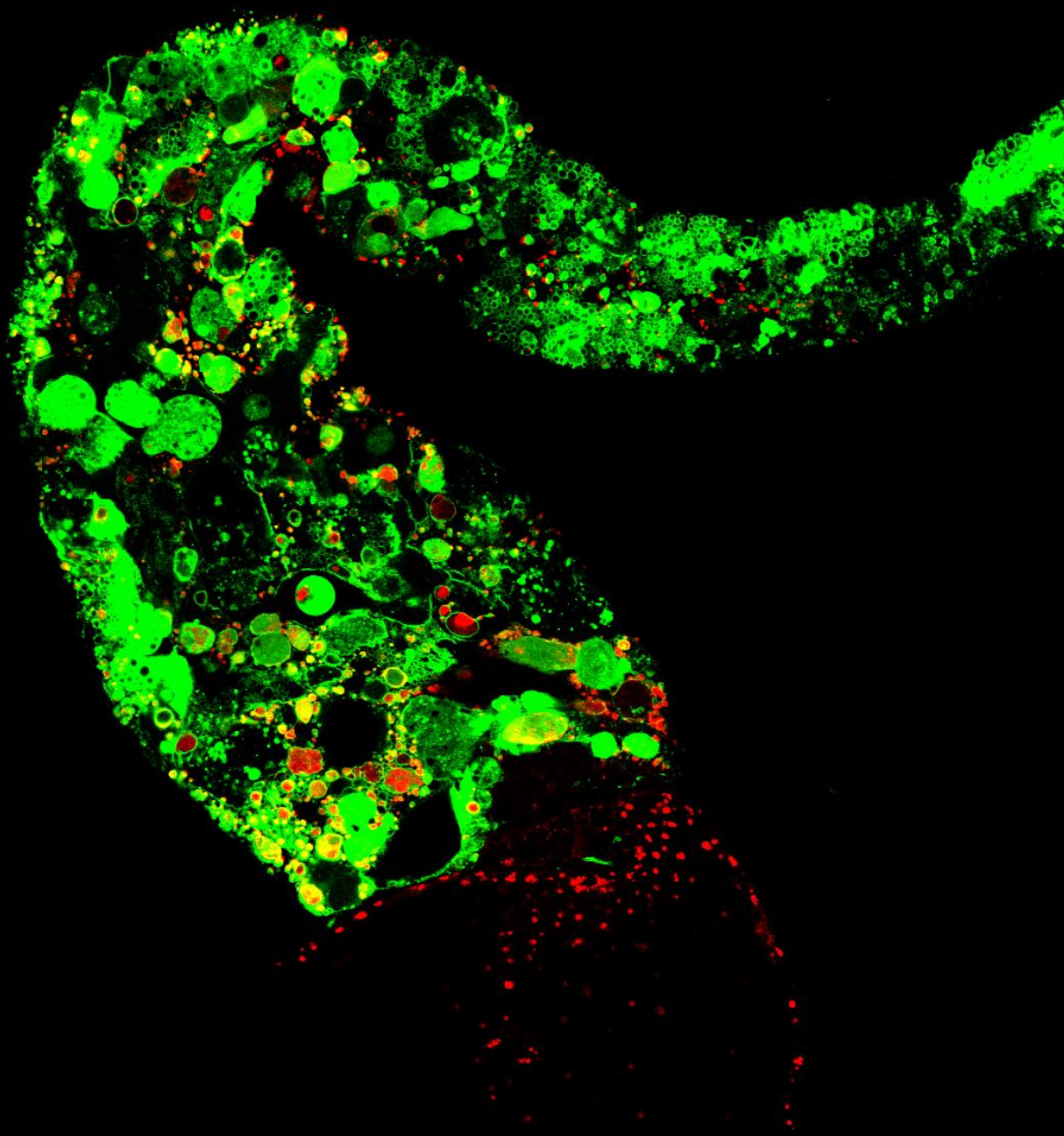
Measurement	Data normally distributed?	Method of analysis
Acidic compartment number	No	Non-parametric
DAPI fluorescence intensity (SC:MC ratio)	No	Non-parametric
Non-acidic compartment number	No	Non-parametric
Nuclear area	Yes	Parametric
Proportion secondary cells positive for a marker (*e.g.* GFP, RFP, EdU, *etc.*)	Yes	Parametric
Secondary cell number	Yes	Parametric
Secondary cell area or volume	Yes	Parametric
Volume of largest acidic compartment	No	Non-parametric

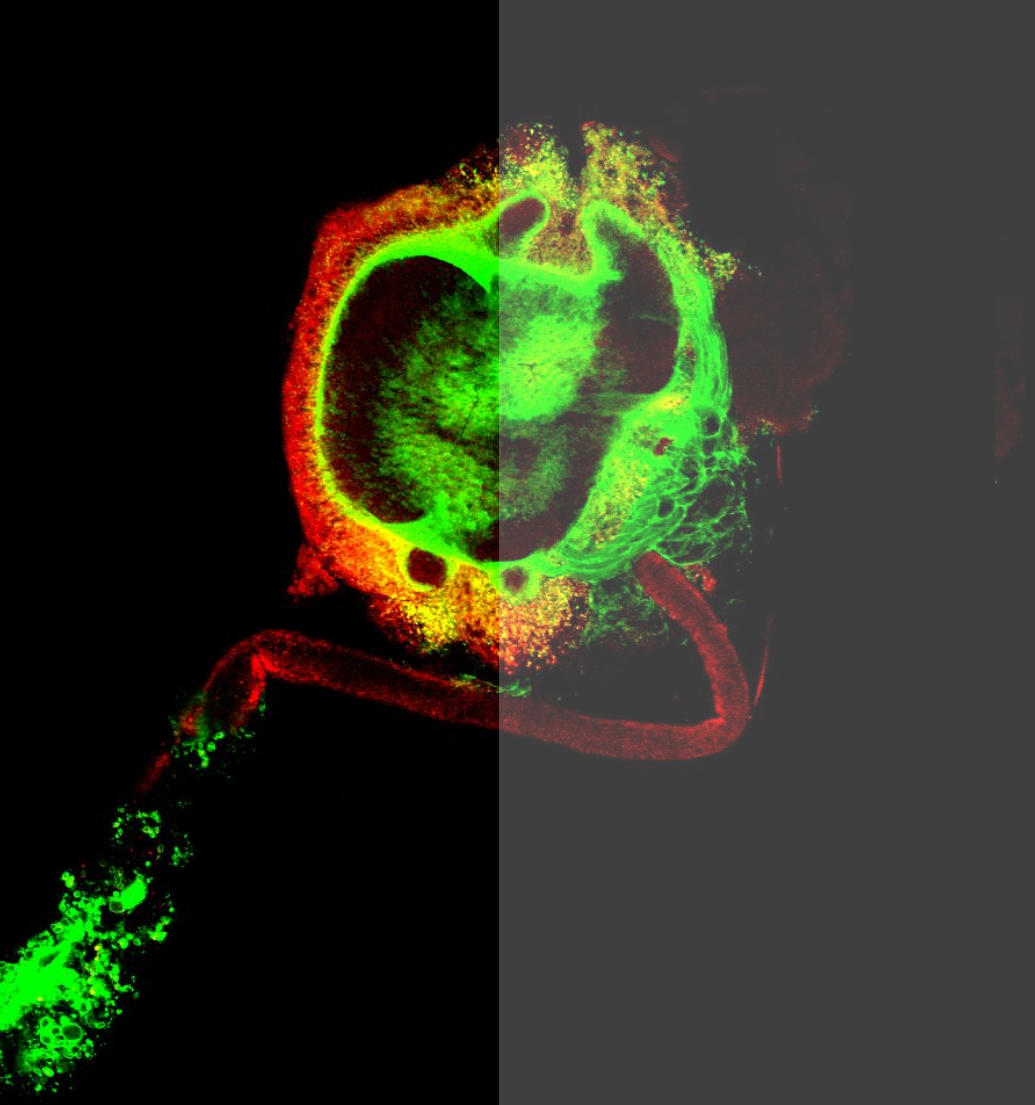
samples (control and *n* number of samples), a one-way ANOVA was used—with either Dunnett's *a posteriori* test for multiple comparisons (comparing the mean of all samples with that of the control) (DUNN, 1961) or Tukey's *a posteriori* test for multiple comparisons (for comparing the mean of all samples) (TUKEY, 1949).

Testing a hypothesis ("$\mathbf{H_1}$") against the null hypothesis ("$\mathbf{H_0}$"), the '*p*' value refers to the probability—not that the H_0 hypothesis is false—but that data as extreme as that observed could have been generated *even if* the H_1 hypothesis was true (FISHER, 1925). A *p*-value of 0.05 therefore implies that data as (or more) extreme than obtained will be generated in 5 % of cases, *even if the null hypothesis is correct*. Even under controlled conditions, using a significance threshold of $p = 0.05$ can therefore result in *as many as 30 % false positives* (COLQUHOUN, 2014). To minimise the risk of false-positive results in this Dissertation, tests were interpreted with caution (especially when yielding *p*-values larger than $p = 0.005$; **TABLE 2. 11.**), on the recommendation of COLQUHOUN (2014) and BENJAMIN *et al.* (2017). Tests yielding *p*-values of $0.001 < p < 0.01$ were considered 'suggestive', on the recommendation of BENJAMIN *et al.* (2017), whereas *p*-values of $p > 0.01$ were typically not considered significant.

TABLE 2. 11. | TYPICAL LEVELS OF STATISTICAL SIGNIFICANCE USED IN THIS DISSERTATION.

Level	*p*-value
'not significant' (n.s)	$p > 0.01$
'suggestive' *	$0.01 > p > 0.001$
**	$0.001 > p > 0.0001$
***	$p < 0.0001$

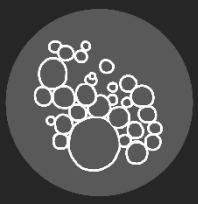




CHAPTER 3

PREVIOUS PAGE:

Composite confocal micrograph of the ejaculatory duct and ejaculatory bulb dissected from a male fly overexpressing wild-type DRONC. The tissues are labelled with a GFP-tagged transmembrane protein (CD63) under *doublesex*-GAL4 control (green), and stained with LysoTracker-Red (red) to label acidic compartments.



CHAPTER 3

CASPASES FUNCTION NON-APOPTOTICALLY TO REGULATE INTRACELLULAR TRAFFICKING IN SECONDARY CELLS

[The flies] will fool you every time

— Thomas H. Morgan
([attributed] in LEWIS, 1998)

Summary

Caspases are proteolytic enzymes that were initially discovered and described as key regulators of programmed cell death (apoptosis). Further characterisation of caspase function has however found them to be important regulators of diverse cellular processes—such as: immunity, fertility, and cell differentiation and morphogenesis. In *D. melanogaster*, the initiator caspase, DRONC (an orthologue of mammalian caspase 9), functions in both apoptotic and non-apoptotic contexts to activate downstream effector caspases, such as drICE. In an effort to characterise novel patterns of *dronc* expression, it was discovered by Dr. Alberto Baena-Lopez (at the Dunn School of Pathology, University of Oxford) that *dronc* is expressed in the secondary cells of the *D. melanogaster* male accessory gland. In this Chapter I show that a novel BMP/EcR signalling axis regulates the expression of *dronc* in secondary cells. In addition, I provide evidence that both DRONC and drICE act as negative regulators of compartment numbers in secondary cells, and show that other proteins known to interact with caspases also have roles in this process. Altogether, the results presented in this Chapter indicate that caspases can function non-apoptotically to regulate intracellular trafficking in secondary cells—with potential implications for secretory cell biology in general.

INTRODUCTION

Proteolytic enzymes (proteases), that are capable of cleaving themselves (and other protein targets) using the proteolytic activity of a nucleophilic cysteine residue, belong to the 'caspase' protein family (ALNEMRI *et al.,* 1996). Capases function in a variety of contexts—both during development and in the adult organism (reviewed by LAMKAMFI *et al*., 2007; FUCHS & STELLER, 2011; CRAWFORD & WELLS, 2011; SHALINI *et al.,* 2015). One of the best characterised of these functions is caspase-mediated regulation of programmed cell death (apoptosis); the process whereby superfluous cells and tissues are eliminated from the organism (FUCHS & STELLER, 2011).

Caspase function in *D. melanogaster*

There are seven caspases in *D. melanogaster*: drICE, DCP-1, DREDD, DRONC, DECAY, STRICA, and DAMM (FRASER & EVAN, 1997; SONG *et al*., 1997; CHEN *et al*., 1998; DORSTYN *et al*., 1999ab; DOUMANIS *et al*., 2001; HARVEY *et*

al., 2001). Caspases are normally present in the cell as latent 'pro-caspases' (zymogens) that are stabilised, maintained, and regulated by interaction with negative effectors of apoptosis, such as the ubiquitin ligase enzyme, *Drosophila* inhibitor of apoptosis protein 1 ("**dIAP1**"). Because of this regulation, caspase zymogens show only low levels of activity—unless activated (KUMAR, 2007). Caspase activation occurs via the zymogen pro-domain, which occurs in two forms: short and long. Long pro-domains contain sub-domains, such as the caspase-recruitment domain ("**CARD**"), and the death-effector domain ("**DED**"), that not only allow the caspases to be recruited to and activated by cell death signalling complexes, but also allow them, in turn, to act on downstream, 'effector' proteins (KUMAR, 2007). Therefore, caspases that are activated through signalling protein complex interactions are typically referred to as 'initiator' caspases, with the other caspases being referred to as 'effectors'.

In *D. melanogaster*, both DRONC and STRICA are initiator caspases with long N-terminal pro-domains that keep the caspase active site inactive (DORSTYN *et al.*, 1999a; DOUMANIS *et al.*, 2001). Under normal conditions, dIAP1 interacts with DRONC to inhibit its activity (KUMAR, 2007). Upon initiation of the cell death machinery, dIAP1 is targeted for proteasomal degradation, allowing the DRONC CARD domain to be recruited into an active cell death signalling complex containing, for example, the CARD-carrying apoptosome component, *Drosophila* apoptotic protease activating factor 1 ("**APAF-1**")-related killer ("**dARK**") (HAY *et al.,* 1995; KHAN & JAMES, 1998; RODRIGUEZ *et al.*, 1999). DRONC interaction with dARK allows DRONC to be activated autolytically, rendering it capable of cleaving downstream effector (executioner) caspases such as drICE and DCP-1 (MEIER *et al.*, 2000; KUMAR, 2007; TAYLOR *et al.,* 2008)—whose short

prodomains otherwise prevent them from becoming active in the absence of interaction with an initiator (KUMAR, 2007).) The ultimate result of apoptotic caspase activation is the initiation of a catalytic cleavage cascade that culminates with the death of the cell (TAYLOR *et al.,* 2008). See **FIGURE 3. 1.**, below, for a simplified model of this process.

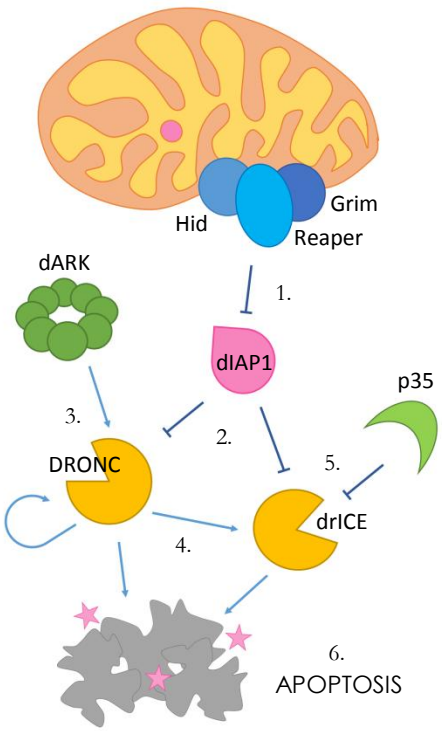


FIGURE 3. 1.| SIMPLIFIED MODEL OF THE APOPTOTIC PATHWAY IN *D. MELANOGASTER.*

An upstream apoptotic signal leads to the activation of the cell death initiators, Reaper, Hid, and Grim, which inhibit the activity of the cell death inhibitor, dIAP1 (1.). Under normal conditions, dIAP1 inhibits the activity of caspases, like DRONC and drICE (2.). When dIAP1 is inhibited, DRONC can become active by interacting with the apoptosome component, dARK (3.), and undergo auto-catalytic activation. As an initiator caspase, active DRONC can activate the effector caspase, drICE (4.)—whose activity can be inhibited by executioner caspase inhibitors, like p35 (5.). Active caspases participate in cleavage cascades, cleaving cellular protein targets, and ultimately bringing about the death of the cell (6.).

Non-apoptotic caspase function in *D. melanogaster* and other organisms

In addition to being key effectors of cell death, caspases participate in diverse cellular processes. Although caspases were first discovered in *Caenorhabditis elegans* (ELLIS & HORVITZ, 1986), many of the non-apoptotic functions of caspases have been described and characterised in *D. melanogaster*. For example, during *D. melanogaster*

oogenesis, DRONC, DCP-1, and STRICA are all required for the successful transfer of nurse cell contents into the developing oocyte (BAUM *et al.*, 2007). Similarly, DRONC, drICE, and the apoptosome component dARK, are required for sperm individualisation in male *D. melanogaster* (ARAMA *et al.*, 2003; 2006). Yet other processes that have been shown to require non-apoptotic caspase activity in the fly include neuroblast differentiation (OUYANG *et al.,* 2011); immunity (LEULIER *et al.,* 2000); synaptic maturation and dendritic pruning in developing neurons (HYMAN & YUAN, 2012); and cell migration and morphogenesis through caspase interaction with the actin cytoskeleton (GEISBRECHT & MONTELL, 2004; OSHIMA *et al.*, 2006). Non-apoptotic caspase activity might therefore be considered a crucial mechanism of cell remodelling; acting as regulators that permit cells to remodel or differentiate, to perform their functions optimally.

Aims of this Chapter

Non-apoptotic caspase activity can be an important regulator of normal cell biology, and although much work has already been done, the topic remains an interesting avenue of research. For example, an outstanding topic concerns the role of non-apoptotic caspase function in specialised secretory cell types.

In an attempt to characterise novel patterns of *dronc* expression in adult *D. melanogaster* using a *dronc* gene trap, one of our collaborators, Dr. Alberto Baena-Lopez (a Principal Investigator at the Sir William Dunn School of Pathology, University of Oxford), discovered that several tissues in the male reproductive system represent previously uncharacterised sites of *dronc* expression (A. BAENA-LOPEZ, unpublished observations). Since the male reproductive system contains specialised secretory cell types, such as the secondary cells of the male accessory

gland, this provided the starting point for an interesting examination of the role of non-apoptotic caspase function in specialised secretory cells. When Dr. Baena-Lopez approached our laboratory with his exciting finding, I was given the opportunity to investigate the role of DRONC in the regulation of secondary cell biology in more detail. This Chapter describes the outcome of these investigations. My initial aims for the work presented in this Chapter were:

- To characterise how the expression of the DRONC caspase is regulated;
- To characterise the role of the DRONC caspase in the regulation of secondary cell biology.

In this Chapter, I describe how a BMP/EcR signalling axis regulates the expression of *dronc* in secondary cells, and also show that non-apoptotic caspase activity can regulate intracellular trafficking in secondary cells. Overall, the results presented in this Chapter implicate non-apoptotic caspase activity as a regulator of secretory cell biology and intracellular trafficking events more generally.

RESULTS

The *Drosophila* Nedd2-like caspase, DRONC, is expressed in the male accessory gland

With Dr. Baena-Lopez having performed the initial work demonstrating that that the *D. melanogaster* Nedd2-like caspase, DRONC, is expressed in the secondary cells of the male accessory gland, my first aim was to characterise the *dronc* expression pattern in the male reproductive system more detail. Although our laboratory had access to a DRONC antiserum (α-DRONC; ARAMA *et al.,* 2003), we have found that antibody staining of antigens in the accessory gland can prove methodologically

difficult (A. LEIBLICH, L. CORRIGAN & M. WAINWRIGHT, personal comments). Indeed, my attempts to visualise DRONC in the reproductive system using antibody staining produced ambiguous results: whereas I could detect α-DRONC–positive *puncta* in the accessory gland that were restricted to secondary cells (**FIGURE 3. 2.**), this kind of punctate staining pattern is hard to interpret—because knock-down of *dronc* was not sufficient to reliably remove this signal (not shown). Fortunately, a *dronc* reporter construct was made available to me (A. BAENA-LOPEZ, unpublished line),

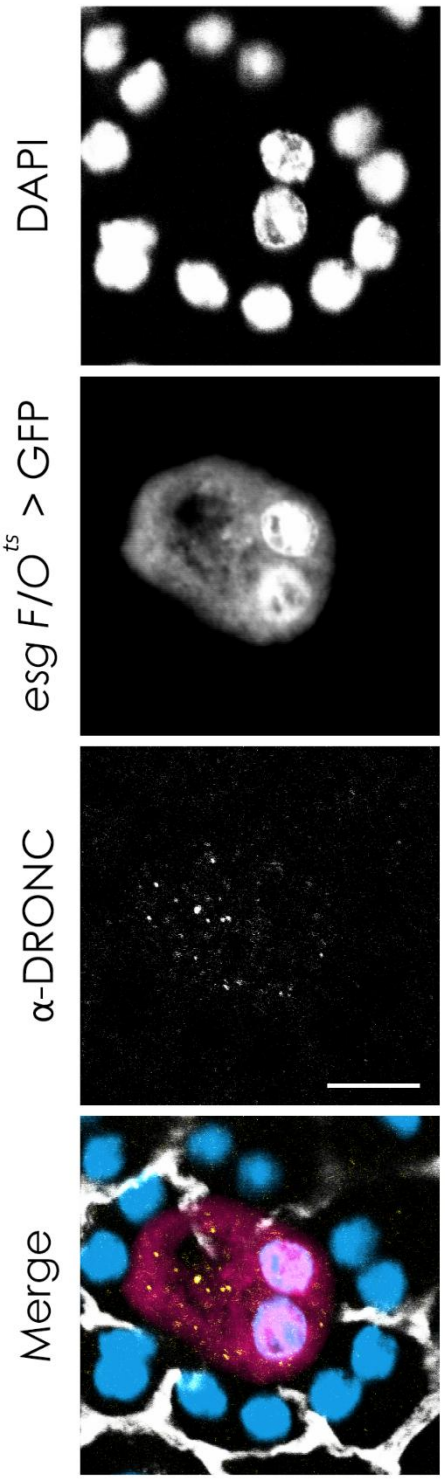


FIGURE 3. 2. | DRONC ANTI-SERUM LABELS PUNCTATE STRUCTURES IN SECONDARY CELLS IN THE *D. MELANOGASTER* MALE ACCESSORY GLAND.

Using a fly line where a temperature shift fills the secondary cell nuclei and cytoplasm with a nuclear GFP (*esg F/O*^{ts} > GFP) allows reliable labelling of secondary cells. Staining these cells in adult males (aged six days), with an antibody that targets DRONC protein, reveals a punctate staining pattern that is confined within the boundary of the secondary cell.

In this figure, the scale bar represents 10 μm and α-FASIII (white) labels main cell junctions. GFP labels the secondary cell (magenta), and DNA is labelled with DAPI (cyan). DRONC antibody staining is shown in yellow.

allowing me to drive the expression of temperature-sensitive GAL4 from the endogenous *dronc* promoter to regulate the expression of an RFP-tagged histone protein ("**RFP^{nls}**"; *dronc^{ts}* > RFP) (**FIGURE 3. 3.**).

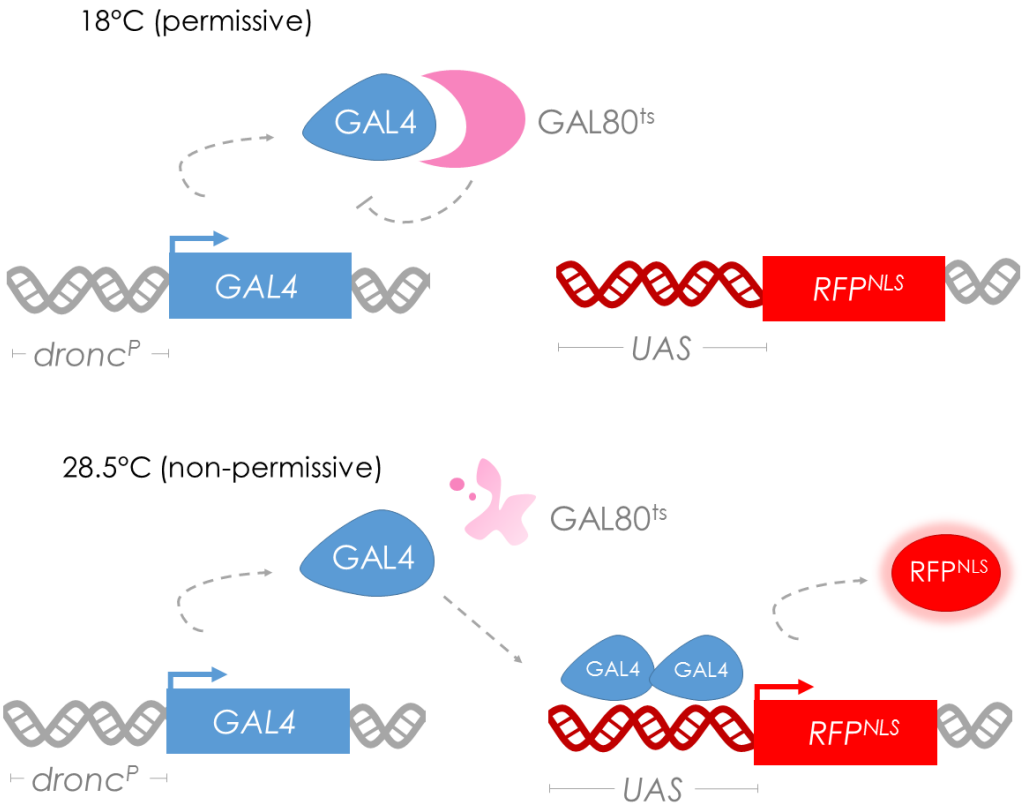


FIGURE 3. 3. | THE LOGIC OF USING *dronc*-GAL4 TO LABEL SECONDARY CELL NUCLEI WITH RFP.

At the permissive temperature, GAL4 is expressed under the control of the *dronc* promoter (*dronc^{P}*), but the activity of GAL4 is inhibited by GAL80^{ts}. At the non-permissive temperature, GAL80^{ts} is rendered inactive, allowing GAL4 to drive the expression of the marker, *UAS*-RFP^{NLS}. As a result, the RFP marker will label the nuclei of cells where the *dronc* promoter is, or recently has been, active.

We have previously shown in our laboratory that secondary cells mature over the first days after eclosion (CORRIGAN *et al.,* 2014; REDHAI, 2016)—the period when the male fly matures sexually (RUHMANN *et al.*, 2016). Based on this knowledge, I was interested to see how the expression of *dronc*^{ts} > RFP changes during this time. What I found is that at the non-permissive temperature (when GAL80^{ts} is inactive, but GAL4 is functional), *dronc*^{ts} > RFP shows low, but persistent, levels of expression in secondary cells; with around 15–20 % of secondary cells showing *dronc*^{ts} > RFP reporter activity at the selected time-points (days one, three, and six) (**SUPPLEMENTARY TABLE 3. 1.,** *p.* 422 (in APPENDIX 1: *p.* 421)).[1] Levels of *dronc* reporter activity may be elevated at day three, but the overall trend is one showing no obvious evidence of elevated levels of *dronc* expression over time ($p = 0.16$) (**FIGURE 3. 4.**). Interestingly, activity from the *dronc*^{ts} > RFP reporter was also observed in the cells of the ejaculatory duct (not shown).

The EcR regulates the expression of *dronc* in secondary cells

The EcR is a steroid-sensitive transcriptional regulator that has been shown to interact with the *dronc* promoter in cell types that are responsive to ecdysone (CAKOUROS *et al.*, 2002; 2004). A previous *D. Phil.* student—and now clinical research fellow—in our laboratory, Dr. Aaron Leiblich, has shown that the EcR is an important regulator of secondary cell biology (LEIBLICH, 2015). With this in mind, I was interested to determine if the EcR regulates the expression of *dronc* also in secondary cells? Again using the *dronc* expression reporter, I determined the

[1] Shapiro-Wilk testing shows that the proportion of secondary cells positive for *dronc* reporter activity is normally distributed ($p < 0.01$), and this type of data has therefore been analysed using parametric methods in this and subsequent assays.

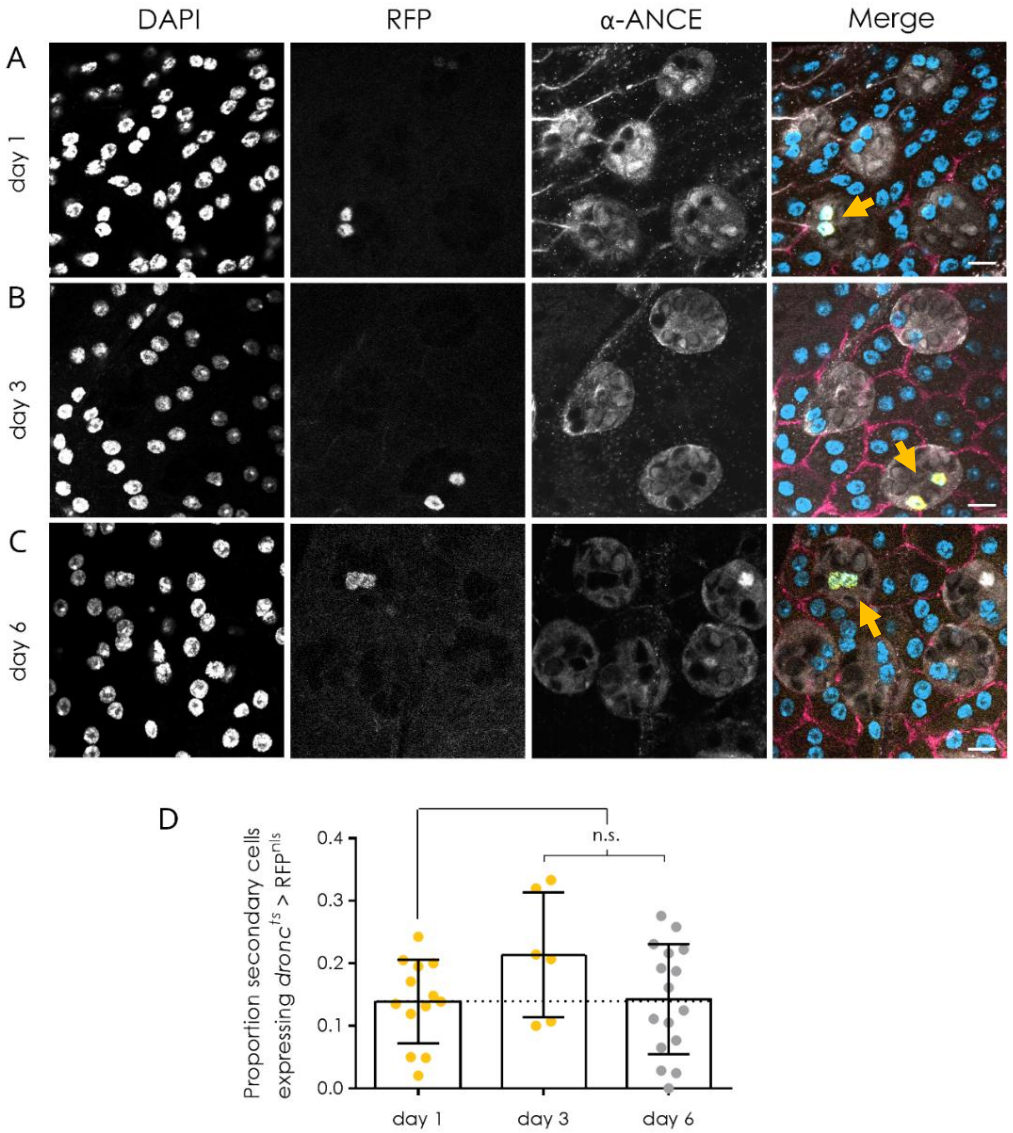


FIGURE 3. 4. | A SIMILAR PROPORTION OF SECONDARY CELLS EXPRESS *dronc* DURING EARLY AGES IN THE ADULT MALE ACCESSORY GLAND.

Secondary cell expression of *dronc* was assessed using using *dronc*^{ts} > RFP at different adult ages.

A: When flies have been kept at the non-permissive temperature (when GAL4 is active) for one day, only a small proportion of secondary cells is positive for *dronc*^{ts} > RFP expression (yellow) (yellow arrow). Note how the α-ANCE staining reveals filamentous extensions between the secondary cells at this age.

B: After three days at the non-permissive temperature, only a small proportion of secondary cells are positive for *dronc*^{ts} > RFP expression (yellow arrow).

C: After six days at the non-permissive temperature, only a small proportion of secondary cells are positive for *dronc*^{ts} > RFP expression (yellow arrow).

D: Scatterplot bar chart plotting the proportion of *dronc*^{ts} > RFP-positive secondary cells at days one and three (yellow) and six (grey) post-eclosion—showing that the proportion does not change significantly over time, as the gland matures. Each dot in the chart corresponds to data obtained from one individual male (see **SUPPLEMENTARY TABLE 3. 1.**, *p.* 422). Day six is considered the control.

In all panels, secondary cells are stained with α-ANCE (white), and α-FASIII (magenta) labels epithelial cell junctions. RFP is shown in yellow. DAPI stains DNA (cyan). The scale bar in panels **A–C** represents 10 μm. In panel **D**, 'n.s' denotes $p > 0.1$; the *p*-value was obtained by one-way ANOVA with a Tukey correction for multiple comparisons (all columns).

proportion of secondary cells expressing *dronc*^{ts} > RFP—both in the control condition, and in the presence of an inverted repeat sequence that targets the region common to all EcR isoforms (COLOMBANI *et al.,* 2005) (*dronc*^{ts} RFP > *Ec*R^{RNAi}). This inverted repeat has previously been shown by Aaron Leiblich to reduce secondary cell *Ec*R expression to undetectable levels and reduce secondary cell growth (LEIBLICH, 2015). Therefore, I hypothesised that if the EcR regulates the expression of *dronc* in secondary cells, I would see reduced expression of the *dronc*^{ts} > RFP reporter when the EcR is knocked down—even if this reduction would also lead to decreased expression of the RNAi itself. Consistent with this hypothesis, I did observe fewer secondary cells that were positive for *dronc*^{ts} > RFP upon co-expression of *Ec*R^{RNAi}, compared to controls at day six ($p < 0.0001$) (**FIGURE 3. 5. A–B, D**). This suggests that the EcR regulates the expression of *dronc* in secondary cells—consistent with observations in other steroid-sensitive tissues and cell types.

The EcR is a nuclear receptor, and, like other nuclear receptors, it has two activation function ("**AF**") domains: one that is isoform-specific (AF1) and one that is highly conserved across isoforms (AF2) (TSAI & O'MALLEY, 1994; CHERBAS *et al.*, 2003). That the EcR isoforms are transcribed from different promoters and have both common and divergent sequence identities and activation functions, allows different EcR isoforms to be both tissue-specific and to regulate different sets of target genes (TALBOT *et al.*, 1993). Previous work has shown that the EcR-B1 is the primary EcR isoform expressed in secondary cells (LEIBLICH, 2015). However, EcR protein levels do not increase when transcripts encoding endogenous EcR isoforms are overexpressed in these cells due to a unique, but uncharacterised, post-transcriptional suppression mechanism (LEIBLICH, 2015). A synthetic EcR isoform, EcR-C, contains only the domains that are shared between the endogenous

EcR isoforms and lacks the amino (N)-terminal domain that confers the isoform-specific AF1 activator function (CHERBAS *et al.*, 2003). However, unlike the endogenous isoforms, expressing the EcR-C in secondary cells increases EcR protein levels and promotes secondary cell growth; suggesting that the N-terminal domain is important for the post-transcriptional regulation of the EcR (LEIBLICH, 2015).

To better understand the role of the EcR in the regulation of *dronc* expression in secondary cells, I used $dronc^{ts}$ > RFP to drive expression of *UAS-EcR-C*. Interestingly, I found that expression of the EcR-C isoform completely abolished the activity of the *dronc* reporter in the accessory gland: with no secondary cells being positive for $dronc^{ts}$ > RFP^{nls} in any of the examined glands (**SUPPLEMENTARY TABLE 3. 2.**, *p.* 422). This suggests that the strong activity of the EcR-C can interfere with the expression of *dronc*—either because excess EcR activity has a negative regulatory effect on *dronc* generally, or because the EcR-C acts differently from the endogenous EcR isoforms when regulating some aspects of secondary cell biology.

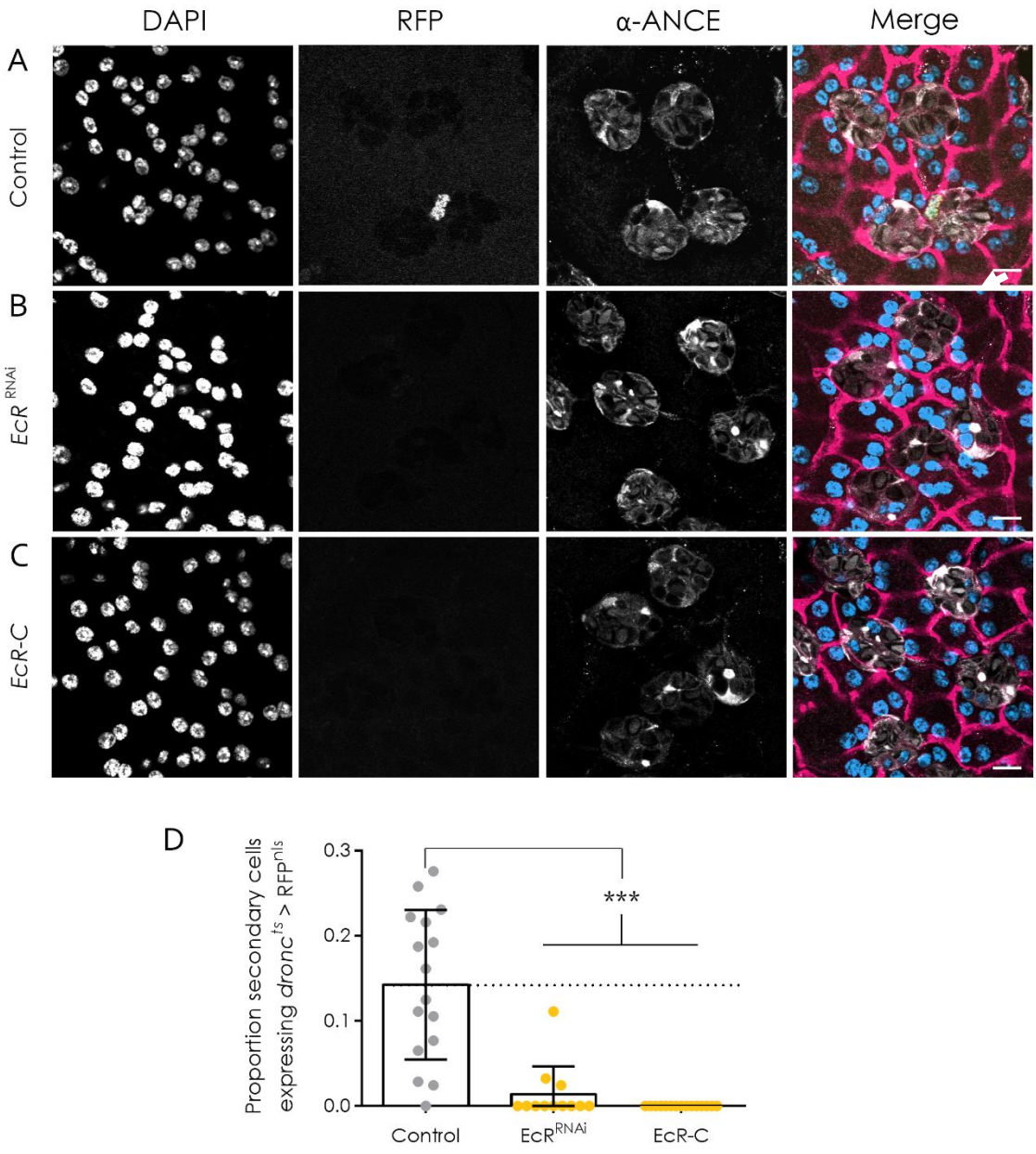
FIGURE 3. 5. | THE EcR REGULATES THE EXPRESSION OF *DRONC* IN SECONDARY CELLS.

Activity from the *dronc* promoter when altering EcR protein levels was assessed at six days at the non-permissive temperature, using $dronc^{ts}$ > RFP.

A: In six-day old males, maintained at 28.5 °C, $dronc^{ts}$ > RFP is detectable in a proportion of secondary cells (arrow).

B: When $dronc^{ts}$ > RFP^{nls} drives the expression of an inverted repeat that targets the *EcR* transcript, the proportion of secondary cells that show evidence of *dronc* reporter activity is reduced.

C: When $dronc^{ts}$ > RFP^{nls} drives the expression of the EcR-C form of the EcR, no secondary cells show any evidence of *dronc* expression.



D: Quantification of the proportion of secondary cells expressing *dronc*^{ts} > RFP^{nls} in different EcR signalling backgrounds. Each dot in the chart corresponds to data obtained from one individual male (see **SUPPLEMENTARY TABLE 3. 2.**, *p.* 422).

Scale bars in panels **A–C** correspond to 10 μm and α-FASIII (magenta) labels epithelial cell junctions. Secondary cells are stained with α-ANCE (white), whereas *dronc*^{ts} > RFP is shown in yellow. DAPI stains nuclei (cyan). In panel **D** *p*-values were obtained by one-way ANOVA with Dunnett's correction for multiple comparisons (all columns compared to the relevant control): *** = $p < 0.001$.

N.B: The value for the control in panel **D** is the same as that for the six-day time-point in the previous figure (**FIGURE 3. 4. D**). This has been taken into account for the statistical analysis.

BMP signalling regulates the expression of *dronc* in secondary cells and is constitutively active at low levels

In addition to describing the EcR is a regulator of secondary cell biology, Dr. Leiblich also found that BMP signalling has a role in this process. BMP signalling promotes secondary cell growth, and stabilises the levels of EcR protein in these cells; acting as a regulator of EcR protein activity (LEIBLICH, 2015). Having found that the EcR regulates the expression of *dronc* in secondary cells, I became curious whether BMP signalling might also be involved. To address this issue, I collaborated with Siamak Redhai (a previous *D. Phil.* student in our laboratory), and parts of the work described in this section was published in REDHAI *et al.* (2016).

To determine if BMP signalling regulates the expression of *dronc*, I used $dronc^{ts}$ > RFP to express either an activated form of the BMP type II receptor, Thickveins (*UAS-tkv*^{Q253D}; WIERSDORFF *et al.,* 1996), or a negative regulator of the pathway, Dad (*UAS-Dad*; TSUNEIZUMI *et al.,* 1997). Quantifying the number of secondary cells expressing $dronc^{ts}$ > RFP in these backgrounds, I found that activating BMP signalling increased the proportion of secondary cells expressing $dronc^{ts}$ > RFP, compared to the control ($p < 0.0001$) (**FIGURE 3. 6. A, C–D**). Conversely, reducing BMP signalling through overexpression of *Dad*, reduced the proportion of secondary cells positive for the $dronc^{ts}$ > RFP^{nls} reporter (**SUPPLEMENTARY TABLE 3. 3.,** *p.* 423).[2] That activated BMP signalling can increase the proportion of secondary cells positive for the *dronc* expression reporter (while reduced signalling blocks the expression), suggests that the expression of *dronc* in secondary cells might indeed require BMP signalling activity. Since *dronc*-GAL4 expression is required to

[2] The low level of expression in the control means a one-way ANOVA with Dunnett's correction for multiple comparisons did not find the decrease significant: $0.01 < p < 0.05$.

express the activated Thickveins receptor in this system, this result suggests that some secondary cells turn on *dronc* expression transiently (as suggested by the low proportion of *dronc*^{ts} > RFP-positive secondary cells in the control), but that this expression is more likely to persist when BMP signalling is activated. Furthermore, since the EcR-B1 isoforms acts downstream of BMP signalling in secondary cells (LEIBLICH, 2015), my data suggest that this isoform (unlike the EcR-C) might positively regulate *dronc*. However, although it would be interesting to determine how activity from the *dronc* promoter is affected by co-expression of different combinations of EcR isoforms and BMP-signalling components, I found such co-expression to be unsuccessful in this system (data not shown), probably due to the number of transgenes present.

The low, but persistent, level of *dronc* expression in secondary cells—which seems to be regulated by BMP signalling—suggested to me that perhaps there is a relationship between BMP signalling activation and *dronc* expression; that perhaps expression of *dronc* in a proportion of secondary cells might mirror the activation of BMP signalling itself? Activated BMP signalling is characterised by translocation of phosphorylated Mad (pMad) protein into the nucleus (DAS *et al.,* 1998; KIM *et al.,* 1997), and pMad is detectable in secondary cells using an antibody that targets the mammalian homologue of the Mad protein (pSMAD 1/5; Cell Signalling) (REDHAI *et al.,* 2016). Working with Siamak Redhai, he and I showed that, indeed, a low proportion of secondary cells show evidence of BMP signalling activation (Mad phosphorylation) at any given time in the accessory glands of virgin males; there being no detectable difference in the proportions of secondary cells staining positive for nuclear pMad at day three, compared to day six ($p > 0.1$) (**FIGURE 3. 6. E**) (**SUPPLEMENTARY TABLE 3. 4.**, *p.* 423) (REDHAI *et al.,* 2016). Further work will be

required to establish whether the cells showing BMP signalling activity (as evidenced by α-pMad staining) also express the *dronc* reporter.

FIGURE 3. 6. | BMP SIGNALLING REGULATES THE EXPRESSION OF *dronc* IN SECONDARY CELLS AND IS ACTIVE AT LOW LEVELS IN VIRGIN GLANDS.

Secondary cell expression of *dronc* when BMP signalling activity is manipulated was assessed at six days at the non-permissive temperature, using $dronc^{ts}$ > RFP.

A: In control glands, low levels of $dronc^{ts}$ > RFP are detectable in secondary cells (yellow arrow).

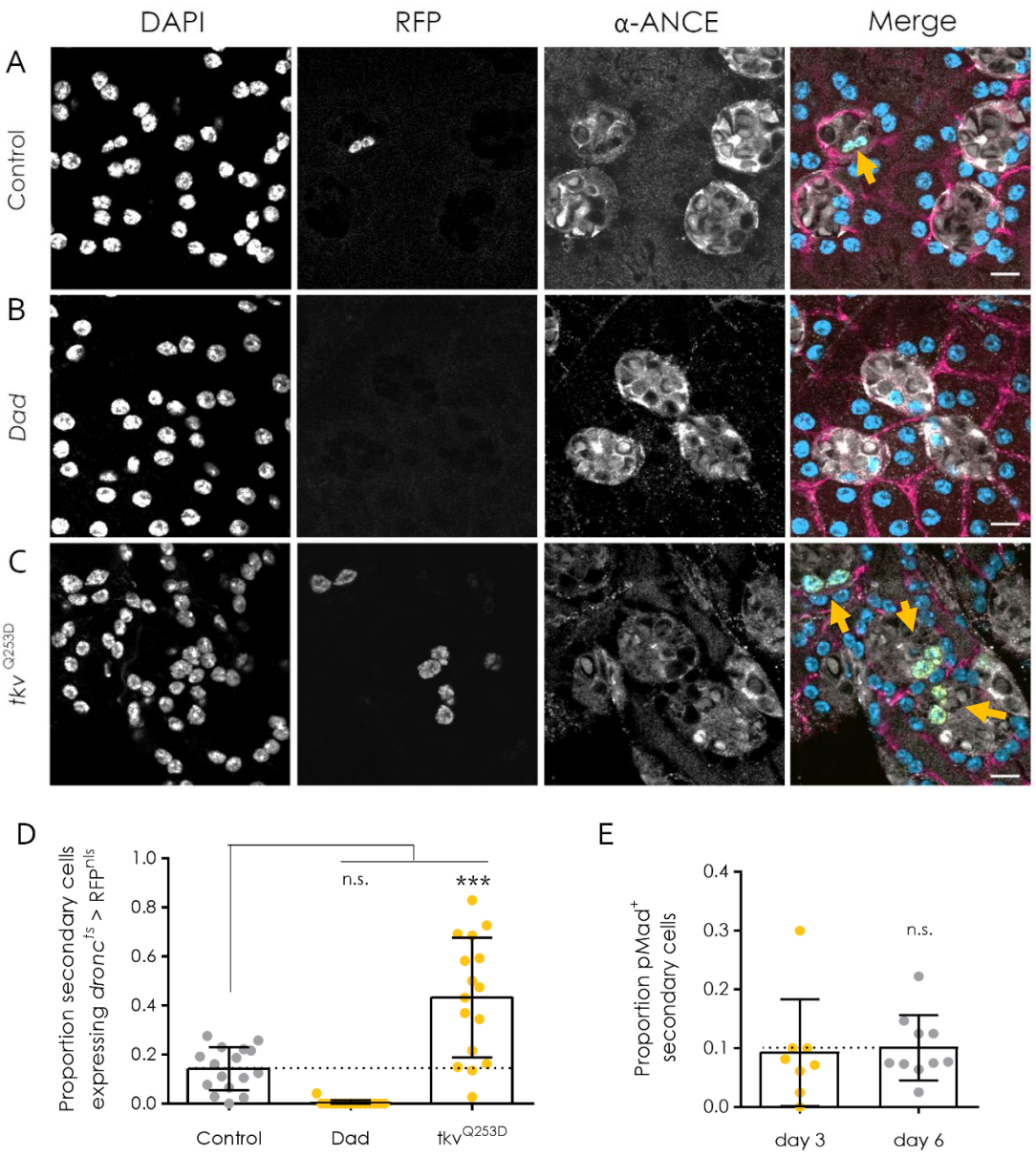
B: When $dronc^{ts}$ > RFP drives the expression of the BMP signalling inhibitor, Dad, no secondary cells show evidence of expression from the *dronc* reporter.

C: When $dronc^{ts}$ > RFP drives the expression of the activated BMP receptor, Tkv^{Q253D}, the proportion of secondary cells expressing the *dronc* reporter is increased (yellow arrows).

D: Quantification of the proportion of secondary cells showing activity from the $dronc^{ts}$ > RFP reporter. Each dot in the chart corresponds to data obtained from one individual male (see **SUPPLEMENTARY TABLE 3. 3.**, *p.* 423).

E: Quantification of the proportion of secondary cells staining positive for phosphorylated Mad protein (pMad) in males at three and six days of age. Each dot in the chart corresponds to data obtained from one individual male (see **SUPPLEMENTARY TABLE 3. 4.**, *p.* 423).

Scale bars in panels **A–C** correspond to 10 μm and α-FASIII (magenta) labels epithelial cell junctions. Secondary cells are stained with α-ANCE (white), and $dronc^{ts}$ > RFP is shown in yellow. DAPI stains DNA (cyan).



In panels **D** and **E**, *p*-values were obtained by one-way ANOVA with Dunnett's correction for multiple comparisons (all columns compared to the relevant control): *** = $p < 0.001$; 'n.s.' denotes $p > 0.1$. Each dot represents the value of an individual accessory gland, and error bars show the standard deviation within the sample. Shapiro-Wilk testing shows that the proportion of secondary cells positive for pMad antibody staining is normally distributed ($p < 0.01$), and this type of data has therefore been analysed using parametric methods.

N.B: The value for the control in panel **D** and the value for the six day virgins in panel **E** are the same**.** This has been taken into account for the statistical analysis.

DRONC regulates the number of non-acidic compartments in secondary cells

Having established that the *dronc* expression reporter is consistently expressed in a proportion of secondary cells during the period of accessory gland maturation after eclosion, and that both BMP signalling and the EcR regulating *dronc* expression, I wondered: what function does DRONC perform in secondary cells? Previously, Dr. Leiblich has shown that secondary cell nuclei grow over time, and that this size increase is regulated by both BMP signalling and the EcR (LEIBLICH *et al.,* 2012; LEIBLICH, 2015). Therefore, I wondered: does DRONC have a role regulating this process? An initial assay showed no role for DRONC in the regulation of secondary cell nuclear size (data not shown)—although this result needs to be replicated to be confirmed. However, with the initial assay assessing the role of DRONC in the regulation of secondary cell nuclear size being negative, I decided to assay another aspect of secondary cell biology: intracellular trafficking (CORRIGAN *et al.*, 2014).

Secondary cells characteristically contain very large intracellular compartments (BAIRATI, 1968; FEDERER & CHEN, 1982; RYLETT & WALKER *et al.,* 2007; CORRIGAN *et al.,* 2014). Our laboratory has shown that these compartments can be labelled with a GFP-tagged form of the transmembrane protein, CD63 (CD63::GFP; PANÁKOVÁ & SPRONG *et al.*, 2005; CORRIGAN *et al.*, 2014). Using this GFP-tagged form of CD63 to mark the membranes of secondary cell intracellular compartments, CORRIGAN *et al.* (2014) showed that BMP signalling regulates the number and the composition of secondary cell intracellular compartments. Therefore, to explore what role DRONC might perform in secondary cells, I asked: does DRONC have a role in regulating secondary cell compartment numbers?

The transcription factor, *doublesex* ("***dsx***") is expressed in secondary cells (LEIBLICH, 2015) and a temperature-sensitive *dsx*-GAL4 (dsx^{ts}) can be used to express *UAS*-CD63::GFP; dsx^{ts} > CD63::GFP) in these cells (CORRIGAN *et al.,* 2014). Together with LysoTracker-Red staining of acidic compartments, the CD63::GFP labelling allows the full complement of secondary cell acidic and non-acidic compartments to be effectively visualised (CORRIGAN *et al.,* 2014). Using confocal microscopy to image glands expressing dsx^{ts} > CD63::GFP and stained with LysoTracker-Red, the CD63::GFP membrane marker typically labels around 40 non-acidic compartments per secondary cell by day 6 post-eclosion (**FIGURE 3. 7. A, C**) (CORRIGAN *et al.*, 2014). An inverted repeat that targets *dronc* is available (*UAS-*$dronc^{RNAi}$; LEULIER *et al.*, 2006), and using dsx^{ts} CD63::GFP allowed me to drive the expression of this construct in secondary cells (dsx^{ts} CD63::GFP > $dronc^{RNAi}$). Doing so, I found that the number of non-acidic, CD63::GFP-positive compartments increases in cells expressing CD63::GFP > $dronc^{RNAi}$, compared to controls (p {day 6} < 0.0001) (**FIGURE 3. 7. B–C**). This suggests that DRONC might normally act as a negative regulator of CD63::GFP-positive non-acidic compartment numbers. To confirm this observation, I decided to make use of an alternative marker for secondary cell non-acidic compartments: a GPI-anchored form of GFP (GPI::GFP) (PANÁKOVÁ & SPRONG *et al.,* 2005). This GPI-tagged GFP molecule is—like CD63::GFP—a membrane marker, with the added benefit that it also labels the secretory granules that are contained within a subset of the secondary cell non-acidic compartments (REDHAI *et al.,* 2016). Similar to *doublesex*, the epidermal growth factor ("**EGF**")-like gene, *spitz* ("***spi***"; RUTLEDGE *et al.*, 1992), is also expressed in secondary cells (LEIBLICH, 2015), and a temperature-sensitive form of *spi*-GAL4

FIGURE 3. 7. | DRONC REGULATES THE NUMBER OF NON-ACIDIC COMPARTMENTS IN SECONDARY CELLS.

The effect of RNAi-mediated knockdown of *dronc* was assessed in secondary cells was assessed using *dsx*^{ts} > CD63::GFP to mark the non-acidic compartments.

A: Expressing *dsx*^{ts} > CD63::GFP in the accessory gland labels secondary cell intracellular compartments (green). LysoTracker-Red (magenta) labels acidic compartments and allows these to be differentiated from the non-acidic compartments.

B: Driving expression of a *UAS-dronc*^{RNAi} hairpin construct using *dsx*^{ts} > CD63::GFP causes the number of CD63::GFP-positive compartments to increase, whereas the volume of the largest Lyotracker-Red–positive compartment is reduced.

C: Scatterplot bar chart plotting the number of CD63::GFP-positive intracellular compartments in control (grey) and *dronc*^{RNAi}-expressing (green) secondary cells at day 3, day 6, and day 15 post-eclosion.

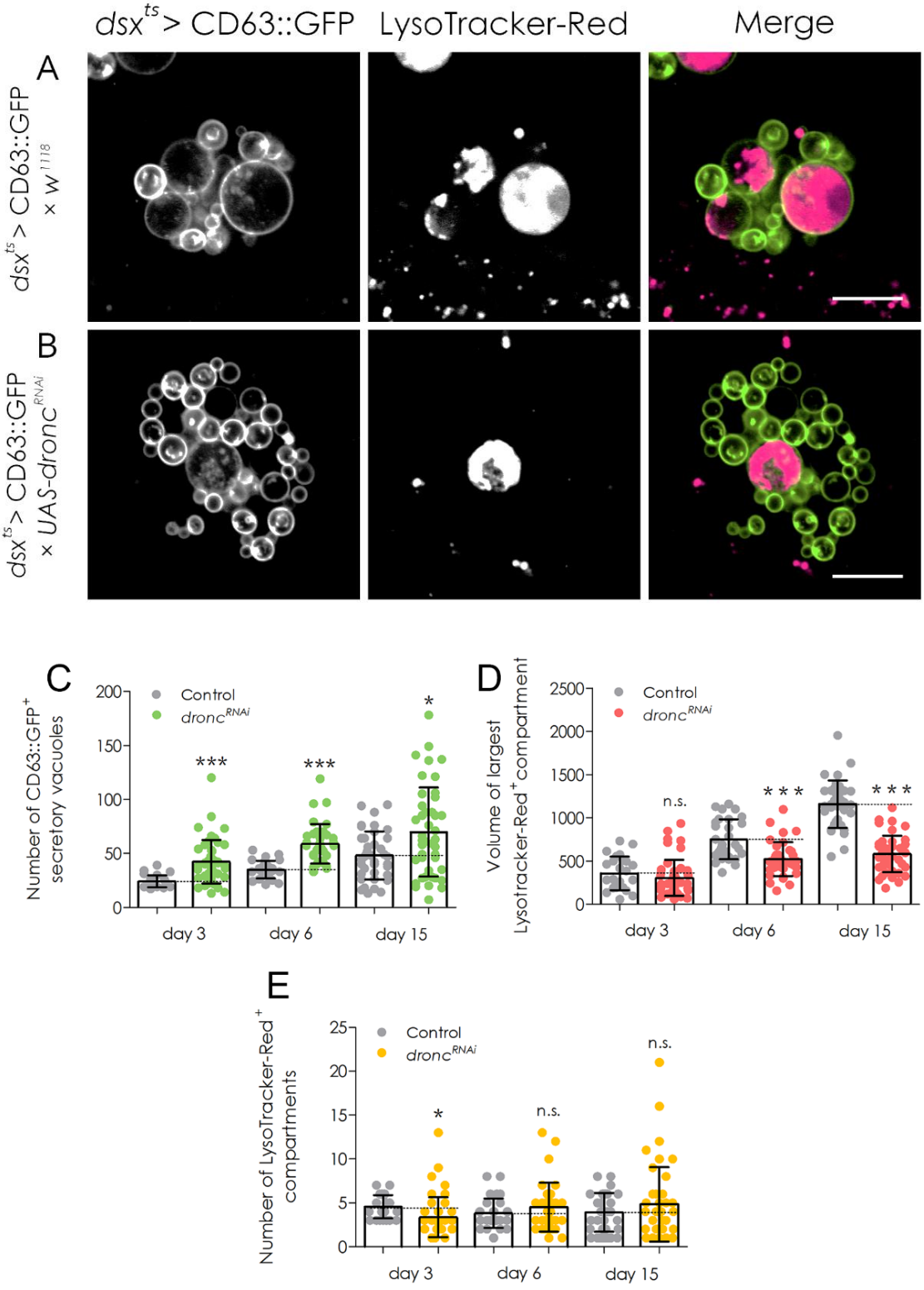
D: Scatterplot bar chart plotting the volume of the largest Lysotracker-Red–positive compartments in control (grey) and secondary cells expressing *dronc*^{RNAi} (red) at day 3, day 6, and day 15.

E: Scatterplot bar chart plotting the number of Lysotracker-Red–positive compartments in control (grey) and *dronc*^{RNAi}-expressing (yellow) secondary cells at day 3, day 6, and day 15.

Image panels **A–B** show representative secondary cells in six-day old males, following a 28.5 °C shift after eclosion. Scale bars in both panels represent 10 μm.

In charts **C–E**, each scatterplot dot represents the sample value of an individual secondary cell, with three cells being quantified from each individual male (see **SUPPLEMENTARY TABLES 3. 5.–3. 7**., *pp.* 423–4). The scatterplot bar represents the average value across all individual secondary cell samples, and error bars show the standard deviation within the sample. Shapiro-Wilk testing shows that secondary cell non-acidic compartment numbers and acidic compartment number and volume is not normally distributed ($p > 0.1$), and these data have therefore been analysed using non-parametric testing methods.

In charts **C** and **E,** p-values were obtained using Mann-Witney U tests: * = $p < 0.01$; *** = $p < 0.0001$. In chart **D**, stars represent values obtained by a Kruskal-Wallis one-way ANOVA, with Dunnett's correction for multiple comparisons (all columns). Treatments that are not different from controls are denoted by 'n.s.'.


dsx^ts > CD63::GFP
LysoTracker-Red
Merge
A
dsx^ts > CD63::GFP × w^1118
B
dsx^ts > CD63::GFP × UAS-dronc^RNAi
C
Number of CD63::GFP+ secretory vacuoles
Control
dronc^RNAi
***
***
*
day 3
day 6
day 15
D
Volume of largest Lysotracker-Red+ compartment
Control
dronc^RNAi
n.s.
***
***
day 3
day 6
day 15
E
Number of LysoTracker-Red+ compartments
Control
dronc^RNAi
*
n.s.
n.s.
day 3
day 6
day 15

(spi^{ts}) allows transgene expression in secondary cells (CORRIGAN, 2014; REDHAI *et al.,* 2016). Similar to when using the dsx^{ts} > CD63::GFP driver, using spi^{ts} GPI::GFP to drive the expression of $dronc^{RNAi}$ (spi^{ts} GPI::GFP > $dronc^{RNAi}$) causes the number of labelled compartments (secretory granules) to increase compared to controls (p {control|$dronc^{RNAi}$} < 0.0001) (**FIGURE 3. 8.**). Altogether, the CD63::GFP and GPI::GFP results (**SUPPLEMENTARY TABLE 3. 5.**, *p.* 423) suggest that the expression of an inverted repeat that targets *dronc* in secondary cells is sufficient to increase the number of large non-acidic compartments—including those containing a GPI::GFP-positive secretory granule. The implication of these observations is that DRONC is a negative regulator of non-acidic and secretory granule-containing compartment numbers in secondary cells.

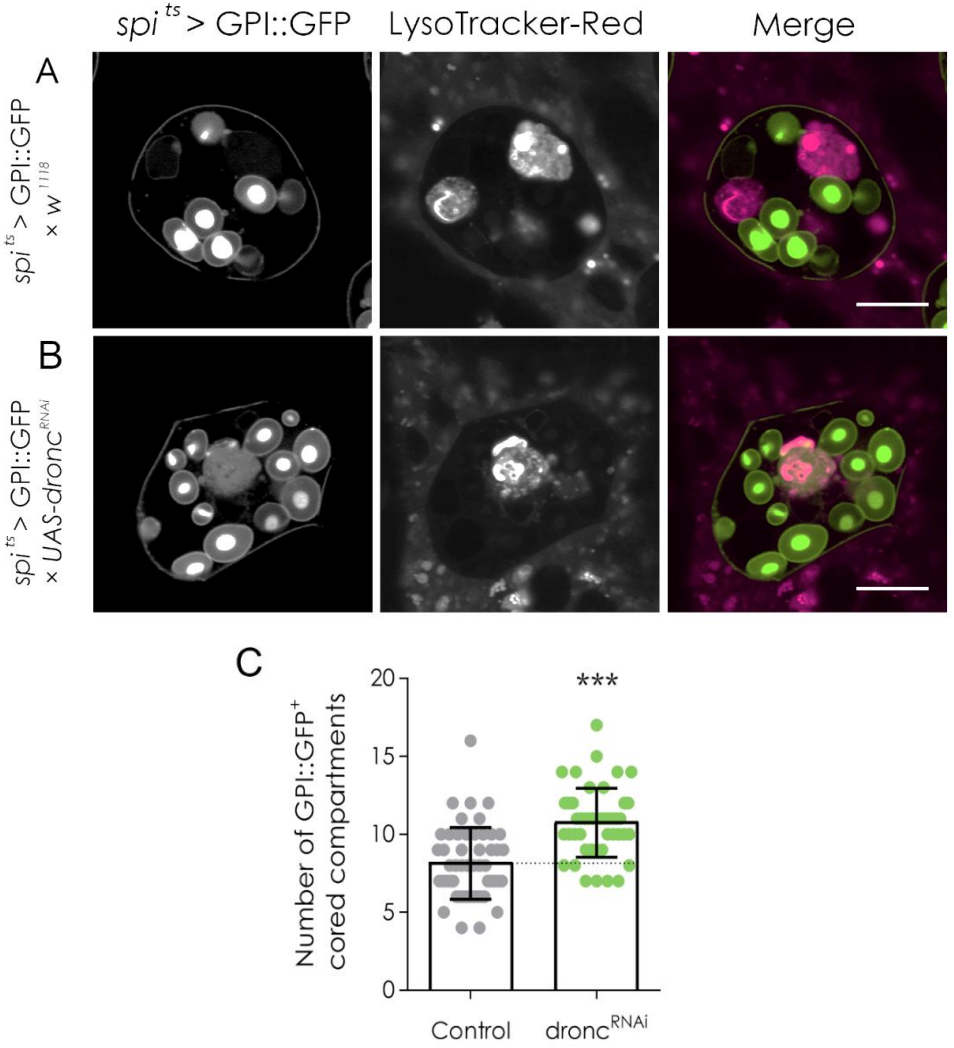


FIGURE 3. 8. | DRONC REGULATES THE NUMBER OF SECONDARY CELL COMPARTMENTS CONTAINING A SECRETORY GRANULE.

RNAi-mediated knockdown of DRONC was induced in secondary cells for six days, before numbers of GPI::GFP-positive secretory granule-containing compartments was assessed.

A: A GPI-anchored form of GFP (GPI::GFP), driven by *spi*^{ts}, labels the dense protein core contained in the non-acidic intracellular compartments (green). LysoTracker-Red stains the acidic compartments (magenta).

B: Using *spi*^{ts} > GPI::GFP to drive expression of *UAS-dronc*^{RNAi} causes the number of intracellular compartments containing a GPI::GFP-positive protein core to increase.

C: Scatterplot bar chart plotting the number of GPI::GFP-positive secretory granule-containing compartments in control (grey) and secondary cells expressing *dronc*^{RNAi} (green) at day six.

The image panels in **A–B** show representative secondary cells on day six post-eclosion. Scale bars in all panels represent 10 μm. In the chart in panel **C**, each scatterplot dot represents the sample value of an individual secondary cell, with three cells quantified from each individual male (see **SUPPLEMENTARY TABLE 3. 5.,** *p.* 423).. The scatterplot bar represents the average value for each sample across all individual secondary cell samples, and error bars show the standard deviation within the sample. Shapiro-Wilk testing shows that secondary cell non-acidic compartment numbers are not normally distributed ($p > 0.1$); these data have therefore been analysed using non-parametric methods. Asterisks represent the *p*-value obtained by a Mann-Witney U test: *** = $p < 0.0001$.

DRONC is a positive regulator of lysosomal growth in secondary cells

Secondary cells contain acidic internal compartments that are positive for the late endosomal and lysosomal marker, RAB7, and the largest of these compartments is thought to represent a lysosome or lysosome-like structure (CORRIGAN *et al.*, 2014; B. KROEGER & K. CARR, personal comments). As the accessory gland matures, the number of acidic compartments in the secondary cell decreases, while the size of the largest acidic compartment increases (CORRIGAN *et al.*, 2014; REDHAI, 2016).

RNAi of *dronc* affects non-acidic compartment numbers in secondary cells. This observation can be interpreted in two ways:

a) DRONC has a role as a regulator of intracellular trafficking (*e.g.* DRONC might co-ordinate compartment trafficking decisions; such as the release of non-acidic (secretory) compartments); or,

b) DRONC has a role suppressing the biogenesis of new, non-acidic compartments.

To investigate this problem further, I decided to determine the effect of knocking-down *dronc* on the number and size of the acidic compartments, using Lysotracker-Red to visualise acidic compartments within secondary cells expressing dsx^{fs} CD63::GFP > $dronc^{RNAi}$. By day 6 of adulthood (at 28.5 °C), secondary cells typically contain 3.8 ± 1.7 (± SD) Lysotracker-Red–positive compartments. This number remains unchanged in secondary cells expressing dsx^{fs} CD63::GFP > $dronc^{RNAi}$ (p {day 6} = 0.4) (**FIGURE 3. 8. E**). However, non-acidic compartments have been shown to

fuse with acidic compartments (CORRIGAN *et al.,* 2014), so the size of the acidic compartment could change if this process is affected.

The volume of the largest acidic compartment normally increases over time in control (dsx^{ts} > CD63::GFP) secondary cells (p {day 3|day 6} < 0.0001; p {day 6|day 15} < 0.05) (**FIGURE 3. 8. D**). If $dronc^{RNAi}$ increased both compartment biogenesis and the frequency of compartment coalescence, I hypothesised that the largest acidic compartment might grow at a faster rate when *dronc* is knocked down, compared to what is observed in controls. Measuring the growth of the largest acidic compartment in the dsx^{ts} CD63::GFP > $dronc^{RNAi}$ shows that—although the largest acidic compartment does grow between days three and six (p {day 3|day6} <0.01)—the compartment experiences no further growth beyond this time (p {day 6|day15} > 0.1) (**FIGURE 3. 7. D**) (**SUPPLEMENTARY TABLES 3. 6.** and **3. 7.**, *p.* 424)—causing its growth to lag behind that in the control cells (p {day 3} > 0.1; p {day 6} > 0.1; p {day 15} < 0.0001). That $dronc^{RNAi}$ inhibits the growth of the largest acidic compartment suggests that DRONC normally might have a role promoting its enlargement. One possible explanation might be that trafficking to the acidic compartments is suppressed when *dronc* is knocked down, leading to the observed accumulation of non-acidic compartments.

A catalytically inactive form of DRONC does not alter non-acidic compartment numbers

DRONC can function both catalytically and non-catalytically in the cell (LAMKANFI *et al.*, 2007; KAMBER KAYA *et al.,* 2017). DRONC constructs with different catalytic activities are available, so, to investigate what effects catalytically active and catalytically inactive forms of DRONC have on secondary cell

intracellular trafficking, I expressed these in secondary cells using the *dsx*^{ts} > CD63::GFP driver. First, I turned to a dominant-negative and catalytically inactive form of DRONC: where the catalytic cysteine ("**C**") residue in the DRONC catalytic (active) site has been replaced with a catalytically inert alanine ("**A**") residue (DRONC$^{C>A}$) (MEIER *et al.*, 2000). If DRONC regulates secondary cell non-acidic compartment numbers/acidic compartment growth via a catalytic mechanism, I expected the catalytically inactive form of DRONC to have a similar effect as the *dronc* hairpin loop (since both interfere with the normal activity of the DRONC caspase).

Expressing DRONC$^{C>A}$ in secondary cells using *dsx*^{ts} > CD63::GFP (*dsx*^{ts} CD63::GFP > DRONC$^{C>A}$), there was no discernible difference in CD63::GFP-positive compartment numbers, compared to control cells, by the six-day timepoint ($p > 0.1$) (**FIGURE 3. 9. A–B, D**). This result is consistent with the hypothesis that DRONC regulates secondary cell non-acidic compartment numbers via a non-catalytic mechanism. Furthermore, the growth of the largest Lysotracker-Red–positive compartment is not reduced in cells expressing *dsx*^{ts} CD63::GFP > DRONC$^{C>A}$, relative to controls ($p > 0.1$) (**FIGURE 3. 9. A–B, E**). Altogether, the data from the catalytically inactive DRONC construct (**SUPPLEMENTARY TABLE 3. 8.**, *p.* 425) suggests that DRONC might regulate secondary cell biology via a non-catalytic mechanism.

Overexpression of a catalytically capacitated form of DRONC reduces secondary cell intracellular compartment numbers and secondary cell viability

To further investigate the role of the catalytic state of DRONC in secondary cell intracellular trafficking, I turned to a form of DRONC that lacks its N-terminal inhibitory caspase pro-domain ($DRONC^{\Delta N}$), and therefore is catalytically capacitated and free to undergo autolytic activation (MEIER *et al.*, 2000). If DRONC is a negative regulator of non-acidic compartment numbers, and if this regulation is catalytic, the catalytically capacitated form of DRONC would be expected to reduce the number of non-acidic compartments in secondary cells. Consistent with this expectation, expressing *dsx*^{ts} CD63::GFP > $DRONC^{\Delta N}$ in secondary cells does reduce the number of CD63::GFP-positive compartments ($p < 0.0001$) (**FIGURE 3. 9. A, C–D**). This decrease is consistent with a catalytic role for DRONC in this process. Like knock-down of *dronc*, activated DRONC also reduces the growth of the largest acidic compartment ($p < 0.001$).

Activated DRONC however also reduces secondary cell viability (**FIGURE 3. 9. G**), which might potentially affect the interpretation of these results. In control glands, secondary cells are invariably healthy on day 3 ($n_{gland} = 14$), and show clearly delineated CD63::GFP-positive intracellular compartments and compartmentalised Lysotracker-Red staining. In glands expressing *dsx*^{ts} CD63::GFP > $DRONC^{\Delta N}$, this proportion is much reduced: with only 29.0 ± 16.3 % (± SD) of secondary cells looking normal and healthy (average: 12/42 healthy cells per gland; $n_{gland} = 8$; $p = 1.31 \times 10^{-12}$ (unpaired, two-tailed Student's *t*-test)). Therefore, although the effects of expressing activated DRONC on secondary cell intracellular trafficking (**SUPPLEMENTARY TABLE 3. 9.**, *p.* 425) are consistent with a catalytic function of

DRONC in these cells, the results are potentially confounded by the reduced viability of the secondary cell population in *dsx*^{ts} CD63::GFP > DRONC$^{\Delta N}$-expressing glands, which might produce similar phenotypes.

FIGURE 3. 9. | EFFECTS OF EXPRESSING CATALYTICALLY ACTIVE AND INACTIVE DRONC CONSTRUCTS IN SECONDARY CELLS.

Different mutant forms of DRONC were expressed in secondary cells using *dsx*^{ts} > CD63::GFP, following a temperature shift upon eclosion. The effects on compartment number and size was assessed at six days.

A: Expressing *dsx*^{ts} > CD63::GFP in control glands labels secondary cell compartments (green). Acidic compartments are labelled with LysoTracker-Red (magenta).

B: Secondary cells expressing a catalytically inactive form of DRONC (*dronc*$^{C>A}$).

C: Relatively normal secondary cell expressing an activated form of DRONC lacking the inhibitory N-terminal prodomain (*dronc*$^{\perp N}$).

D: Bar chart plotting the number of CD63::GFP-positive secretory vacuoles in secondary cells expressing either catalytically inactive or activated DRONC constructs at day six.

E: Scatterplot chart plotting the volume of the largest LysoTracker-Red–positive compartment in secondary cells expressing either catalytically inactive or activated DRONC constructs at day six.

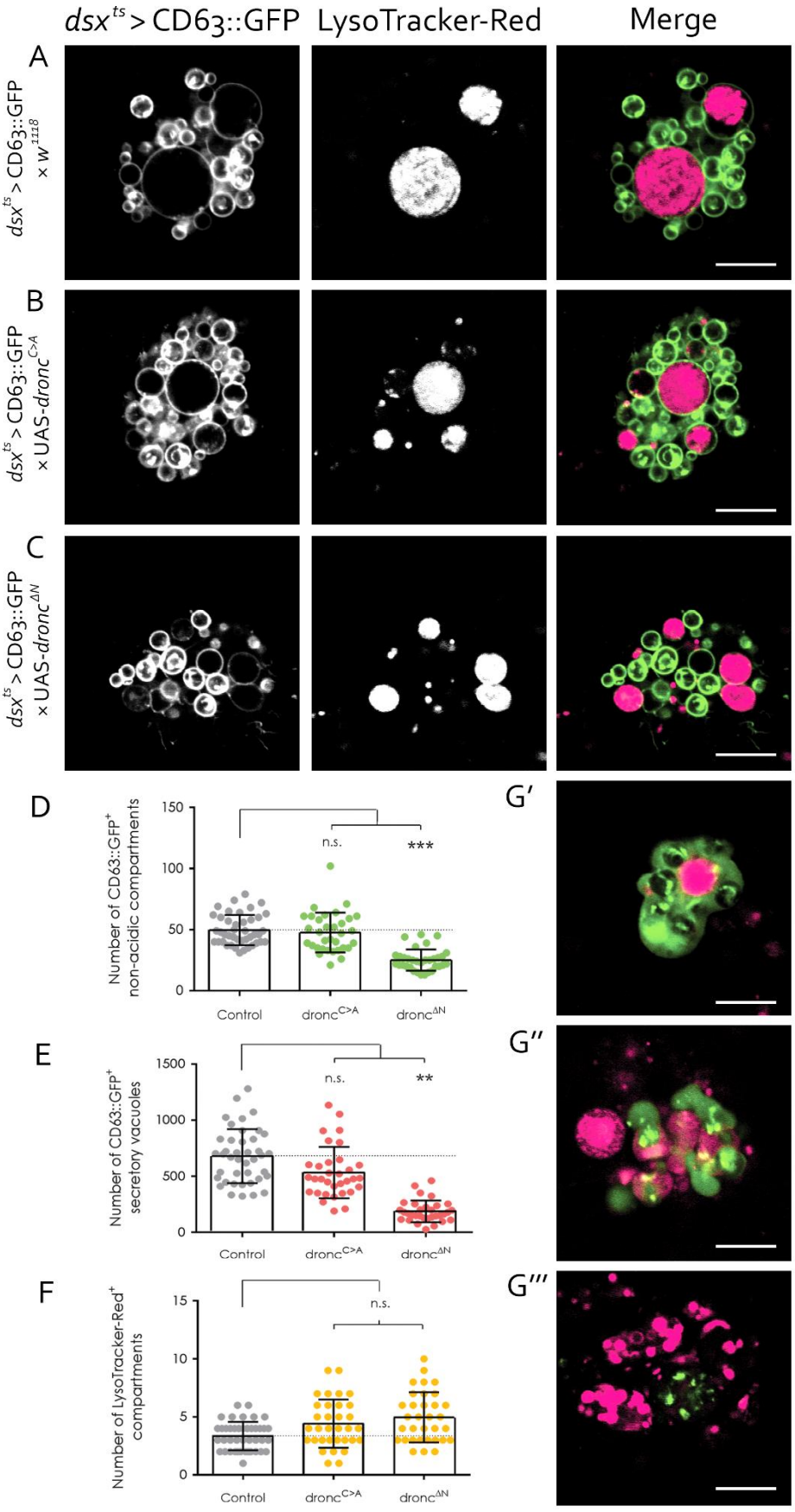
F: Bar chart plotting the number of LysoTracker-Red–positive compartments in secondary cells expressing either catalytically inactive or activated DRONC constructs at day six.

G: Secondary cells expressing activated DRONC show reduced viability, and adopt abnormal morphologies, including: reduced size and cytoplasmic CD63::GFP (**G'**); cytoplasmic CD63::GFP and intracellular acidification (**G"**); and, secondary cell 'atrophy'—accompanied by increased acidity of neighbouring main cells (**G"'**).

Image panels **A–C** and **G** show representative secondary cells on day six post-eclosion. Scale bars in all panels represent 10 μm.

In charts **D–F**, each scatterplot dot represents the sample value of an individual secondary cell, with three cells being quantified from each individual male (see **SUPPLEMENTARY TABLES 3. 8.–3. 9.**, *p.* 425). The scatterplot bar represents the average value across all individual secondary cell samples, and error bars show the standard deviation within each sample.

Asterices represent *p*-values obtained by Kruskal-Wallis one-way ANOVA with Dunnett's correction for multiple comparisons (all test columns compared to the control): ** $p < 0.001$; *** $p < 0.0001$. Treatments that do not differ from the control are denoted by 'n.s.'.


dsx^ts > CD63::GFP
LysoTracker-Red
Merge
A
dsx^ts > CD63::GFP × w^1118
B
dsx^ts > CD63::GFP × UAS-dronc^C>A
C
dsx^ts > CD63::GFP × UAS-dronc^ΔN
D
Number of CD63::GFP+ non-acidic compartments
150
100
50
0
n.s.
***
Control
dronc^C>A
dronc^ΔN
E
Number of CD63::GFP+ secretory vacuoles
1500
1000
500
0
n.s.
**
Control
dronc^C>A
dronc^ΔN
F
Number of LysoTracker-Red+ compartments
15
10
5
0
n.s.
Control
dronc^C>A
dronc^ΔN
G′
G″
G‴

Overexpression of *dronc* reduces secondary cell viability

Being an initiator caspase, and therefore a positive regulator of cell death, DRONC is both necessary and sufficient to induce cell death in *D. melanogaster* (DORSTYN *et al.*, 1999a). Yet, as shown above, secondary cells normally express *dronc* while also remaining viable; suggesting that secondary cells are able to regulate the catalytic activity of DRONC to avoid adverse effects. Alternatively, secondary cells may be refractory to the catalytic activity of DRONC, although expression of an active construct ($dronc^{\Delta N}$) appears to induce severe distress in the cells, leading to what looks like cell death (**FIGURE 3. 9. G'–G'"**).

To determine to what extent secondary cells can regulate the catalytic capacitation of DRONC, I tested whether the ectopic expression of wild-type *dronc* is sufficient to reduce secondary cell viability. By day three of adulthood, secondary cells are typically full of CD63::GFP-positive non-acidic compartments that fill the interior of the cell, alongside one or two large acidic compartments (**FIGURE 3. 10. A**). Although secondary cells are flanked by main cells in the epithelium, these are not immediately obvious when using LysoTracker-Red, since the acidic compartments in main cells are normally small and inconspicuous. When I used dsx^{ts} CD63::GFP to overexpress wild-type *dronc* (*UAS-dronc*; QUINN *et al.*, 2000), the accessory gland epithelium appeared decidedly different: with the secondary cells being obviously adversely affected and no healthy-looking secondary cells remaining in the accessory gland epithelium ($n_{gland} = 14$) (**FIGURE 3. 10. B**). Indeed, *no* secondary cells seem to remain in the epithelium, and, the only trace of their previous location at the distal tip of each gland being a scattering of CD63::GFP-positive *puncta*, surrounded by main cells that stain strongly with LysoTracker-Red—indicating higher lysosomal activity perhaps associated with the phagocytic activity the death

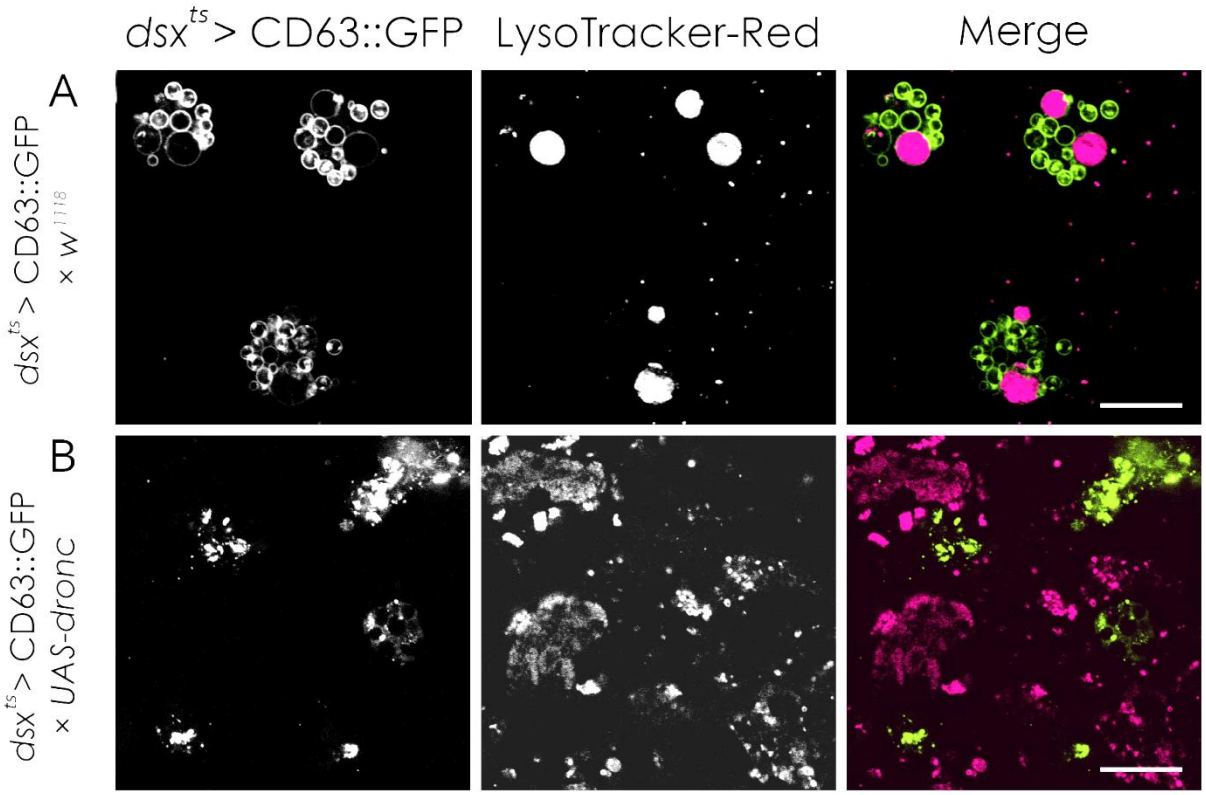


FIGURE 3. 10. | OVEREXPRESSION OF *dronc* REDUCES SECONDARY CELL VIABILITY.

Wild-type *dronc* was expressed in secondary cells using *dsx*^{ts} > CD63::GFP, following a temperature shift upon eclosion.

A: Expressing *dsx*^{ts} > CD63::GFP in control glands labels secondary cell compartments (green). Acidic compartments are labelled with LysoTracker-Red. Note only faint LysoTracker-Red staining (magenta) of the main cells flanking the secondary cells.

B: Secondary cells expressing wild-type DRONC (*dronc*) in secondary cells using *dsx*^{ts} > CD63::GFP completely eliminates normal-lloking secondary cells from the accessory gland epithelium. The surrounding main cells show strong LysoTracker-Red staining.

Image panels show representative secondary cells on day three post-eclosion. Scale bars represent 20 μm.

of a cell can induce in its epithelial neighbours (FRANC, 2002). Overall, this suggests that overexpression of *dronc* is not well-tolerated by secondary cells.

DRONC is a negative regulator of the secondary cell population in the maturing accessory gland epithelium

While characterising the role of DRONC in the regulation of secondary cell biology, I noticed that when I used dsx^{fs} CD63::GFP to express the inverted repeat that targets *dronc*, there seemed to be noticeably more secondary cells populating the accessory gland epithelium. This suggested to me that perhaps DRONC has a role in regulating the numbers of secondary cells in the adult gland. Intrigued, I decided to quantify secondary cell numbers in both control glands and glands where *dronc* has been knocked down in secondary cells.

Using dsx^{fs} CD63::GFP to label secondary cells with GFP, I stained the glands with LysoTracker-Red to better visualise the accessory gland epithelium. Doing so, I found that the individual arm of a control accessory gland in three-day old adults typically contains around 44.9 ± 4.0 secondary cells (**FIGURE 3. 11. A, E**)—a number that is in agreement with numbers reported in the literature previously (*e.g.* 43 ± 9 in BERTRAM *et al.,* 1993). However, when I used dsx^{fs} CD63::GFP to express the *dronc* inverted repeat in secondary cells, I found a significant increase in the number of secondary cells contained within each gland ($p < 0.0001$) (**FIGURE 3. 11. B, E**). This is intriguing, since it implies that the presence of a *dronc* hairpin affects secondary cell numbers. However, it is feasible that different transgenic lines—which will have diverged genetically due to genetic drift and frequent population bottlenecks—could harbour genetic variation that affect secondary cell numbers. Therefore, to further investigate the difference in secondary cell numbers between control accessory

glands and glands with secondary cells expressing *UAS-dronc*^{RNAi} are due to the RNAi, I decided to quantify the number of secondary cells in the male accessory glands of the RNAi parent line. Doing so, I found that secondary cell numbers in this line were indistinguishable from those of the driver line ($p > 0.1$) (**FIGURE 3. 11. D–E**). This result further strengthens the possibility that DRONC has a role negatively regulating the size of the secondary cell population in the accessory gland epithelium. When does this regulation take place? Is the presence of DRONC in secondary cells sufficient to reduce secondary cell numbers at any time, or, is there a window of time during which secondary cells are particularly sensitive to the action of DRONC? To investigate, I decided to see how an overnight pulse (day two to day three) of *dronc*^{RNAi} transgene expression would affect secondary cell numbers. Quantifying these, I again found that secondary cell numbers in flies shifted overnight were not different from those of the control glands kept at 29 °C since eclosion ($p > 0.1$) (**FIGURE 3. 11. C, E**). This might suggests that DRONC regulates secondary cell numbers sometime prior to the end of day two post-eclosion (**SUPPLEMENTARY TABLE 3. 10.,** *p.* 426), but most likely demonstrates that an overnight pulse of *dronc*^{RNAi} expression is not sufficient for the phenotype to develop.

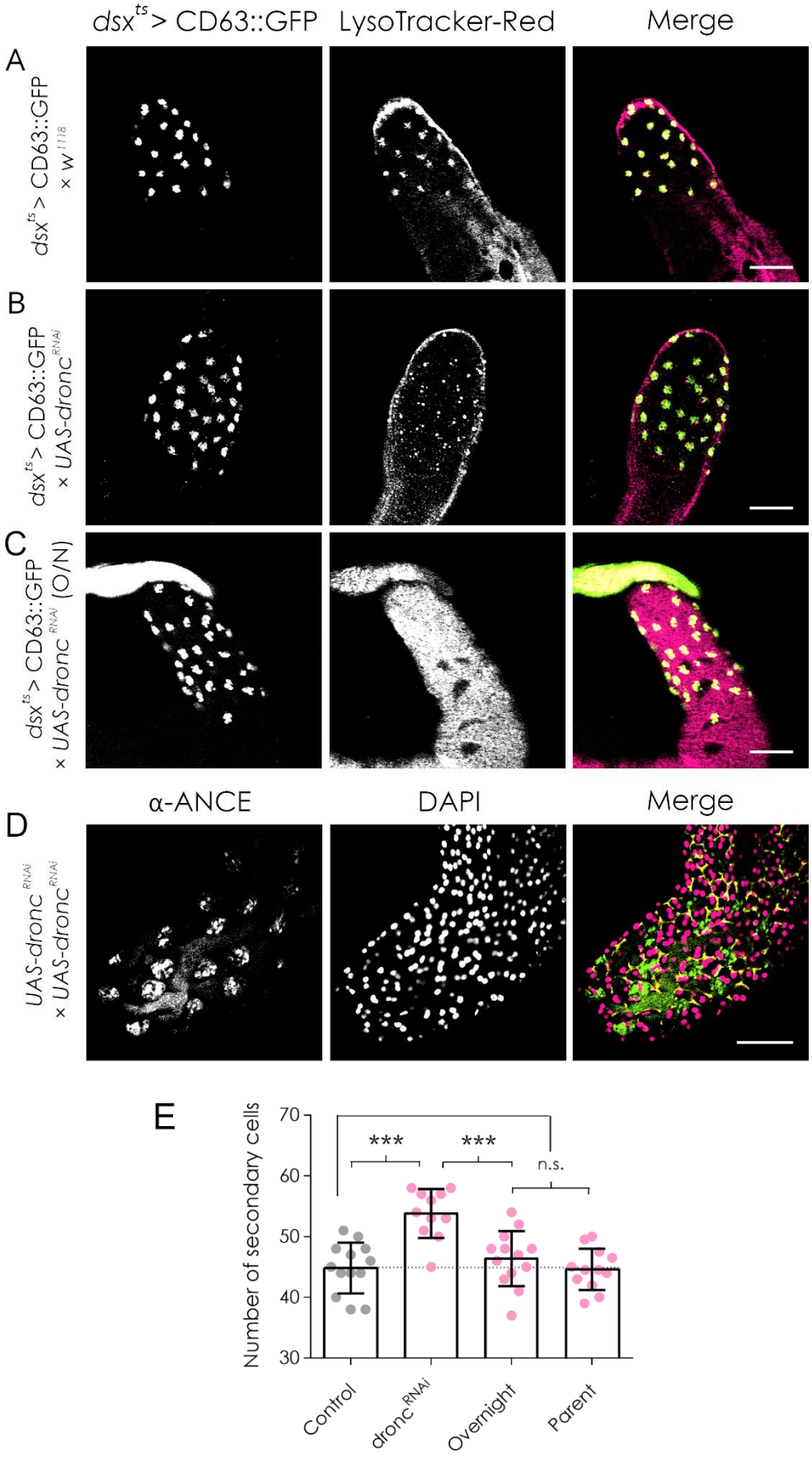

dsx^ts > CD63::GFP
LysoTracker-Red
Merge
A
dsx^ts > CD63::GFP × w^1118
B
dsx^ts > CD63::GFP × UAS-dronc^RNAi
C
dsx^ts > CD63::GFP × UAS-dronc^RNAi (O/N)
α-ANCE
DAPI
Merge
D
UAS-dronc^RNAi × UAS-dronc^RNAi
E
Number of secondary cells
70
60
50
40
30
***
***
n.s.
Control
dronc^RNAi
Overnight
Parent

FIGURE 3. 11. | DRONC REGULATES SECONDARY CELL NUMBERS.

Secondary cell numbers in the accessory gland were determined after RNAi of *dronc* was expressed in secondary cells for three days using dsx^{ts} > CD63::GFP, following a temperature shift to 28.5 °C at eclosion.

A: Expressing dsx^{ts} > CD63::GFP in control accessory glands labels secondary cells (green), which are obvious at the distal tip of the gland. Acidic compartments are labelled with LysoTracker-Red (magenta), which provides an outline of the accessory gland.

B: Accessory gland where secondary cells are expressing an inverted repeat that targets *dronc*.

C: Accessory gland where secondary cells have experienced an overnight (~16 hour) expression of the *dronc* RNAi construct. The structure that is evident above the accessory gland represents a part of the ejaculatory duct that has been bent into that position by the mounting procedure.

D: Accessory gland dissected from a male of the *dronc* dsRNA donor line and stained with ANCE antiserum to visualise secondary cells (green).

E: Bar chart plotting the number of secondary cells observed in each of the genotypes presented above.

Image panels **A–D** show representative accessory glands on day three post-eclosion. Scale bars in panels **A–C** represent 100 μm, whereas the scale bar in panel **D** represents 50 μm.

In chart **E**, each scatterplot dot represents the sample value of an individual accessory gland, each obtained from an individual male (see **SUPPLEMENTARY TABLE 3. 10.**, *p.* 426). The scatterplot bar represents the average value, and error bars show the standard deviation within each sample. Shapiro-Wilk testing shows that secondary cell numbers are normally distributed ($p < 0.01$), and these data were therefore analysed usinf parametric testing methods. Asterices represent *p*-values obtained by one-way ANOVA with Tukey's correction for multiple comparisons (all columns): *** $p < 0.0001$. Treatments that do not differ from the control are denoted by 'n.s.'.

The DRONC downstream effector, drICE, regulates intracellular compartment numbers in secondary cells

During apoptotic cell death, initiator caspases cleave downstream effector caspases to initiate an apoptotic cascade that culminates with the execution of cell death through the cleavage of key cellular targets (DORSTYN *et al.*, 1999a). DRONC is an initiator caspase, and the *D. melanogaster* interleukin-1 β-converting enzyme ("**drICE**") is a downstream effector caspase that is cleaved by DRONC in both apoptotic and non-apoptotic contexts (MEIER *et al.*, 2000; ARAMA *et al.*, 2003; DORSTYN & KUMAR, 2008). Having shown above that DRONC has a role regulating the number and composition of secondary cell intracellular compartments, I was interested to determine if drICE has a similar function.

FIGURE 3. 12. | THE EFFECTOR CASPASE, drICE, REGULATES SECONDARY CELL COMPARTMENT NUMBERS.

RNAi-mediated knockdown of *drice* was induced in secondary cells at eclosion using dsx^{ts} > CD63::GFP, and compartment numbers and their size was determined after six days at the non-permissive temperature.

A: Expressing dsx^{ts} > CD63::GFP in the accessory gland labels secondary cell intracellular compartments (green). LysoTracker-Red (magenta) labels acidic compartments.

B: Secondary cell expressing a *UAS-drice*^{RNAi} construct using dsx^{ts} > CD63::GFP.

C: Scatterplot bar chart plotting the number of CD63::GFP-positive intracellular compartments in control (grey) and *drice*^{RNAi}-expressing (green) secondary cells at day six post-eclosion.

D: Scatterplot bar chart plotting the volume of the largest Lysotracker-Red–positive compartments in control (grey) and secondary cells expressing *drice*^{RNAi} (red) at day six.

E: Scatterplot bar chart plotting the number of Lysotracker-Red–positive compartments in control (grey) and *drice*^{RNAi}-expressing (yellow) secondary cells at day six.

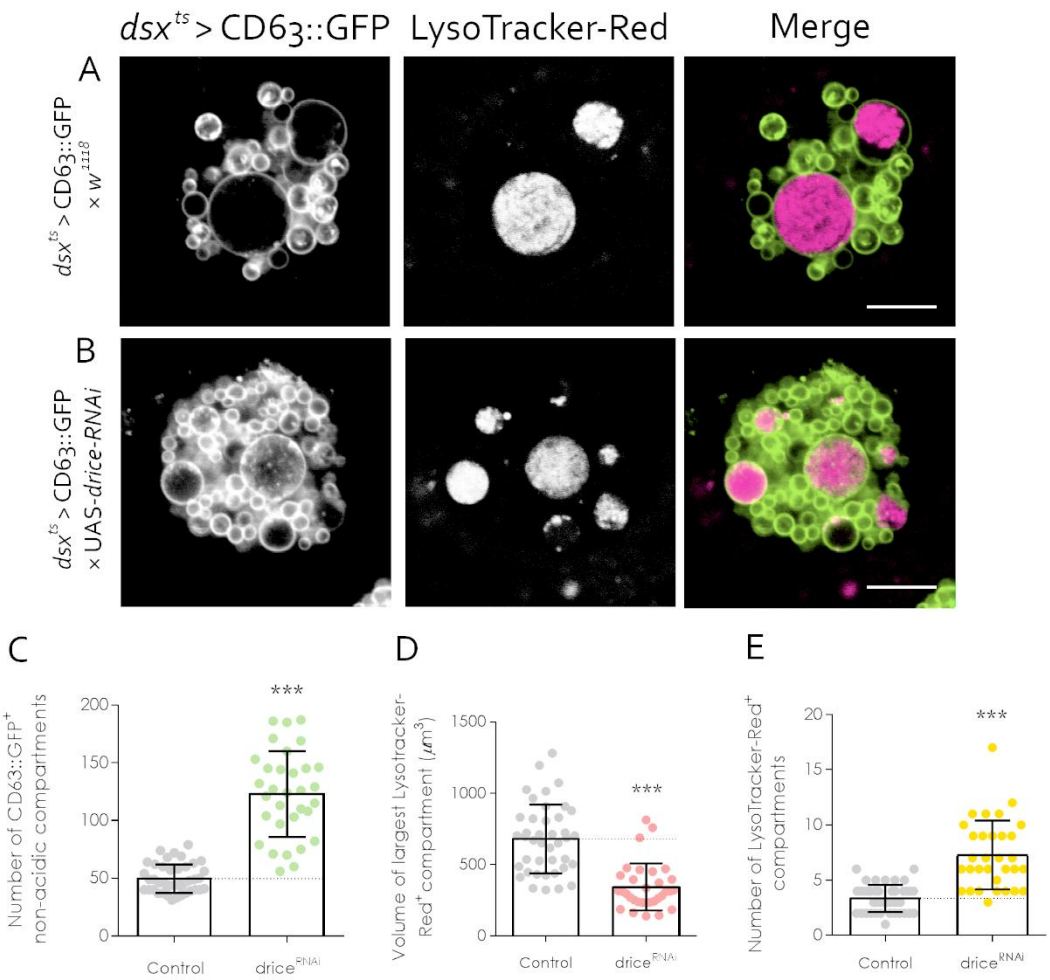


Image panels **A–B** show representative secondary cells on day six post-eclosion. Scale bars in both panels represent 10 μm.

In charts **C–E**, each scatterplot dot represents the sample value of an individual secondary cell, with three cells being quantified from each individual male (see **SUPPLEMENTARY TABLE 3. 11.**, *p.* 426). The scatterplot bar represents the average value across all individual secondary cells samples, and error bars show the standard deviation within the sample. In panels **C–E**, *p*-values were obtained by Kruskal-Wallis one-way ANOVA with Dunn's correction for multiple comparisons (all columns compared to control): *** < 0.0001. Treatments that are not different from controls are denoted by 'n.s.'.

N.B. The control in this figure is the same as shown in **FIGURE 3. 9.**, since the experiments were performed at the same time. This has been taken into account for the statistical analysis.

To determine if drICE has a role regulating secondary cell biology, I used dsx^{ts} CD63::GFP to express an inverted repeat that targets *drice* ($drice^{RNAi}$; DIETZL *et al.,* 2007) in secondary cells (dsx^{ts} CD63::GFP > $drice^{RNAi}$). Similar to when $dronc^{RNAi}$ is expressed in secondary cells, the expression of $drice^{RNAi}$ affected the growth of the largest acidic compartment in the cell; with its growth being much reduced compared to controls ($p < 0.0001$) (**FIGURE 3. 12. A–B, D**). The number of acidic compartments was also increased when $drice^{RNAi}$ is expressed ($p < 0.0001$) (**FIGURE 3. 12. A–B, E**). Similar to when the inverted repeat that targets *dronc* is expressed in secondary cells, RNAi of *drice* caused a significant increase in the numbers of non-acidic CD63::GFP-positive compartments ($p < 0.0001$) (**FIGURE 3. 12. A–C**). Altogether, this indicates that drICE might be a negative regulator of CD63::GFP-positive non-acidic compartment numbers and a positive regulator of acidic compartment growth (**SUPPLEMENTARY TABLE 3. 11.**, *p.* 426).

The apoptosome component, dARK, regulates secondary cell intracellular compartment numbers

Although traditionally associated with cell death, the apoptosome—a protein complex with a role in caspase capacitation—can also drive non-apoptotic caspase activation to regulate cellular processes such as spermatogenesis (ARAMA *et al.*, 2003; 2006; D'BROT *et al.*, 2013). The *D. melanogaster* APAF-1–related killer, dARK, is the main structural component of the fly apoptosome and associates with the DRONC N-terminal caspase-recruitment domain (CARD) to activate DRONC activity (RODRIGUEZ *et al.*, 1999; DORSTYN & KUMAR, 2008; YUAN *et al.*, 2011). Previously in this Chapter, I have shown that DRONC has a role in regulating intracellular compartment numbers, although the requirement for the

catalytic activation of DRONC remains ambiguous. To address the question of DRONC activity/capacitation from another angle, I decided to investigate if dARK is required for the regulation of intracellular trafficking in secondary cells; *i.e.* whether endogenous caspase activation is important for this process. To test this, I expressed a *dark*^{RNAi} construct (NI *et al.*, 2011) in secondary cells using *dsx*^{ts} CD63::GFP. However, contrary to what would be expected if dARK is required for caspase activation, the number of CD63::GFP-positive compartments seemed to be reduced in secondary cells expressing *dark*^{RNAi} ($p < 0.0001$) (**FIGURE 3. 13. B–C**). However, both the number of acidic compartments and lysosomal volume remained unchanged by this treatment ($p > 0.1$) (**FIGURE 3. 13. B, D–E**). Altogether, these results (**SUPPLEMENTARY TABLE 3. 12.**, *p.* 427) suggest that dARK might have a role as a positive regulator of CD63::GFP-positive secretory compartment numbers, but with no major effect on lysosomal growth.

Role for caspase inhibitors in the regulation of secondary cell intracellular trafficking

The *Drosophila* inhibitor of apoptosis protein 1 (dIAP1) regulates DRONC protein levels and is crucial for cell survival (HAY *et al.*, 1995; MURO *et al.,* 2002). dIAP1 is an E3-ubiquitin ligase that normally binds to and inhibits active caspases through ubiquitination, but which is also capable of ubiquitinating inactive DRONC and drICE zymogens. This further inhibits their pro-apoptotic activities, to promote their stability and keep them in a catalytically inactive state (LEE *et al.,* 2011). Having found a role for the apoptosome component, dARK, in the regulation of intracellular trafficking in secondary cells (but with the requirement for DRONC catalytic activity remaining ambiguous), I decided to overexpress dIAP1 in secondary

cells (*UAS-diap1*; GUO *et al.*, 2004) using dsx^{ts} CD63::GFP (dsx^{ts} CD63::GFP > *diap1*). If overexpression of dIAP1 stabilises the non-catalytic form of DRONC and drICE, this treatment might have the same effect on intracellular trafficking as the catalytically inactive form of DRONC (*i.e.* no effect) (*p.* 95).

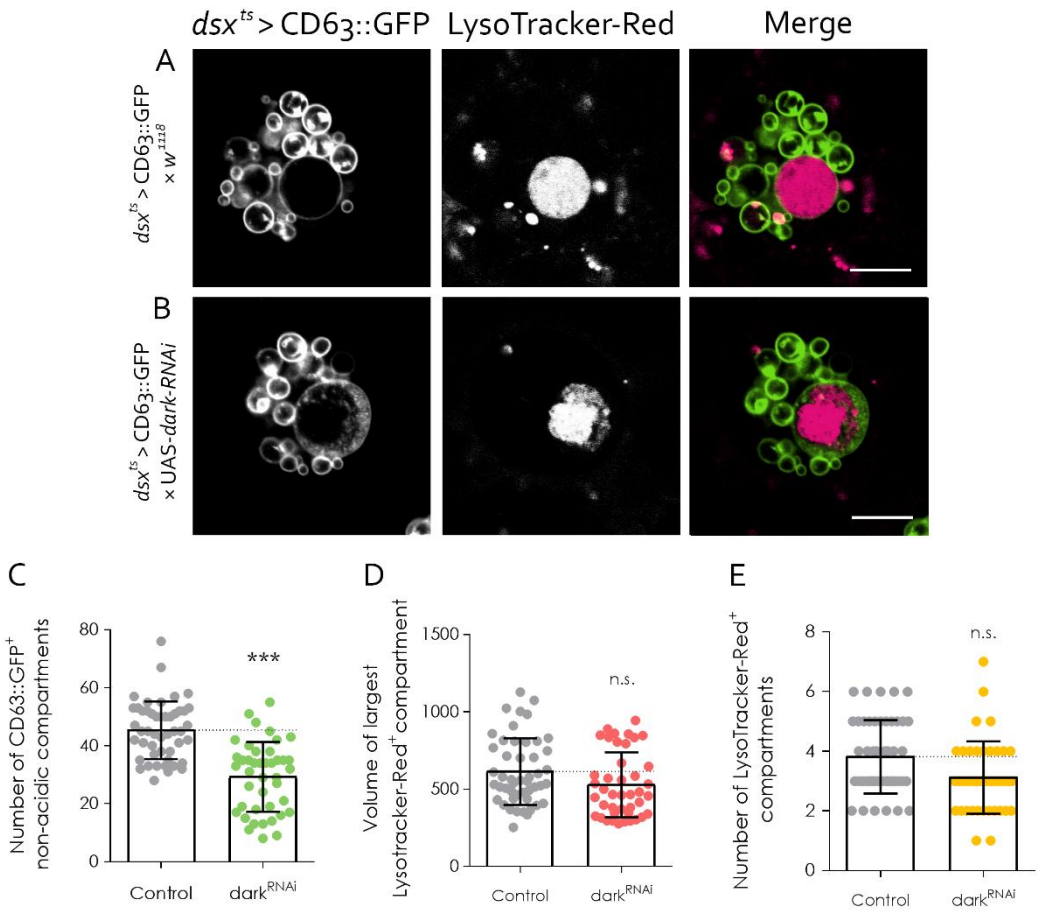
Conversely, if DRONC regulates trafficking catalytically (*viz.* DRONC is normally catalytically active and dIAP1 inhibits this), dIAP1 might be expected to have the same effect on intracellular trafficking as the expression of the *dronc* hairpin loop (*p.* 88). Contrary to both expectations, *diap1* expression in secondary cells *decreased* the number of non-acidic CD63::GFP-positive compartments ($p < 0.0001$) (**FIGURE 3. 14. A–C**). In addition, *diap1* overexpression also had no effect on either the number of acidic compartments, or the volume of the largest one ($p > 0.1$) (**FIGURE 3. 14. A–C**). All these effects were similar to those observed when *dark* was knocked down (**FIGURE 3. 14. C**). These results (**SUPPLEMENTARY TABLE 3. 13.**, *p.* 427) suggest that dIAP1, like dARK, might be a positive regulator of CD63::GFP-positive non-acidic compartment numbers, but with no major effect on the acidic compartment complement.

FIGURE 3. 13. | THE APOPTOSOME COMPONENT, dARK, REGULATES INTRACELLULAR TRAFFICKING.

RNAi-mediated knockdown of *dark* was induced in secondary cells at eclosion using dsx^{ts} > CD63::GFP, and compartment numbers and their size was determined after six days at the non-permissive temperature.

A: Expressing dsx^{ts} > CD63::GFP in the accessory gland labels secondary cell intracellular compartments (green). LysoTracker-Red (magenta) labels acidic compartments.

B: Secondary cell expressing a *UAS-dark*^{RNAi} construct using dsx^{ts} > CD63::GFP.



C: Scatterplot bar chart plotting the number of CD63::GFP-positive intracellular compartments in control (grey) and *dark*^{RNAi}-expressing (green) secondary cells at day six post-eclosion.

D: Scatterplot bar chart plotting the volume of the largest Lysotracker-Red–positive compartments in control (grey) and secondary cells expressing *dark*^{RNAi} (red) at day six.

E: Scatterplot bar chart plotting the number of Lysotracker-Red–positive compartments in control (grey) and *dark*^{RNAi}-expressing (yellow) secondary cells at day six. Each scatterplot dot corresponds to data obtained from an individual secondary cell, with three cells quantified from each individual male (see **SUPPLEMENTARY TABLE 3. 12.**, *p.* 427).

Image panels **A–B** show representative secondary cells on day six post-eclosion. Scale bars in both panels represent 10 μm. In panels **C–E**, *p*-values were obtained by Kruskal-Wallis one-way ANOVA with Dunn's correction for multiple comparisons (all columns compared to control): *** < 0.0001. Treatments that are not different from controls are denoted by 'n.s.'.

DISCUSSION

Caspases were initially described as active participants in the execution of cell death. Contemporary understanding of caspase function is more nuanced; recognising caspases as important regulators also of non-apoptotic cell biology that are involved in diverse cellular processes such as cell remodelling and cellular differentiation (CRAWFORD & WELLS, 2011; HYMAN & YUAN, 2012; GALLUZZI *et al.,* 2012). Our collaborator, Dr. Baena-Lopez, made the initial discovery that the *D. melanogaster* initiator caspase, *dronc*, is expressed in the secondary cells of the male accessory gland. Contributing to this exciting discovery, I have investigated the regulation of *dronc* in more detail. The work presented in this Chapter indicates that caspases can function non-apoptotically to regulate secondary cell biology in the male *D. melanogaster* accessory gland, and that they can also affect their survival.

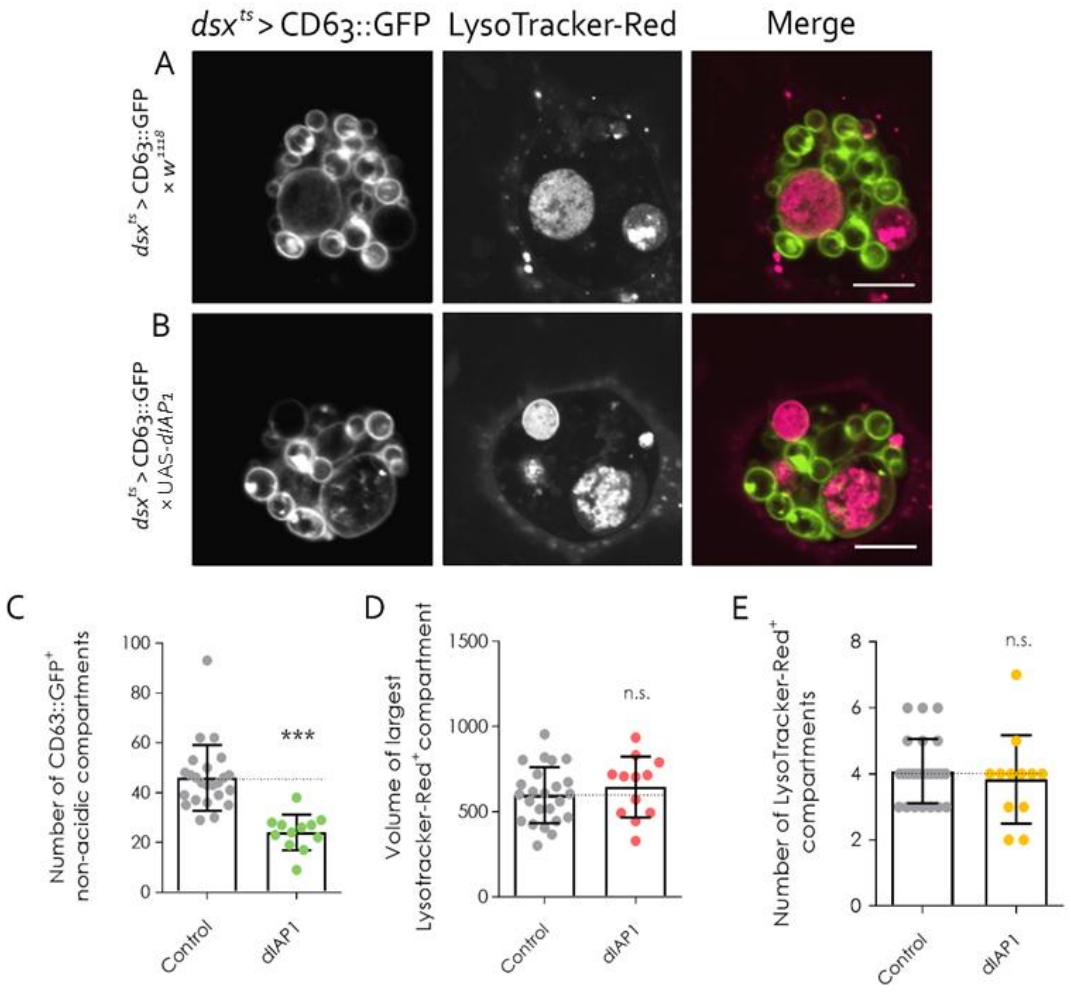
FIGURE 3. 14. | THE CASPASE INHIBITOR, dIAP1, REGULATES INTRACELLULAR TRAFFICKING.

UAS-diap1 was expressed in secondary cells using dsx^{ts} > CD63::GFP, following a temperature shift upon eclosion. The effects on compartment number and size was assessed at six days.

A: Expressing dsx^{ts} > CD63::GFP in the accessory gland labels secondary cell intracellular compartments (green). LysoTracker-Red (magenta) labels acidic compartments.

B: Secondary cell expressing *UAS-diap1* using dsx^{ts} > CD63::GFP.

C: Scatterplot bar chart plotting the number of CD63::GFP-positive intracellular compartments in control (grey) and *diap1*-expressing (green) secondary cells at day six post-eclosion.



D: Scatterplot bar chart plotting the volume of the largest LysoTracker-Red–positive compartments in control (grey) and secondary cells expressing *diap1* (red) at day six.

E: Scatterplot bar chart plotting the number of LysoTracker-Red–positive compartments in control (grey) and *diap1*-expressing (yellow) secondary cells at day six. Each scatterplot dot corresponds to data obtained from an individual secondary cell, with three cells quantified from each individual male (see **SUPPLEMENTARY TABLE 3. 13.**, *p.* 427).

Image panels **A–B** show representative secondary cells on day six post-eclosion. Scale bars in both panels represent 10 μm. In charts **C–E**, *p*-values were obtained by a Mann-Witney U test: *** < 0.0001. Treatments that are not different from controls are denoted by 'n.s.'.

The initiator caspase, DRONC, is expressed in the male accessory gland and is regulated by a BMP/EcR signalling axis

The *D. melanogaster* male accessory gland is steroid-sensitive, and it has been shown previously that the EcR regulates the expression of *dronc* in other fly tissues that are sensitive to ecdysone (CAKOUROS *et al.,* 2004). The EcR itself is a potent regulator of secondary cell biology, and it appears to be regulated by BMP signalling through a novel, post-transcriptional mechanism to form a BMP/EcR signalling axis (LEIBLICH, 2015). Work in this Chapter has established that the BMP/EcR axis is capable of regulating the expression of *dronc* in secondary cells. This suggests that the BMP/EcR signalling axis regulates the expression of *dronc* in secondary cells, and *dronc* represents the first EcR target gene to be identified in this cell type.

In addition to finding that activity from the *dronc* expression reporter decreases in the presence of an inverted repeat that targets the *EcR* transcript, I have also shown that expressing a synthetic isoform of the EcR (EcR-C) is sufficient to abolish the expression of *dronc* in secondary cells. The EcR-C isoform lacks the EcR N-terminal domain that confers the activation function that is specific to each isoform (AF1); only retaining the EcR DNA-binding domain and the hormone-dependent activator functions that are conserved across EcR isoforms (AF2). That the EcR-C cannot drive the expression of the *dronc* expression reporter in secondary cells therefore suggests that the hormone- and DNA-binding activities of the EcR alone are insufficient to drive expression of *dronc* in secondary cells, and that the isoform-specific activator function of endogenous EcR isoforms is required for the expression of *dronc*. Like other nuclear-hormone receptors, the EcR can function as both a repressor and activator of promoter activity (CHERBAS *et al.,* 1993), and it is feasible that the EcR-C might function as a repressor of *dronc* transcription in

secondary cells even in the presence of its usual activator, the steroid hormone ecdysone. Unfortunately, experiments to further address this question using the *dronc*^{fs} reporter were unsuccessful due to the low number of *UAS*-constructs that is tolerated by the driver, and any resolution will therefore require inquiry using a different system.

Different EcR isoforms are expressed in different tissues, where they help confer differential tissue-level responses to the presence of ecdsyone. TALBOT *et al.* (1993) showed that the EcR-A isoform is enriched in the imaginal tissues of the larva, whereas the EcR-B1 isoform is enriched in larval tissues that will undergo programmed cell death during metamorphosis. As the initiator caspase, DRONC is an active participant in this process, and is stereotypically expressed in tissues that contain the EcR-B1 isoform (CAKOUROS *et al.,* 2004). Previous work in our laboratory identified the EcR-B1 isoform as the main EcR isoform in secondary cells (LEIBLICH, 2015)—consistent with the expression of *dronc* in this cell type. However, the male accessory gland develops as an imaginal structure in the *D. melanogaster* larva, and would be expected to contain the EcR-A isoform during development, which would not typically be associated with the expression of *dronc*. So, how do we reconcile this discrepancy? It is possible that the EcR isoform complement changes in the accessory gland throughout development, and, indeed, SHARMA *et al.* (2017) have recently proposed that the EcR is a survival factor in the accessory gland, as the expression of a dominant-negative form of the EcR-A isoform during development causes dramatic cell death of the epithelial tissues in the male accessory gland. This suggests that the EcR-A might be required in the accessory gland precursor tissues to protect them from cell death during the steroid hormone pulses that occur during metamorphosis, and only thereafter acquire a

different complement of EcR isoforms, with the EcR-B1 becoming specifically dominant in the secondary cells. To determine if this is the case, a more detailed investigation of EcR isoform expression- and staining patterns throughout development would need to be undertaken in the accessory gland. This has potentially interesting implications for how the regulation of a steroid hormone-responsive organ changes during development. In addition, an altered complement of EcR isoforms in immature and mature male accessory glands might also help explain how the function of the organ is so different between its developmental stages; with the male accessory gland not producing protein until late in metamorphosis/soon after eclosion. Because of its role regulating secondary cell compartment numbers, DRONC might be an example of an adult-specific gene product. It would be interesting to determine if the onset of *dronc* expression in the accessory gland coincides with the onset of adult secondary cell functions—such as secretion.

Together with Siamak Redhai (in work published in REDHAI *et al.,* 2016), he and I have shown that BMP signalling in the secondary cells is activated upon mating. This signalling activates the BMP/EcR signalling axis (see LEIBLICH, 2015; and CHAPTER 5). In this Chapter, I have provided evidence that activating BMP signalling can increase the number of secondary cells expressing the *dronc* reporter—with the caveat that the nature of the reporter (as a *dronc*-GAL4 construct) complicates the interpretation of my experiments. Having established that the BMP/EcR axis regulates the expression of *dronc* in secondary cells, my results suggest the intriguing possibility that *dronc* expression levels respond to the mating status of the male fly. It would be interesting to determine if any such increase in *dronc* expression can be seen when using a multiple mating protocol in conjunction

with the *dronc* expression reporter. For these experiments, it will be important to develop a *dronc* reporter that does not express GAL4—so other transgenes can be manipulated in secondary cells using an independent GAL4-driver.

Activity from the *dronc* expression reporter and what it might mean for our understanding of secondary cell biology

Levels of *dronc* reporter activity appear to not change much over the first days of adulthood—presumably reflecting the expression pattern of *dronc* itself. However, it will not have escaped notice that the proportion of secondary cells showing activation of the *dronc* expression reporter remains low throughout the time-points assayed in this Chapter; an observation that merits further attention of its own. As part of the work presented in this Chapter, I have shown that secondary cells remain sensitive to ectopic expression of *dronc*; with overexpression of wild-type DRONC being sufficient to eliminate secondary cells from the accessory gland epithelium, and with expression of a catalytically capacitated form of DRONC being sufficient to reduce the proportion of healthy-looking secondary cells. Yet, secondary cells do express *dronc* endogenously, and DRONC seems to be required for the normal function of all secondary cells. Altogether, these results imply that levels of DRONC protein might be tightly controlled within secondary cells—ensuring that they are neither too low, nor too high, for cell function to be optimised. That only a subset of secondary cells are positive for *dronc* reporter activity at any given time suggests that *dronc* gene expression might occur either in intermittent 'bursts', or that the reporter construct does not reflect the normal patterns of *dronc* gene expression.

Levels of DRONC protein are normally tightly regulated in cells, as a result of continuous protein turnover (WILSON, GOYAL & DITZEL *et al.,* 2002). This kind

of post-translational protein turnover does however not explain the low (transcriptional) activity of the expression reporter. The *dronc* reporter itself is formed by a regulatory *dronc*-GAL4 construct that has been genetically combined with a *UAS*-regulated fluorescent histone marker (A. BAENA-LOPEZ, unpublished). When the *dronc* promoter is active, GAL4 will increase the production of a RFP-tagged histone protein—resulting in the formation of fluorescent nucleosomes. However, DNA is very dynamic, and in addition to 'breathing', nucleosome remodelling occurs in response to promoter melting and gene transcriptional activity, and it has been estimated that nucleosomes in active genes turn over multiple times during each cell cycle in cultured *D. melanogaster* S2 cells (DEAL *et al.,* 2010). Although it is unlikely that the entire nuclear genome is active in *D. melanogaster* secondary cells, it is plausible that histone turnover might affect the detection of a fluorescent nucleosomal marker. This line of reasoning might provide further support for the 'burst' hypothesis of *dronc* promoter activity in secondary cells; since continuous activity from the *dronc* promoter would result in a continuous supply of RFP-tagged histone protein to replenish the histone pools turned over as part of normal nuclear activities. Therefore, although further work (*e.g.* quantitative reverse-transcription PCR; "**qRT-PCR**") will be needed to firmly establish how the activity from the *dronc* promoter changes over time in secondary cells, I believe that the evidence presented in this Chapter supports the 'burst' hypothesis, and that *dronc* protein might be produced 'on demand' in secondary cells—and then rapidly turned over, perhaps in response to BMP/EcR axis activation.

The reader might recall that more secondary cells seem to be present in the male accessory gland after RNAi of *dronc*. Although it remains to be determined how the number of secondary cells changes during the time around eclosion (from before

secretion begins, to after the gland has matured fully), it seems likely that some secondary cells are lost as the accessory gland matures, and that DRONC might have a role in this process. The activity of the EcR (on its own, or in conjunction with BMP signalling) might also be important—and it will be interesting to determine how the signalling axis and its components behave at this critical time. Interesting in this context, the proportion of secondary cells positive for *dronc* reporter activity increases after BMP signalling activation. Working on such glands, I have observed that a subset of secondary cells appear 'refractionally' different when viewed through differential interference contrast ("**DIC**") Nomarski microscopy (APPENDIX 2, *p.* 428). This kind of refractionally-different cell can be indicative of cell death, and DIC microscopy can be used to reliably detect dead cells ('cell corpses') in living tissue (GUMIENNY *et al.,* 1999). It would be interesting to quantitatively employ this method in the accessory gland, perhaps in combination with antibody staining for the presence of activated caspases (*i.e.* activated caspase-3) or RNAi of *dronc*, to determine to what extent activated BMP signalling might reduce the viability of secondary cells and if this happens via a BMP/EcR/DRONC regulatory axis.

It appears as if DRONC protein levels might be tightly regulated in secondary cells, because they are sensitive to elevated levels of the caspase. If *dronc* expression levels are elevated as the result of normal BMP signalling activity (as we have seen), it is possible that BMP signalling activation represents a form of 'antagonistic pleiotropy' (WILLIAMS, 1957) in secondary cells; where BMP signalling activation is not only required for the normal function of the cells—but might also have deleterious consequences that reduce secondary cell viability. Indeed, secondary cell viability (as evidenced by an increase in the proportion of refractionally-different

cells) does seems to be reduced after mating in wild-type accessory glands (when we know that BMP signalling is activated; REDHAI *et al.,* 2016), but not when the BMP inhibitor, *Dad*, is overexpressed (J. HELLBERG & C. WILSON, unpublished observations; see APPENDIX 2, *p.* 428). Although these observations require quantitative corroboration, another suggestive observation is that BMP signalling activation (through either mating or tkv^{Q253D} expression) can cause secondary cells to delaminate from the accessory gland epithelium (LEIBLICH *et al.*, 2012; LEIBLICH, 2015). Typically, these delaminated cells contain granular cytoplasm and signs of active JNK (stress) signalling (APPENDIX 2, *p.* 428). This suggests that activated BMP signalling correlates with signs of cellular stress, although the causality remains to be established. Yet, it might be interesting to examine this BMP-viability relationship in more detail, since a new theory of ageing (the 'hyperfunction theory of ageing') proposes that the age-related functional decline of cells and tissues might be the inevitable result of normal cellular functions (BLAGOSKLONNY, 2013).

Caspases have a role regulating the number of secondary cell secretory compartments and the size of the largest endolysosomal compartment

DRONC seems to affect the size and number of intracellular compartments labelled by both the CD63::GFP and GPI::GFP membrane markers in secondary cells. DRONC also appears to reduce the size of the largest acidic compartment. To avoid potentially confounding effects, these results need to be confirmed in cells lacking membrane markers—for example through the use of antibodies specific to these compartments (*e.g.* α-ANCE (RYLETT & WALKER, 2007), or α-chaoptin (FUJITA *et al.,* 1982; M. WAINWRIGHT & C. WILSON, unpublished observations)).

Circumstantial evidence that also might be of interest in this context involves the EcR itself: where expression of the EcR-C (which, as you might recall, abolishes *dronc* reporter activity) causes numbers of secondary cell exocytic compartments to increase (J. HELLBERG & A. LEIBLICH, unpublished observations)—a phenotype very similar to that observed when *dronc* itself is knocked-down. Altogether, the evidence I have presented so far suggests that DRONC has a role in regulating compartment numbers in secondary cells—and that the BMP/EcR axis also participates in this process. Knockdown of the DRONC downstream effector caspase, drICE, has similar effects; suggesting that secondary cell compartment numbers might be co-ordinated through non-apoptotic caspase interactions. Yet, it is important to emphasise that *dronc* does not appear to be involved in all of the secondary cell functions regulated by the BMP/EcR signalling axis. For example, I found that manipulating *dronc* and *drICE* does not appear to have an effect on nuclear growth (not shown); a key cell biological process regulated by the BMP/EcR (LEIBLICH *et al.*, 2012; LEIBLICH, 2015). This might explain why the EcR-C—which otherwise promotes growth (LEIBLICH, 2015)—seems to suppress *dronc*; suggesting that the properties of the EcR required to drive growth can be de-coupled from the properties required to regulate *dronc*.

That DRONC appears to have a role regulating non-acidic compartment numbers suggests at least two possible modes of action for the caspase:

a) DRONC could have a negative role in the biogenesis of secretory compartments (DRONC inhibits their accumulation); or,
b) DRONC regulates the trafficking of these compartments within the cell (DRONC promotes their mobility).

Based on the results presented in this Chapter, I believe mechanism (b) provides at least part of the explanation—particularly when considered alongside failure of the largest acidic compartment to grow when *dronc* is lost from the cell, since non-acidic compartments have previously been observed to fuse with acidic compartments (CORRIGAN *et al.,* 2014). In some cell types, compartments carrying secretory granules can be selectively targeted for degradation—to avoid the build-up of secreted components during period of low demand and/or to avoid the premature activation of their precursor enzyme (zymogen) contents (VACCARO, 2012). Recent work in our laboratory suggests that secondary cells might use 'zymophagy' to regulate their secretory output (KROEGER, 2017). If such autophagy of secretory granules (and the exocytic compartments that carry them) is responsible for the growth of the acidic compartments in secondary cells, it is plausible that any intervention that might prevent this trafficking from happening (such as losing DRONC activity) might also reduce the growth of the acidic compartment. However, it should be noted that my work does not entirely exclude the possibility that these effects result from an apoptotic role for DRONC, and this would need to be confirmed through future work.

DRONC protein abundance, rather than its catalytic activity, might be more important for secretory compartment regulation

Expression of an activated form of DRONC that lacks its inhibitory N-terminal prodomain reduces non-acidic compartment numbers and might indicate that DRONC regulates intracellular trafficking *catalytically*. However, as I have also shown in this Chapter, activated DRONC (or just overexpression of the wild-type protein) has an adverse effect on secondary cell viability—which might have confounding

effects on the intracellular trafficking in secondary cells. Conversely, the expression of dominant-negative catalytically inactive form of DRONC causes an early increase in non-acidic compartment numbers. However, unlike in the *dronc* knock-down, this increase is not maintained; suggesting that DRONC potentially regulates some aspects of secondary cell biology non-catalytically and that the *presence* of the caspase itself is more important. Indeed, there is evidence demonstrating that DRONC is capable of processing its downstream effector drICE even when not catalytically capacitated (DORSTYN & KUMAR, 2008). In addition, there are several different cleavage forms of DRONC—of which only some are associated with cell death (MURO *et al.,* 2004). Therefore, it is possible that secondary cells maintain DRONC in an active, but non-apoptotic, form that allows it to act as a regulator of compartment numbers (via, or alongside, drICE)—without compromising secondary cell viability. (In the absence of staining for the activity of activated caspases, an apoptotic role for these can however not be completely ruled out.)

DRONC is capable of catalysing its own catalytic activation, and it has been suggested that this activation might occur in a concentration-dependent manner (DORSTYN & KUMAR, 2008). Overexpression of *dronc* and expression of an activated form of DRONC are both sufficient to reduce secondary cell viability—suggesting that levels of DRONC protein are normally tightly regulated in secondary cells to prevent ectopic caspase activation. This idea is supported by my analysis of the *dronc* reporter line. One candidate for regulating DRONC protein levels is the *Drosophila* inhibitor of apoptosis protein 1 (dIAP1) (HAY *et al*., 1995). As mentioned above, dIAP1 is an E3-ubiquitin ligase that normally binds to and inhibits active caspases, but which is also capable of promoting the stability of inactive DRONC and drICE zymogens to inhibit their pro-apoptotic activities (LEE *et al.,* 2011).

Overexpression of dIAP1 reduces non-acidic compartment numbers, and also has no effect on the growth of the largest acidic compartment. This is potentially consistent with a model where the presence of DRONC is more important than the catalytic nature of its activity. To wit, potentially increased levels of catalytically inactive DRONC (as a result of dIAP1-mediated stabilisation) could be causing non-acidic compartment numbers to decrease—the opposite effect to *dronc* knock-down.

The activity of the *D. melanogaster* apoptosome is normally required for the activity of DRONC and drICE—in both apoptotic and non-apoptotic contexts (RODRUGUEZ *et al.,* 1999; ARAMA *et al.,* 2006; D'BROT *et al.,* 2013). Since DRONC appears to be a positive regulator of compartment numbers, dARK (the main apoptosome structural component) might have been expected to behave similarly—especially since dARK is required for the activation of DRONC (QUINN *et al.,* 2000). However, *dARK* knock-down had the opposite effect on non-acididc compartment numbers, compared to knock-down of *dronc* and *drICE*. In this regard, dARK has also been shown to engage in a mutually antagonistic relationship with DRONC: where activated DRONC cleaves dARK to prevent further caspase activation (AKDEMIR *et al.,* 2006), and where dARK promotes the degradation of DRONC (SHAPIRO *et al.,* 2008). Indeed, in the absence of dARK, the DRONC zymogen has been shown to accumulate to high levels (MURO *et al.*, 2002). This might partly explain the effects of *dark* knock-down in secondary cells—although the absence of size changes in the acidic compartments of knock-down cells suggest that the trafficking into these compartments is not detectably affected.

Tangentially, there are reports in the literature of non-apoptotic apoptosome and dARK/APAF1 functions, such as in sperm individualisation (HUH *et al.,* 2003) and DNA-damage checkpoint inhibition (ZERMATI & MOUHAMAD *et al.,* 2007).

For example, the transport and Golgi organisation ("**TANGO**") gene, *TANGO7*, was originally identified as a regulator of ER-to-Golgi protein trafficking (BARD *et al.,* 2006), but has since been proposed to also act as a regulator of non-apoptotic apoptosome function (D'BROT *et al.,* 2013). Interestingly, TANGO7 has a role promoting the accumulation of DRONC protein in S2 cells (CHEW *et al.,* 2009)—providing an attractive possibility to further probe the role of DRONC accumulation in secondary cells through study of TANGO7. A *TANGO7* gene trap is available (SAROV *et al.,* 2016), and, using this, I have determined that TANGO7 is present at high levels in both main cells and secondary cells (see APPENDIX 3, *p.* 434). High levels of *TANGO7* expression in the male accessory gland is in agreement with transcriptome data (CHINTAPALLI *et al.*, 2007), and makes me believe that the role of this molecule in secondary cell biology merits further investigation—for example by using the *TANGO7* RNAi construct that is available (NI *et al.,* 2010). By combining knock-down of different genes in the caspase regulatory network, it should be possible to determine whether they function together to control secondary cell compartments, or whether they have independent roles that merit further characterisation.

Do caspases regulate secretion from secondary cells by mobilising the actin cytoskeleton?

Secondary cells are specialised secretory cells. Through my work, I have provided some evidence to suggest that DRONC might have a role regulating the elimination of secretory compartments from secondary cells; suggesting the intriguing possibility that what DRONC regulates is aspects of secondary cell *secretion*. But what is the mechanism by which this potential regulation might be orchestrated?

Eukaryotic cells contain an extensive, fibrillar, intracellular scaffolding network that supports the three-dimensional shape of the cell, and help govern its behaviour (ALBERTS *et al.,* 2002). This intracellular meshwork constitutes the cytoskeleton. Despite the rigidity implied by the name, the cytoskeleton is a highly dynamic structure; being constantly dis- and re-assembled to facilitate cell functions; allowing the cell to extend protrusions to interact with its environment, and providing the networked substrate upon which intracellular trafficking takes place (ALBERTS *et al.,* 2002). In mammalian *β*-cells, the release of secretory granules containing the pro-hormone, insulin, is negatively regulated by the actin cytoskeleton—which acts as an internal barrier to prevent the recruitment of secretory compartments to the cell plasma membrane (ORCI *et al.,* 1972; LI *et al.,* 1994). Similarly, secretion from resting acinar cells in the rat parotoid and submandibular salivary glands is inhibited by actin-containing microfilaments that coat the luminal plasma membrane; forming a barrier that prevents secretory vesicle fusion (SEGAWA & YAMASHIMA, 1989). More recently, both TRAN *et al.* (2015) and ROUSSO *et al.* (2016) have shown that glue secretion from the cells in the *D. melanogaster* larval salivary gland requires a similar clearance of apical actin before exocytic compartment fusion to the plasma membrane can occur; showing that secretion requires modification to the actin cytoskeleton in animals as diverse as mammals and flies.

Intriguingly, actin has previously been shown to be a caspase substrate; being cleaved by the mammalian drICE homologue, ICE (MASHIMA *et al.,* 1997). Consistent with this, dIAP1 has been shown to promote the stability of the actin cytoskeleton and its assembly (GEISBRECHT & MONTELL, 2004; OSHIMA *et al.,* 2006). In view of this, it is possible that DRONC regulates intracellular trafficking in secondary cells, at least partly, by acting to mobilise secretory

compartments through non-apoptotic regulation of the actin cytoskeleton (**FIGURE 3. 15.**). However, whether manipulating the activity or availability of DRONC or drICE in secondary cells actually affects the release of secondary cell-specific proteins into the accessory gland lumen is something that remains to be established. Initial steps to investigate this possibility would involve characterising the role of caspase-mediated secretion in secondary cells, and how actin cytoskeleton dynamics contribute to this process. One simple initial avenue of investigation would include looking at the effect of knocking-down the RAS homolog ("**RHO**") protein 1, ("**RHO1**"), in secondary cells—to see how this treatment affects secondary cell intracellular compartment numbers and secretion. This is because *Rho1* manipulations have been shown to affect the size of the salivary gland lumen in larval *D. melanogaster*, by affecting actin accumulation at the salivary gland cell apical plasma membrane (XU *et al.,* 2011). Unfortunately, actin visualisations in secondary cells have been marred by a failure of common actin stains (*e.g.* phalloidin (WIELAND & GOVINDAN, 1974) and Life-Act Red (RIEDL *et al.*, 2008)) to satisfactorily label the secondary cell interior (A. LEIBLICH, M. WAINWRIGHT & I. STEFANA, personal comments). Additional future work that will be important to carry out will be to rule out an apoptotic role for DRONC and related caspases in the regulation of secondary cell biology. For example, it would be particularly interesting to differentiate between possible apoptotic roles for the caspase during the maturation of the male accessory gland (to regulate secondary cell numbers), and potentially non-apoptotic roles in the mature gland (to regulate secondary cell biology).

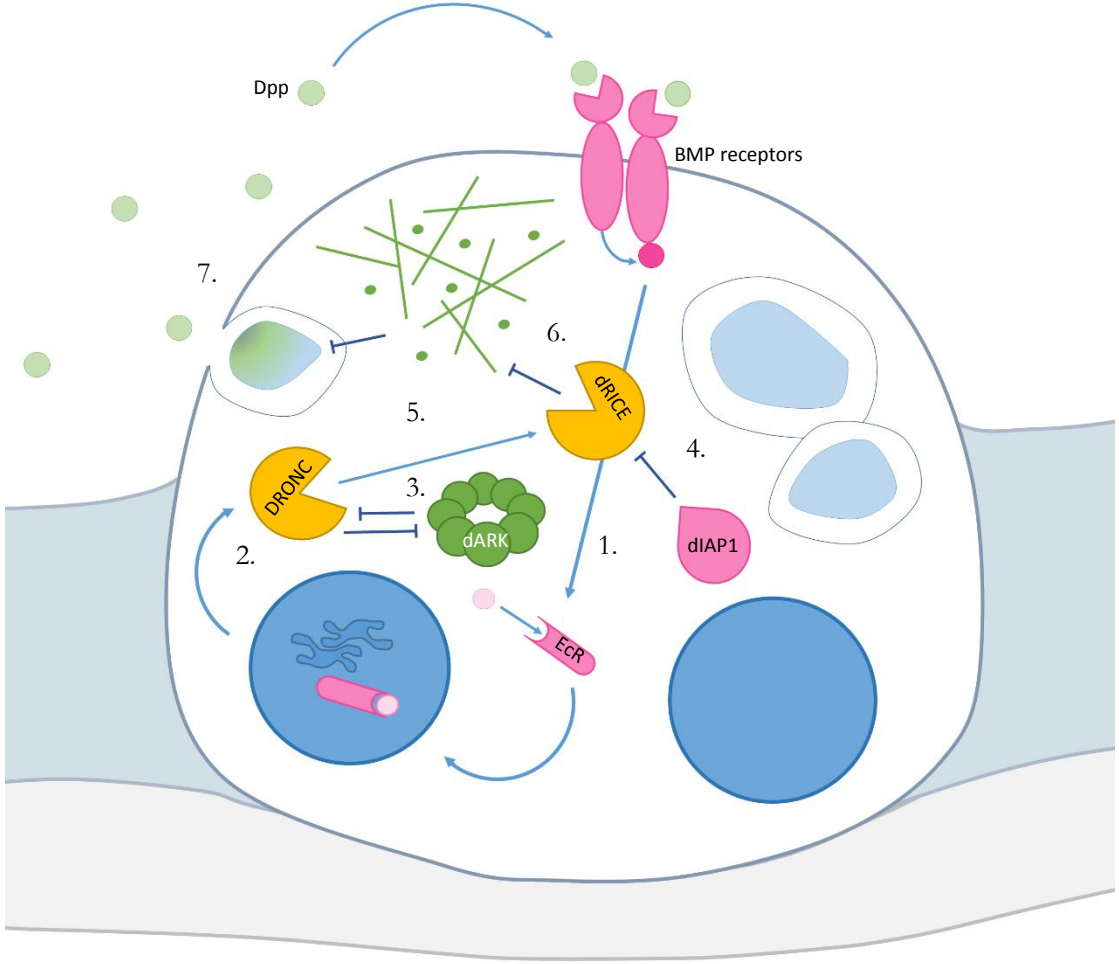
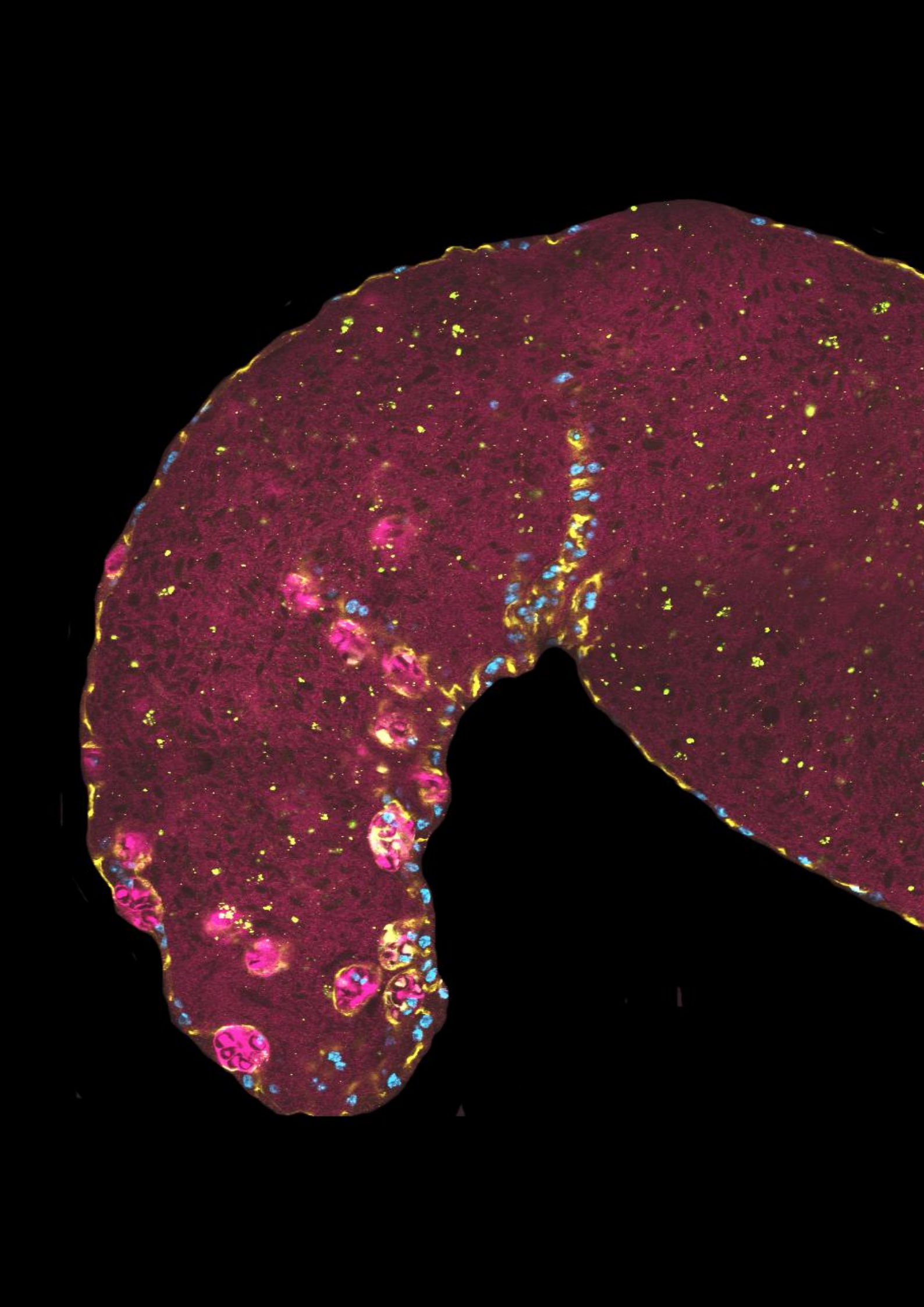


FIGURE 3. 15. | A HYPOTHETICAL MODEL OF HOW NON-APOPTOTIC CASPASE FUNCTION MIGHT REGULATE INTRACELLULAR TRAFFICKING IN SECONDARY CELLS.

Activated BMP signalling stabilises the EcR (1.) to regulate the transcription of the caspases, DRONC and drICE (2.). DRONC is engaged in a mutually antagonistic relationship with the apoptosome component, dARK (3.), and the caspase inhibitor, dIAP1, also negatively regulates caspase function (4.). As an initiator caspase, DRONC regulates the activity of the effector caspase, drICE (5.) and collectively, the caspases negatively regulate the actin cytoskeleton (green meshwork) (6.). This liberates exocytic compartments and allows them to fuse with the secondary cell plasma membrane to deliver their contents into the accessory gland lumen (7.).

Concluding remarks

In this Chapter I have built upon a collaborator's work showing that the *D. melanogaster* initator caspase, DRONC, is expressed and present in the male accessory gland—specifically in the secondary cells. As part of this study, I have shown that the caspases DRONC, and its downstream effector, drICE, both appear to be positive regulators of intracellular trafficking in secondary cells—and that the apoptosome component, dARK, and the caspase inhibitor, dIAP1, might negatively regulate this process Altogether, my results suggest that caspases can function non-apoptotically to potentially regulate intracellular trafficking in secondary cells in the *D. melanogaster* male accessory gland—and I speculate that levels of DRONC protein are crucial for the proper regulation of this process. In addition, I have shown that expression of the DRONC caspase in secondary cells is regulated by the BMP/EcR signalling axis that has been identified previously in secondary cells; and provided evidence that this regulatory axis independently controls different cell biological processes in this cell type. Overall, the results presented in this Chapter suggest that caspases might have a role in regulating the secretory pathway and its interactions with the endolysosomal system in secondary cells of the *D. melanogaster* male accessory gland. The wider implications of this work are that non-apoptotic (and potentially non-catalytic) caspase activity might also regulate secretion from other secretory cell types in other animals; providing interesting avenues of research for non-apoptotic caspase activity as a more general regulator of secretory function in organs such as the human pancreas or prostate gland.





CHAPTER 4

PREVIOUS PAGE:

Composite confocal micrograph showing a male accessory gland with secondary cells expressing a GFP-tagged form of Hedgehog (HhN::GFP; green). Secondary cells also release the protease, ANCE (shown in magenta), and secondary cell-derived Hedgehog is dispersed among ANCE-negative flocculates in the gland lumen. Epithelial cell junctions are stained with α-FASIII (shown in yellow) and DNA is stained with DAPI (shown in cyan).



CHAPTER 4

HEDGEHOG SIGNALLING IS ACTIVE IN SECONDARY CELLS AND REGULATES NUCLEAR GROWTH

If you know what you're going to find,
you're not doing research.

— Peter A. Lawrence
(in GARWOOD, 2011)

Summary

Hedgehog signalling is one of the key developmental signalling pathways, with roles in the patterning and maintenance of tissue. Diverse glandular tissues also depend on Hedgehog signalling activity for their specification and function, and the pathway has been shown to interact with steroid signalling in steroid-sensitive tissues. In *D. melanogaster,* Hedgehog signalling has been shown to be active in both testes and ovaries. In this Chapter, I describe some of the changes that the male accessory gland undergoes as the male becomes fully sexually competent, and I provide evidence that Hedgehog signalling becomes active in secondary cells during this time. I also demonstrate that Hedgehog signalling is an important regulator of secondary cell nuclear size. Furthermore, the Hedgehog receptor, *patched*, is expressed in neurons innervating both the male accessory gland and the sperm-storage organs of the female reproductive tract. Overall, the findings presented in this Chapter show that Hedgehog signalling regulates secondary cell biology in *D. melanogaster*; with potential implications the function of male reproductive glands in other animals.

INTRODUCTION

The Hedgehog signalling pathway is one of the key pathways of embryonic development, and was initially discovered by Nüsslein-Volhard and Wieschaus in their Nobel Prize-winning genetic screen in the late 1970's (NÜSSLEIN-VOLHARD & WIESCHAUS, 1980). Hedgehog signalling has since been shown to be involved in diverse developmental processes—such as setting up the patterning of the vertebrate facial structures (XAVIER *et al.,* 2016), and regulating the elongation of the vertebrate limb and specifying digit identity (TICKLE & TOWERS, 2017). In the adult, Hedgehog signalling also has important roles maintaining stem cell populations to regulate tissue homeostasis (PETROVA & JOYNER, 2014). In contemporary research, the focus on Hedgehog signalling has however evolved beyond understanding its diverse roles in development, to understanding its role in cancer ætiology—where developmental processes seem to become co-opted to instead drive the development of pathology (I ALTABA *et al.,* 2004; BARAKAT *et al.,* 2010; BLAGOSKLONNY, 2013).

The Hedgehog signalling pathway is eponymous with the Hedgehog signalling molecule, which is the pathway ligand. As a molecule, Hedgehog is a small (45 kDa)

peptide that undergoes post-translational modifications that endow it with both cholesterol and palmitic acid residues. These lipid modifications make part of the Hedgehog ligand highly hydrophobic, and alter the capacity of the molecule to diffuse through tissue, to regulate tissue patterning (see CHAPTER 1: *p.* 14, for more detail). Before vertebrate orthologues of *hedgehog* were discovered, work on Hedgehog signalling primarily focussed on its role setting up the segmentation patterning of the *D. melanogaster* embryo. Aside from its role patterning the embryo and the imaginal discs of the larva, Hedgehog signalling regulates stem cell biology and tissue maintenance in adult *D. melanogaster*; with roles regulating tissue damage-responses in the gut (TIAN & SHI *et al.,* 2015), and in maintaining stem cell populations in the ovary and testis (ZHANG & KALDERON, 2001; AMOYEL *et al.,* 2013). Hedgehog has also been shown to function as a circulating endocrine hormone in *D. melanogaster,* which regulates the timing of adult metamorphosis (RODENFELS *et al.,* 2014).

In mammals, Hedgehog signalling has a similar role in regulating stem cell biology to maintain tissue homeostasis in diverse organs, such as the brain, skin, bone, and gut (reviewed by PETROVA & JOYNER, 2014). Diverse glandular and endocrine tissues also benefit from Hedgehog signalling activity. For example, Hedgehog signalling helps specify and direct the development of mammalian sebaceous glands (ALLEN *et al.,* 2005). Vertebrate orthologues of *hedgehog* are also expressed in both the developing and adult tissues of the gastric epithelium (VAN DEN BRINK *et al.,* 2007); liver (OMENETTI *et al.*, 2011); pituitary gland (PYCZEK *et al.*, 2016); adrenal gland (SWIERCZYNSKA *et al.,* 2013); and exocrine pancreas (THOMAS *et al.,* 2000). In the pituitary, Hedgehog signalling has a role in hormone production (PYCZEK *et al.,* 2016), and in the pancreas, Hedgehog regulates the processes of insulin transcription and secretion from the pancreatic β-cells (THOMAS *et al.,* 2000). Because of its role

regulating stem cell proliferation and maintenance, ectopic Hedgehog signalling has also been implicated in various childhood and adult cancers in humans; such as basal cell carcinoma (ORO *et al.*, 1997; EPSTEIN, 2008), but also other cancers, like medulloblastoma (RAFFEL *et al.*, 1997), pancreatic adenocarcinoma (THAYER & DI MAGLIANO, 2003), and cancers of the breast (KUBO *et al.,* 2004), and prostate (KARHADKAR *et al.,* 2004). Whereas Hedgehog signalling is commonly paracrine during development, it often appears to be autocrine in cancers of secretory tissue (RUBIN & DE SAUVAGE, 2006); being produced by cells that also are sensitive to it. Interestingly, it has been suggested that basal cell carcinoma might result from constitutive Hedgehog signalling activation in the hair follicle epithelium—beyond that which is required for the normal cycling of the hair follicle itself (HUTCHIN *et al.,* 2005).

Hedgehog signalling in the male reproductive system and associated glands

In the developing *D. melanogaster* larva, Hedgehog signalling is important for the patterning of the imaginal discs (as we saw in CHAPTER1: *p.* 15). One of these is the genital disc; the imaginal disc that gives rise to the fly *terminalia*: the *analia* and *genitalia,* and their accessory tissues. Hedgehog signalling is important for the correct patterning of these (SÁNCHEZ *et al.,* 1997; EMERALD & ROY, 1998; SÁNCHEZ & GUERRERO, 2001). Despite the developmental differences between flies and mammals, a role for Hedgehog signalling during genital development is conserved. For example, Hedgehog regulates both the development and adult function of the male reproductive tract in mammals (PODLASEK *et al.,* 2003; VEZINA & BUSHMAN, 2007; CHEN *et al.*, 2011).

Sonic hedgehog ("***SHH***") is one of several vertebrate orthologues of *hedgehog*. In the developing male reproductive system, *SHH* has a role in paracrine tissue patterning. The function and maintenance of reproductive tissues and organs in the adult male also requires SHH. In mammals, *SHH* is expressed in the 'genital tubercle'; the precursor of the mammal penis and accessory sex organs (including the prostate gland) (PODLASEK *et al.,* 2003; CHEN *et al.*, 2011). Unlike in *D. melanogaster*, where the morphological differentiation of the adult sex organs is mostly complete upon eclosion, the main morphological differentiation events of the mammalian sex organs occur after birth—but before sexual maturity. In rats, expression of *SHH* in penile tissue increases as the animal matures, and the expression remains high in adult individuals, where it has a role in maintaining the erectile function of the organ (PODLASEK *et al.,* 2003). Similarly, the prostate is formed *in utero* in mammals, upon stimulation by fœtal androgens (MARKER *et al.,* 2003), and paracrine SHH signalling is required for the proper patterning of prostate tissue (BERMAN *et al.*, 2005). As with the penis, the development of the prostate is not complete until the male reaches sexual maturity (MARKER *et al.*, 2003). In mice, SHH signalling in the prostate persists into adulthood (albeit at low levels), where it has an important role maintaining tissue homeostasis and preventing ductal overgrowth (a type of tissue hypertrophy) (LIM & SHIN *et al.,* 2014). However, although SHH is important for the morphogenesis of the prostate in humans, levels decline after the initial, developmental, morphogenetic events, and higher levels can be detected again only in the adult human organ (VEZINA & BUSHMAN, 2007). This suggests that Hedgehog signalling might have a role maintaining the function of prostate tissue also in humans.

A relationship between androgen and Hedgehog signalling activity has been demonstrated in the developing mouse prostate, where androgen stimulation is not

required for the expression of *SHH* during prostate morphogenesis, but is nevertheless sufficient to increase levels of available *SHH* transcript via the androgen receptor ("**AR**") (PODLASEK *et al.,* 1999). In addition, there seems to be a role for androgens in directly influencing the *locale* of *SHH* expression in the developing prostate (LAMM *et al.,* 2002). A relationship between Hedgehog signalling and the androgen receptor in mice is intriguing, since the ecdysone receptor, EcR, binds to the *hedgehog* gene promoter after ecdysone treatment (SEDKOV & CHO *et al*., 2003). Not only does this cast *hedgehog* as a target gene of steroid signalling in *D. melanogaster*, but it also suggests that the relationship between male steroid hormone signalling and *hedgehog* promoter activity might be conserved between flies and mammals. Indeed, the *D. melanogaster* larval salivary gland is sensitive ecdysone (BIYASHEVA & DO *et al.,* 2001), and the tissue has been shown to be a site of active Hedgehog signalling (ZHU *et al*., 2003). Similarly, Hedgehog signalling has also been implicated in oogenesis in the *D. melanogaster* ovary (ZHANG & KALDERON, 2000)—another steroid-sensitive organ (BOWNES, 1989; CARNEY & BENDER, 2000). However, although Hedgehog signalling is important for the normal functioning of the testes in adult flies (AMOYEL *et al.,* 2013), a role for Hedgehog signalling has yet to be identified and described in the male reproductive glands of adult *D. melanogaster*. Like the male reproductive glands of mammals, the male accessory glands in the fly are however sensitive to the activity of steroid hormones (YAMAMOTO *et al.,* 1988; HERNDON *et al*., 1997).

Molecular regulation of Hedgehog signalling

The Hedgehog protein undergoes post-translational modification to yield a functional lipoprotein. The Hedgehog precursor peptide is capable of catalysing its own cleavage

(autoproteolysis), to form N-terminal and C-terminal fragments (BRISCOE & THÉROND, 2013). Following the autoproteolytic cleavage event, the C-terminal Hedgehog fragment recruits cholesterol and catalyses its attachment onto the 19 kDa N-terminal Hedgehog fragment (PORTER *et al.*, 1996). The N-terminal fragment is lipidated a second time in the ER, where the acetyltransferase, Skinny hedgehog ("**Ski**")[1] (LEE & TREISMAN, 2001), attaches a palmitic acid residue to the N-terminus of the N-terminal Hedgehog fragment, to yield the final, doubly-lipidated, Hedgehog lipoprotein (BRISCOE & THÉROND, 2013). Whereas the mature Hedgehog protein is destined for release, the C-terminal cleavage fragment is removed from the cell by proteasomal degradation (BRISCOE & THÉROND, 2013).

Lipidated Hedgehog has a strong affinity for the lipids of the cell membrane (WENDLER, FRANCH-MARRO & VINCENT, 2006), and it is targeted to lipid-raft microdomains (RIETVELD *et al.*, 1999). Therefore, the molecule needs to be actively released from the cell surface to enable its spread through tissue. There are several processes by which Hedgehog can be released from the cell. For example, aggregated Hedgehog monomers can be released either as soluble multimers (BRISCOE & THÉROND, 2013), or as part of lipoprotein particles following interaction of the Hedgehog multimers with apolipoprotein-recruiting glypicans, such as Dally (AYERS & GALLET *et al.*, 2010). Mature Hedgehog can also be released from the cell on membrane-bound exovesicles derived from the endolysosomal system in *D. melanogaster* S2 cells (PARCHURE *et al.,* 2015). Hedgehog is not thought to be released on exosomes (a type of exovesicle), at least not in the developing larval wing

[1] Ski is known also as 'Raspberry' or 'Sightless' (WENDLER, FRANCH-MARRO & VINCENT, 2006). According to the *D. melanogaster* gene expression atlas, FlyAtas (CHINTAPALLI *et al.,* 2007), *Ski* mRNA is enriched in the male accessory gland—relative to other tissues in the adult fly.

disc (PANÁKOVÁ & SPRONG *et al.,* 2005). Another mechanism by which mature Hedgehog can be released from the cell is through interaction of its cholesterol moiety with the multipass transmembrane protein, Dispatched ("**Disp**")[2] (BURKE *et al.,* 1999).

Once released from its site of production, the movement of the Hedgehog molecule through tissue is restricted by membrane proteins on recipient cells (BRISCOE & THÉROND, 2013). The Hedgehog receptor, Patched, is one such protein, and it can form a multi-protein receptor-complex together with its co-receptors: Interference Hedgehog ("**Ihog**") and Brother of Ihog ("**Boi**") (CAMP *et al.*, 2014). In its unbound state, Patched acts as a repressor of Hedgehog signalling—both by sequestering the Hedgehog molecule itself, but also by inhibiting another transmembrane protein, Smoothened ("Smo"). Smoothened is a member of the G-protein–coupled receptor ("**GPCR**") superfamily, and is the activator of the Hedgehog signalling pathway (ALCEDO *et al.,* 1996; VAN DEN HEUVEL & INGHAM, 1996). The mechanism by which Patched inhibits Smoothened is not yet known. However, like Dispatched, Patched is a multipass membrane protein belonging to the resistance-nodulation division ("**RND**") transporter family, complete with a sterol-sensing domain ("**SSD**") (TSENG *et al.,* 1999; BRISCOE & THÉROND, 2013). Such transporters have a role transporting lipophilic proteins across the lipid bilayer (PIDDOCK, 2006). Smoothened can be inhibited chemically, for example by the alkaloid sterol, cyclopamine (a potent Hedgehog signalling inhibitor derived from the wild California grass, *Veratrum californicum*) (INCARDONA *et al.,* 1998). Therefore, it is feasible that Patched functions as a transporter of an endogenous sterol ligand that is

[2] Like Ski, dispatched mRNA is enriched in the D. melanogaster adult male accessory gland. The transcripts of two related RND-family proteins, *patched*, and the Niemann-Pick type C-1a (*NPC1a*) gene, are also enriched (FlyAtalas, CHINTAPALLI *et al.,* 2007).

capable of inhibiting the activity of Smoothened (EATON, 2008). Such a function is consistent with observations made in tissue culture, where small amounts of Patched can inhibit higher levels of Smoothened protein ('non-stoichiometric inhibition') (TAIPALE *et al.,* 2002).

Where Hedgehog is present, it binds to Patched. The resultant receptor·ligand complex is internalised, preventing Patched from inhibiting Smoothened; allowing Smoothened to transduce the Hedgehog signal and activate the signalling pathway (BRISCOE & THÉROND, 2013). Smoothened has a C-terminal tail that extends into the cytoplasm and permits Smoothened monomers to dimerise and interact with intracellular components of the Hedgehog signalling pathway (ZHAO, TONG & JIANG, 2007). Several kinases (*e.g.* protein kinase A ("**PKA**"); casein kinase Iα ("**CkIα**"); and the GPCR kinase 2 ("**GPCRK2**") are present in the cytoplasm, active in the pathway, and capable of phosphorylating Smoothened (BRISCOE & THÉROND, 2013). Phosphorylation of the Smoothened C-terminal tail facilitates its association with a Hedgehog signalling complex ("**HSC**"). This intracellular protein complex is associated with microtubules and includes the mobile, microtubule-associated protein, Costal-2 ("**Cos-2**"); the kinase, Fused ("**Fu**"), and its inhibitor, Suppressor of Fused ("**Su(Fu)**"); and the transcription factor, Cubitus interruptus (Ci) (BRISCOE & THÉROND, 2013).[3]

Cubitus interruptus is a Gli-family transcription factor, and is responsible for regulating the expression of Hedgehog target genes (STRINGINI & COHEN, 1997).

[3] Many of the Hedgehog signalling pathway genes are named after wing, rather than embryonic, phenotypes: mutation of *coastal-2* causes hypertrophy of the costal part of the wing (WHITTLE, 1979); mutation of *fused* causes a fusion of the third and fourth veins of the wing (LYNCH, 1919); mutation of *cubitus interruptus* causes an interruption of the fourth vein in the wing (the '*cubitus*') (TINIAKOW & TERENTIEVA, 1933).

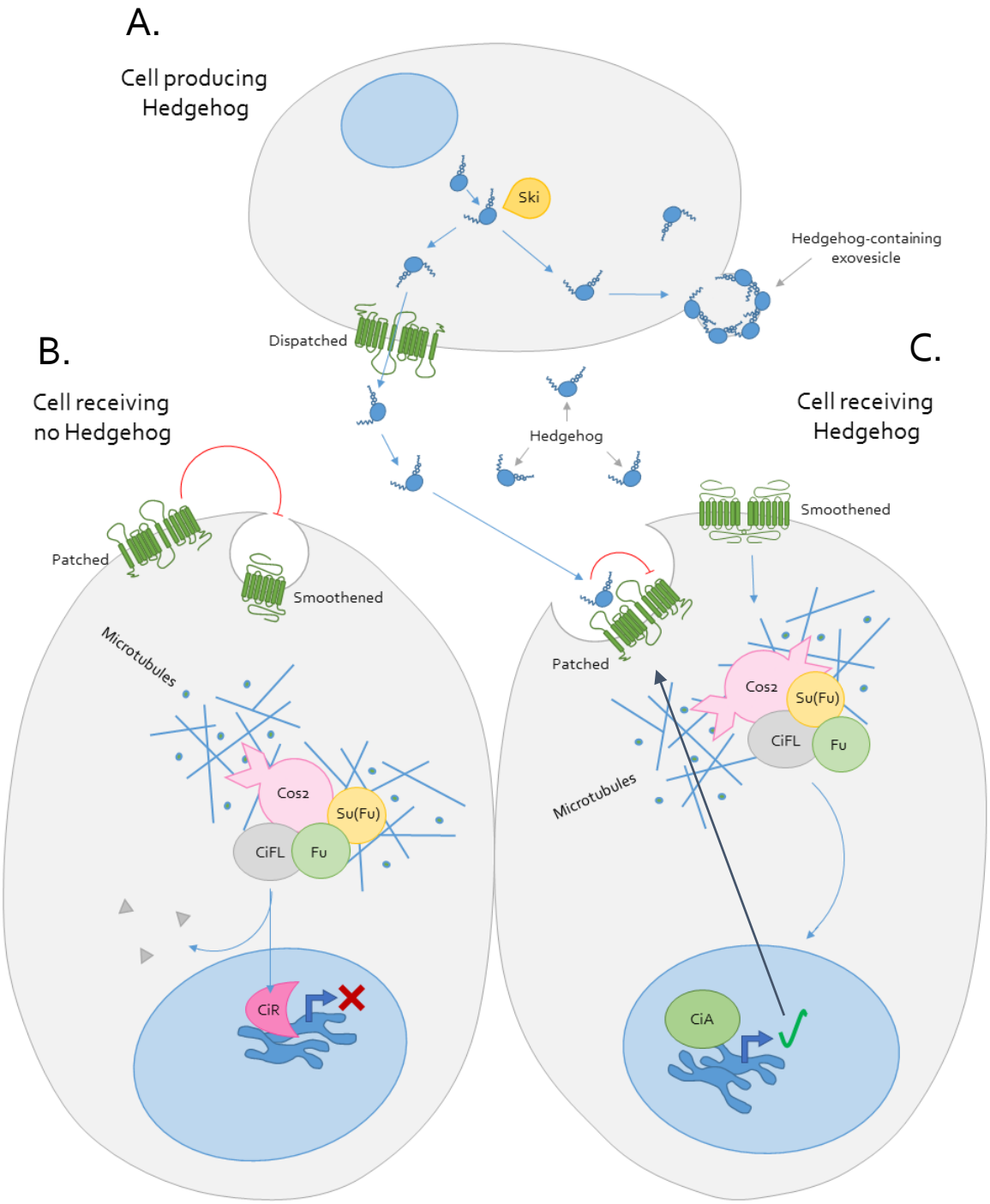
FIGURE 4. 1. | THE HEDGEHOG SIGNALLING PATHWAY IN *D. MELANOGASTER.*

A: The Hedgehog molecule is synthesised as a precursor that undergoes auto-catalytic cleavage to generate C- and N-terminal fragments. The C-terminal fragment recruits cholesterol, which is attached to the N-terminal fragment. The acetyltransferase, Skinny hedgehog (Ski), attaches a palmitic acid residue onto the N-terminal fragment; generating doubly-lipidated and mature Hedgehog protein. Mature Hedgehog has an affinity for the cell membrane, and is released from the cell either in the form of exovesicles, or via the small molecule-transporter, Dispatched.

B: In cells receiving no Hedgehog protein, the transmembrane protein, Patched, inhibits the activity of the Hedgehog signalling activator, Smoothened. Patched inhibiting Smoothened causes Smoothened to translocate to the interior of the cell, preventing it from activating the Hedgehog signalling pathway. The Hedgehog signalling complex (HSC), which, among other components, include the microtubule-binding protein, Costal-2 (Cos2), and the kinase, Fused (Fu) and its inhibitor, Suppressor of Fused (Su(Fu)), is located on microtubules. In the absence of a Hedgehog signal, the HSC is inactive, and the transcription factor, Cubitus interruptus (Ci) is modified from its full-length form (CiFL) to remove its transactivation domain; causing it to function as a repressor (CiR). The CiR can translocate to the nucleus, where it represses the activity of Hedgehog target genes.

C: In cells receiving Hedgehog protein, Hedgehog binds its receptor, Patched—which sequesters Hedgehog to limit its spread through tissue. Hedgehog binding to Patched relieves the Patched inhibition of Smoothened, allowing Smoothened to localise to the cell membrane and dimerise. Dimeric Smoothened interacts with the components of the HSC, causing Ci to be released, complete with its transactivation domain; allowing Ci to function as an activator (CiA). CiA translocates to the nucleus, where it promotes the transcription of Hedgehog target genes. One of the Hedgehog target genes is *patched*, whose transcription forms a negative feedback loop to limit the activity of the pathway

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A.
Cell producing Hedgehog
Ski
Hedgehog-containing exovesicle
Dispatched
Hedgehog
B.
Cell receiving no Hedgehog
Patched
Smoothened
Microtubules
Cos2
Su(Fu)
CiFL
Fu
CiR
C.
Cell receiving Hedgehog
Smoothened
Patched
Cos2
Su(Fu)
CiFL
Fu
Microtubules
CiA

Ci is bifunctional and exists in two forms: an activator form ("**CiA**"; containing both DNA-binding and activator domains), and a repressor form ("**CiR**"; containing only the DNA-binding domains) (ALEXANDRE *et al.,* 1996; AZA-BLANC *et al.*, 1997). Both the activator and repressor forms of Ci are derived from the full-length Ci protein ("**CiFL**"). In the absence of Hedgehog signalling (and thus Smoothened activity), CiFL is preferentially processed into CiR through a kinase-dependent catalytic process that removes the Ci C-terminal transactivation domain (BRISCOE & THÉROND, 2013). Conversely, when Hedgehog signalling is active, Smoothened associates with the Hedgehog signalling complex, allowing the sequential phosphorylation of Fused and Costal-2—leading to the release of CiA from the HSC (BRISCOE & THÉROND, 2013). Upon release, both CiR and CiA translocate to the nucleus, where they bind Hedgehog-responsive target genes (*i.e.* those containing a Ci DNA-binding motif), to either repress or to promote their expression (ALEXANDRE *et al.,* 1996; AZA-BLANC *et al.*, 1997). Thus, either absence or presence of the Hedgehog ligand promotes Ci-dependent changes in transcription. Please see **FIGURE 4. 1.** for an illustration of the logic of the Hedgehog signalling pathway.

Aims of this Chapter

The *D. melanogaster* male accessory gland shares similarities with the larval salivary gland. For example, both organs are steroid-sensitive, and contain cells that are specialised for secretion. In addition, the male accessory gland shares some functional similarities with the prostate gland of mammals; producing many of the proteins of the ejaculate. Reading about the development of the male reproductive systems of *D. melanogaster* and mammals, I was intrigued by their shared requirement for Hedgehog signalling. That Hedgehog signalling is maintained in adult tissue in

mammals—where it seems to be important for male reproductive function—made me curious if this is the case also in *D. melanogaster*. However, although Hedgehog signalling has been shown to be active in both the larval salivary glands and the adult testes, there is a no literature describing a role for Hedgehog signalling in the adult male accessory glands.

The aims I set for the work presented in this Chapter were:

- To establish whether Hedgehog signalling is active in the adult male accessory gland in *D. melanogaster*; and,
- To establish the role of Hedgehog signalling in secondary cell function.

In this Chapter, I describe some of the changes that the accessory gland epithelium and its secretions undergo as the gland matures and the male approaches full sexual maturity. In addition, I show that Hedgehog is present in the accessory gland and that *hedgehog* is expressed in the secondary cells—during the period when the accessory glands mature after eclosion. Furthermore, I also show that Hedgehog protein can be released into the seminal fluid, and provide data suggesting that Hedgehog signalling might be autocrine, and that it regulates the size of the secondary cell nuclei. The findings presented in this Chapter suggest a role for Hedgehog signalling in the regulation of accessory gland biology—with potential implications for the reproductive function of both male and female *D. melanogaster,* and the regulation of secretory cell function more generally.

RESULTS

The size and secretory content of the male accessory gland increases over time

Previous work in our laboratory has demonstrated that secondary cells undergo a process of maturation during the first few days of adult life (CORRIGAN *et al.*, 2014; LEIBLICH *et al.*, 2012). These changes correlate with the time it takes for the gland itself to mature (RUHMANN *et al.,* 2016). Yet, to my knowledge, no work has been undertaken to show how accessory gland growth and maturation affects the epithelial tissues in the gland at the microscopic level.[4] To rectify, and to characterise the development of the male accessory gland on the tissue-level, I reared wild-type (w^{1118}) flies and dissected males at set time points: at eclosion (0 hours), on day 1 (24 hours), day 3 (72 hours), and day 6 (144 hours). Already during the dissection process, it was clear that the gland increases in size over time: with individual glands increasing in both length and apparent secretory content as the male ages after eclosion. In males that have just eclosed from their *puparia*, the glands are very small and contain very few secretions (**FIGURE 4. 2. A**). However, by day three, the glands have increased in both volume and size—to the extent that they occupy what represents a large fraction of the total abdominal content (**FIGURE 4. 2. C**).

The protease, ANCE, is expressed in secondary cells (RYLETT *et al.,* 2007), and ANCE anti-serum (α-ANCE) can be used to specifically label these cells in the accessory gland (LEIBLICH *et al.,* 2012). In addition, an antibody against the cell-

[4] Do however see RUHMANN *et al.* (2016) for a description of how the size and function of the *D. melanogaster* male accessory gland changes during the process of maturation.

junction protein, Fasciclin III ("**FASIII**") (α-FASIII; PATEL *et al.*, 1987) can be used to label cell junctions in the epithelia of the *D. melanogaster* male reproductive system (LEIBLICH *et al.*, 2012). Confocal examination of fixed glands stained with α-ANCE and α-FASIII allowed description of the morphological changes that occur to the accessory gland epithelium as the gland matures. In the glands of flies that have just eclosed (and still by day one), the cells in the secretory epithelium appear columnar (**FIGURE 4. 2. A**). The main cell nuclei are also not uncommonly stacked one on top of the other. Secondary cell nuclei are located basally (**FIGURE 4. 3.**).

The contents of the male accessory gland are heterogeneous and contain what has been interpreted as dispersed protein 'flocculates' (BAIRATI, 1968; FEDERER & CHEN, 1982; SCHMIDT *et al.*, 1985). A subset of these structures can be labelled with lipophilic markers and stains (CORRIGAN, 2014); suggesting that the non-flocculent fraction might represent the aqueous phase of the gland secretions. This phase makes up the bulk of the contents of the gland lumen, and it stains strongly with α-ANCE—allowing the flocculent (immiscible) secretory fraction to be resolved as α-ANCE–negative spaces (**FIGURE 4. 2.**). In very young flies (aged less than 24 hours) the secretions appear relatively homogenous, with only few flocculates present. The flocculate fraction however increases over time, making up a significant proportion of the secretions by day three and six (**FIGURE 4. 2. C–D**). The total secretory volume has also increased significantly by this time, stretching and distending the accessory gland epithelium. This epithelial stretching flattens the tissue into a more squamous conformation; so that the two nuclei in each main cell and secondary cell appear in the same focal plane (**FIGURE 4. 3. C**).

FIGURE 4. 2. | THE ADULT MALE ACCESSORY GLAND MATURES OVER TIME.

A: The accessory gland of the just-eclosed male (0 hours; 0 days) is small and stains strongly with α-FASIII (white). The luminal volume of the gland is small, and the aqueous phase of the seminal fluid is stained with α-ANCE (magenta)—with the immiscible phase visible as negative spaces. The epithelium is near-columnar in appearance. DAPI stains DNA (cyan).

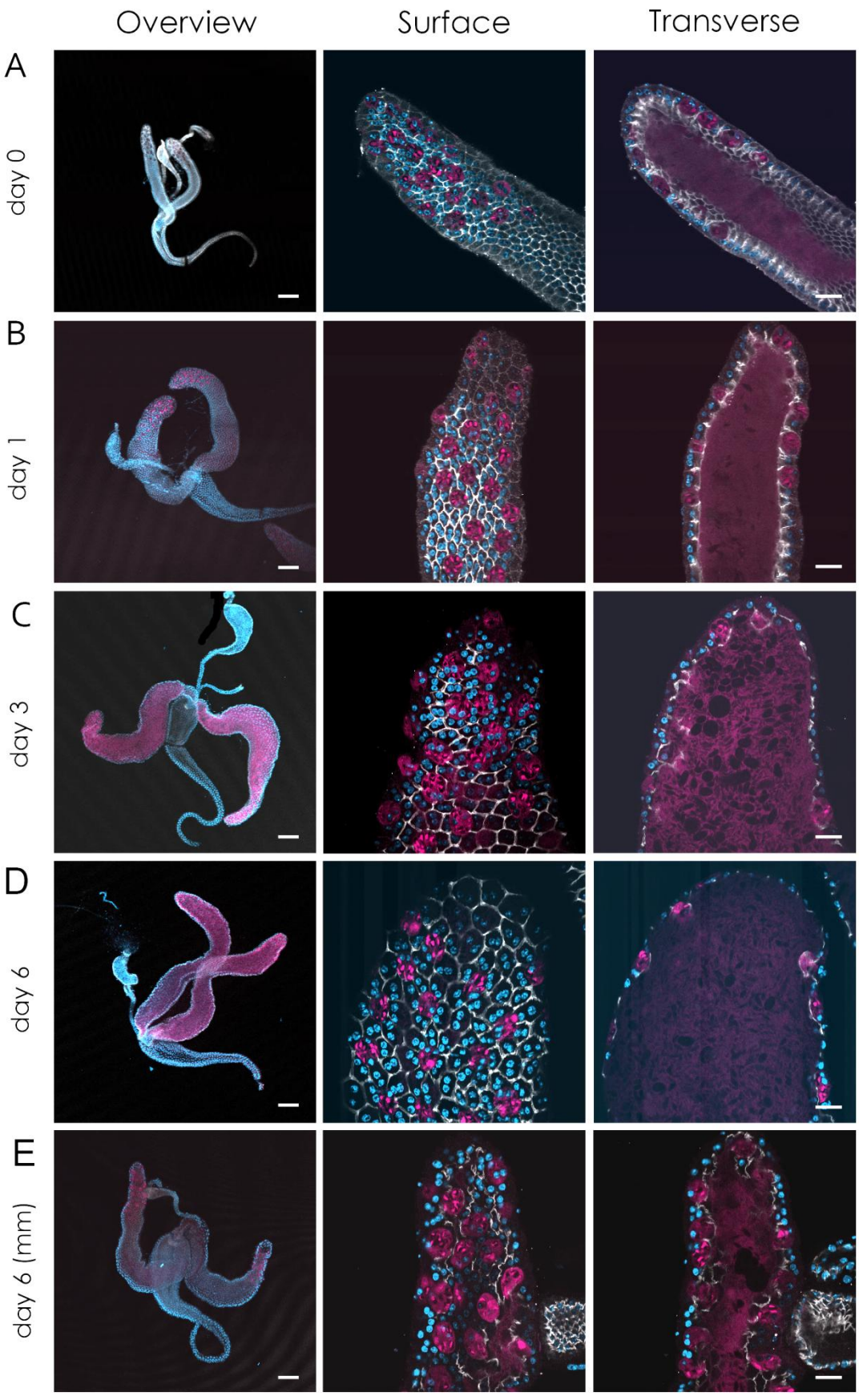
B: The accessory gland of a male dissected 24 hours post-eclosion (day 1) contains secretions that show some heterogeneity, as evidenced by α-ANCE staining.

C: The accessory gland of the three-day old male (72 hours) has filled up with heterogeneous secretions and the epithelium has changed in appearance from columnar to more squamous. The aqueous phase of the secretions stains with α-ANCE and reveals larger, immiscible (non-aqueous) structures as negative paces in the staining pattern.

D: The accessory gland of a six-day old male (144 hours) is much distended with secretion products, and the epithelium is stretched to accommodate these.

E: The gland of a six-day old male that has been kept together with multiple females contains a much-reduced amount of accessory gland secretions, allowing the epithelium to revert to a more columnar conformation, and cell junctions appear more irregular. The tissue in this panel that stains strongly with α-FASIII (white), to the bottom right, is part of a seminal vesicle.

In the overview panels, the scale bar corresponds to 100 μm, whereas the scale bar in the close-up panels corresponds to 20 μm.


Overview
Surface
Transverse
A
day 0
B
day 1
C
day 3
D
day 6
E
day 6 (mm)

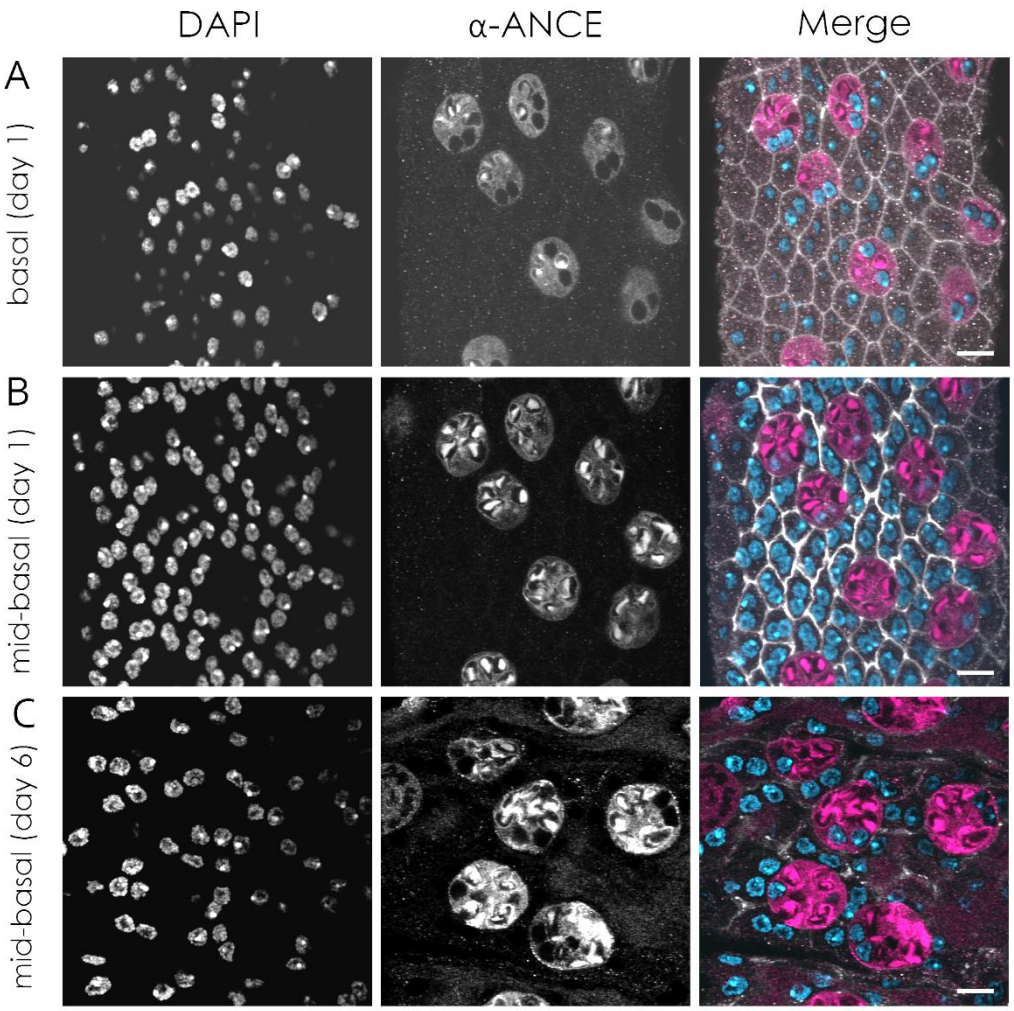


FIGURE 4. 3. | THE APICO-BASAL POLARITY OF SECONDARY CELL NUCLEI CHANGES OVER TIME—PRESUMABLY DUE TO STRETCHING OF THE EPITHELIUM.

A: Extremely basal view of the male accessory gland epithelium of a one-day old fly, showing the basal localisation of secondary cell nuclei relative to those of the main cells. Note the epithelial cell junctions stained with α-FASIII (white).

B: View of the same gland as shown in panel **A**, but in a more apical plane (mid-basal), showing the localisation of the main cell nuclei. Note how only some of the secondary cell nuclei are visible in this plane.

C: Mid-basal view of the male accessory gland epithelium of a six-day old fly, showing how the nuclei of both secondary cells and main cells are localised in the same mid-basal plane. Also note how the cell junctions appear more stretched (faint α-FASIII staining).

In all panels, the scale bar corresponds to 10 μm, while α-ANCE stains secondary cells (magenta) and DAPI stains cell nuclei (cyan).

The *D. melanogaster* male accessory gland is a novel site of *hedgehog* gene expression

Based on the analogous functions of the prostate and the accessory gland and the developmental similarities of the two organs, I hypothesised that a role for Hedgehog signalling might be conserved, and that Hedgehog be important also for accessory gland biology in adult male *D. melanogaster*. In the GAL4/*UAS* system, expression of the transcription factor GAL4 under a specific promoter allows GAL4 to drive the transcription of transgenes containing the GAL4-binding *UAS* sequence motif (BRAND & PERRIMON, 1993). To determine if *hedgehog* is expressed in the *D. melanogaster* male reproductive system, I used a *hh*-GAL4 promoter fusion (an enhancer trap) that permits expression of GAL4 under the control of the endogenous *hedgehog* gene promoter (SZUPLEWSKI *et al.,* 2009), and crossed it to a line containing an *UAS*-GFP^{nls} reporter construct (NEUFELD *et al.*, 1998). In the F_1 progeny resulting from this cross, *UAS-GFP^{nls}* will be transcribed wherever the *hh-GAL4* gene is expressed. To examine the expression pattern of *hh*-GAL4 > *UAS*-GFP^{nls} (*hh* > GFP) in the adult male reproductive system, I dissected male reproductive tracts from six-day old flies and examined them using confocal microscopy. This examination revealed that

hh > GFP was present in the male reproductive system at this age: with GFP being observed at the apical tip of the testes (where the cyst stem cells are located; AMOYEL *et al.*, 2013) (**FIGURE 4. 4. A**)—as well as near the distal ends of the accessory glands (where the secondary cells are located) (**FIGURE 4. 4. C**). No GFP was observed in the epithelia of either the seminal vesicles or the ejaculatory duct (**FIGURE 4. 4. B–D**).

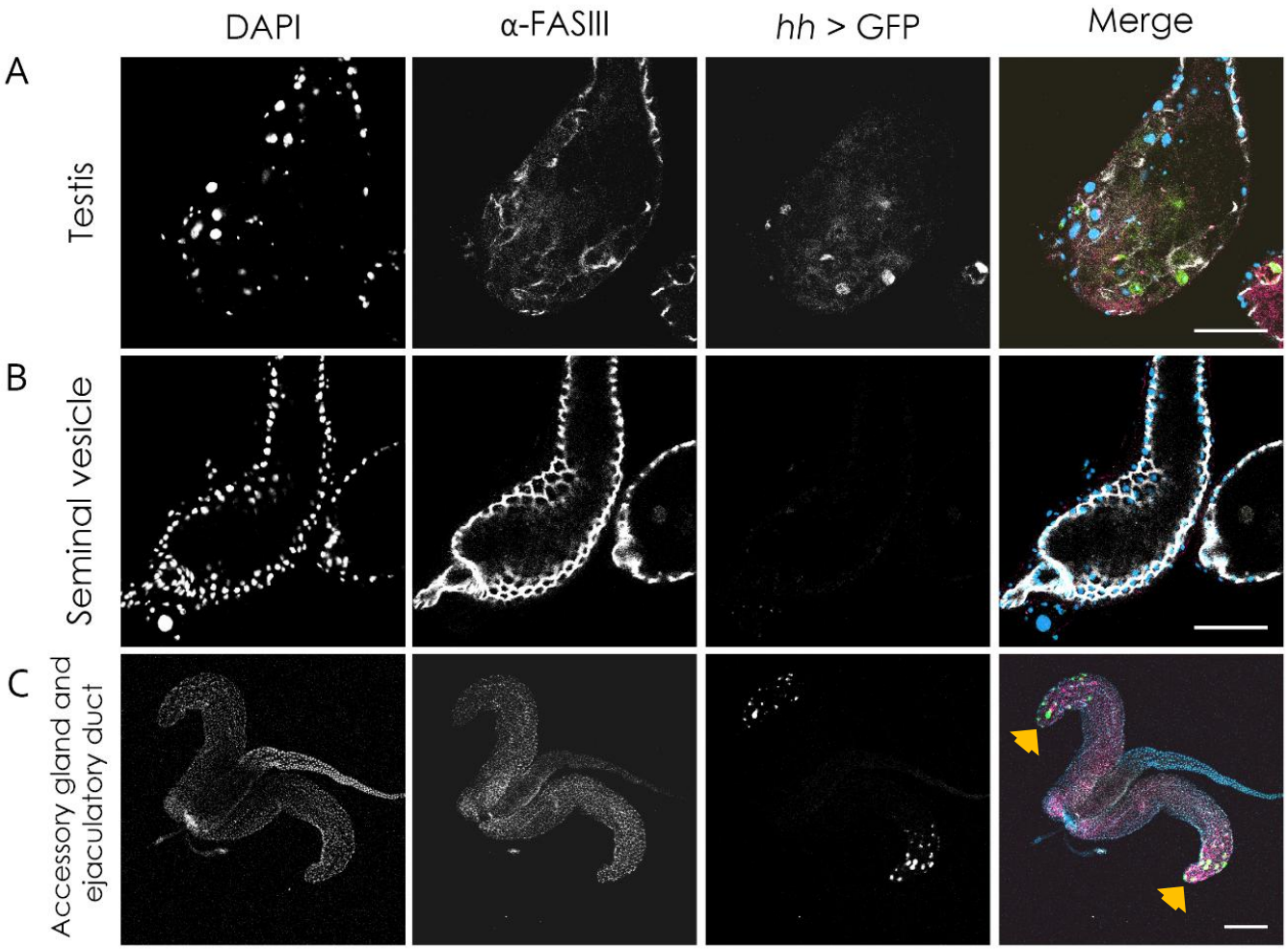


FIGURE 4. 4. | SECONDARY CELLS IN THE MALE ACCESSORY GLAND ARE A SITE OF *hh* > GFP EXPRESSION.

A: Confocal image panels showing the expression of *hh* > GFP (green) at the apical tip of the testis (where the cyst stem cells are typically located).

B: Confocal image panels showing the lack of *hh* > GFP expression in the tissues of the seminal vesicles.

C: Confocal image panels showing that *hh* > GFP (green) is expressed in the secondary cells at the distal tip of each accessory gland (yellow arrows), but is absent from the cells making up the ejaculatory duct.

In all panels, α-FASIII (white) labels main cell junctions. Cells positive for *hh* > GFP are shown in green, whereas α-ANCE (pink) stains secondary cells and the accessory gland seminal fluid, as well as other structures elsewhere in the male reproductive system. DAPI stains cell nuclei (cyan). In panels **A–B** the scale bar corresponds to 50 μm, whereas the scale bar corresponds to 200 μm in panel **C**.

The number of secondary cells expressing *hh* > GFP increases over time

Examining accessory glands stained with α-ANCE and expressing *hh* > GFP, I noticed that secondary cell *hh* > GFP expression was variable at day three post-eclosion: whereas some secondary cells showed strong *hh* > GFP expression (sufficient to allow GFP^{nls} to accumulate in the cytoplasm), other secondary cells showed more moderate *hh* > GFP expression that restricted the protein to the nuclei (**FIGURE 4. 5.**). I also observed that some secondary cells did not seem to express *hh* > GFP at all. Looking at glands from more flies, I decided to quantify the proportion of secondary cells expressing *hedgehog* in the gland. Counting the total number of secondary cells that were positive for *hh* > GFP, and dividing this number by the total number of secondary cells in the gland (as determined by α-ANCE staining), I determined the proportion of secondary cells positive for *hh* > GFP expression in each gland.

That not all secondary cells express *hh* > GFP by day three of adulthood suggested to me that perhaps the level of secondary cell *hh* > GFP expression changes over time. To investigate, I dissected accessory glands at specific time-points: *viz,* at eclosion (0 hours) and on days 1 (24 hours), 3 (72 hours), and 6 (144 hours) post-eclosion. Staining these glands with α-ANCE and quantifying the proportion of secondary cells expressing *hh* > GFP, revealed that the expression of *hh* > GFP begins after eclosion in secondary cells—and that it increases over time (**FIGURE 4. 6.** and **FIGURE 4. 7.**). Importantly, at eclosion, there was no detectable GFP present in the gland. However, by day 1 post-eclosion, a small proportion of secondary cells were positive for *hh* > GFP expression ($p\ \{d0|d1\} < 0.0001$), increasing further by day 3

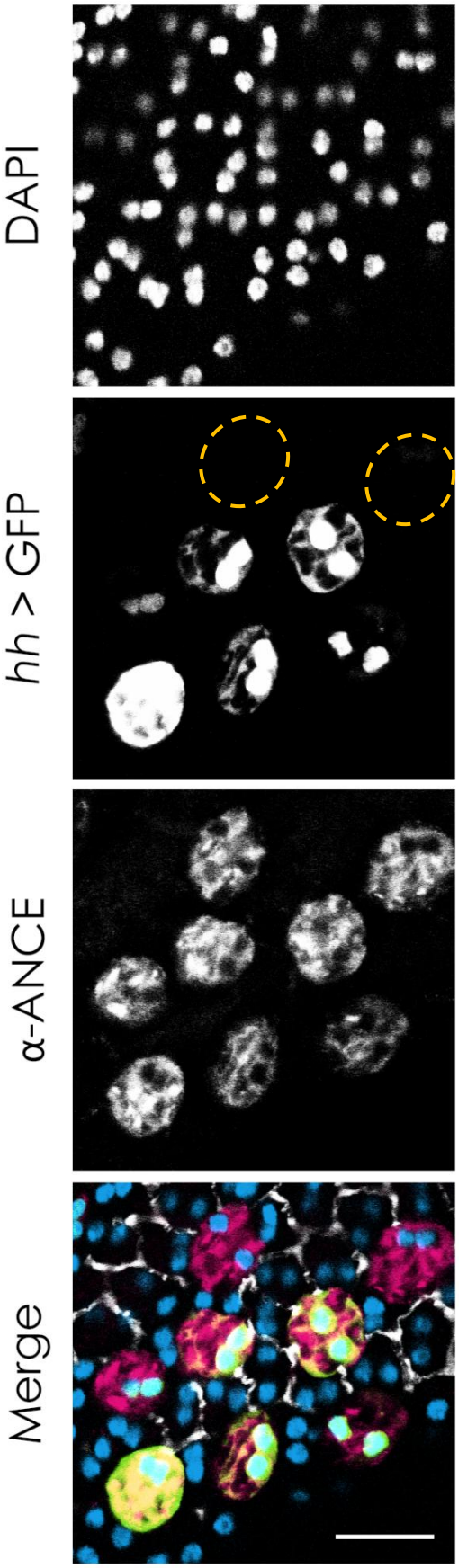


FIGURE 4. 5.| SECONDARY CELLS SHOW VARIABLE LEVELS OF *hh* > GFP EXPRESSION.

At 144 hours (day 6) post-eclosion, the amount of *UAS*-GFP^{nls} expression driven by *hh*-GAL4 seems to vary between individual secondary cells: whereas some secondary cells are sufficiently saturated with GFP^{nls} to fill the cytoplasm, GFP^{nls} is restricted to the nuclei in other cells, suggesting that the overall expression is lower in these. Some secondary cells are also devoid of *hh* > GFP^{nls} (circled).

In this figure, α-FASIII (white) labels main cell junctions. Cells positive for *hh* > GFP are shown in green, and α-ANCE stains all secondary cells (pink). DAPI labels epithelial nuclei (cyan). The scale bar corresponds to 20 μm.

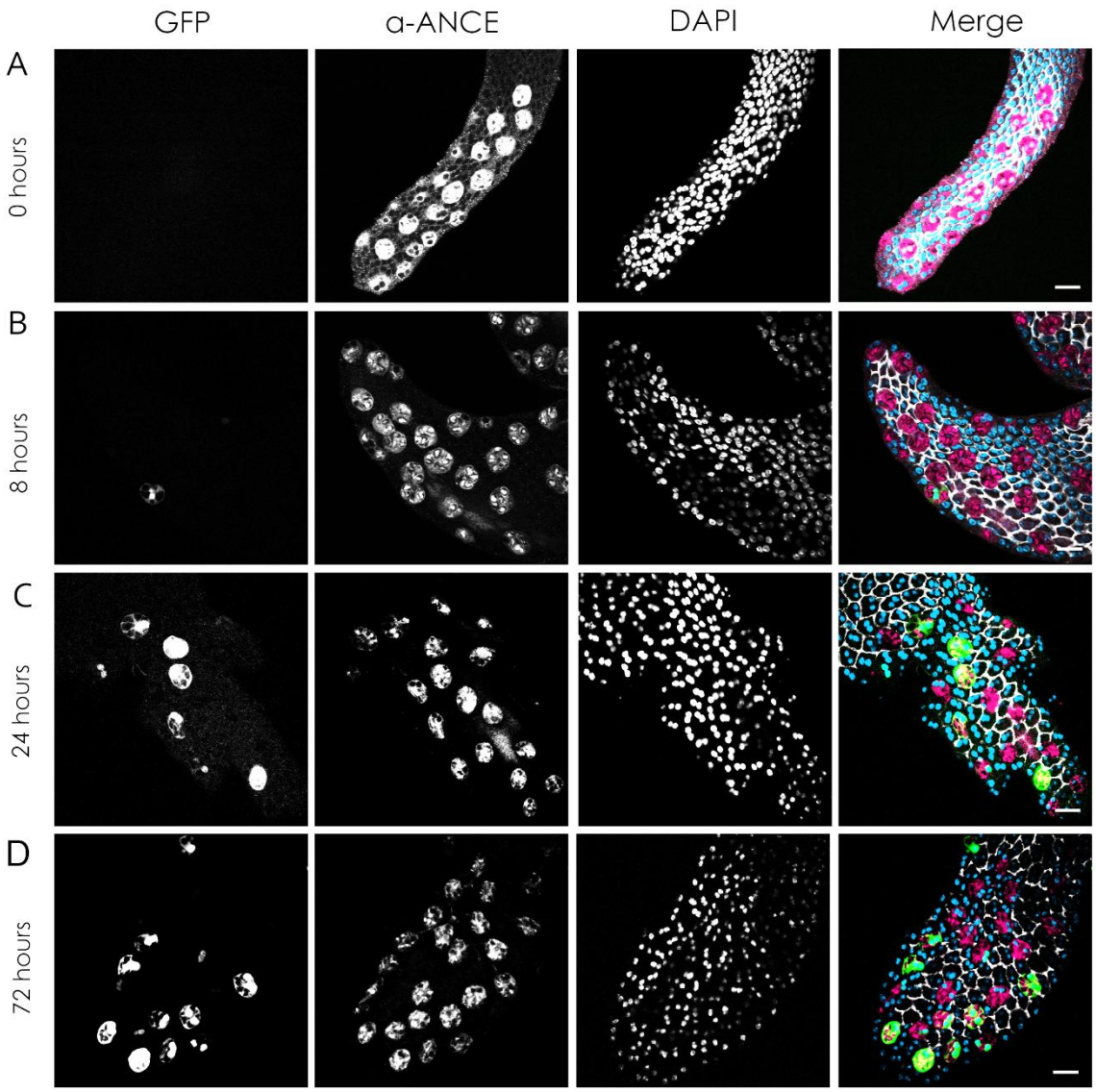


FIGURE 4. 6. | SECONDARY CELLS SHOW CUMULATIVELY INCREASING *hh* > GFP EXPRESSION.

Secondary cells express *hh* > GFP and the proportion of *hh* > GFP$^+$ secondary cells in the accessory gland increases over time.

A: At eclosion (0 hours) there is no detectable *hh* > GFP in the accessory gland, although secondary cells can be detected by staining with α-ANCE.

B: By 8 hours post-eclosion, the first secondary cells positive for *hh* > GFP (green) appear.

C: By 24 hours post-eclosion, the proportion of secondary cells in the gland that are positive for *hh* > GFP has increased further.

D: By day 3 (72 hours) post-eclosion, a large proportion of secondary cells express *hh* > GFP.

In all panels, secondary cells are stained with α-ANCE (pink), and α-FASIII (white) labels main cell junctions. DAPI stains DNA (cyan). Scale bars in panels **A–D** correspond to 20 μm.

post-eclosion (p {d1|d3} < 0.0001). No further change was observed beyond this point (p {d3|d6} > 0.01) (**FIGURE 4. 6.**)—although I admittedly never looked at flies aged ten days, which I should have done.

Having narrowed the onset of *hh* > GFP expression to the first 24 hours post-eclosion, I wondered: when does *hh* > GFP first become detectable in the secondary cells? To answer this question, I dissected flies at an arbitrary initial time-point (eight hours post-eclosion) and was fortunate to find that *hh* > GFP expression is just becoming detectable in the accessory gland at this time (**FIGURE 4. 6.**). The GFP molecule undergoes several post-translational modifications that are required for full fluorescence to develop, and it has been estimated that it takes between 50–120 minutes for full GFP fluorescence to develop in *D. melanogaster* tissues (HAZELRIGG *et al.*, 1998). Using these numbers to extrapolate from the onset of *hh* > GFP detection at 8 hours post-eclosion in accessory gland, it can be estimated that secondary cell *hedgehog* gene expression begins at approximately 4–6 hours post-eclosion. Please see **SUPPLEMENTARY TABLE 4. 1.** (*p.* 438; APPENDIX 5) for a summary of *hh* > GFP expression in secondary cells over time.

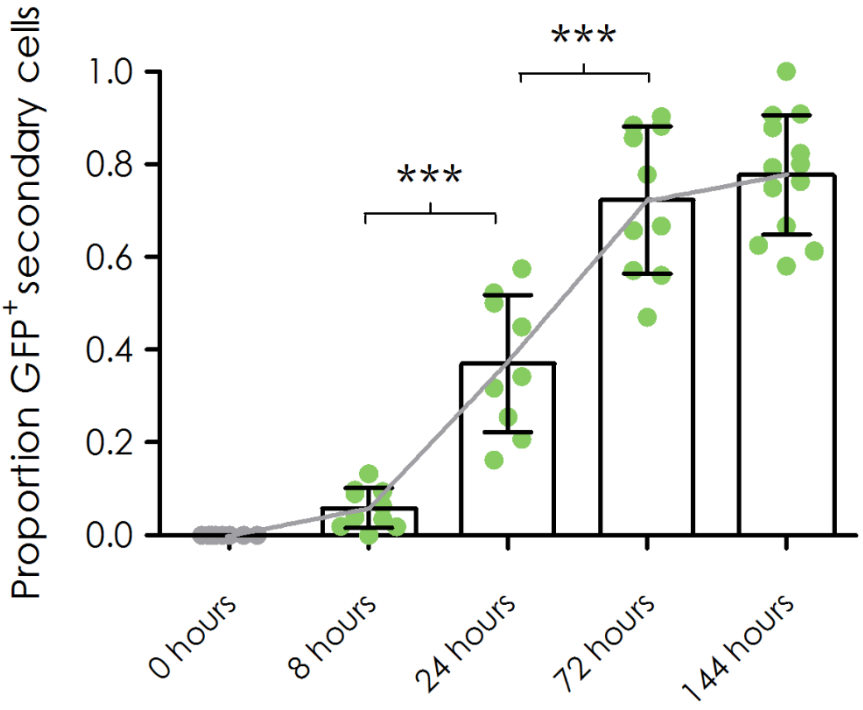


FIGURE 4. 7. | SECONDARY CELL *hh* > GFP EXPRESSION INCREASES OVER TIME.

Scatterdot bar-chart showing how the expression of *hedgehog* > GFP in secondary cells begins after eclosion and increases over the first days after eclosion (green). Each dot in the chart corresponds to data obtained from one individual male (see **SUPPLEMENTARY TABLE 4. 1.**, *p.* 438). The number of stars in the figure represents the *p*-value obtained by one-way ANOVA with a Bonferroni correction for multiple comparisons (all columns): *** = $p < 0.001$. Shapiro-Wilk testing shows that the proportion of secondary cells positive for *hh* > GFP expression is normally distributed ($p < 0.01$).

GFP-tagged forms of Hedgehog are carried on secretory granules and are released into the seminal fluid

Since *hedgehog* is expressed in the secondary cells of the male accessory gland (and that this expression seems to begin after eclosion), I reasoned that *hedgehog* might be required for a function specific to the adult. To better understand what this function might be, I decided to determine where in the secondary cells Hedgehog localises. To do so, I made use of a GFP-tagged form of Hedgehog (Hh::GFP; TORROJA *et al.*, 2004) and expressed it in the secondary cells of adult males by using the temperature-sensitive *dsx*-GAL4/GAL80^{ts} driver (*dsx*^{fs}; *dsx*^{fs} > Hh::GFP). Doing so, and imaging accessory glands live[5] (stained with LysoTracker-Red to visualise intracellular acidic compartments), I found that some Hh::GFP goes into the secondary cell acidic compartments. Hh::GFP also labels the limiting membrane of the large secondary cell intracellular secretory compartments—as well as being present on the secretory granules contained within these compartments (**FIGURE 4. 8. A**). That Hh::GFP is carried within the secretory granule—which contains protein cargo to be secreted from the secondary cell—suggested to me that Hh::GFP is released from the secondary cell into the seminal fluid. Dissecting glands expressing *dsx*^{fs} > Hh::GFP in paraformaldehyde and staining these with α-ANCE (to label the seminal fluid), confirmed that Hh::GFP is indeed released from the secondary cells into the accessory gland lumen (**FIGURE 4. 9. A**)—where luminal Hh::GFP appears as discrete GFP-positive *puncta*. Interestingly, when accessory glands expressing *dsx*^{fs} > Hh::GFP are imaged live and stained with LysoTracker-Red, Hh::GFP appears to make its way from the secondary cells into the surrounding main cells (**FIGURE 4. 8. A**; **FIGURE 4. 10.**)—

[5] Live imaging allows for better detection of faint GFP signals in secondary cells (S. REDHAI & B. KROEGER, personal comments).

with the concentration of Hh::GFP-positive main cells seemingly decreasing with increasing distance from the Hh::GFP-expressing secondary cell (**FIGURE 4. 10. B**). (I admit this data needs to be verified further to render it more convincing, but I find it interesting enough to merit inclusion here—to serve as a foundation for future work.) Altogether, these observations suggest that Hh::GFP behaves like a secondary cell secretory product, and provides tentative evidence for a role of Hedgehog not only as a molecule with the potential to pass to other cells in the male accessory gland epithelium, but also as a novel component of the *D. melanogaster* seminal fluid.

The Hedgehog molecule is post-translationally modified to yield a mature Hedgehog protein containing both cholesterol and palmitic acid moieties that are required for the molecule's full activity and spread through tissue (BRISCOE & THÉROND, 2013). Finding that wild-type GFP-tagged Hedgehog labels secondary cell secretory compartments and that it is released into the seminal fluid, I wondered if the Hedgehog protein needs to be lipid-modified for this localisation pattern to develop? To investigate, I turned to three differentially-lipidated GFP-tagged Hedgehog constructs that are available (**FIGURE 4. 11.**). The first, HhN::GFP (TORROJA *et al.,* 2004), constitutes only the N-terminal part of the Hedgehog molecule and its palmitic acid residue, and lacks the cholesterol moiety. HhN::GFP is, however, signalling competent, since the cholesterol is thought to only maximise Hedgehog signalling activity without being absolutely required for its function (WENDLER & FRANCH-MARRO & VINCENT, 2006). Consistent with this, when expressed in secondary cells using dsx^{ts} (dsx^{ts} > HhN::GFP), I find that this construct behaves similar to wild-type Hedgehog: labelling the limiting membrane of the secondary cell secretory compartments and being carried on their secretory granules. The molecule also goes into the secondary cell acidic compartments, and is released

into the gland lumen as punctate structures (**FIGURE 4. 8. B**; **FIGURE 4. 9. B–C**). However, I also found that HhN::GFP is present only in the secondary cells and does not go into the surrounding main cells (**FIGURE 4. 8. B**); suggesting that the cholesterol moiety might be important for the extracellular spread of Hedgehog in the accessory gland epithelium. The second GFP-tagged Hedgehog construct, HhC85S::GFP (SIERRA *et al.,* 2004), contains a cysteine → serine mutation at amino acid position 85 ("**C85S**") that prevents palmitylation of Hedgehog. As a result, the HhC85S::GFP

FIGURE 4. 8. | THE HEDGEHOG MOLECULE LABELS SECONDARY CELL INTRACELLULAR COMPARTMENTS AND IS CARRIED ON SECRETORY GRANULES.

GFP-tagged Hedgehog protein was expressed in secondary cells for three or six days using a temperature-sensitive *dsx*-GAL4/GAL80^{ts} driver. LysoTracker-Red (pink) stains the acidic compartments.

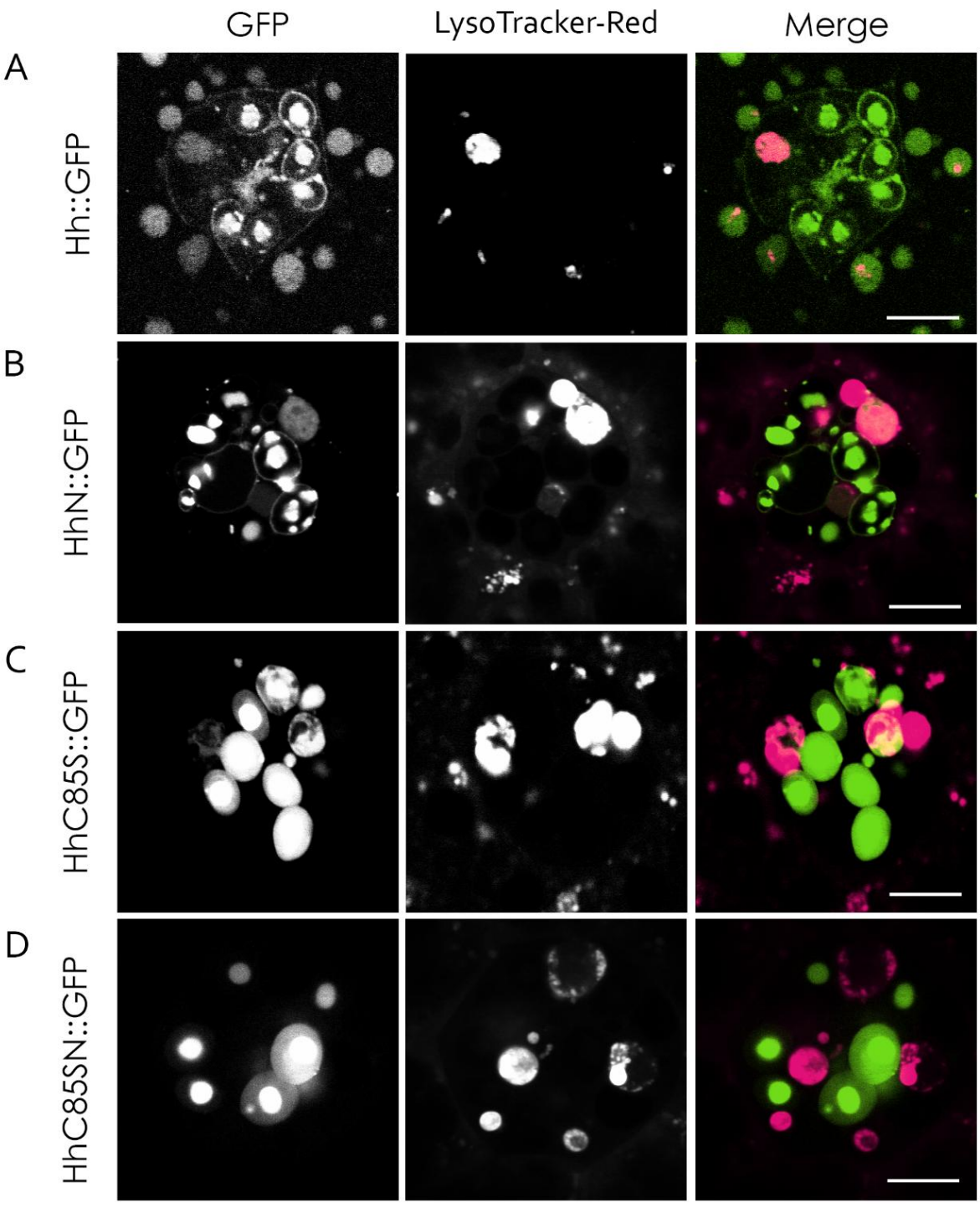
A: Hh::GFP (green) labels the limiting membrane of the secondary cell secretory (non-acidic) compartments after three days, and is carried on secretory granules. Hh::GFP also seem to go into the secondary cell acidic compartments—as well as those of the main cells. (It is worth noting that the main cell acidic compartments appear unusually large in this genotype, suggesting that Hedgehog ligand uptake has an effect on their function.)

B: HhN::GFP (green) after six days labels the limiting membrane of the secondary cell exocytic compartments, as well as being carried on the secretory granules. HhN::GFP seems to be excluded from the main cells.

C: HhC85S::GFP (green) after six days, labels secondary cell exocytic compartments, and is carried on the secretory granules, but the distribution of this form is more diffuse throughout the compartments and it does not appear to go into either main cells or the secondary cell acidic compartments.

D: HhC85SN::GFP (green) after six days, is diffusely distributed throughout the exocytic compartments and their secretory granules. It is excluded from the secondary cell acidic compartments and the surrounding main cells.

Scale bars in all panels correspond to 10 μm.


GFP
LysoTracker-Red
Merge
A
Hh::GFP
B
HhN::GFP
C
HhC85S::GFP
D
HhC85SN::GFP

protein contains only the Hedgehog cholesterol moiety (**FIGURE 4. 11. C**). This cholesterol-only form of Hedgehog is strongly signalling impaired, since the palmitic acid residue is important for the Hedgehog signalling activity (WENDLER & FRANCH-MARRO & VINCENT, 2006). When expressed in secondary cells, HhC85S::GFP labels the secretory compartments and their secretory granules. However, this labelling is different from that observed with either the Hh::GFP or the HhN::GFP constructs; with the labelling being more diffuse and distributed throughout the entire secretory compartment, as if HhC85S::GFP is not properly

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FIGURE 4. 9. | THE HEDGEHOG MOLECULE IS RELEASED FROM SECONDARY CELLS INTO THE ACCESSORY GLAND LUMEN.

When expressed in secondary cells for six days using *dsx*^{ts}, GFP-tagged Hedgehog is released into the accessory gland lumen, where its distribution seems to depend on its lipid modification.

A: Hh::GFP (green) contains both an N-terminal palmitic acid residue and a C-terminal cholesterol moiety, and is released as discrete *puncta* into the gland lumen (stained with α-ANCE; pink).

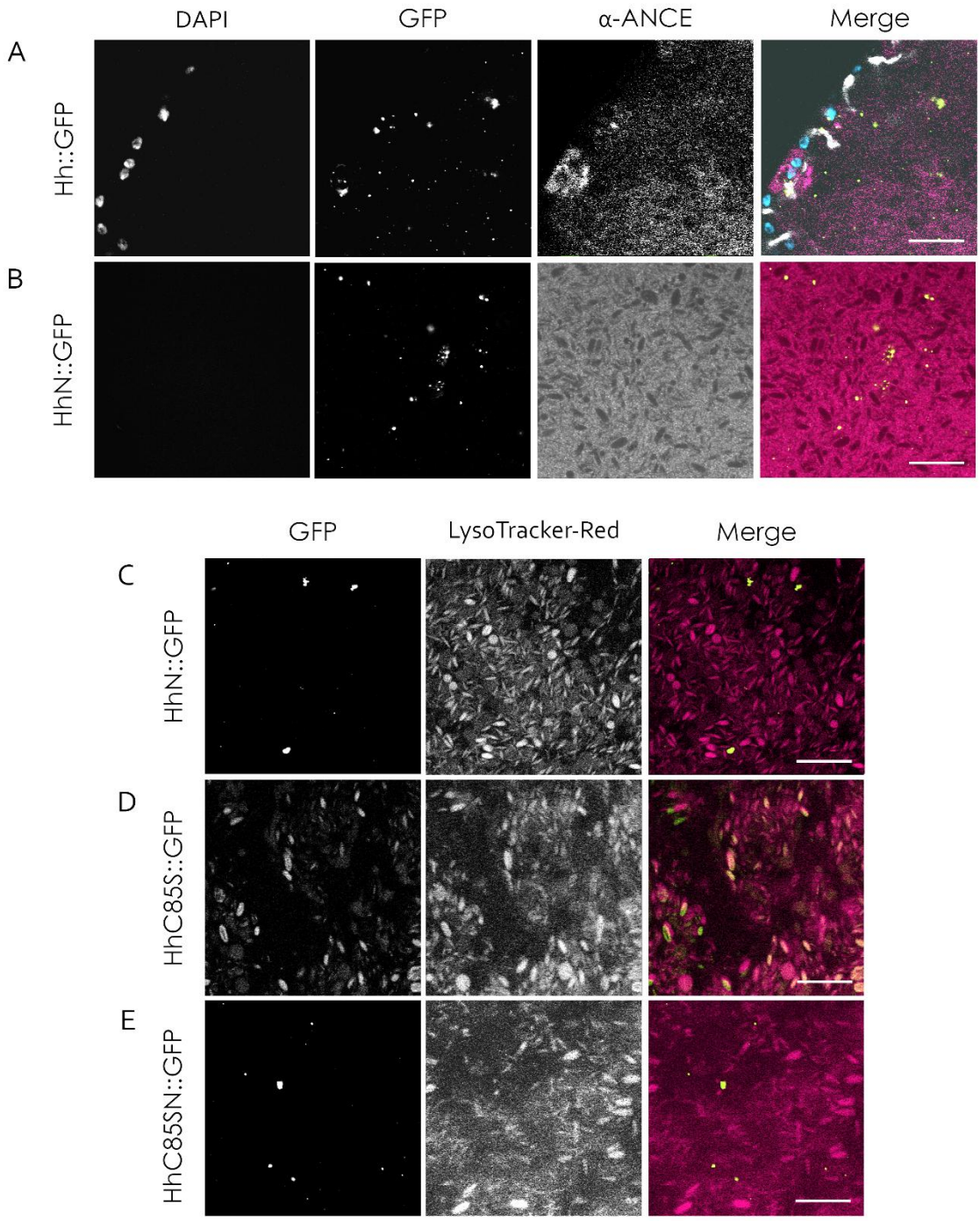
B: HhN::GFP (green) behaves similarly to wild-type Hh::GFP: being released as punctate structures into the gland lumen. These *puncta* do not appear to associate with the cigar-shaped (ovoid) hydrophobic protein flocculates therein (visible as negative spaces in the α-ANCE stain; magenta).

C: When imaged in live tissue, HhN::GFP again appears as discrete *puncta* that do not co-localise with the ovoid flocculates (stained with LysoTracker-Red; magenta).

D: HhC85S::GFP (green) is released from the secondary cells, although it does not appear as discrete *puncta*, but rather seems to associate with a subset of the LysoTracker-Red–positive ovoid flocculates (magenta).

E: HhC85SN::GFP (green) appears as (sparsely distributed) *puncta* that do not appear to associate with the LysoTracker-Red–labelled ovoid flocculates (magenta).

Scale bars correspond to 20 μm in all panels, and in panel **A,** α-FASIII labels main cell junctions (white).


DAPI
GFP
α-ANCE
Merge
A
Hh::GFP
B
HhN::GFP
GFP
LysoTracker-Red
Merge
C
HhN::GFP
D
HhC85S::GFP
E
HhC85SN::GFP

packaged into the secretory granule (**FIGURE 4. 8. C**). Interestingly, HhC85S::GFP seems to be excluded from the secondary cell acidic compartments, and, like HhN::GFP, HhC85S::GFP does not appear to go into the main cells; suggesting that the palmitic acid residue might be important for the extracellular spread of Hedgehog. What is more, whereas HhC85S::GFP is released from the secondary cells into the accessory gland lumen, it is not present in the lumen as discrete *puncta*. Rather, HhC85S::GFP appears to associate with the cigar-shaped (ovoid) flocculates present in the non-aqueous phase of the seminal fluid (**FIGURE 4. 9. D**). The third Hedgehog construct, HhC85SN::GFP (a gift from I. GUERRERO), lacks both the palmitic acid and cholesterol modifications of the wild-type molecule; severely impacting the signalling activity of the Hedgehog molecule. When expressed in secondary cells, HhC85SN::GFP behaves very similar to HhC85S::GFP regarding its localisation within the secondary cells (**FIGURE 4. 8. D**)—but not regarding its distribution within the accessory gland lumen, where it appears as sparse, but discrete, *puncta* (**FIGURE 4. 9. E**). Altogether, this suggests that the lipid modifications of the Hedgehog molecule are important for the function and distribution of Hedgehog protein in the male accessory gland.

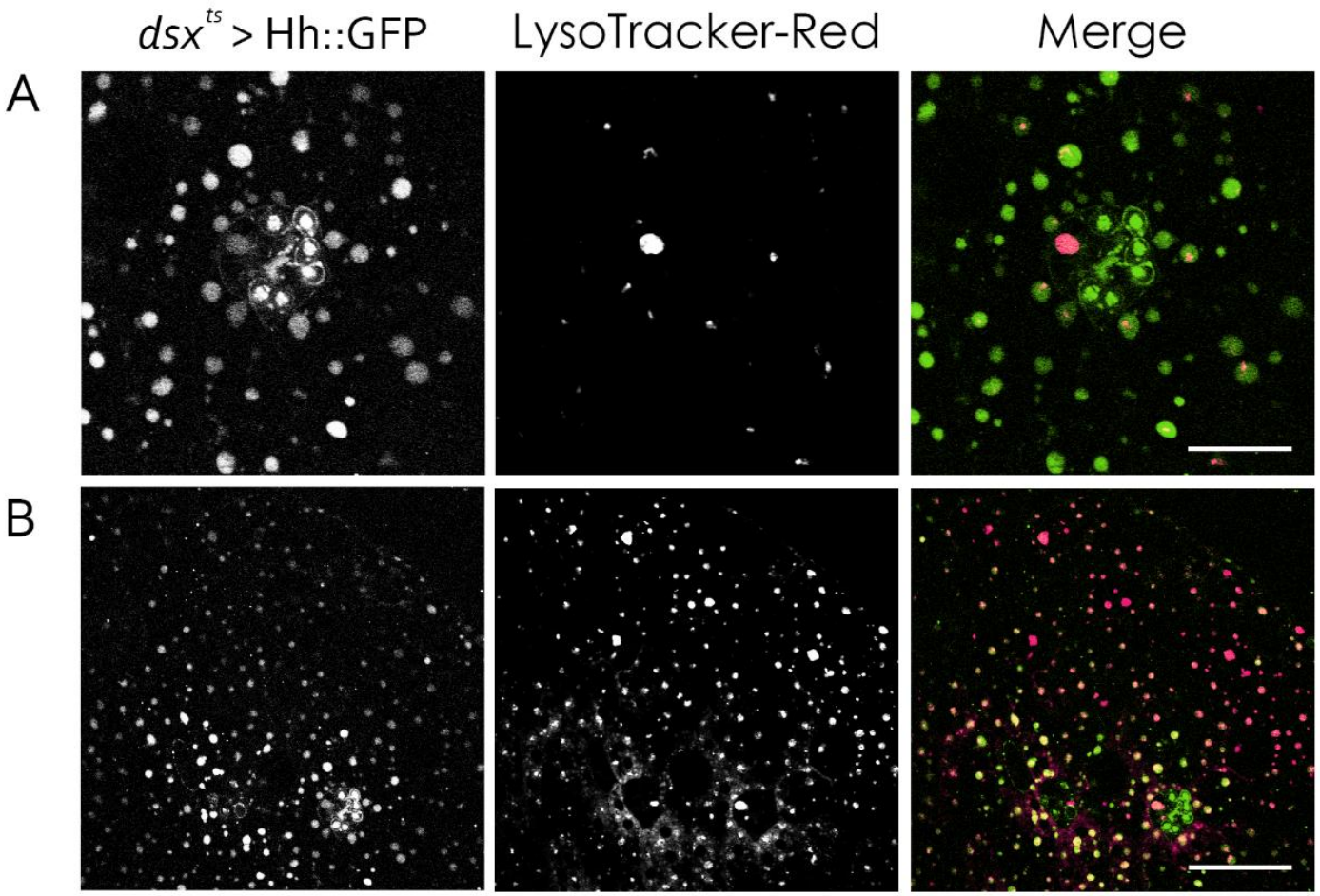


FIGURE 4. 10. | THE HEDGEHOG MOLECULE SEEMS TO BE TRANSFERRED FROM SECONDARY CELLS INTO SURROUNDING MAIN CELLS.

When expressed in secondary cells for three days using *dsx*^{ts}, GFP-tagged Hedgehog labels secondary cell intracellular compartments and appears to make its way into the surrounding main cells.

A: Hh::GFP (green) labels the limiting membrane of the secondary cell secretory (non-acidic) compartments, and is carried on their secretory granules. Hh::GFP also seems to go into large main cell compartments—many of which are stained with LysoTracker-Red (magenta).

B: A lower-powered image of the same field of cells as shown in panel **A** shows more clearly that Hh::GFP (green)seems to be present in the main cell acidic compartments (pink)—and that it is more highly concentrated in the most nearby main cells.

The scale bar in panel **A** corresponds to 20 μm and 50 μm in panel **B**.

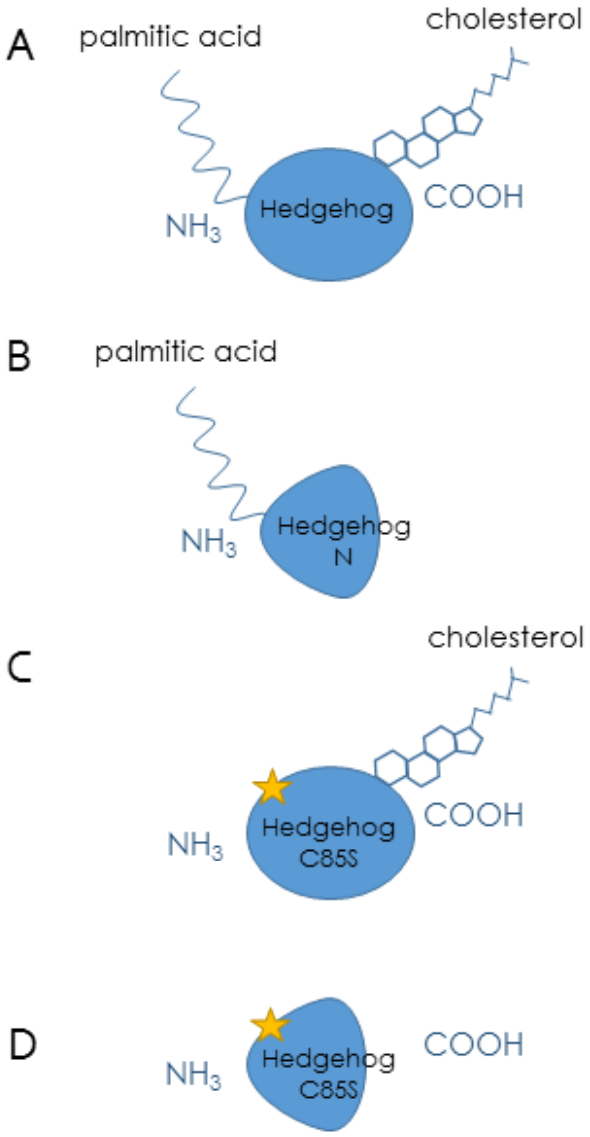


FIGURE 4. 11.| DIFFERENT HEDGEHOG CONSTRUCTS CARRY DIFFERENT COMBINATIONS OF LIPID MODIFICATIONS.

A: Wild-type Hedgehog (Hh) contains both an N-terminal palmitic acid residue and a C-terminal cholesterol moiety.

B: The N-terminal form of Hedgehog (HhN) contains only the N-terminal palmitic acid residue.

C: The form of Hedgehog carrying a cysteine → serine mutation (HhC85S) carries only the C-terminal cholesterol moiety.

D: The N-terminal form of Hedgehog carrying a cysteine → serine mutation (HhC85SN) carries none of the lipid moieties of the wild-type molecule.

The Hedgehog receptor, *patched*, is also expressed in the male accessory gland

Patched (Ptc) is the receptor of the Hedgehog ligand and is expressed by cells that are sensitive to Hedgehog signalling (CAPDEVILA *et al.*, 1994; TABATA & KORNBERG, 1994). Knowing that secondary cells are a site of *hedgehog* expression, and that secondary cells might release Hedgehog into the accessory gland lumen, I wanted to establish where in the accessory gland *patched* was expressed. To do so, I used a *ptc*-GAL4 enhancer trap (HINZ *et al.*, 1994) to drive expression of *UAS*-GFP^{nls} (*ptc* > GFP)—as described for *hedgehog*, above (*p.* 151). Dissecting glands expressing *ptc* > GFP revealed that *ptc* > GFP is expressed in a similar pattern to *hh* > GFP: namely, in the secondary cells (**FIGURE 4. 12.**). However, unlike *hh* > GFP, *ptc* > GFP is also expressed in the tissue of the seminal vesicles and *vas deferens* (**FIGURE 4. 12.**)—as well as being expressed in neurons innervating the proximal accessory gland (**FIGURE 4. 13.**). Focusing on the secondary cells (**FIGURE 4. 14.**), *ptc* > GFP is expressed in all secondary cells in six-day old males, with all α-ANCE–positive secondary cells also showing strong expression of *ptc* > GFP (**FIGURE 4. 14. E**).

The number of secondary cells expressing *hh* > GFP increases over the first six days post-eclosion, as we have seen previously (**FIGURE 4. 6.** (*p.* 135) and **FIGURE 4. 7.** (*p.* 137)). Therefore, I was curious if *ptc* > GFP expression would show a similar pattern? To investigate, I dissected glands expressing *ptc* > GFP at various time-points post-eclosion, *viz.*, at 0 hours, 8 hours, 24 hours, 72 hours (day 3), and 144 hours (day 6). Using α-ANCE to label secondary cells, I determined what proportion of secondary cells expressed *ptc* > GFP at these time-points (**FIGURE 4. 15.**; **SUPPLEMENTARY TABLE 4. 2.**, *p.* 438). Unlike *hh* > GFP, *ptc* > GFP was already detectable is a small proportion of secondary cells at eclosion (**FIGURE 4. 14. A**). This proportion increased

rapidly over the first 8 hours after eclosion (p {0hr|8hr} < 0.0001) (**FIGURE 4. 14. B**). By 24 hours post-eclosion the majority of secondary cells were positive for *ptc* > GFP (**FIGURE 4. 14.. C**), and *ptc* > GFP expression remained high thereafter (p {d3|d6} > 0.1) (**FIGURE 4. 14. D–E**). Altogether, this suggests that *ptc* > GFP expression follows a similar pattern to *hh* > GFP overall (**SUPPLEMENTARY TABLE 4. 2.**, *p.* 438); with the expression beginning around or just after eclosion. However, it seems as if the proportion of *ptc* > GFP-positive cells increases much faster than the proportion of *hh* > GFP-positive cells (compare **FIGURE 4. 7.** (*p.* 157) and **FIGURE 4. 15.**)—perhaps because of differences in the behaviour of the enhancer trap lines.

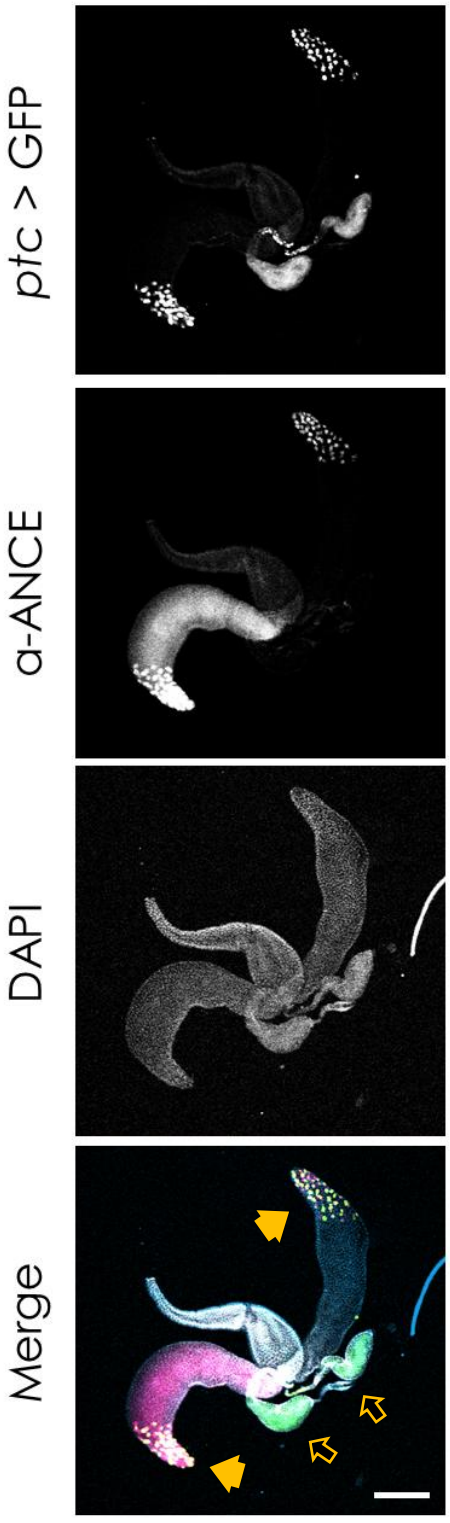


FIGURE 4. 12. | IN THE MALE REPRODUCTIVE TRACT, *ptc* > GFP IS EXPRESSED IN BOTH SECONDARY CELLS AND SEMINAL VESICLES.

A confocal image of the male accessory gland, seminal vesicles and ejaculatory duct, showing expression of *ptc* > GFP (green) in secondary cells (yellow arrows), and the tissues of the seminal vesicles and *vas deferens* (black arrows). DAPI stains DNA (cyan), and α-FASIII stains epithelial cell junctions (white). α-ANCE stains both secondary cells and the aqueous phase of the seminal fluid (pink). The scale bar corresponds to 200 μm.

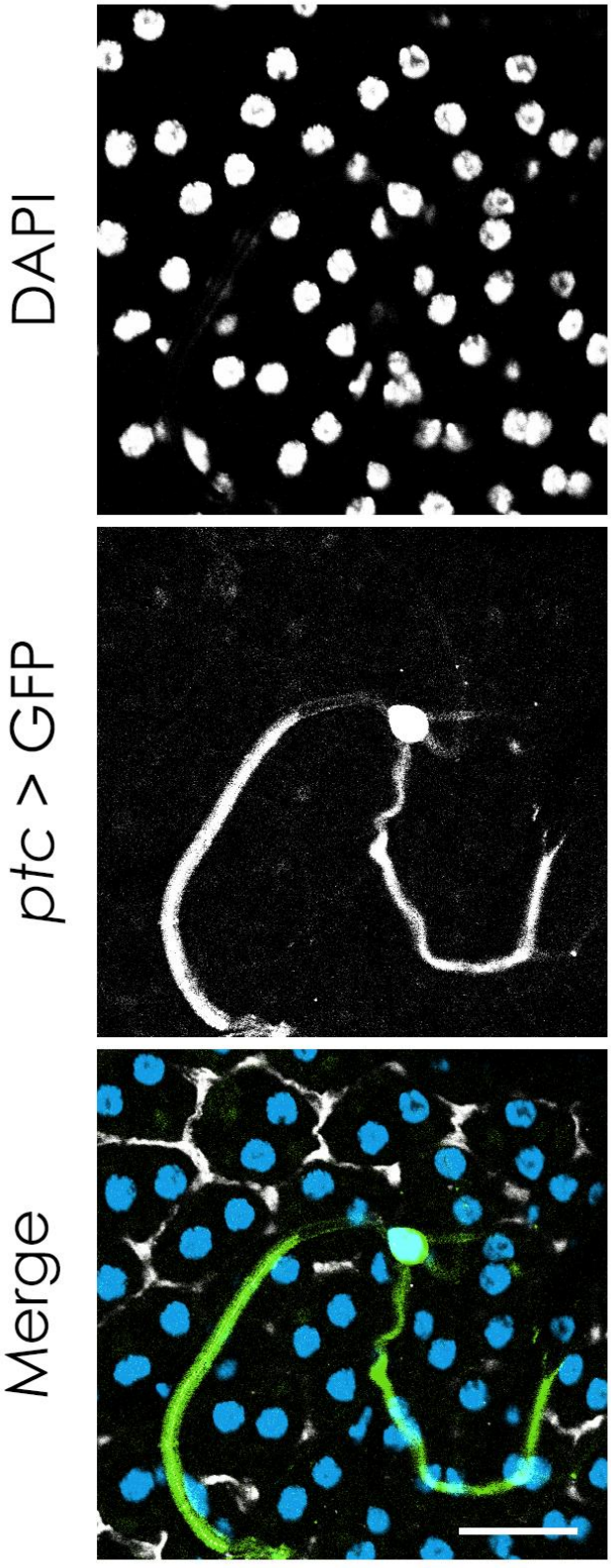


FIGURE 4. 13. | NEURONS ASSOCIATED WITH THE PROXIMAL MALE ACCESSORY GLAND EXPRESS *ptc* > GFP.

A confocal image of the proximal male accessory gland, close to where the gland attaches to the ejaculatory duct, showing expression of *ptc* > GFP (green) in a neuron with both cell body and extensions in close association with the tissue. DAPI stains DNA (cyan), and α-FASIII stains epithelial cell junctions (white). The scale bar corresponds to 20 μm.

FIGURE 4. 14. | SECONDARY CELLS IN THE MALE ACCESSORY GLAND ARE A SITE OF *ptc* > GFP EXPRESSION IN ADULT *D. MELANOGASTER*.

Secondary cells express *ptc* > GFP and the proportion of *ptc* > GFP^+ secondary cells in the accessory gland increases over time.

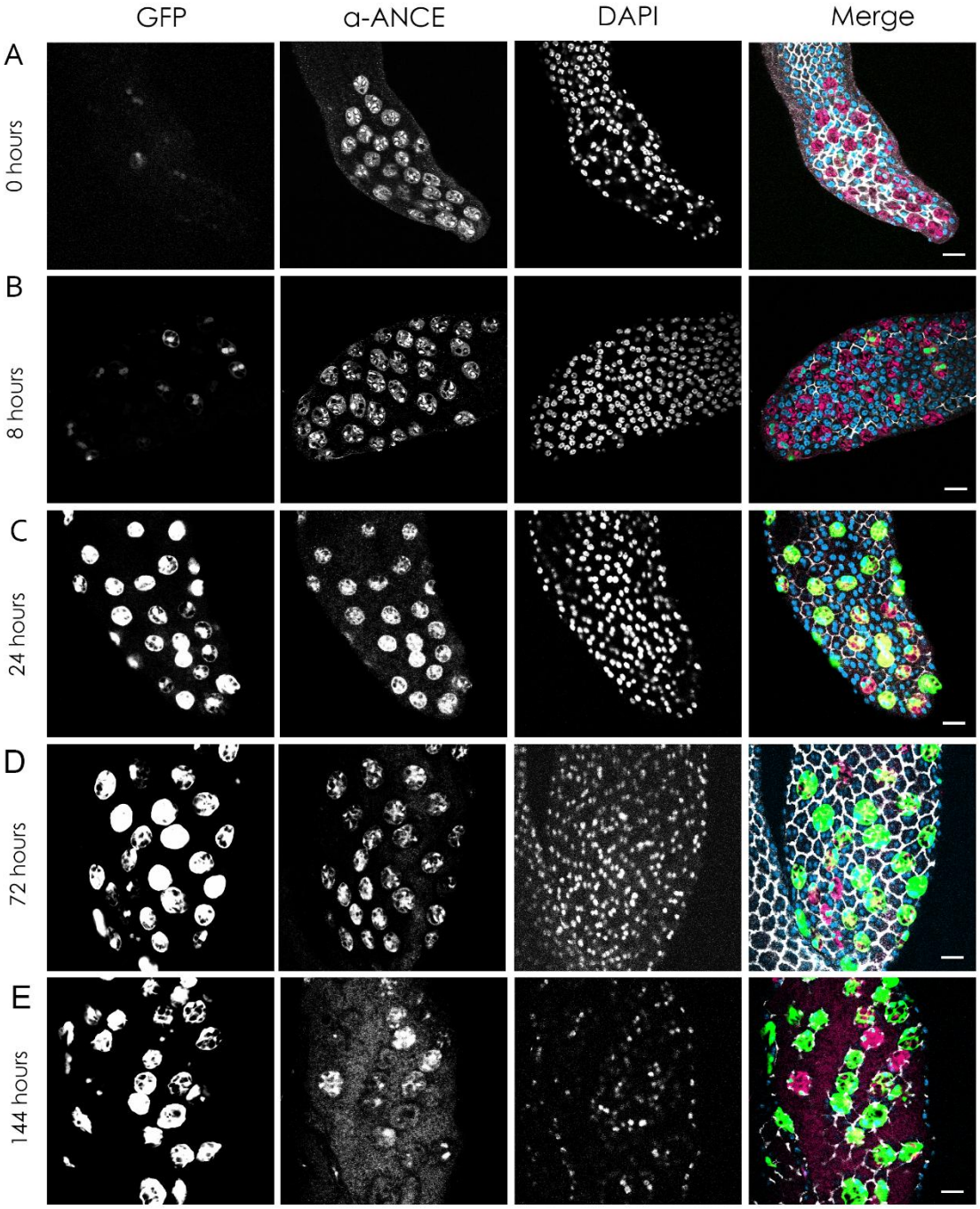
A: At eclosion (0 hours) there is already detectable *ptc* > GFP in the accessory gland, where secondary cells have been stained with α-ANCE.

B: By 8 hours post-eclosion, greater numbers of secondary cells positive for *ptc* > GFP are evident.

C: By 24 hours post-eclosion, *ptc* > GFP is detected in all secondary cells in the gland.

D–E: The high proportion of secondary cells positive for *ptc* > GFP is maintained through days 3–6 (72–144 hours).

In all panels, secondary cells are stained with α-ANCE (magenta), and α-FASIII (white) labels cell junctions. Scale bars in all panels correspond to 20 μm.


GFP
a-ANCE
DAPI
Merge
A
0 hours
B
8 hours
C
24 hours
D
72 hours
E
144 hours

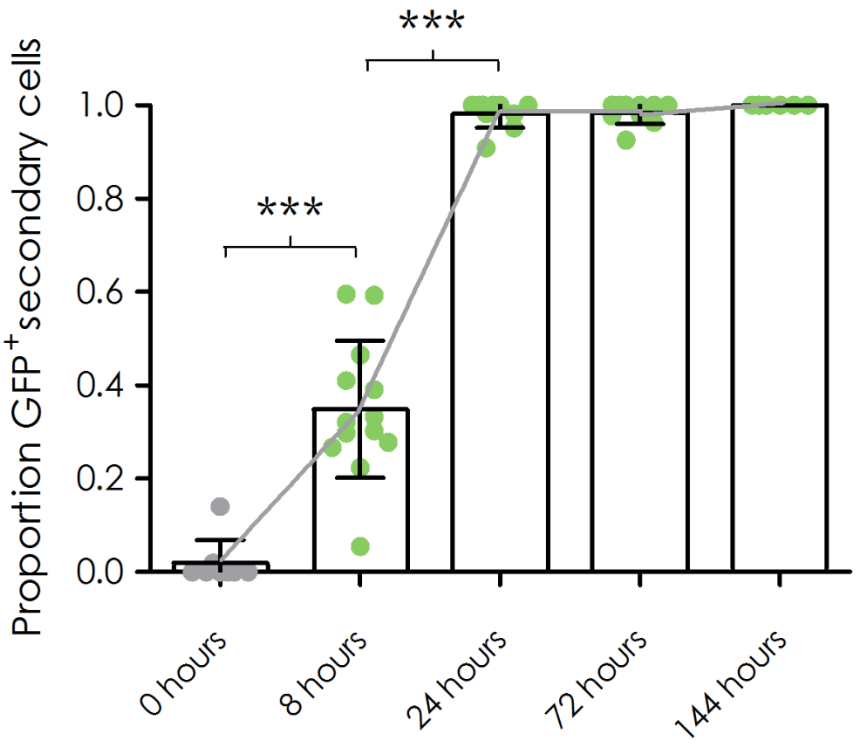


FIGURE 4. 15. | SECONDARY CELL *ptc* > GFP EXPRESSION INCREASES OVER TIME.

A scatterdot bar-chart showing how secondary cell *patched* gene expression begins after eclosion and increases over the first days post-eclosion. Each dot in the chart corresponds to data obtained from one individual male (see **SUPPLEMENTARY TABLE 4. 2.**, *p.* 438). The number of stars in the figure represents the *p*-value obtained by one-way ANOVA with a Bonferroni correction for multiple comparisons (all columns): *** = $p < 0.001$.

Hedgehog might signal in an autocrine fashion in secondary cells

That secondary cells both release Hedgehog into the seminal fluid and express the Hedgehog receptor, *patched*, suggests that Hedgehog signalling might be autocrine in this cell type. In the absence of Hedgehog, Patched is typically located on the plasma membrane of the receiving cell, as well as on its internal compartments (ZHU *et al.,* 2003; LU *et al.,* 2006). Conversely, in the presence of Hedgehog, Patched is internalised and depleted from the cell membrane of the receiving cell (LU *et al.,* 2006). Thus, to determine if Hedgehog signalling might, in fact, be autocrine in secondary cells, I decided to characterise the subcellular localisation of Patched in secondary cells using a GFP-tagged form of Patched (Ptc::GFP; ZHU *et al.,* 2003). I reasoned that, if Hedgehog signalling is active in secondary cells, Patched would be expected to be absent from the secondary cell plasma membrane. To investigate, I used dsx^{ts} to express Ptc::GFP (dsx^{ts} > Ptc::GFP) in secondary cells. Imaging these cells live (after six days of continuous expression at the non-permissive temperature), I found that this GFP-tagged form of Patched labels secondary cell internal compartments—but that it also seems to be absent from the secondary cell plasma membrane (**FIGURE 4. 16. A**). Unfortunately, cells expressing this GFP-tagged form of Patched appear quite sick, and seem to contain abnormal amounts of internal membrane. Perhaps this is to be expected of overexpression of a signalling pathway component. However, the labelling pattern of Ptc::GFP is consistent with the localisation of Patched bound to Hedgehog, suggesting that Hedgehog signalling might be active in secondary cells, and that it might be autocrine. It should be noted, however, that in this scenario, Patched would be expected to be highly overexpressed, and, therefore, the intracellular localisation of Ptc::GFP may not be fully explained by ligand-binding alone. To follow up on these

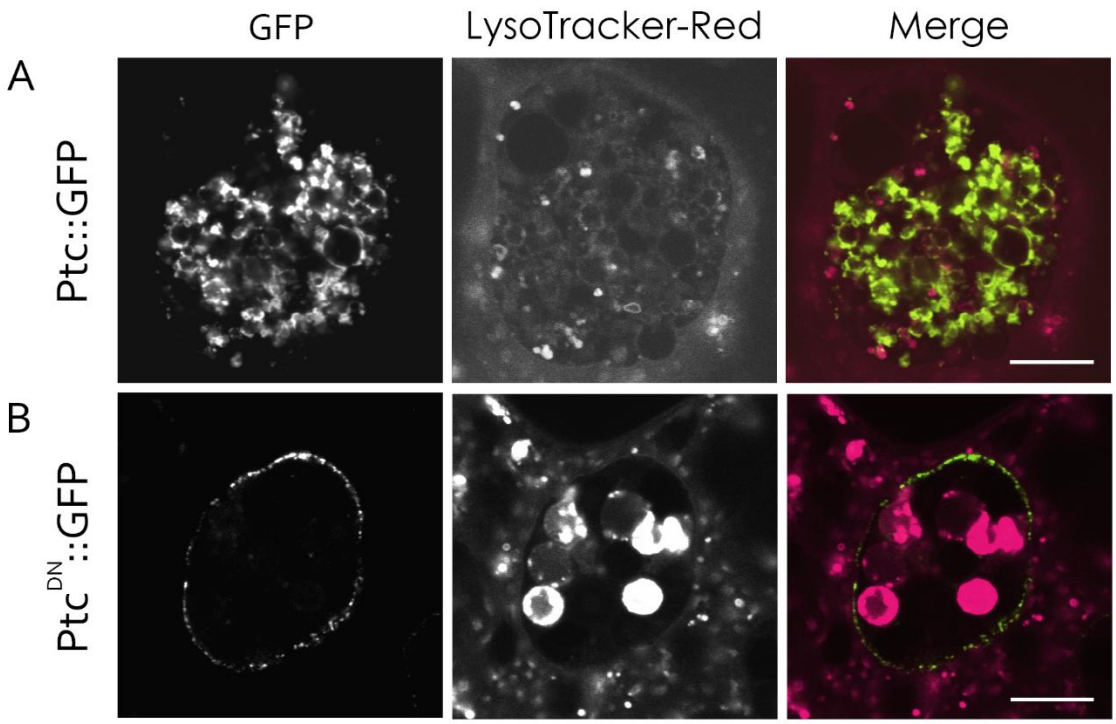


FIGURE 4. 16. | THE SUBCELLULAR PROTEIN LOCALISATION OF THE HEDGEHOG RECEPTOR, PATCHED, SUGGESTS THAT HEDGEHOG SIGNALLING IS ACTIVE IN SECONDARY CELLS.

When expressed in secondary cells for six days using *dsx*^{ts}, GFP-tagged Patched constructs indicate that Hedgehog signalling is active in this cell type.

A: Wild-type GFP-tagged Patched (Ptc::GFP) (green) labels intricate secondary cell internal compartments. The construct appears to be absent from the secondary cell plasma membrane—which can be perceived faintly in the LysoTracker-Red channel (magenta). (Do however note that the secondary cell contains an atypical amount of internal membrane, and must therefore be considered sick. It would have been more informative for the behaviour of this construct to have been assessed following a brief pulse of transgene expression.)

B: A GFP-tagged form of dominant-negative Patched (Ptc^{DN}::GFP) (green)—which cannot be activated by Hedgehog signalling—labels the secondary cell plasma membrane. Acidic compartments are stained with LysoTracker-Red (magenta).

Scale bars in both panels correspond to 10 μm.

observations, I made use of a dominant-negative form of YFP(GFP)-tagged Patched protein (Ptc^{DN}::GFP; ZHU *et al.*, 2003) that permits activation of Hedgehog signalling even in the absence of Hedgehog (JOHNSON *et al.,* 2000). This dominant-negative Patched protein is unaffected by Hedgehog, and remains on the plasma membrane of the receiving cell—even when Hedgehog is present.[6] When expressed in secondary cells using dsx^{fs} (dsx^{fs} > Ptc^{DN}::GFP), Ptc^{DN}::GFP is present in a dotted pattern on the secondary cell plasma membrane (**FIGURE 4. 16. B**). This pattern is consistent with what ZHU *et al.* (2003) observed upon expression of this construct in the salivary gland cells of the *D. melanogaster* larva: "[dominant-negative Ptc::GFP being distributed in] a discontinuous, dotted pattern along the plasma membrane" (*p.* 1243). Altogether, GFP-tagged Patched appears competent to label the plasma membrane of secondary cells (as attested by the behaviour of dominant-negative Patched)—and the absence of wild-type (GFP-tagged) Patched from the secondary cell plasma membrane indicates that Hedgehog signalling is normally active in the male accessory gland.

Patched is an inhibitor of the transmembrane protein, Smoothened—in addition to being the Hedgehog receptor. Hedgehog binding to Patched prevents Patched from inhibiting Smoothened. This allows Smoothened (and Hedgehog signalling) to become active. In the absence of Hedgehog, Patched keeps Smoothened restricted to compartments in the cell interior, and Smoothened is only present on the plasma membrane after Hedgehog binding to Patched. Therefore, if Hedgehog signalling is active in secondary cells, a GFP-tagged form of Smoothened would be expected to label the secondary cell plasma membrane. However, expressing a GFP-tagged form of wild-type Smoothened (Smo::GFP; ZHU *et al.,* 2003) using dsx^{fs} (dsx^{fs} > Smo::GFP) does not give this result. Rather, Smo::GFP seems to label only internal compartments

[6] Recall that the Hedgehog-Patched interaction follows a negative logic; where Patched is a suppressor of the Hedgehog signalling pathway, and where Hedgehog is an inhibitor of Patched.

in the secondary cells (**FIGURE 4. 17.**). In addition, this construct also seems to label the surface if the secondary cell secretory granules faintly. This would not typically be expected of a transmembrane protein. However, high-resolution 3D-SIM microscopy of live secondary cells has suggested that small vesicles bud off from the limiting membrane of the secretory compartments and traffic toward the secretory granule (KROEGER, 2017). These tiny vesicles form bead- or foam-like structures on the surface of the granule, and common-

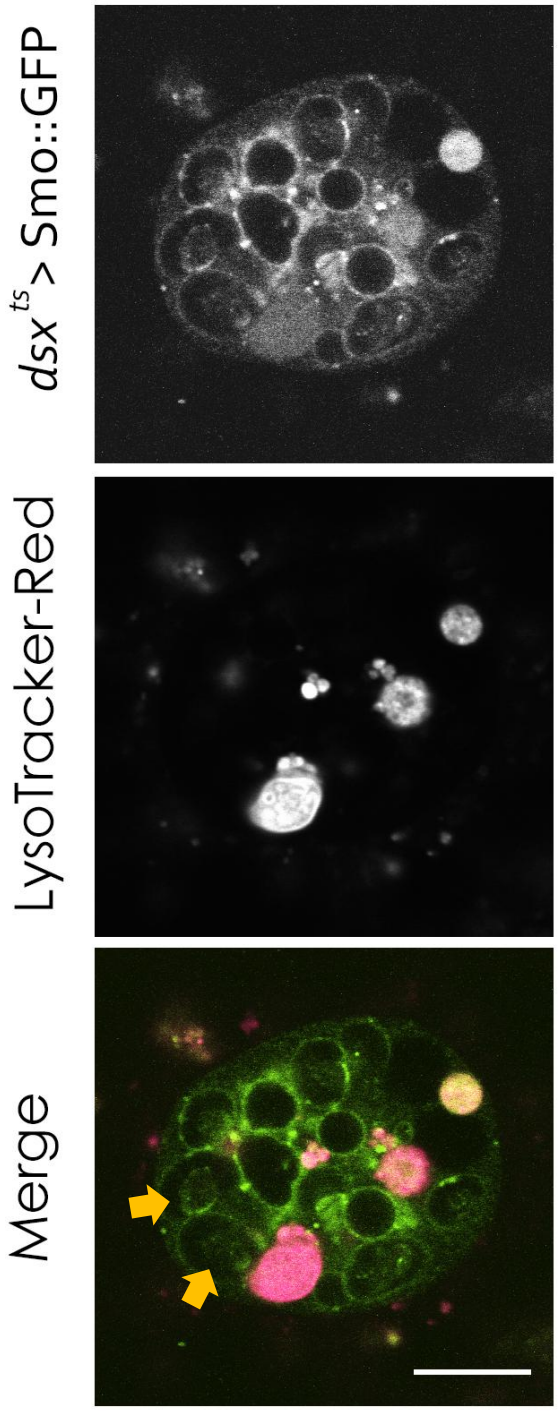


FIGURE 4. 17. | A GFP-TAGGED FORM OF SMOOTHENED LABELS SECONDARY CELL INTRA-CELLULAR COMPARTMENTS.

When expressed in secondary cells for six days using *dsx*^{ts}, GFP-tagged Smoothened (green) labels secondary cell intracellular compartments and appears to be absent from the secondary cell plasma membrane. However, the molecule appears to be present on the surface of secretory granules (yellow arrows), perhaps representing accumulation of small vesicles derived from the limiting membrane of the compartment. (As with the Ptc::GFP contrast in the previous figure, **FIGURE 4. 16.**, it would have been more informative to image cells after a brief pulse of transgene expression.) LysoTracker-Red (magenta) labels acidic compartments. The scale bar corresponds to 10 μm.

ly incorporate GFP-tagged transmembrane proteins (like CD63::GFP, or GPI::GFP, for example) (KROEGER, 2017). Therefore, it is possible that a GFP-tagged transmemebrane protein like Smoothened would behave similarly. Overall, the evidence from the Smo::GFP line must however be considered weak regarding its implications for Hedgehog signalling in secondary cells, since it is likely that overexpression of the protein changes the behaviour of the cells significantly. Indeed, it would have been more informative to image secondary cells after a brief pulse of Smo::GFP (or Ptc::GFP) transgene expression, rather than after six days of continuous expression as I have done here.

hedgehog and *patched* are expressed in the *D. melanogaster* female reproductive tract

That GFP-tagged Hedgehog is released into the seminal fluid when expressed in secondary cells suggests that Hedgehog might be delivered to the female upon mating. This motivated me to look at the female reproductive tract, to see if any of the tissues therein express the Hedgehog receptor, Patched. To this end, I used *ptc-GAL4* to drive expression of *UAS-GFP*, and characterised its expression pattern in the female reproductive tract on day six of adulthood (**FIGURE 4. 18.**). Focussing on the uterus and the associated sperm-storage organs, I observed *ptc* > GFP expression in the neurons innervating the latter: *viz.*, the seminal receptacle and the *spermathecae* (**FIGURE 4. 19.**). Since Patched is the Hedgehog receptor, and the neurons innervating the female sperm-storage organs have been shown to be important for the female post-mating response, this makes it theoretically possible that the female might be able to respond to Hedgehog ligand delivered by the male via the seminal fluid. However, *patched* is also a target gene of Hedgehog signalling, and is induced by high concentrations of

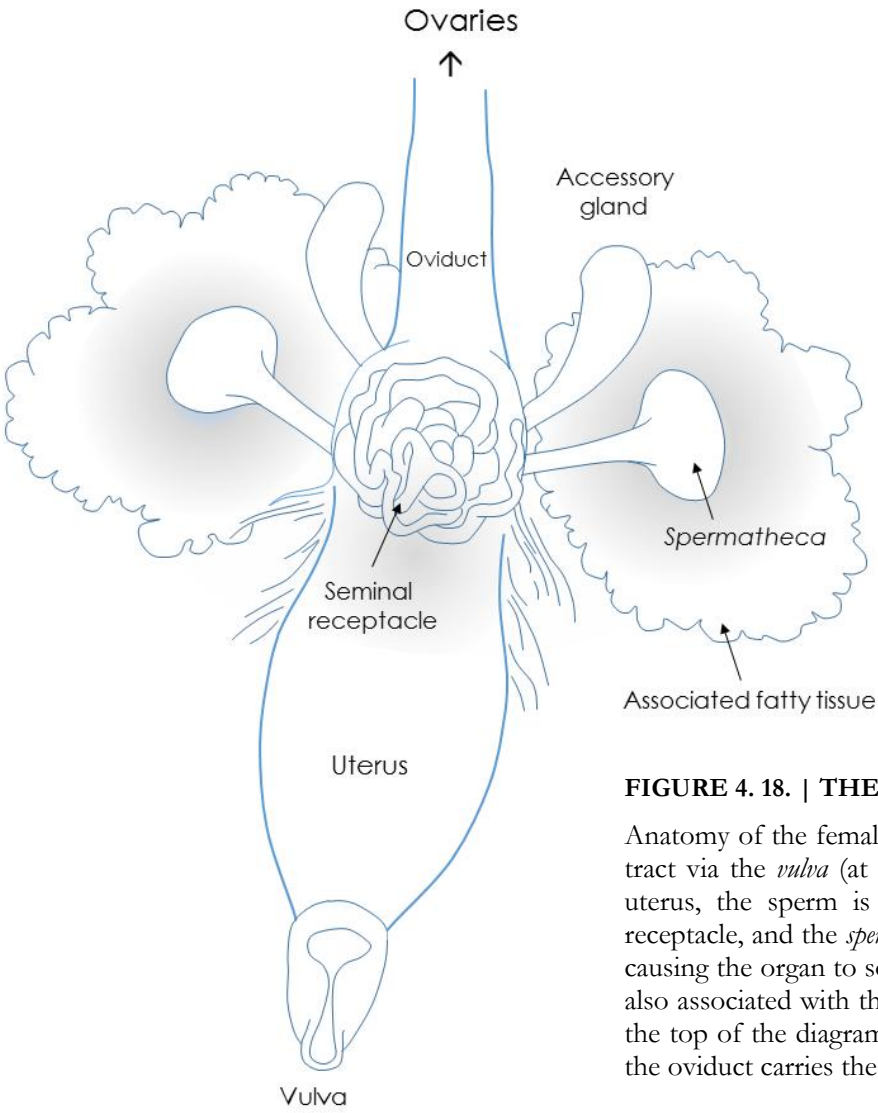


FIGURE 4. 18. | THE *D. MELANOGASTER* FEMALE REPRODUCTIVE TRACT.

Anatomy of the female reproductive tract. The male ejaculate enters the female reproductive tract via the *vulva* (at the bottom of the diagram), to be deposited in the uterus. From the uterus, the sperm is further localised to the female sperm storage organs: the seminal receptacle, and the *spermathecae* (*s. spermatheca*). The *spermatheacae* are surrounded by fatty tissue, causing the organ to somewhat resemble a flower or mushroom. Female accessory glands are also associated with the reproductive system. The oviduct is located anterior to the uterus (at the top of the diagram) and connects the uterus with the ovaries (not shown). At ovulation, the oviduct carries the egg into the uterus, to be fertilised, and oviposited.

Hedgehog protein (HEPKER & WANG *et al.*, 1997). Therefore, it is also possible that the neurons of the female reproductive tract express *patched* because Hedgehog is already present in the tissue. Examining the expression pattern of *hh-GAL4 > UAS-GFP* (*hh* > GFP) in the female reproductive tract reveals that *hedgehog* is indeed expressed in the same tissues that are innervated by the *ptc* > GFP-positive neurons: the seminal receptacle and the *spermathecae* (**FIGURE 4. 20.**). Altogether, this suggests that both *hedgehog* and *patched* are expressed in the sperm storage organs of the female reproductive tract—prompting questions for future work. For example, is Hedgehog signalling in the female sperm-storage organs required for her reproductive functions? Can male-derived Hedgehog complement these?

Hedgehog regulates secondary cell nuclear growth

Hedgehog signalling regulates cell growth (DUMAN-SCHEEL *et al.,* 2002). Therefore, I was curious to determine if Hedgehog can regulate cell growth also in the accessory gland. Previous work has shown that secondary cell nuclei grow in response to various growth stimuli (*e.g.* BMP signalling; LEIBLICH *et al.*, 2012); prompting suggestions that secondary cell nuclear growth can be used as a proxy for secondary cell growth. Thus, to determine if Hedgehog regulates secondary cell growth, I decided to examine the role of Hedgehog in the regulation of secondary cell nuclear growth.

Secondary cells express the snail transcription factor, *escargot* ("***esg***") at high levels (LEIBLICH *et al.*, 2012), and the temperature-sensitive *esg FLP-Out* system ("***esg F/O^{ts}***") (JIANG *et al.*, 2009) can be used to stably and reliably drive transgene expression in secondary cells (LEIBLICH *et al.*, 2012). Briefly, in the *esg F/O^{ts}* system (**FIGURE 4. 21.**), *esg*-GAL4 is combined with a temperature-sensitive form of the GAL4

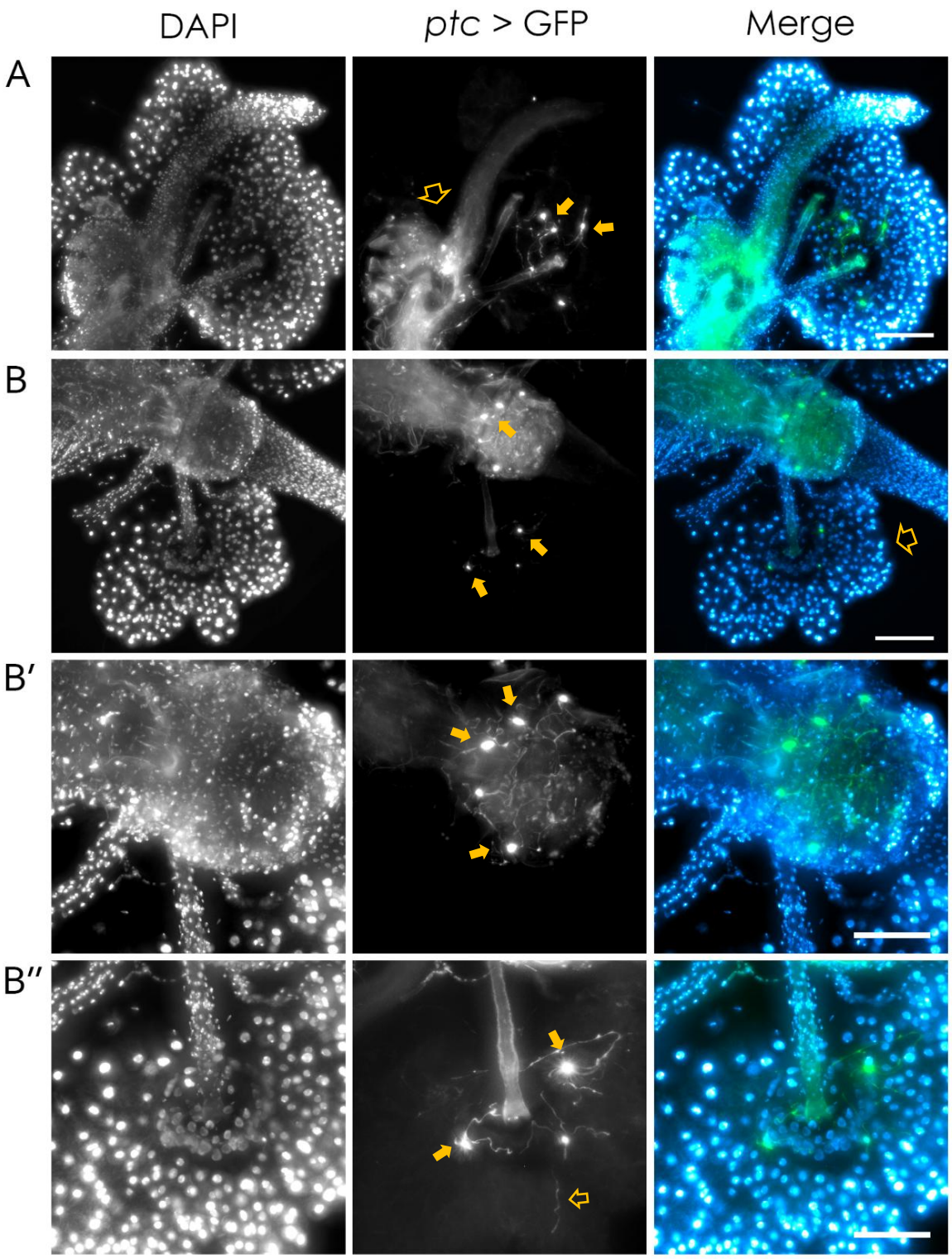

DAPI
ptc > GFP
Merge
A
B
B′
B″

FIGURE 4. 19. | *ptc* > GFP IS EXPRESSED IN NEURONS INNERVATING THE FEMALE SPERM-STORAGE ORGANS.

A: Using *ptc*-GAL4 to express *UAS*-GFP (green) reveals that *ptc* > GFP is expressed in neurons innervating the *spermatheca* (yellow arrows). The green staining in the oviduct (towards the top of the panel) and uterus (bottom left of panel) represents artefactual autoflourescence (black arrow).

B: A dorsal view of the female reproductive tract shows that *ptc* > GFP is expressed in neurons innervating the *spermatheca* (centre of panel; yellow arrows), as well as the seminal receptacle or proximal part of the uterus (towards the top of the panel). The flower-shaped tissue (black arrow) that surrounds the *spermatheca* is easily visible.

B': An enlarged view of (**B**), providing a better view of the neurons innervating the seminal receptacle or proximal part of the uterus (arrows). Note how the presumed cell bodies seem to stain brighter with GFP than the fine tendril-like projections emanating from them.

B": An enlarged view of (**B**), providing a better view of the neurons innervating the tissue surrounding the *spermatheca* (yellow arrows). Note the fine GFP-positive projections emanating from the neurons (black arrow).

Scale bars in panels **A–B** correspond to 100 μm, whereas the scale bars in panels **B'–B"** correspond to 50 μm.

These images were acquired in collaboration with Benjamin R. Kroeger (a *D. Phil.* student in our laboratory), at the University of Oxford Micron Imaging Facility, Department of Biochemistry.

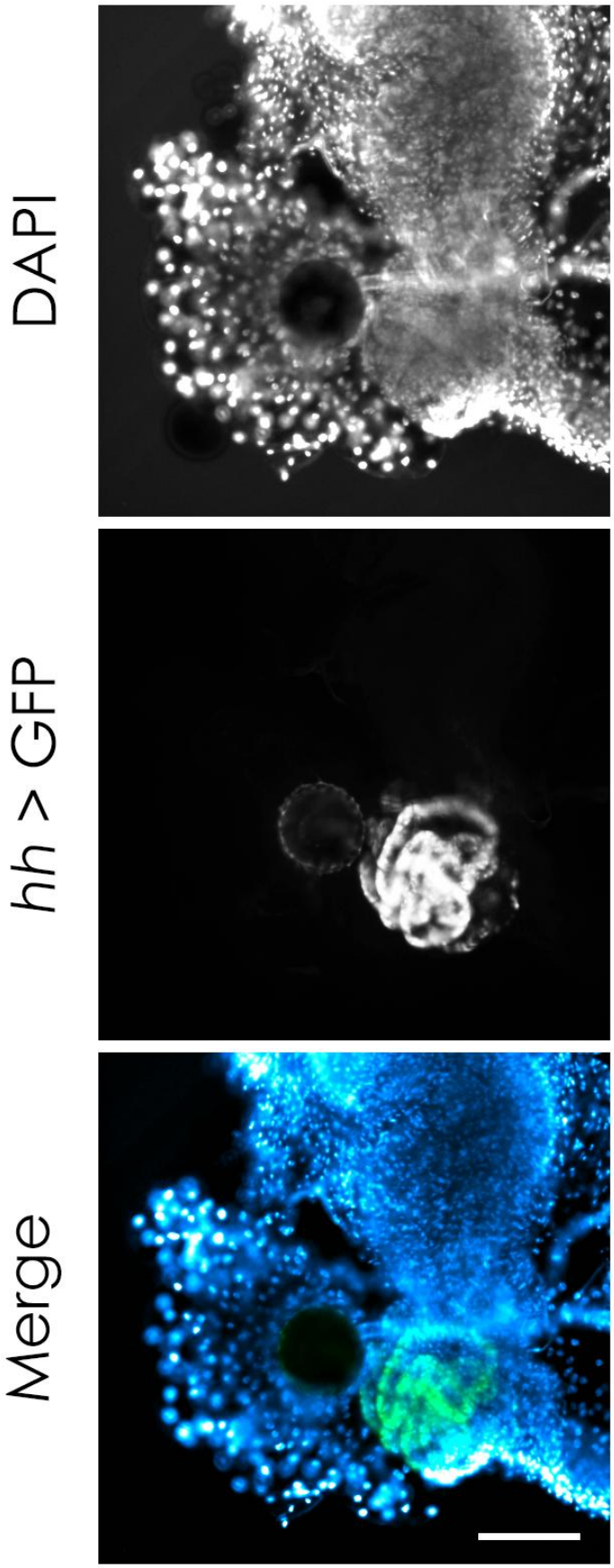


FIGURE 4. 20. | *hh* > GFP IS EXPRESSED IN THE FEMALE SPERM-STORAGE ORGANS.

Using *hh*-GAL4 to express *UAS*-GFP (green) reveals that the tissues of both the *spermatheca* and the seminal receptacle express *hh* > GFP. DAPI is shown in cyan. The type of cells stained with GFP is not known.

The scale bar in this figure corresponds to 100 μm.

This image was acquired in collaboration with Benjamin R. Kroeger (a *D. Phil.* student in our laboratory), at the University of Oxford Micron Imaging Facility, Department of Biochemistry.

inhibitor, GAL80^{ts}, driven by a tubulin promoter (*tub*-GAL80^{ts}) (MCGUIRE *et al.*, 2003; SUSTER *et al.*, 2004). At the non-permissive temperature (28.5 °C), GAL80^{ts} can no longer inhibit GAL4 activity, allowing *esg*-GAL4 to drive expression of a FLP recombinase (*UAS-FLP*). This catalyses a recombination event between two *FRT* sites flanking a CD2 coding sequence (*FRT*-CD2-*FRT*), that lie upstream of the GAL4 coding sequence. The *GAL4* transgene is associated with an *actin* ("***act***") promoter, so that the FLP-mediated recombination event generates functional *act*-GAL4. This new *act*-GAL4 driver can then be used to express both *UAS*-driven reporters and transgenes (JIANG *et al.*, 2009). When used in secondary cells, *esg F/O*^{ts} is combined with *UAS-GFP*^{nls} (*esg F/O*^{ts} > GFP) to label the cells and set them apart from the surrounding epithelium (LEIBLICH *et al.*, 2012).

To determine if Hedgehog is involved in the regulation of secondary cell nuclear growth, I used *esg F/O*^{ts} > GFP to drive expression of three different inverted repeats that target *hedgehog* mRNA (*hh*^{RNAi}s: I, II, and III) (*esg F/O*^{ts} > *hh*^{RNAi}) (NI *et al.,* 2008; NI *et al.*, 2010). In the *esg F/O*^{ts} background, secondary cell nuclei are smaller than main cell nuclei at eclosion, but experience age-dependent growth as the accessory gland matures, so that, by day six, secondary cell nuclei are larger than those of the surrounding main cells (LEIBLICH *et al.*, 2012). In flies whose secondary cells have expressed *hh*^{RNAi} throughout the first six days have nuclei smaller than those of controls ($p < 0.001$) (**FIGURE 4. 22.; SUPPLEMENTARY TABLE 4. 3.**, *p.* 439). Importantly, *hh*^{RNAi} expression in secondary cells has no effect on main cell nuclear size (**FIGURE 4. 22. E**); suggesting that the growth-inhibitory effects of knocking down *hedgehog* are specific to secondary cells. No change in the area of the secondary cells was observed in cells expressing

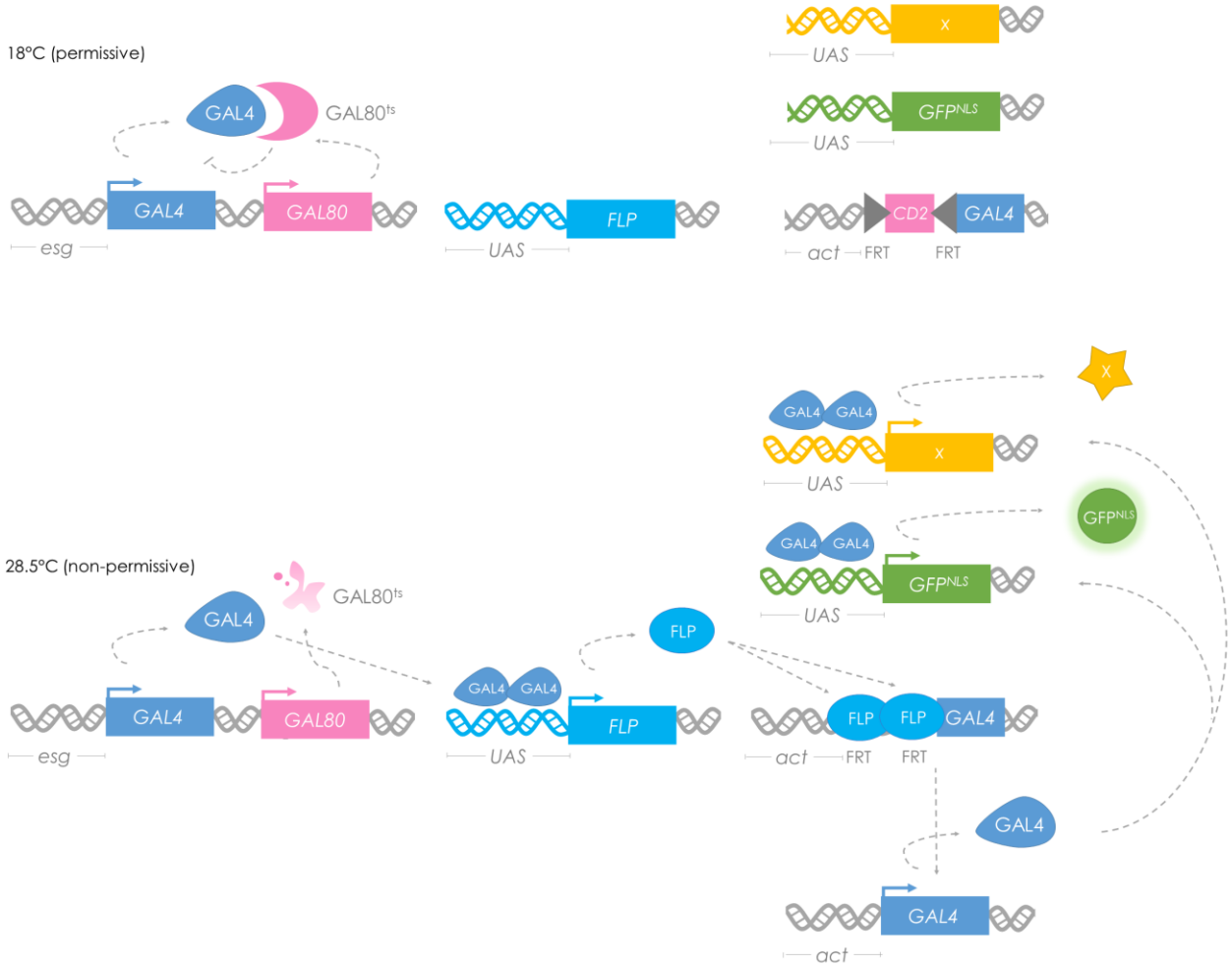


FIGURE 4. 21. | THE *esg* F/O^{ts} SYSTEM.

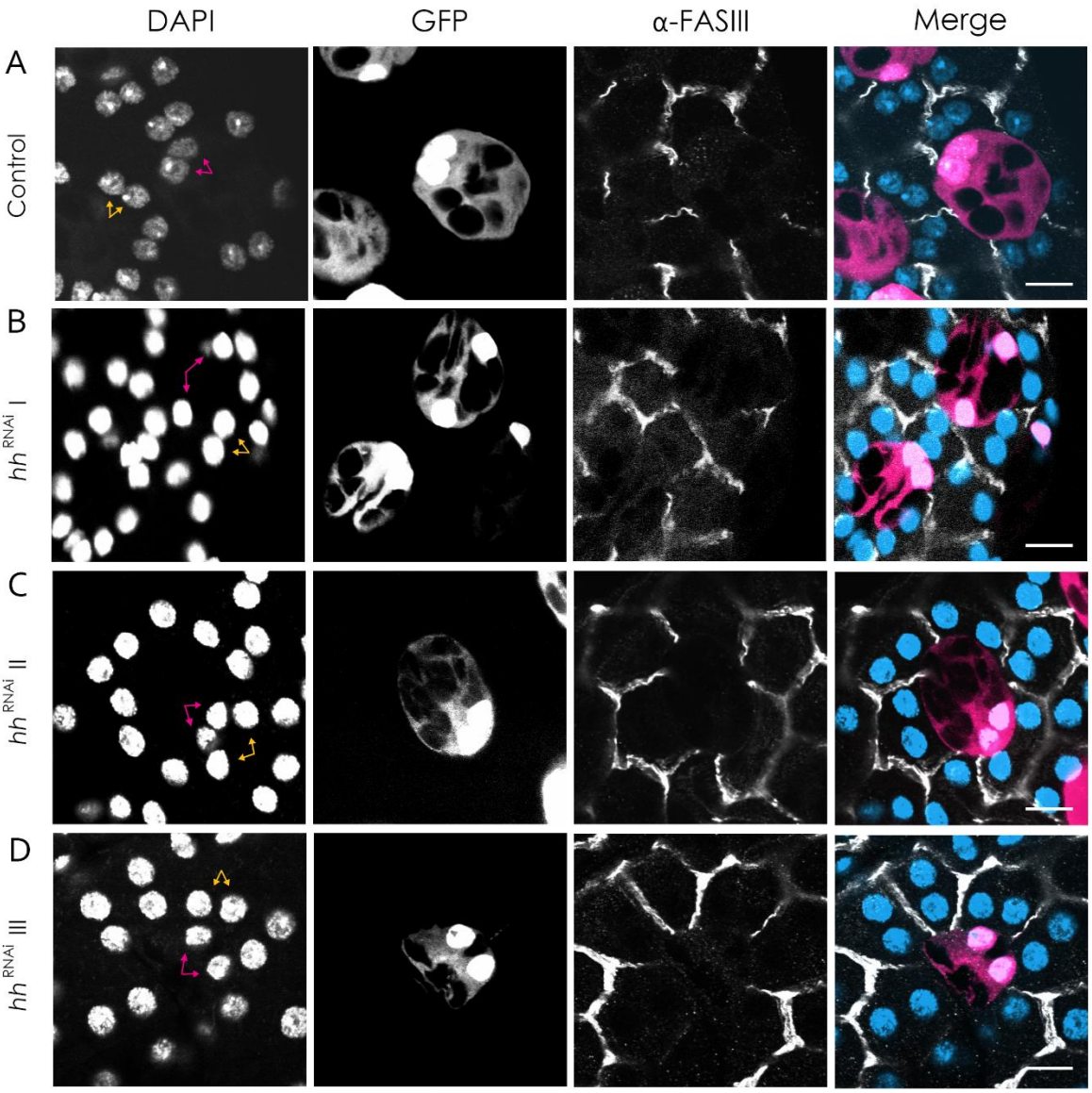
A schematic showing the genetic logic of the *esg^{ts} F/O* system. The chromosome II derived from the driver stock parent carries the genes for *esg*-GAL4; the GAL4-inhibitor, *tub*-GAL80^{ts}; and *UAS*-GFP^{nls}. The driver parent chromosome III carries *UAS*-FLP and an *actin* promoter-driven *CD2* gene, flanked by FRT sites and upstream of GAL4. Transgenes of interest can be present on any non-driver parent chromosome. At the permissive temperature, GAL80^{ts} inhibits the activity of *esg*-GAL4 and the *esg^{ts} F/O* system is inactive. At the non-permissive temperature, GAL80^{ts} can no longer inhibit *esg*-GAL4 activity, and *esg*-GAL4 can regulate the expression of *UAS*-FLP. FLP activity on the FRT sites that separate the *actin* promoter and the *gal4* gene causes FRT sites to be excised (flip-out event), allowing stable GAL4 expression to be driven by the *actin* promoter—even in the absence of *esg-gal4* gene activity. Expression from *act*-GAL4 is then used to drive expression of the *UAS*-GFP^{nls} reporter and the transgene(s) of interest, contributed by the non-driver parent chromosome.

hh^{RNAi}, relative to controls (**FIGURE 4. 23.**; **SUPPLEMENTARY TABLE 4. 4.**, *p.* 439).[7] Altogether, the data suggests that *hedgehog* gene expression is required in secondary cells, where it regulates secondary cell nuclear size—but without obviously affecting the size of the secondary cell itself. From this, it also appears that the size of the secondary cell itself is not 100 % dependent on the size of its nuclei.

Ectopic expression of *hedgehog* reduces secondary cell nuclear size

Having established that secondary cell nuclear size is affected when *hedgehog* is knocked-down, I was curious to establish what effect *hedgehog* overexpression would have. Recall that a GFP-tagged version of the Hedgehog molecule is available where the GFP tag does not interfere with Hedgehog signalling activity (WENDLER & FRANCH-MARRO & VINCENT, 2006). Using *esg* F/O^{ts} > GFP to drive expression of Hh::GFP (*esg* F/O^{ts} GFP > Hh::GFP), I found that—similar to when *hedgehog* is knocked down—overexpression of *hedgehog* also causes secondary cell nuclei to decrease in size, relative to the control ($p < 0.0001$) (**FIGURE 4. 24. A–B; FIGURE 4. 25.**). Finding that overexpression of wild-type (GFP-tagged) Hedgehog can affect secondary cell nuclear growth, I wondered: does this phenotype require fully functional Hedgehog protein? To investigate, I turned to the three GFP-tagged Hedgehog constructs shown in **FIGURE 4. 11.** (*p.* 168). As mentioned, HhN::GFP is signalling competent and shows a similar localisation pattern to wild-type Hedgehog when expressed in secondary cells. Consistent with these previous observations, HhN::GFP (like wild-type Hedgehog), reduces secondary cell nuclear size when overexpressed in

[7] In retrospect, secondary cell volume would have made a better measurement, but I also do not think measuring the area rather than the volume makes that much of a difference concerning the results.


DAPI
GFP
α-FASIII
Merge
A
Control
B
hh RNAi I
C
hh RNAi II
D
hh RNAi III

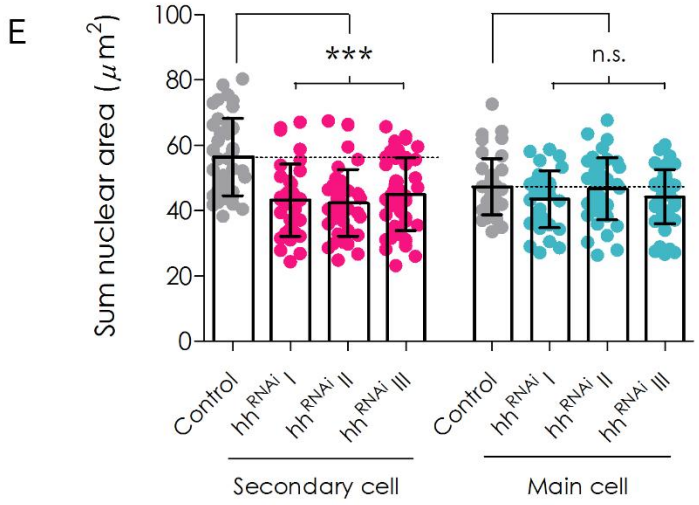

E
Sum nuclear area (μm2)
100
80
60
40
20
0
***
n.s.
Control
hh RNAi I
hh RNAi II
hh RNAi III
Control
hh RNAi I
hh RNAi II
hh RNAi III
Secondary cell
Main cell

FIGURE 4. 22. | HEDGEHOG REGULATES SECONDARY CELL NUCLEAR SIZE.

Using the *esg F/O*^{ts} system to express inverted repeats targeting *hedgehog* in secondary cells reduces secondary cell nuclear size after six days at the non-permissive temperature.

A: In controls, the nuclei of secondary cells expressing *esg F/O*^{ts} > GFP^{nls} (marked with pink arrows) are larger than the nuclei of the surrounding main cells (marked with yellow arrows).

B–D: The nuclei of secondary cells (pink arrows) expressing different inverted repeats targeting *hedgehog* (*hh*^{RNAi} I–III) are smaller than the nuclei of the surrounding main cells (yellow arrows).

E: When *esg F/O*^{ts} is used to express *hh*^{RNAi} in secondary cells, only the size of secondary cell nuclei is affected—with the nuclei of surrounding main cells showing no difference in size compared to those in controls. Each dot in the chart corresponds to data obtained from one individual cell, with three cells quantified per male (see **SUPPLEMENTARY TABLE 4. 3.**, *p.* 439).

Scale bars in panels **A–D** correspond to 10 μm and α-FASIII labels main cell junctions.

In panel **E**, the number of stars represent the *p*-value obtained by one-way ANOVA with Dunn's correction for multiple comparisons (all columns compared to the relevant control): *** = $p < 0.001$.

secondary cells ($p < 0.0001$) (**FIGURE 4. 24. C; FIGURE 4. 25.**). The second construct, HhC85S::GFP, is signalling-impaired—and as we have seen previously (**FIGURE 4. 8.** (*p.* 161) and **FIGURE 4. 9.** (*p.* 163)) it behaves differently from both wild-type GFP-tagged Hedgehog and HhN::GFP. When expressed in secondary cells, HhC85S::GFP also reduces secondary cell nuclear size relative to controls ($p < 0.001$) (**FIGURE 4. 24. D; FIGURE 4. 25.**). Finally, the third construct, HhC85SN::GFP, lacks both the palmitic acid and cholesterol modifications; severely impacting the signalling activity of the molecule. When expressed in secondary cells, this construct appears to reduce

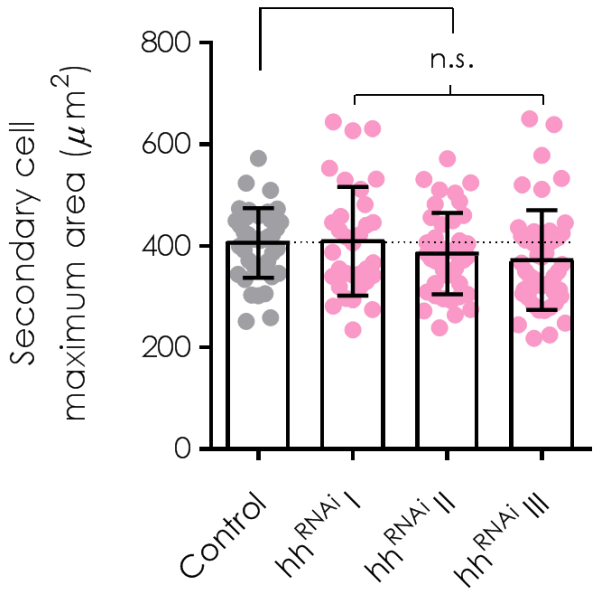


FIGURE 4. 23. | KNOCKING DOWN *hedgehog* DOES NOT AFFECT THE MAXIMUM AREA OF SECONDARY CELLS.
Using the *esg F/O*^{ts} system to express inverted repeats targeting *hedgehog* in secondary cells does not affect the largest cross-sectional area of secondary cells (in images captured parallel to the epithelial surface). Each dot in the chart corresponds to data obtained from one individual cell, with three cells quantified per male (see **SUPPLEMENTARY TABLE 4. 4.**, *p.* 439). Statistical significance was obtained by one-way ANOVA with Dunn's correction for multiple comparisons (all columns compared to the relevant control): 'n.s.' = $p > 0.1$.

secondary cell nuclear size slightly, but not significantly ($p < 0.05$) (**FIGURE 4. 25.**); suggesting that this form of Hedgehog is less competent at reducing secondary cell nuclear size. Altogether, these data (**SUPPLEMENTARY TABLE 4. 5.**, *p.* 439) suggest that post-translational lipid modification might be important for the ability of Hedgehog to regulate secondary cell nuclear size, but that the modifications are not crucial for the nuclear size phenotype to develop after Hedgehog has been overexpressed. This implies that the action of Hedgehog is local and short-range—since the lipid modifications typically affect the long-range spread of Hedgehog through tissue.

Dispatched is required for secondary cell nuclear growth

So far, I have shown that the Hedgehog molecule is produced by secondary cells and that it is released into the gland lumen. I have also demonstrated that Hedgehog is required for secondary cell nuclear growth, and that this is likely to result from local Hedgehog signalling. In addition, I have shown that the Hedgehog receptor, Patched, is specifically expressed by secondary cells in the *D. melanogaster* male accessory gland. Altogether, these findings suggest that Hedgehog needs to be released from, and be taken up again, by secondary cells in order for Hedgehog signalling to become active. Dispatched is a transmembrane molecule that is involved in Hedgehog secretion (BURKE *et al.,* 1999). Interestingly, *dispatched* mRNA is significantly enriched in the male accessory gland—in comparison to the whole fly carcass (FlyAtlas; CHINTAPALLI *et al.*, 2007). This suggests a possible role for Dispatched in the male accessory gland, and perhaps that it might be relevant for the secretion of Hedgehog in

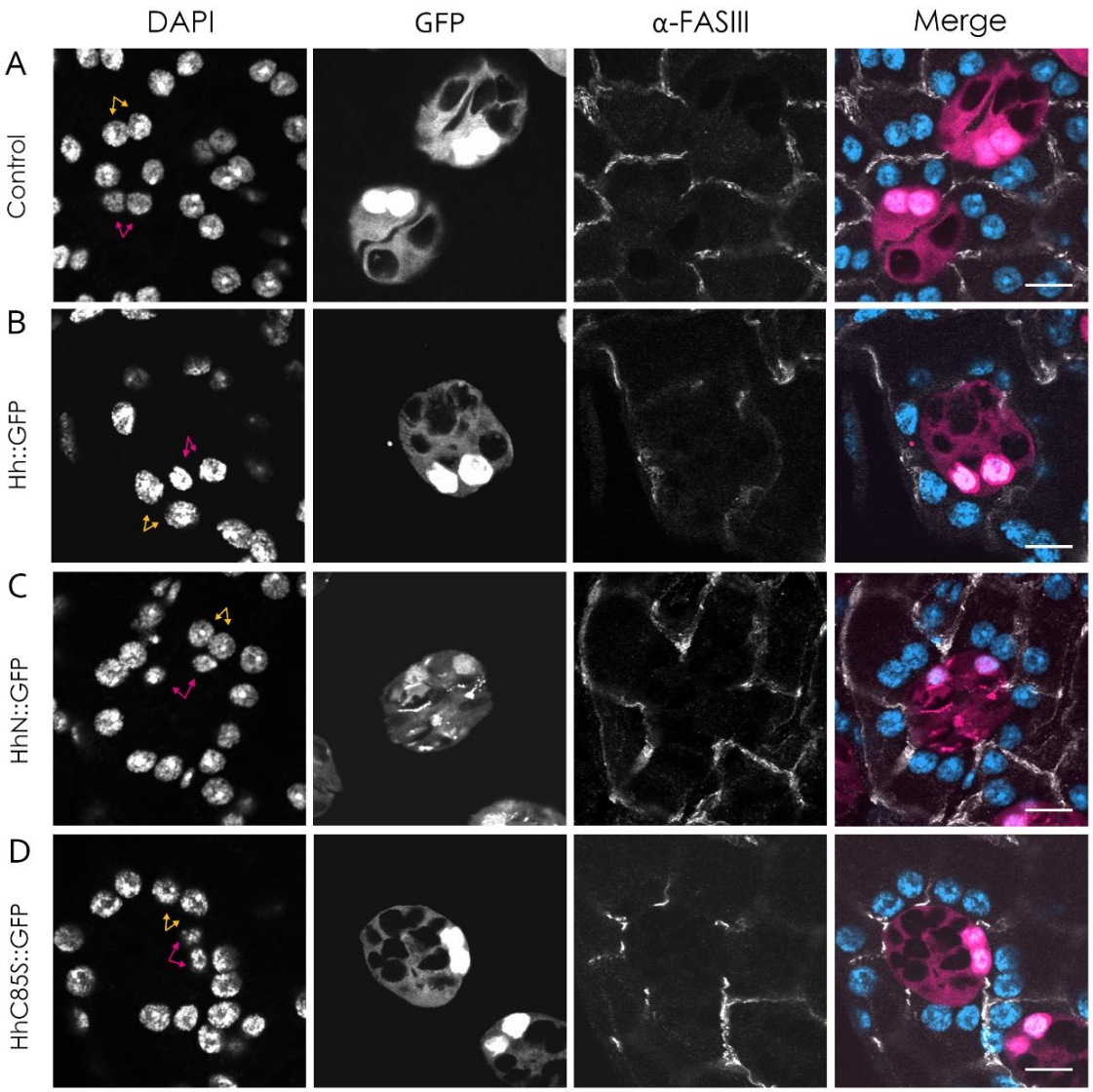


FIGURE 4. 24. | SECONDARY CELL NUCLEAR SIZE IS REDUCED UPON ECTOPIC EXPRESSION OF GFP-TAGGED HEDGEHOG.

GFP-tagged forms of Hedgehog were expressed in secondary cells for six days using the *esg^{ts} F/O* system.

A: In the accessory glands of control males, secondary cell nuclei (marked with pink arrows) are similar in size to those of the surrounding main cells (yellow arrows).

B: Secondary cell nuclei appear smaller than those of the surrounding main cells upon ectopic expression of a GFP-tagged form of wild-type Hedgehog (Hh::GFP).

C: Expression of a GFP-tagged form of Hedgehog that lacks the palmitoyl moiety (HhN::GFP) reduces secondary cell nuclear size—and Hh::GFP can also be observed internally within secondary cells as brighter *puncta* in the GFP channel.

D: Expression of a cholesterol-deficient form of Hedgehog (HhC85S::GFP) reduces secondary cell nuclear size.

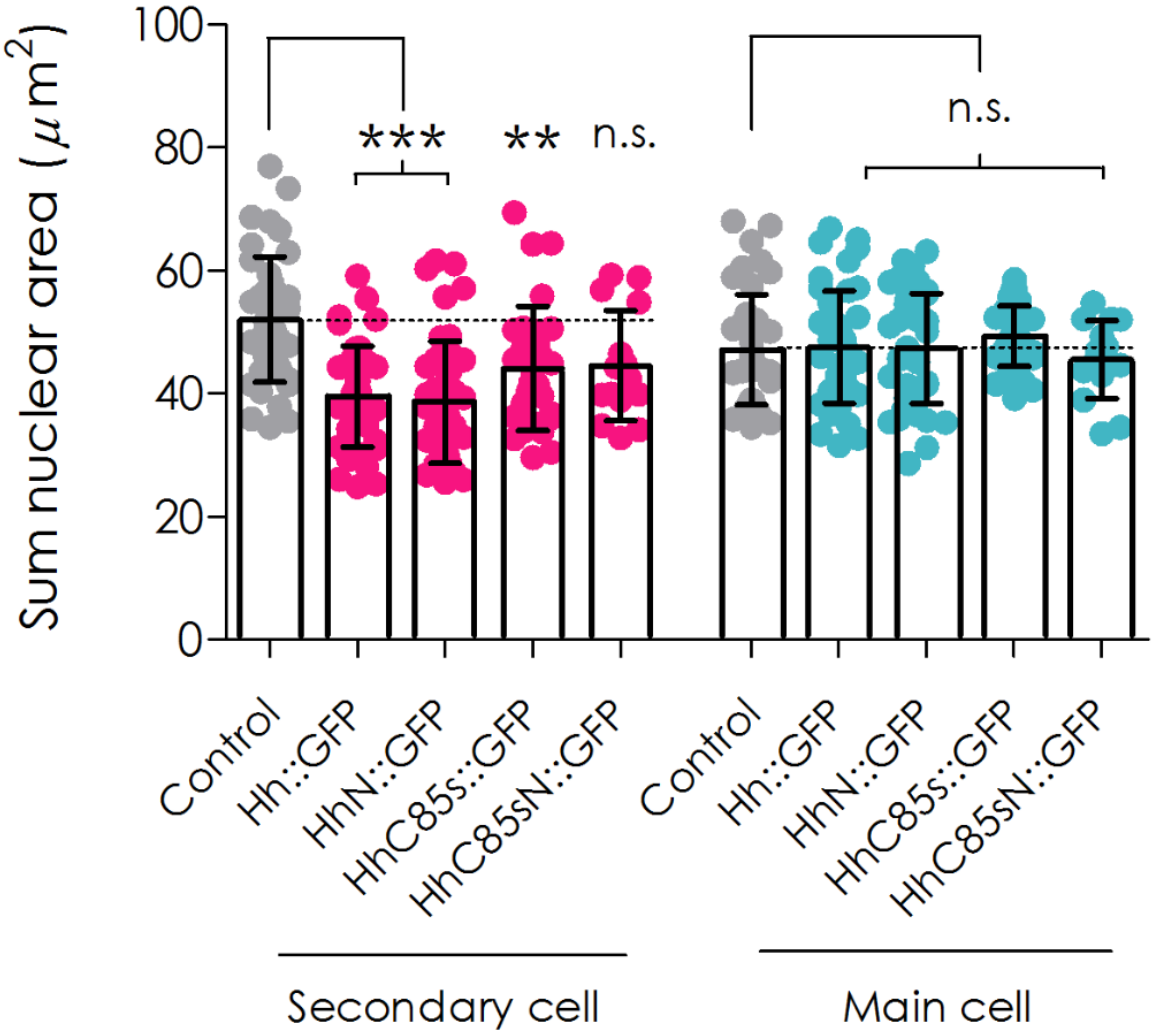
Scale bars in all panels correspond to 10 μm and α-FASIII (white) labels epithelial cell junctions.

N. B. The double mutant, HhC85SN::GFP is not shown in this figure due to a lack of high-quality images of secondary cells in this genotype. I had intended to re-image the genotype to this end, but failed to accomplish this due to time-constraints.

FIGURE 4. 25. | ECTOPIC EXPRESSION OF GFP-TAGGED FORMS OF HEDGEHOG AFFECTS SECONDARY CELL NUCLEAR SIZE.

Using the *esg*^{ts} *F/O* system to express GFP-tagged forms of Hedgehog specifically reduces secondary cell—and not main cell—nuclear size. Each dot in the chart corresponds to data obtained from one individual cell, with three cells quantified per male (see **SUPPLEMENTARY TABLE 4. 5.**, *p.* 439).

The number of stars represent the *p*-value obtained by one-way ANOVA with Dunnett's correction for multiple comparisons (all cell type-specific columns compared to the relevant control): ** = $p < 0.01$; *** = $p < 0.001$.



this tissue. To investigate if Dispatched has a role in the regulation of secondary cell nuclear growth, I used *esg F/O*^{ts} to express two different RNAis targeting *dispatched*: *disp*^{RNAi} I and *disp*^{RNAi} II (NI *et al.*, 20011; 2009). The secondary cell sum nuclear area was reduced in cells expressing either of the two inverted repeats targeting *dispatched* (*esg*^{ts} *F/O* > *disp*^{RNAi}), although *disp*^{RNAi} II showed the only significant reduction ($p < 0.0001$) (**FIGURE 4. 26.**). Since knock-down of *dispatched* can affect secondary cell nuclear size (**SUPPLEMENTARY TABLE 4. 6.**, *p.* 440), Hedgehog release might be important for secondary cell nuclear growth.

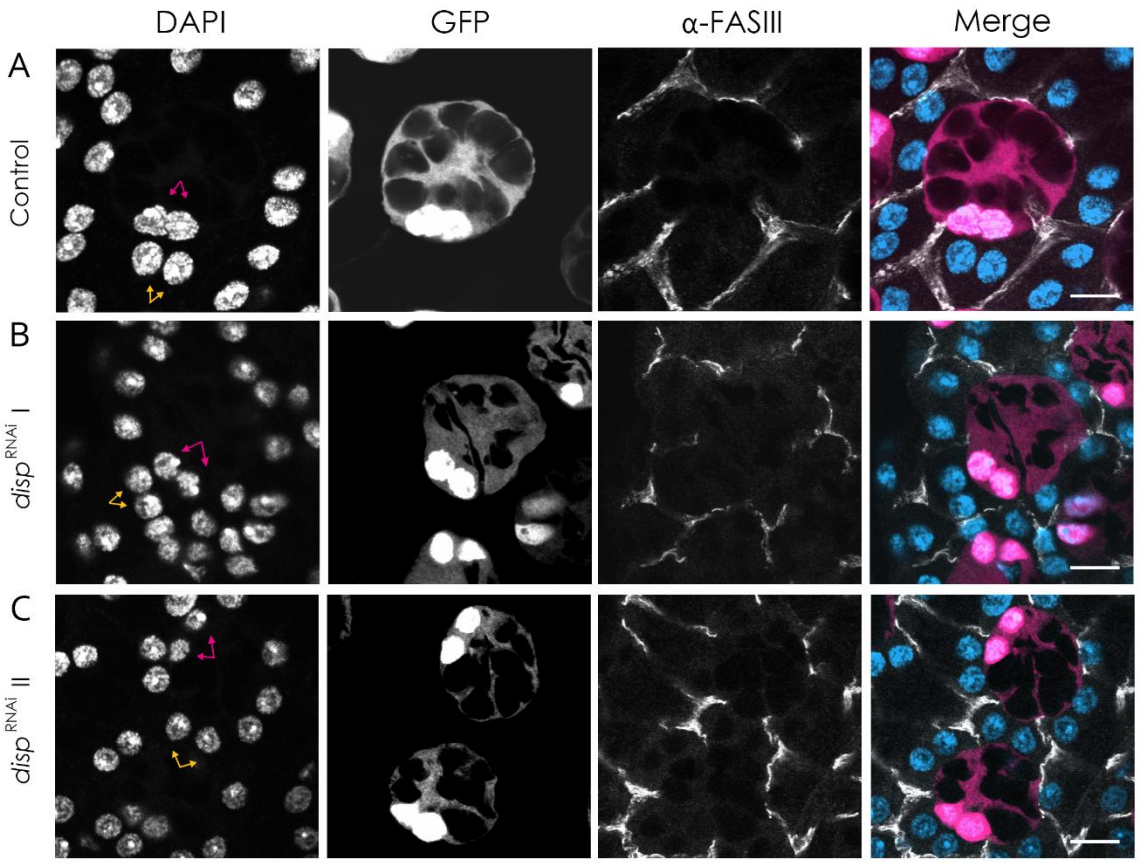
Components of the Hedgehog signalling pathway are required for secondary cell nuclear growth

Having established that Hedgehog regulates secondary cell nuclear growth, I next decided to investigate if downstream components of the Hedgehog signalling pathway are also involved in the regulation of secondary cell nuclear size. The transmembrane protein, Smoothened, is involved in transducing the Hedgehog signal and would therefore be expected to act as a positive regulator of secondary cell nuclear growth—similar to Hedgehog. To test this hypothesis, I used a *smo*^{RNAi} (NI *et al.*, 2009) to knock down the expression of *smoothened* in secondary cells using *esg F/O*^{ts} (*esg F/O*^{ts} > *smo*^{RNAi}). Consistent with expectation, expressing *smo*^{RNAi} in secondary cells reduced secondary cell nuclear size ($p < 0.0001$)—while main cell nuclear size was unaffected ($p > 0.1$) (**FIGURE 4. 27. A–B, D; SUPPLEMENTARY TABLE 4. 7.,** *p.* 440). This suggests that expression of *smoothened* is required for secondary cell nuclear growth, and further implicates Hedgehog signalling in this process.

Patched inhibits the activity of Smoothened non-stoichiometrically. As a result, Patched is capable of suppressing Smoothened activity even when the latter protein is

present in excess (TAIPALE *et al.,* 2002). To investigate the effect of ectopic activation of Smoothened on secondary cell nuclear size, I used *esg F/O*^{ts} to express a *smoothened* cDNA (*esg F/O*^{ts} > *smo*) (INGHAM *et al.,* 2000). Similar to knock-down of *smoothened*, the sum area of nuclei in secondary cells expressing *esg F/O*^{ts} > *smo* were much reduced compared to controls ($p < 0.0001$) (**FIGURE 4. 28. A, C–D**). However, closer inspection of secondary cells expressing the *smoothened* cDNA suggests that cell viability was affected; with the normally nuclear and cytoplasmic GFP^{nls} being visible only in granulate *puncta* throughout a reduced cell body, and wholly excluded from the nuclei (**FIGURE 4. 27. C**). This phenotype is similar to that observed in response to overexpression of *dronc* in CHAPTER 3 (**FIGURE 3. 10. B**, *p.* 101)—perhaps suggesting that overexpression of *smoothened* affects cells sufficiently for them to adopt an abnormal morphology.

The activity of Smoothened is repressed in the absence of Hedgehog signalling by the activity of the transmembrane protein, Patched. Having determined that both Hedgehog and Smoothened are required for secondary cell nuclear growth, and that overexpressing either can limit secondary cell nuclear growth, I hypothesised that Patched might function in a similar (but opposite) way. Using the *esg F/O*^{ts} system to express two different inverted repeats targeting *patched* (*esg*^{ts} *F/O* > *ptc*^{RNAi}: I and II) (NI *et al.,* 2009; 2010), I found that both RNAi lines reduced the sum area of secondary cell nuclei ($p < 0.0001$) (**FIGURE 4. 28.; SUPPLEMENTARY TABLE 4. 8.,** *p.* 440). Intriguingly, the size of main cell nuclei was also affected by this treatment ($p < 0.0001$) (**FIGURE 4. 28. D**). Altogether, this suggests that *patched* is necessary for secondary cell nuclear growth, and that it also has an effect on the growth of main cell nuclei. Altogether, it appears that excess activation of the Hedgehog signalling pathway


DAPI
GFP
α-FASIII
Merge
A
Control
B
disp RNAi I
C
disp RNAi II

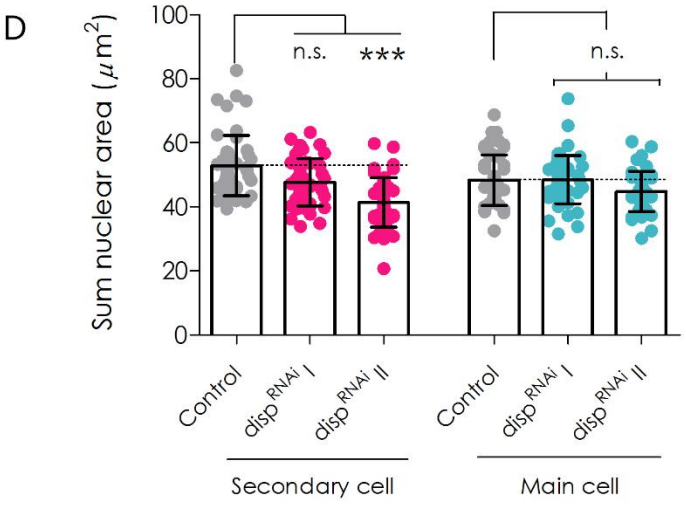

D
Sum nuclear area (μm²)
100
80
60
40
20
0
n.s.
***
n.s.
Control
disp RNAi I
disp RNAi II
Control
disp RNAi I
disp RNAi II
Secondary cell
Main cell

FIGURE 4. 26. | DISPATCHED HAS A ROLE IN THE REGULATION OF SECONDARY CELL NUCLEAR SIZE.

Secondary cell nuclear size is affected by *disp*^{RNAi}, expressed for six days using the *esg F/O*^{ts} system.

A: Secondary cell nuclei (marked with pink arrows) are similar in size to those of the surrounding main cells (marked with yellow arrows) in a control accessory gland.

B: The nuclei of secondary cells expressing an inverted repeat targeting *dispatched* (*disp*^{RNAi} I) are similar in size to those of the surrounding main cells.

C: A different *dispatched* RNAi (*disp*^{RNAi} II) reduces secondary cell nuclear size compared to controls.

D: Quantifying the nuclear size of both secondary cells and main cells shows that *disp*^{RNAi} II reduces the size of secondary cell nuclei, while main cell nuclei are unaffected. Each dot in the chart corresponds to data obtained from one individual cell, with three cells quantified per male (see **SUPPLEMENTARY TABLE 4. 6.**, *p.* 440).

Scale bars in panels **A–C** correspond to 10 μm and α-FASIII (white) labels cell junctions

In panel **D**, the number of stars represent the *p*-value obtained by one-way ANOVA with Dunnett's correction for multiple comparisons (all cell type-specific columns compared to the relevant control): *** = $p < 0.001$.

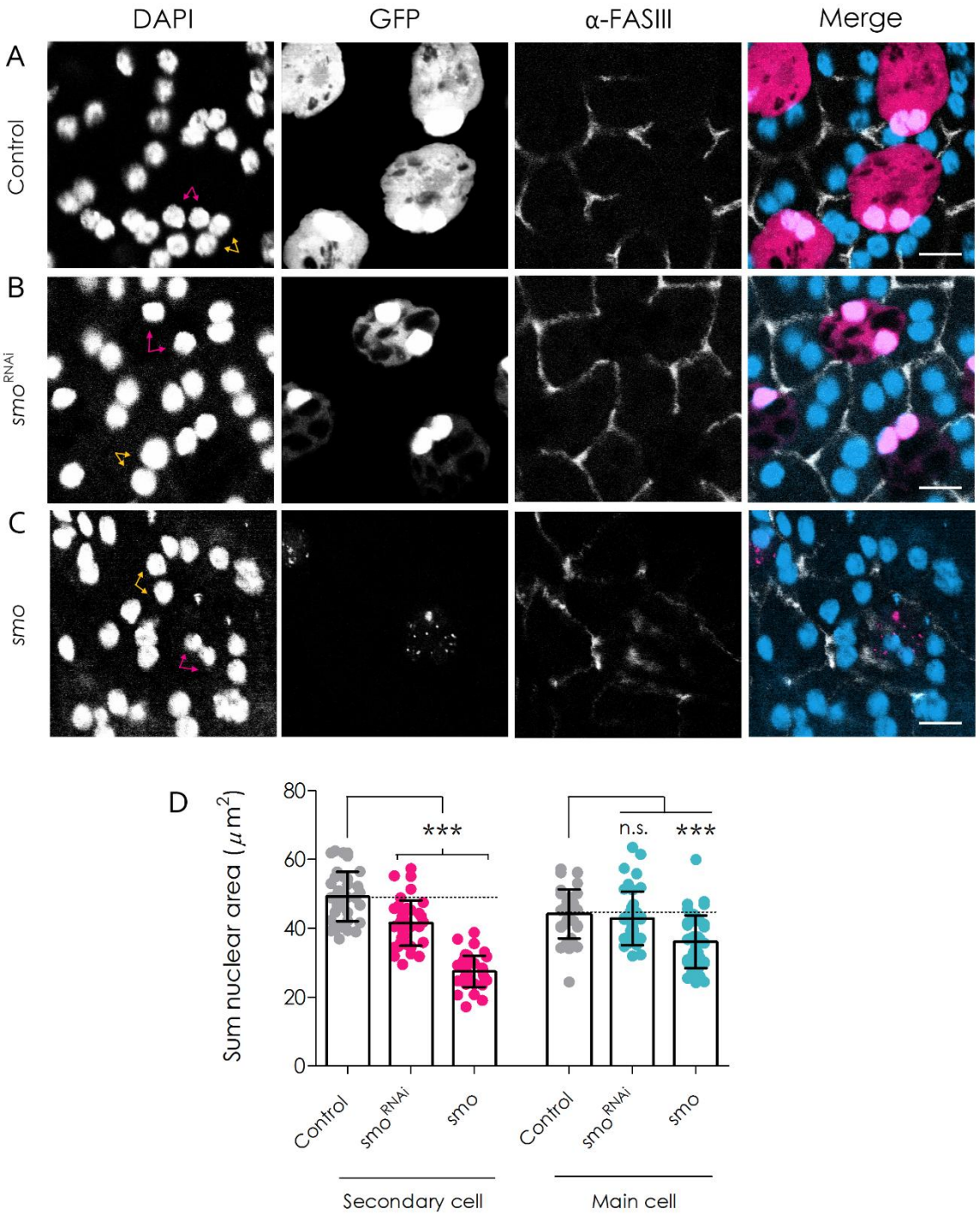

DAPI
GFP
α-FASIII
Merge
A
Control
B
smo RNAi
C
smo
D
Sum nuclear area (μm²)
80
60
40
20
0
***
n.s.
***
Control
smo RNAi
smo
Control
smo RNAi
smo
Secondary cell
Main cell

FIGURE 4. 27. | SECONDARY CELL NUCLEAR SIZE IS REGULATED BY SMOOTHENED.

Secondary cell nuclear size is affected by expression of smo^{RNAi} and a *smoothened* cDNA using the *esg F/O*^{ts} system.

A: Secondary cell nuclei (marked with pink arrows) are similar in size to those of the surrounding main cells (marked with yellow arrows) in a control accessory gland.

B: The nuclei of secondary cells expressing an inverted repeat targeting *smoothened* are smaller than those of the surrounding main cells.

C: Using a *smoothened* cDNA to overexpress Smoothened seems to affect secondary cell viability. They contain only dispersed, granular GFP that is excluded from the nuclei. Secondary cell nuclei also appear much reduced in size, compared to both the controls (in **A**) and those of the surrounding main cells.

D: Quantifying the nuclear size of both secondary cells and main cells shows that whereas smo^{RNAi} only reduces the size of secondary cell nuclei, overexpression of a *smo* cDNA reduces the nuclear size of both secondary cells and main cells. Each dot in the chart corresponds to data obtained from one individual cell, with three cells quantified per male (see **SUPPLEMENTARY TABLE 4. 7.**, p. 440).

Scale bars in panels **A–C** correspond to 10 μm and α-FASIII (white) labels cell junctions.

In panel **D**, the number of stars represent the *p*-value obtained by one-way ANOVA with Dunnett's correction for multiple comparisons (all columns compared to the relevant control): *** = $p < 0.001$; 'n.s.' = $p > 0.1$.

FIGURE 4. 28. | PATCHED REGULATES SECONDARY CELL NUCLEAR SIZE.

Secondary cell nuclear size is affected by two different ptc^{RNAi} constructs, expressed for six days using the *esg F/O*^{ts} system.

A: Secondary cell nuclei (marked with pink arrows) are similar in size to those of the surrounding main cells (marked with yellow arrows) in a control accessory gland. The 'third' DNA accumulation in the DAPI channel in the secondary cell in this panel is in fact the nucleus of a muscle cell that is visible in the same *z*-plane.

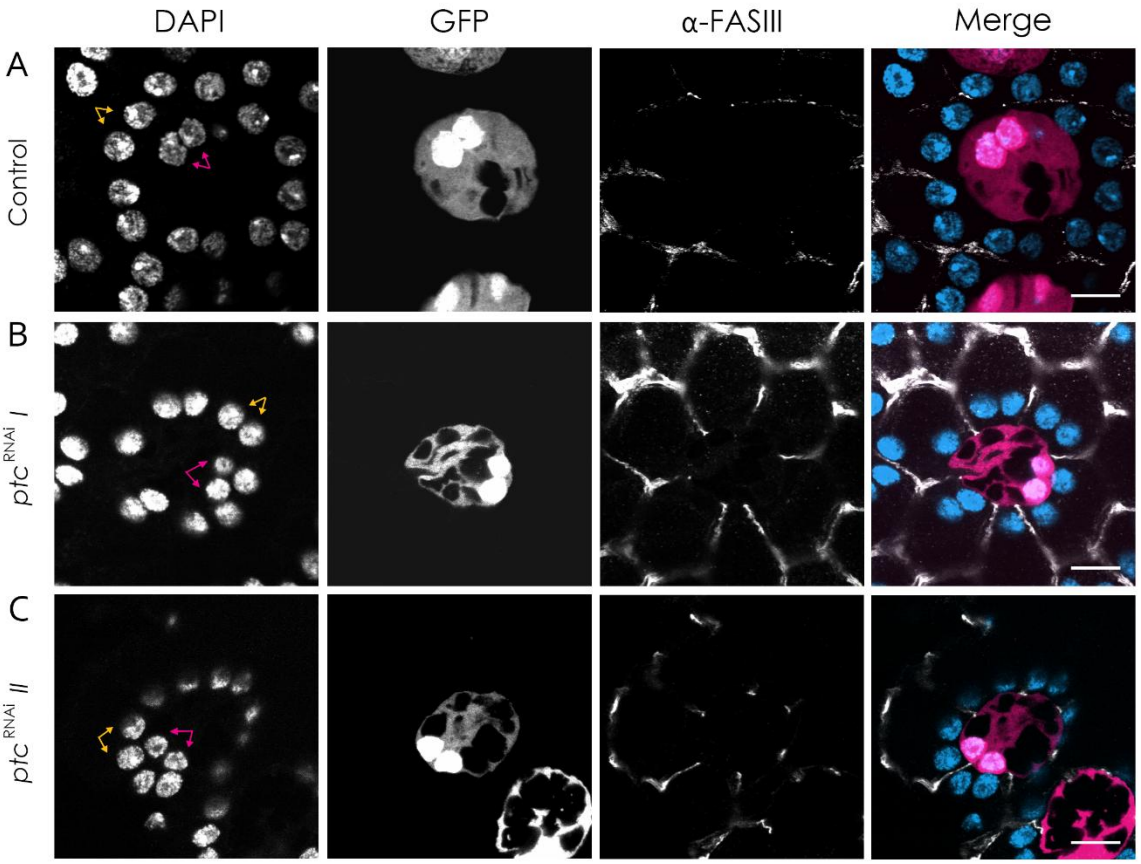
B: The nuclei of secondary cells expressing an inverted repeat targeting *patched* (ptc^{RNAi} I) are smaller than those of the surrounding main cells.

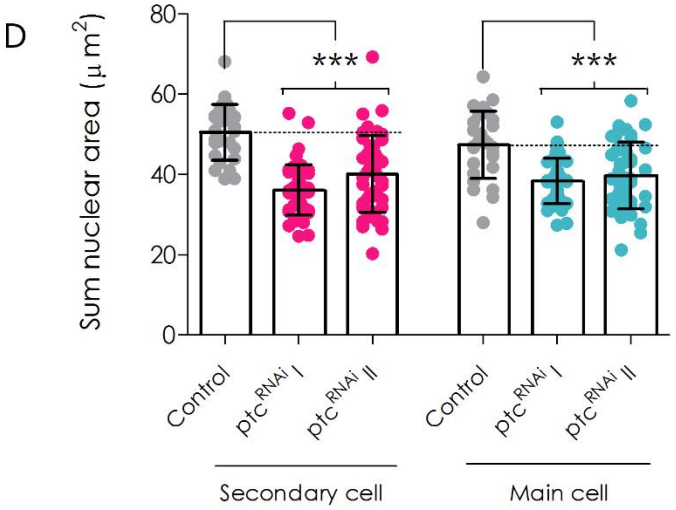
C: A different *patched* RNAi (ptc^{RNAi} II) has the same effect and reduces secondary cell nuclear size compared to controls.

D: Quantifying the nuclear size of both secondary cells and main cells shows that ptc^{RNAi} reduces the size of both secondary cell and main cell nuclei. Each dot in the chart corresponds to data obtained from one individual cell, with three cells quantified per male (see **SUPPLEMENTARY TABLE 4. 8.**, *p.* 440).

Scale bars in panels **A–C** correspond to 10 μm and α-FASIII (white) labels cell junctions.

In panel **D**, the number of stars represent the *p*-value obtained by one-way ANOVA with Dunnett's correction for multiple comparisons (all columns compared to the relevant control): *** = $p < 0.001$.


DAPI
GFP
α-FASIII
Merge
A
Control
B
ptc RNAi I
C
ptc RNAi II


D
Sum nuclear area (μm²)
80
60
40
20
0
***
***
Control
ptc RNAi I
ptc RNAi II
Control
ptc RNAi I
ptc RNAi II
Secondary cell
Main cell

can be sufficient to reduce secondary cell nuclear size. Importantly, transgene overexpression using the *esg* F/O^{ts} system does not always reduce secondary cell nuclear size (see CHAPTER 5 for several examples of this).

The Hedgehog-dependent transcription factor, Cubitus interruptus, is necessary and sufficient for secondary cell nuclear growth

The transcription factor Cubitus interruptus (Ci) is converted from a transcriptional repressor (Ci^R) into a Hedgehog-responsive transcriptional activator (Ci^A) in response to Hedgehog signalling via Smoothened. If the age-dependent nuclear size increase in secondary cells depends on Hedgehog signalling via Smoothened to activate Ci, secondary cells expressing an inverted repeat targeting *ci* would be expected to behave similar to when expression of *hedgehog* or *smoothened* is knocked down, *i.e.* secondary cell nuclei would be expected *not* to grow. To test this hypothesis, I looked at the effect of expressing three different ci^{RNAi}s in secondary cells using *esg* F/O^{ts} (*esg* F/O^{ts} > ci^{RNAi} I–III) (NI *et al.*, 2008; 2009). Consistent with *ci* being required for secondary cell nuclear growth, secondary cells expressing inverted repeats targeting *ci* showed reduced nuclear growth compared to controls—in two out of the three *RNAi* lines tested (**FIGURE 4. 29.**; **SUPPLEMENTARY TABLE 4. 9.,** *p.* 441). That *esg* F/O^{ts} > ci^{RNAi} can reduce secondary cell nuclear size suggests that Hedgehog might regulate secondary cell nuclear growth via Ci.

If expression of *ci* is required for normal secondary cell nuclear growth, I hypothesised that overexpression of *ci* would be sufficient to drive this growth. To test this hypothesis, I used two different *ci* overexpression lines: one that drives expression of a *ci* cDNA (*UAS-ci*) (DOMINUEZ *et al.,* 1996), and one that combines the Ci zinc-finger DNA-binding domain with a potent *Herpes simplex* virus transcriptional activator

domain (*UAS*-Ci::VP16) to force the overexpression of genes regulated by Ci (ALEXANDRE *et al.*, 1996). Whereas *esg F/O*^{ts} > *ci* did not seem to affect secondary cell nuclear growth ($p > 0.1$) (**FIGURE 4. 30. A–B, D**), forced overexpression of Ci-regulated genes using *esg F/O*^{ts} to express CiVP16 in secondary cells (*esg*^{ts} *F/O* > Ci::VP16) seemed sufficient to drive an increase in secondary cell nuclear size ($p < 0.0001$) (**FIGURE 4. 30. A, C–D; SUPPLEMENTARY TABLE 4. 10.,** *p.* 441). Expression of Ci::VP16 also affects main cell nuclear size (**FIGURE 4. 30. D**), and it is interesting to note that this phenotype is similar to the two conditions where secondary cell nuclei decrease in size—despite Hedgehog signalling being increased (*e. g.* overexpression of *smo* (**FIGURE 4. 27. D**: *p.* 197) and knock-down of *ptc* (**FIGURE 4. 28. D**: *p.* 199)). Altogether, these results suggest that Ci is both required and sufficient for the regulation of secondary cell nuclear growth, and that the processing of functional Ci might still take place—even when the protein is overexpressed.

DISCUSSION

Hedgehog signalling is one of the key developmental pathways. In mammals, it has a role specifying gland development and regulating the secretory function of glands. Although Hedgehog signalling has a role patterning the genital disc in *D. melanogaster*, the only described function for Hedgehog in the adult fruit fly concerns stem cell maintenance in the testes. In this Chapter, I have described additional roles for Hedgehog signalling in adult male reproductive tissues in *D. melanogaster*; showing that *hedgehog* is expressed in the secondary cells and that the Hedgehog protein is likely to be released into the *D. melanogaster* seminal fluid. Altogether, the data presented in this

FIGURE 4. 29. | CUBITUS INTERRUPTUS IS REQUIRED FOR SECONDARY CELL NUCLEAR GROWTH.

Secondary cell nuclear size is affected by *ci*^{RNAi} expression, using the *esg F/O*^{ts} system.

A: Secondary cell nuclei (marked with pink arrows) are similar in size to those of the surrounding main cells (marked with yellow arrows) in a control accessory gland.

B: The nuclei of secondary cells expressing an inverted repeat targeting *ci* (*ci*^{RNAi} I) are smaller than those of the surrounding main cells.

C: A different *ci* RNAi (*ci*^{RNAi} II) has the same effect and reduces secondary cell nuclear size compared to controls.

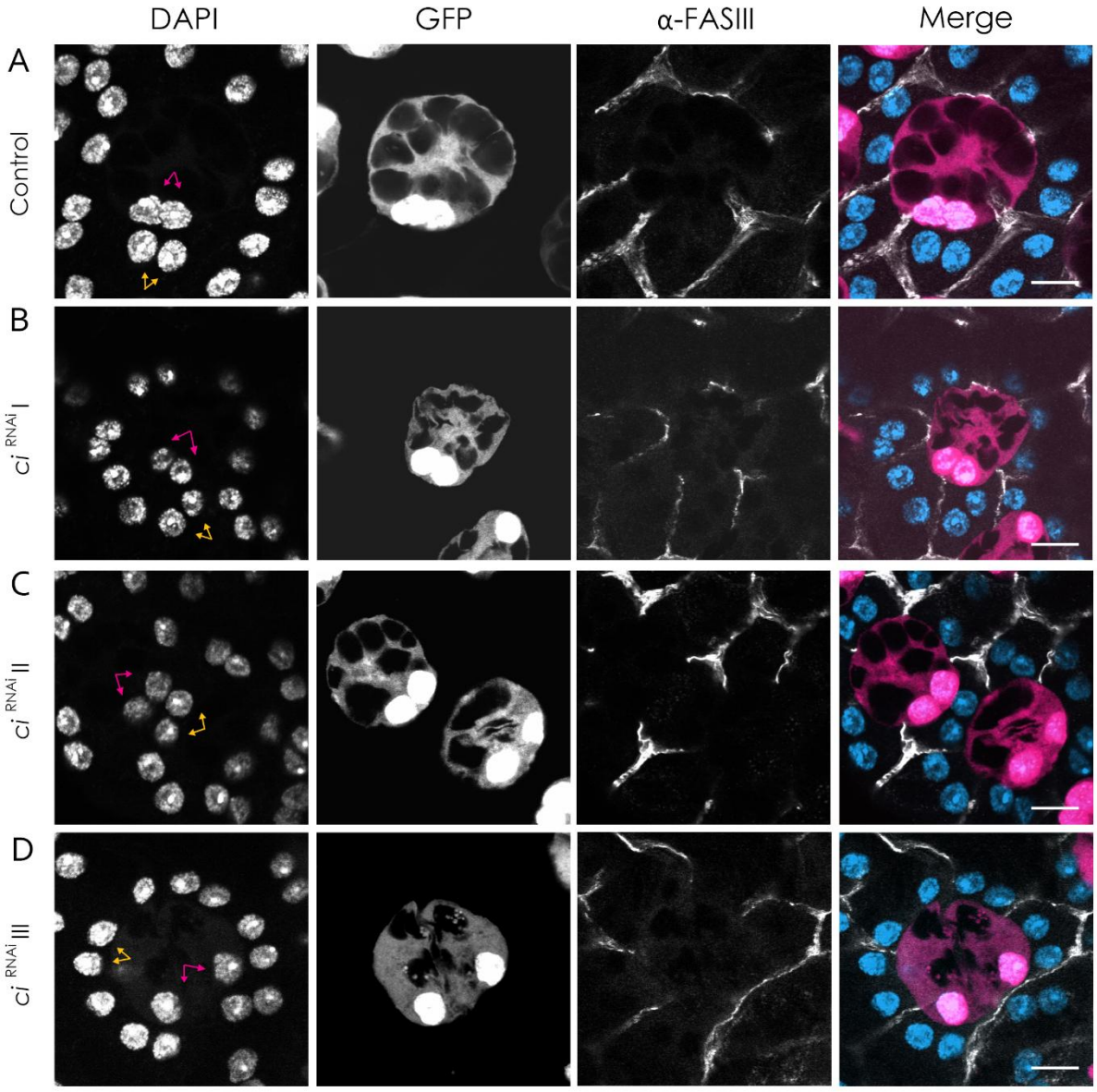
D: A third *ci* RNAi (*ci*^{RNAi} III) does not affect secondary cell nuclear size.

E: Quantifying the nuclear size of both secondary cells and main cells shows that *ci*^{RNAi} I and II reduce the size of secondary cell nuclei. Each dot in the chart corresponds to data obtained from one individual cell, with three cells quantified per male (see **SUPPLEMENTARY TABLE 4. 9.**, *p.* 441).

Scale bars in panels **A–D** correspond to 10 μm and α-FASIII (white) labels main cell junctions

In panel **E**, the number of stars represent the *p*-value obtained by one-way ANOVA with Dunnett's correction for multiple comparisons (all cell type-specific columns compared to the relevant control): *** = $p < 0.0001$.

N. B. The panels and values for the controls in this figure are the same as for **FIGURE 4. 26.** (*p.* 194). This has been taken into account for the statistical analysis.


DAPI
GFP
α-FASIII
Merge
A
Control
B
ci RNAi I
C
ci RNAi II
D
ci RNAi III

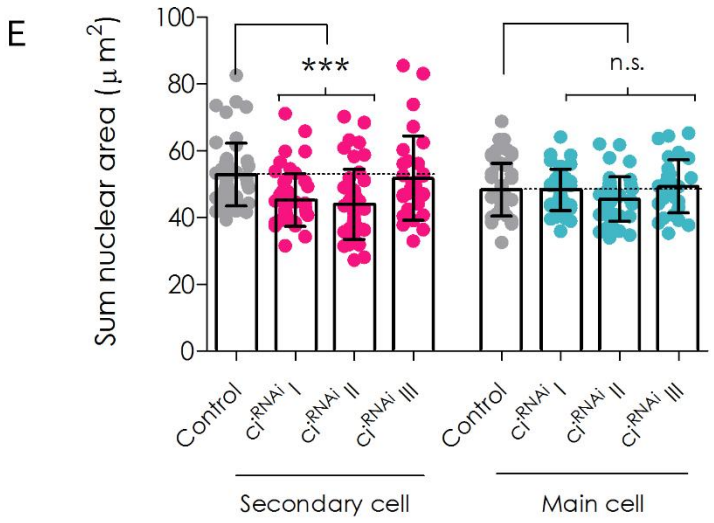

E
Sum nuclear area (μm2)
100
80
60
40
20
0
***
n.s.
Control
ci-RNAi I
ci-RNAi II
ci-RNAi III
Control
ci-RNAi I
ci-RNAi II
ci-RNAi III
Secondary cell
Main cell

FIGURE 4. 30. | CUBITUS INTERRUPTUS REGULATES SECONDARY CELL NUCLEAR SIZE.

Secondary cell nuclear size can be increased by expressing a modified form of the transcription factor, Cubitus interruptus for six days, using the *esg F/O*^{ts} system.

A: Secondary cell nuclei (marked with pink arrows) are similar in size to those of the surrounding main cells (marked with yellow arrows) in a control accessory gland.

B: The nuclei of secondary cells expressing a *ci* cDNA are not significantly different from those of the surrounding main cells.

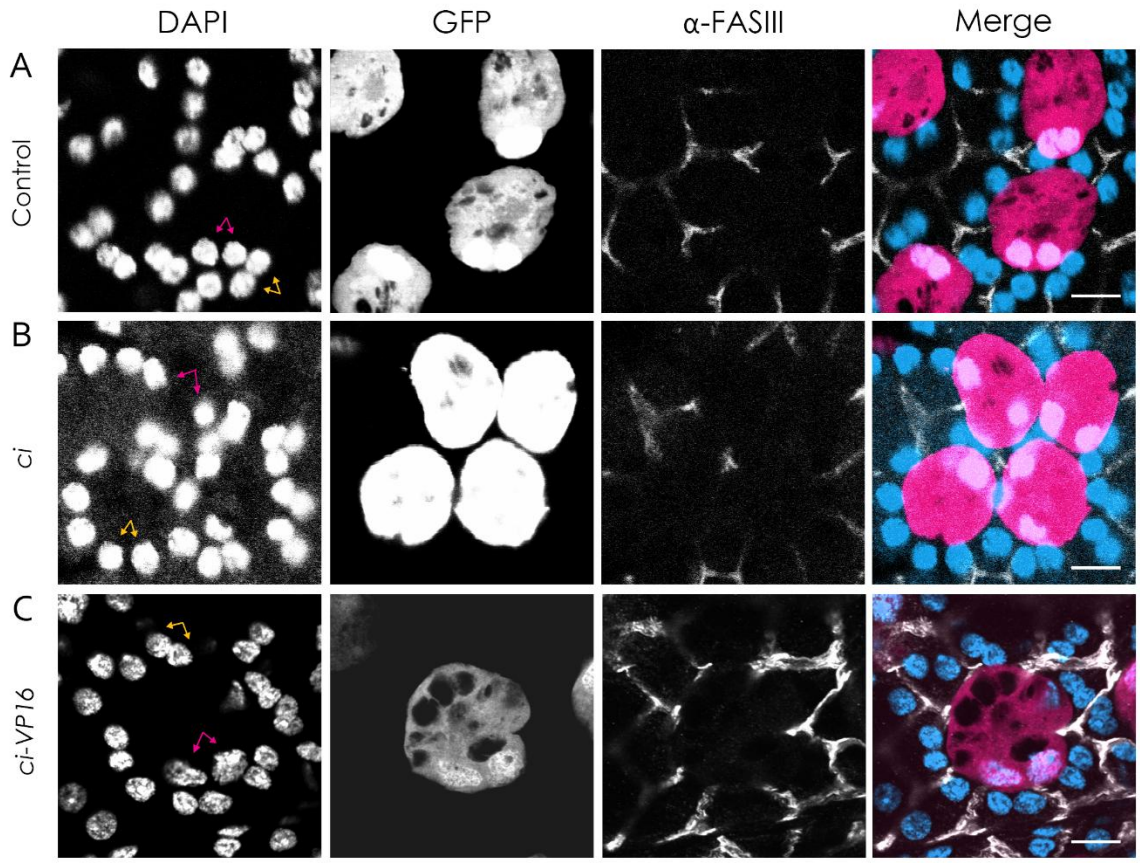
C: A fusion protein consisting of the Ci DNA-binding domain and the activation domain from a *Herpes simplex* virus forces overexpression of Ci-regulated genes in secondary cells, and can drive nuclear growth.

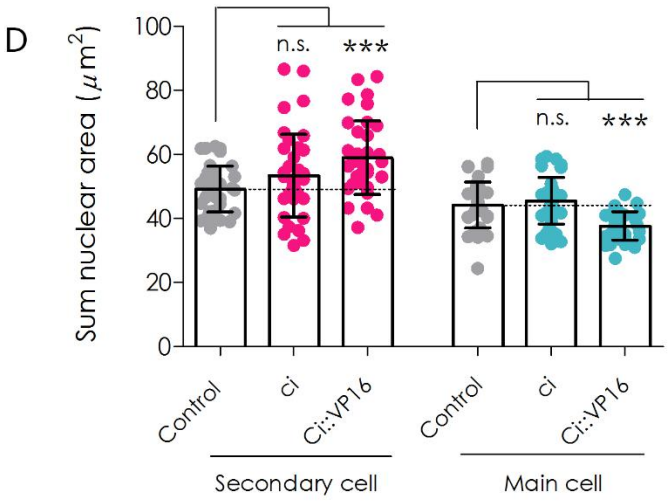
D: Quantifying the nuclear size of both secondary cells and main cells shows that whereas overexpression of a *ci* cDNA has no effect on either secondary cell or main cell nuclear size, overexpression of Ci-regulated genes (via Ci::VP16) in secondary cells is sufficient to drive both secondary cell nuclear growth and cause main cell nuclei to decrease in their cross-sectional area. Each dot in the chart corresponds to data obtained from one individual cell, with three cells quantified per male (see **SUPPLEMENTARY TABLE 4. 10.**, *p.* 441).

Scale bars in panels **A–C** correspond to 10 μm and α-FASIII (white) labels main cell junctions

In panel **D**, the number of stars represent the *p*-value obtained by one-way ANOVA with Dunnett's correction for multiple comparisons (all cell type-specific columns compared to the relevant control): *** = $p < 0.001$.

N.B: The panels and values for the controls in this figure are the same as for **FIGURE 4. 27.** (*p.* 197)**.** This has been taken into account for the statistical analysis.


DAPI
GFP
α-FASIII
Merge
A
Control
B
ci
C
ci-VP16


D
Sum nuclear area (μm²)
100
80
60
40
20
0
n.s.
***
n.s.
***
Control
ci
Ci::VP16
Control
ci
Ci::VP16
Secondary cell
Main cell

Chapter shows that Hedgehog signalling is important for secondary cell nuclear growth; suggesting that Hedgehog signalling might have a role regulating the reproductive function of the male fruit fly.

The structural complexity of the seminal fluid increases as the adult male fruit fly matures

Although the adult period of a fruit fly is considered to begin upon its eclosion from the *puparium*, it is not fully mature at this point. In this Chapter, I have shown that there are corresponding changes in both the epithelial organisation and the composition of the accessory gland secretions as it increases in size while the male fruit fly matures. In *D. melanogaster* , the reproductive success of the male scales with the amount of seminal fluid he delivers to the female: with a larger ejaculate resulting in a more successful mating (BANGHAM *et al.,* 2003; LINKLATER *et al.,* 2007; WIGBY & SIROT *et al.,* 2009). The importance of ejaculate size provides an explanation for the reduced reproductive capacity of the young *D. melanogaster* male. A similar relationship has been observed in the stalk-eyed fly (*C. dalmanni*): where there is a positive relationship between the size of an individual male's accessory glands and the time it takes him to reach sexual maturity (BAKER *et al.,* 2003). A similar relationship seems to exist also in rodents, where the size of the male reproductive glands seems to scale with the volume of the ejaculate, and also the success of the mating event itself (RAMM *et al.,* 2005; LEMAÎTRE *et al.*, 2010). This suggests that there might be a general relationship between the volume of the ejaculate and the reproductive success of male animals.

From my work, and that of others, it appears as if most of the accessory gland secretions in *D. melanogaster* accumulate during the first three days after eclosion. As

these secretions accumulate they also become increasingly heterogeneous through the several different protein fractions they contain (flocculate, immiscible, and membrane-derived) (BAIRATI, 1968; FEDERER & CHEN, 1982; SCHMIDT *et al.,* 1985; CORRIGAN, 2014). The flocculate fraction of the seminal fluid can be labelled using lipophilic markers, such as a GPI-anchored form of GFP (CORRIGAN, 2014). Previous workers have speculated that these structures are formed from lipophilic proteins that have coalesced together into 'flocculates' (FEDERER & CHEN, 1982; SCHMIDT *et al.*, 1985).

The structural complexity of the secretions in the *D. melanogaster* male accessory gland increases as they accumulate. I think this suggests that the structural heterogeneity of the ejaculate might be an additional factor worth to consider beyond its mere volume or absolute protein content. However, although it is beyond the scope of the work performed in our laboratory, it would be interesting to determine if the structural complexity of the seminal fluid is important in the reproduction of not just flies, but also other animals. For example, I think it is possible that this structural complexity contributes to the male reproductive function by keeping the proteins in the aqueous and lipophilic phases separate (to avoid the premature mixing of, for example, proteases and their protein targets) prior to their delivery to the female during mating. Interestingly, REZAEI *et al.* (2015) find that although the accessory gland protein volume does not change much over the lifespan of the male fly, his fertility goes down with age. It would be interesting to determine if this is due to some sort of 'de-potentiation' of the seminal fluid components, perhaps as they mix over time. However, the lipophilic flocculates remain (visually) intact even in old glands, although some concentration of the gland protein content has been observed (M. WAINWRIGHT & C. WILSON, personal comments).

Finally, although the formation of these lipophilic structures within the seminal fluid could be without an adaptive effect on reproduction, it is interesting to consider that macro-structural features are found also in the seminal fluid in humans (BRODY *et al.,* 1981). Exploration of the function and characteristics of these structures in reproduction would represent an interesting avenue for future work. To the extent that the *D. melanogaster* model allows, such work is already being performed in the Wilson laboratory by our postdoctoral researcher, Dr. Mark Wainwright.

Is secondary cell production of Hedgehog protein a cause or an effect of secondary cell maturation?

Secondary cells do not express *hedgehog* on eclosion, although the expression seems to begin in the hours following this event. Admittedly this evidence was obtained using a *hh*-GAL4 enhancer trap, and I am happy to acknowledge that it might not reflect the genuine expression pattern or timing of endogenous *hedgehog*. Still, the proportion of secondary cells positive for *hedgehog* increases over time, and the majority of secondary cells are positive for *hedgehog* by the third day of adulthood. This transition correlates with the period when the accessory gland fills up with secretions, as the male approaches maturity (RUHMANN *et al*., 2016). During this time, the secondary cells themselves also mature: their nuclei growing larger, and their secretory compartments increasing in number (LEIBLICH *et al*., 2012; CORRIGAN *et al.,* 2014). Altogether, these observations suggest that the cell biology of the secondary cells changes as the male matures fully, and that Hedgehog signalling might be part of this process. In addition, I think that steroid signalling might also be involved.

In mammals, an interaction between steroid signalling and the vertebrate *hedgehog* orthologue, *Sonic hedgehog* (*SHH*) is important for the development of the male

reproductive organs (PODASLEK *et al.,* 2003; BERMAN *et al.*, 2005; CHEN *et al.*, 2011). In *D. melanogaster*, the *hedgehog* gene promoter contains EcR binding motifs, and the EcR can regulate the expression of *hedgehog* via a co-factor: the methyltransferase, Trithorax-related ("**TRR**") (SEDKOV & CHO *et al.,* 2003). Incidentally, I have found that the expression of an inverted repeat targeting *TRR* is sufficient to reduce the nuclear size of secondary cells (APPENDIX 6: *p.* 442). Although this is not evidence of a conclusive relationship, it does merit further investigation into the role of steroids and/or the EcR in the regulation of *hedgehog* gene expression in secondary cells. For example, it might be interesting to determine how knock-down of either the *EcR* or *TRR* affects the expression of *hedgehog* via qRT-PCRs of accessory gland material.

Evidence against any steroid-dependent regulation of *hedgehog* in secondary cells might come through the lack of any obvious increases in the whole-animal ecdysone titre during the fruit fly's first days of adult life (HANDLER, 1982). However, steroid-independent activities of the EcR have been observed, for example in the absence of the EcR co-receptor, Ultraspiracle (Usp), in the larval wing disc (SCHUBIGER & TRUMAN, 2000). Interestingly, the EcR seems to act independently of Ultraspiracle in the male accessory gland (LEIBLICH, 2015; SHARMA *et al.,* 2017). Further work will be needed to better explore the relationship between steroid signalling and *hedgehog* gene expression in the male accessory gland—if such a relationship does indeed exist. Nevertheless, the data presented in this Chapter suggests that Hedgehog signalling is active in the secondary cells of the mature *D. melanogaster* male, where we know the EcR to be an important regulator (LEIBLICH, 2015).

Failing to ascertain how the production of Hedgehog protein in secondary cells might be regulated, we can nevertheless speculate. For example, might Doublesex have a role in regulating the expression of *hedgehog*? In genetically male *D. melanogaster* larvae,

the male accessory glands develop from the genital (imaginal) disc. Like other imaginal discs, the genital disc is patterned by morphogens (including Hedgehog and Dpp), and its genital identity and sex-specific development pattern are defined, respectively, through the expression of the homeobox gene, *Abdominal-B* ("***Abd-B***"), and the sex-determining gene, *doublesex* (SÁNCHEZ & GUERRERO, 2001). The presence of the male isoform of Doublesex, Dsx^{M}, renders the tissue able to respond to Hedgehog signalling (SÁNCHEZ & GORFINKIEL & GUERRERO, 2001). Expression of both *Abd-B* and *doublesex* is maintained in the secondary cells of adult male accessory gland (GLIGOROV & SITNIK *et al.,* 2013; CORRIGAN *et al.,* 2014). This suggests that *doublesex* might have a role in the regulation of Hedgehog signalling in these cells. It would be interesting to explore this through further work.

Might Hedgehog signalling itself be involved in regulating BMP signalling in secondary cells? In many imaginal discs, Hedgehog signalling induces the expression of *dpp* (STRINGINI & COHEN, 1997). This is true also in the genital disc (SÁNCHEZ & GUERRERO, 2001). How the expression of *dpp* in the male accessory gland is regulated has not been examined in detail. However, in collaboration with Siamak Redhai, he and I have shown that a *dpp*-GAL4 gene trap (dpp^{blk}-GAL4; STAEHLING-HAMPTON *et al.,* 1994) is expressed by every secondary cell by day six of the male's adult life (REDHAI *et al.,* 2016). Unfortunately, I have noticed that the dpp^{blk} gene trap line is quite sick. Due to the rush to get my laboratory work finished by the end of my *D. Phil.*, I did not manage to raise enough flies of this frail genotype for any quantitative measurements to be made of how the expression of dpp^{blk} varies over time in secondary cells. From the few flies I did dissect and examine, it does however seem as if the dpp^{blk}-GAL4 gene trap is not expressed in the accessory gland at early time-points (preliminary data not shown). Of course, these observations do of course need

to be verified and properly quantified for any reliable conclusions to be made. However, if *dpp* is indeed not present in the accessory gland at early ages (*i.e.* during the first days after eclosion), this would be consistent with the temporal expression pattern of *hedgehog* in this tissue. We already know that BMP signalling is an important regulator of secondary cell biology. This indicates that it might be worthwhile to further explore the genetic interaction between Hedgehog signalling and the expression of *dpp* in these cells. Indeed, a lack of BMP signalling activity in secondary cells has previously been shown to reduce the male's ability to induce the full post-mating response in his mate (LEIBLICH *et al.*, 2012). This suggests that Hedgehog signalling might indeed be an important regulator of secondary cell maturation: by acting as a (possibly steroid-sensitive) regulator of BMP signalling activity. This would form an interesting topic for future work.

Is secondary cell-derived Hedgehog a seminal fluid component—or an accessory gland signalling factor?

Many accessory gland-derived seminal fluid proteins have important roles in *D. melanogaster* by promoting the male's reproductive success through stimulating the female's fecundity and other aspects of her post-mating response (AVILA *et al.,* 2011). In this Chapter I have provided data suggesting that the Hedgehog protein is produced by secondary cells; that it is packaged into their secretory granules; and that it is present in the seminal fluid. Technically, this makes Hedgehog a secreted accessory gland protein (an 'Acp'). But what might this mean for the *role* of the Hedgehog protein in male reproduction? I can think of two possible scenarios:

a) Hedgehog promotes male fertility by acting as an *inter*-sexual (male → female) reproductive protein; it being transferred in the seminal fluid to *directly* affect

reproduction by promoting male- and female-specific functions like sperm capacitation in the female reproductive tract, or female ovulation rates; *or*,

b) Hedgehog promotes male fertility by acting as an *intra*-sexual (male only) regulatory protein that affects male reproductive function *indirectly*: promoting secondary cell and/or accessory gland function.

Although Hedgehog seems to be present in the seminal fluid, I have no evidence to suggest that the main fate of the protein is to be transferred to the female. Experiments investigating any such roles are marred by the lack of good drivers that are specific to the secondary cells in the male reproductive system, when *hedgehog* is expressed also in the testes (**FIGURE 4. 4.,** *p.* 152). Therefore, any driver seeking to tease out the role of secondary cell-derived Hedgehog must ideally be exclusively expressed in the secondary cells and not affect testes function. I think the best approach to ensure this is to make use of the 'split-GAL4 system' (LUAN *et al.,* 2006; PFEIFFER *et al.,* 2010).

Typically, the promoter regions of traditional ('first-generation) GAL4 lines are near-universally expressed in more than one cell type. This is certainly true for the drivers commonly used in our laboratory: *spitz*-GAL4, *escargot*-GAL4, and *doublesex*-GAL4. In the split-GAL4 system, however, the *GAL4* gene coding region is split into two parts that are driven by different promoters: one driving the expression of the GAL4 activation domain, and the other driving the expression of the GAL4 DNA-binding domain. Individually, the GAL4 activation- or DNA-binding domains are not able to promote *UAS*-dependent gene expression, and functional GAL4 protein is only present in cell types where both domains are transcribed at the same time (LUAN *et al.*, 2006). Because of the need for two different promoters to drive the production

of functional GAL4 protein, the split-GAL4 system makes it much easier to identify genuinely cell-specific promoter combinations.

Using the split-GAL4 system to selectively knock down *hedgehog* in secondary cells would *theoretically* make it possible to figure out if seminal fluid-contained Hedgehog protein has any role regulating, for example, the female post-mating response. In practice, however, the question is more complicated, and it is difficult to tease out the exact contributions of secondary cell-derived Hedgehog on reproductive function. My work suggests that secondary cells (which do seem to modify important seminal fluid protein components (GLIGOROV & SITNIK *et al.*, 2013; SITNIK & GLIGOROV *et al.*, 2016)) might require Hedgehog to themselves function optimally. Experimental transplantation of seminal fluid into the female reproductive tract is equally sensitive to optimal secondary cell function, and can therefore also not be considered a good solution to the problem. Similarly, the ectopic expression of *hedgehog* in the female reproductive tract is marred by my findings that the *hedgehog* gene seems to be expressed endogenously in at least some of these tissues. As a result, an inter-sexual role for male-derived Hedgehog protein in fruit fly reproduction remains unknown, and I am not quite sure how this might best be tested? Ideally (and with a lot of luck), perhaps a combination of bioinformatics and targeted mutagenesis might reveal if there are any *hedgehog* enhancer elements that specifically regulate the gene's expression in the male or female reproductive tracts to permit the generation of genetic alleles.

If we cannot easily test the inter-sexual role of Hedgehog protein, how might we go about thinking about the potential intra-sexual role? In the male accessory gland, although the epithelium secretes into the gland lumen, the epithelium remains exposed to the lumen contents. There is evidence that the movement of the epithelial secretions is not unidirectional (epithelium → lumen), but that the lumen contents can actually be

re-absorbed by the epithelium (lumen → epithelium) for processing (GLIGOROV & SITNIK *et al.*, 2013; SITNIK & GLIGOROV *et al.,* 2016). In addition, there are anecdotal reports of lumen protein contents appearing more concentrated with age (M. WAINWRIGHT, personal comments). Under such a model of bi-directional movement of lumen contents (epithelium ↔ lumen), any secreted protein has the potential to become a signalling molecule. My work suggests that Hedgehog might be a case-in-point.

Based on the data resulting from my work (although Hedgehog, of course, might perform multiple roles in the male accessory gland), a possible scenario is that Hedgehog regulates male reproductive function by acting as an intra-sexual regulator of secondary cell function and/or nuclear growth. Hedgehog appears to be carried in secondary cell secretory granules (implying it is destined to be released from the cell), and it is also present in the accessory gland lumen (implying that it is, in fact, released). In addition, the Hedgehog receptor, Patched, is present on the secondary cell plasma membrane (allowing the molecule to signal back into the cell). As we also have seen in this Chapter, Hedgehog (and other components of the Hedgehog signalling pathway) has a role regulating secondary cell nuclear size. Secondary cell nuclei grow over time (LEIBLICH *et al.,* 2012), and this growth happens during the time when the secondary cells fill up with compartments (CORRIGAN *et al.,* 2014) and the accessory gland lumen fills up with secretions (RUHMANN *et al.,* 2016). Altogether, a model emerges where secondary cells produce Hedgehog protein (which is released into the lumen, independently or via Dispatched), allowing Hedgehog to interact with Patched and signalling back into the secondary cell to regulate processes involved in secondary cell nuclear growth (**FIGURE 4. 31.**).

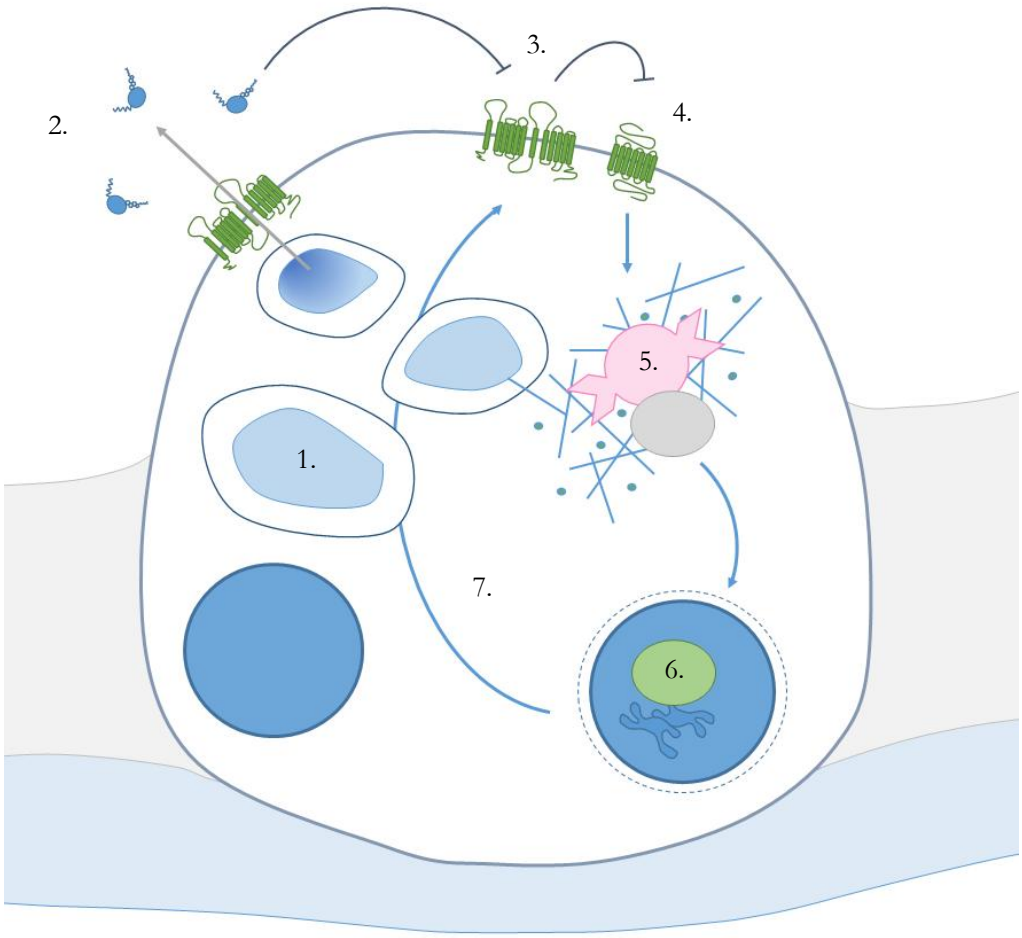


FIGURE 4. 31. | HEDGEHOG SIGNALLING IN THE *D. MELANOGASTER* MALE ACCESSORY GLAND.

A diagram showing how Hedgehog signalling might regulate secondary cell biology in the male accessory gland. The Hedgehog protein is produced by secondary cells, and carried on secretory granules (1.). Hedgehog protein is released from secondary cells—possibly through a process that might require the transmembrane protein, Dispatched (2.). Hedgehog protein in the accessory gland lumen can interact with the Hedgehog receptor, Patched; inhibiting the receptor (3.). Hedgehog binding to Patched relieves Patched inhibition of the Hedgehog signal activator, Smoothened (4.). Activated Smoothened can signal into the secondary cell, activating the Hedgehog signalling complex (HSC) (5.), containing the microtubule-associated protein Cos-2 (pink) and the Hedgehog-dependent transcription factor, Ci (grey). Smoothened activation of the HSC allows Ci to be released from the HSC, and translocate to the nucleus (6.), where Ci can function as an activator, CiA (green), to regulate Hedgehog-dependent gene expression. The activity of the CiA also causes secondary cell nuclei to grow. One of the Hedgehog target genes is *patched*, and its expression completes a negative feedback loop to reduce Hedgehog signalling activity (7.).

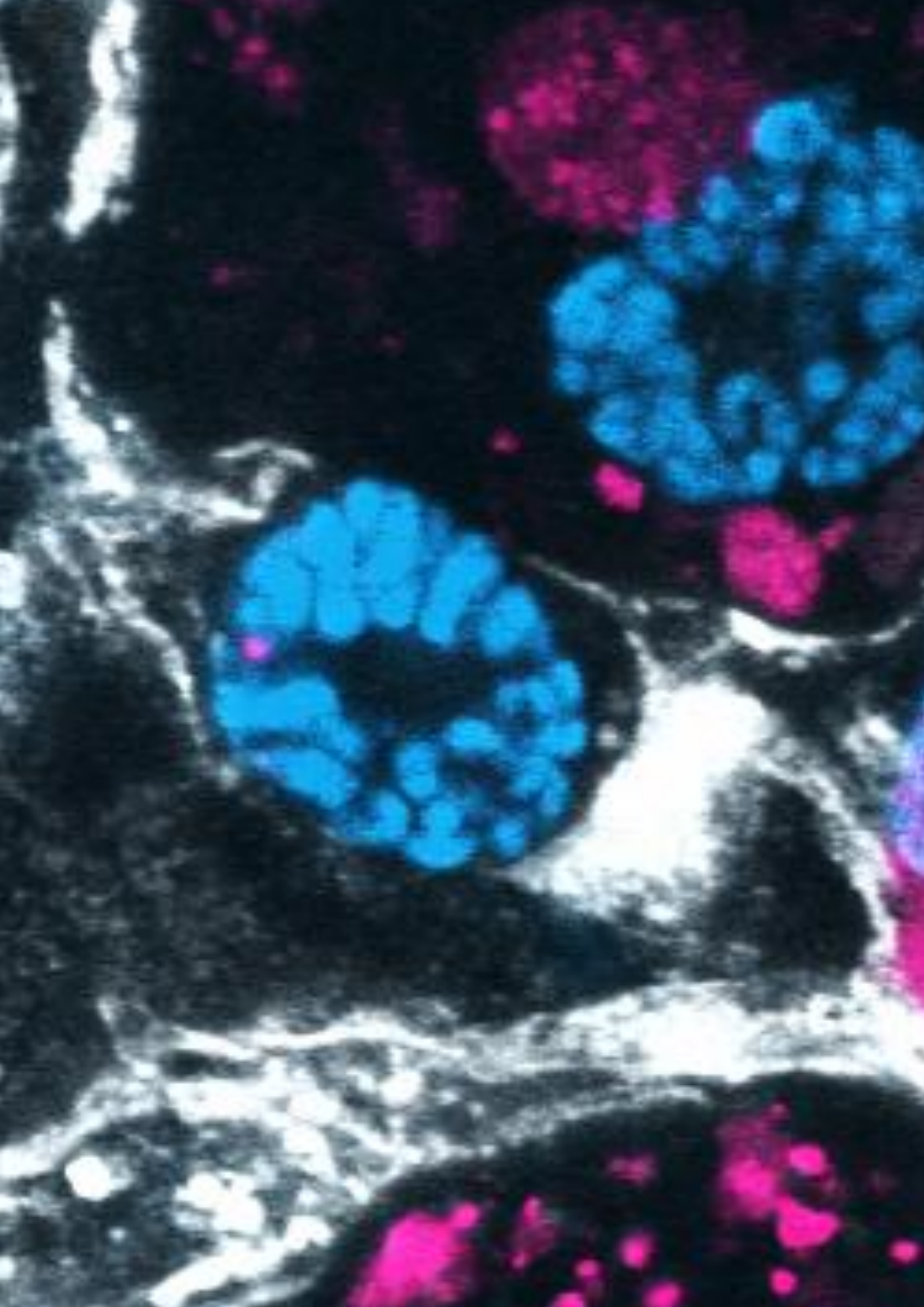
Previous work in our laboratory has shown that manipulations that decrease secondary cell nuclear size also reduce the male's ability to induce the full post-mating response in his mate (LEIBLICH *et al.,* 2012). In addition, work by others has shown that other manipulations affecting secondary cell development and/or function have similar effects (*e.g.* MINAMI *et al.,* 2012). This makes sense, since secondary cells seem to help process main cell secretion products (GLIGOROV & SITNIK *et al.*, 2013), and the protein cocktail composing the accessory gland secretions is crucial for male reproductive success (XUE & NOLL, 2000). Interestingly, the work in this Chapter suggests that Hedgehog might also be transferred between secondary cells and main cells, and that ectopic activation of the Hedgehog signalling pathway can reduce main cell nuclear size. Altogether, this suggests that Hedgehog signalling might have a role regulating secondary cell function—and male reproductive function, by extension. According to this logic, I think Hedgehog might act within the male accessory gland as an intra-sexual signalling factor; promoting secondary cell function and affecting male reproductive function only indirectly. This scenario yields further questions: Is the size of the accessory gland epithelial nuclei a key indicator of male reproductive capacity? What is the mechanism underlying the secondary cell nuclear growth? I do not want to discuss these questions in too much detail here, but would rather refer the reader on to the next Chapter (CHAPTER 5). This next Chapter is dedicated to exploring the regulation of secondary cell nuclear size and its implications for male reproductive function in more detail.

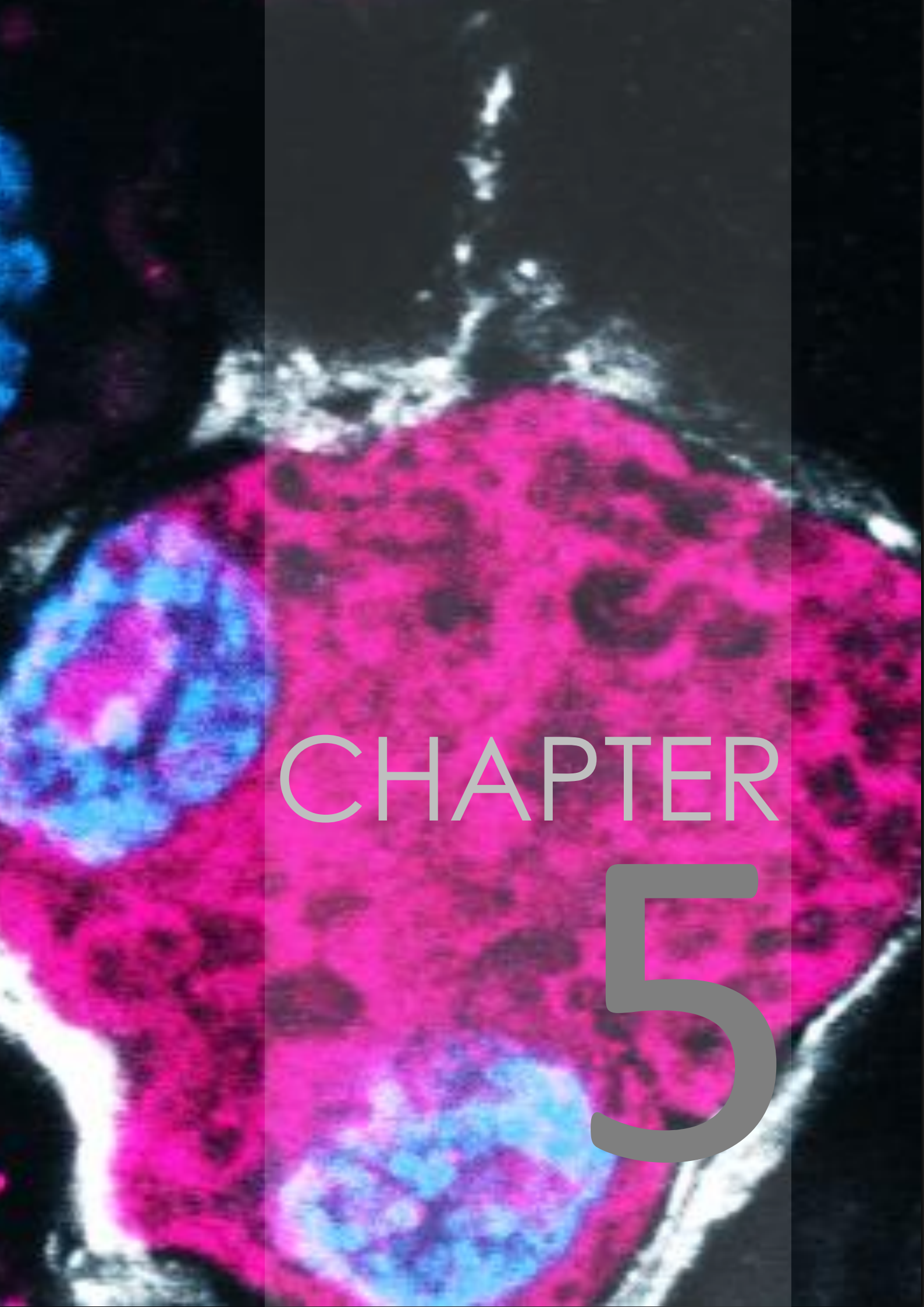
Concluding remarks

In this Chapter I have described some of the changes that the male accessory gland undergo as the male matures after eclosion; showing that the accessory gland fills up with secretions of increasing heterogenity. This Chapter has also explored a role for Hedgehog signalling in male accessory gland function; showing that a *hedgehog* enhancer trap is expressed in the secondary cells, and that this expression begins after eclosion and increases over time. In the context of male maturation after eclosion, the expression pattern of the *hedgehog* enhancer trap suggests that Hedgehog signalling might be important for male reproductive function. Hedgehog protein seems to be packed into the secondary cell secretory granules—finding its way not only into the accessory gland lumen, but also possibly signalling back into both secondary cells and the surrounding main cells.

In this Chapter I have also shown that Hedgehog and other components in the Hedgehog signalling pathway have a role regulating secondary cell nuclear size. Further work will be needed to explore the potential regulatory relationship between steroid signalling, Hedgehog signalling, and BMP signalling in the secondary cells, to determine if Hedgehog protein helps secondary cells to mature—or if its production is a sign of secondary cell maturity. However, from the work presented in this Chapter, it seems likely that the Hedgehog protein is produced by secondary cells and that it helps promote their nuclear growth and the function of these. I think it is likely that the protein is an intra-sexual signalling molecule that promotes male reproductive function through its effect on secondary cell biology. How Hedgehog signalling might promote the growth of the secondary cell nuclei, and what impact this might have on the

function of secondary cells and the accessory gland itself, will be explored in more detail in the next Chapter.





CHAPTER 5

PREVIOUS PAGE:

Two secondary cells stained with α-ANCE (magenta) showing greatly enlarged nuclei (stained with DAPI, cyan) as the result of *cyclin D* and *CDK4* overexpression. Cell junctions are stained with α-FASIII and shown in white.



CHAPTER 5

A BMP/EcR SIGNALLING AXIS DRIVES ENDOREPLICATION IN SECONDARY CELLS IN RESPONSE TO MATING

The only true voyage of discovery […] would be not to visit strange lands but to possess other eyes.

— Marcel Proust
(in PROUST, 1923 [2003])

Summary

Work in our laboratory has shown that BMP signalling regulates the growth of secondary cell nuclei, and that the EcR also drives growth in collaboration with the cell cycle regulators, E2F1 and RBF. In this Chapter, I demonstrate that mating stimulates the growth of secondary cell nuclei via activation of BMP signalling that promotes an increase in the secondary cell DNA content. This growth is mediated by regulators of the cell cycle, and I show that both Cyclin D and Cyclin E have roles in this process; promoting the synthesis of new DNA. Working with E2F1 and RBF, I further show that secondary cell nuclei can grow via two different mechanisms—both of which I think might increase the biosynthetic potential of the cells. As a result of the work presented in this Chapter, I propose that secondary cells grow through a variant of the cell cycle called the 'endocycle'; revealing a potential mechanism by which secretory cells in male reproductive glands can respond to physiological signals to match their biosynthetic activity to the male's reproductive demand.

INTRODUCTION

Secondary cell nuclei are dynamic, and increase in size in response to both cellular and physiological stimuli. Some of these stimuli were introduced already in CHAPTER 1 (*p.* 37): where I describe results from our laboratory that show BMP signalling to be a key pathway involved in the regulation of secondary cell nuclear growth (LEIBLICH *et al.*, 2012). However, as we saw in the previous Chapter, Hedgehog signalling also has a role regulating this process. In addition, repeated copulations can also promote secondary cell nuclear growth (LEIBLICH *et al.*, 2012). Exactly how these different inputs regulate the growth of secondary cell nuclei is not well understood.

Binucleation, polyploidy, and mechanisms of cell growth

Binucleation occurs in metabolically active and stressed tissues

Both main cells and secondary cells are binucleate (BAIRATI, 1968). This arrangement can be physiologically relevant, and it occurs in an assortment of cell types (see **TABLE 5. 1.**). Yet, the exact reasons why a cell would benefit from having more than one nucleus are not fully understood (AHUJA *et al.*, 2007). However, it

has been suggested that binucleation of the epithelial cells in the accessory gland maximises the flexibility of the tissue; accommodating the repeated stretching and contraction that occurs as the gland fills up with secretions and then expels these during mating (TANIGUCHI *et al.*, 2012). Another proposal suggests that metabolically active cells might benefit from having two or more nuclei—since each doubling of the number of nuclei represents an effective doubling of the DNA content to support the synthesis of new RNA and protein (AHUJA *et al.,* 2007). For example, gene expression profiling in human and rat hepatocytes and cardiomyocytes (two polynucleate cell types) has shown an association between DNA content increases and the expression of genes involved in metabolism and stress-resistance; promoting the survival of cells in stressed tissues (ANATSKAYA & VINOGRADOV, 2007). Interestingly, at least the main cells in the accessory gland epithelium experience high levels of ER stress as a result of their high synthetic and secretory activity (CHOW *et al.,* 2015), and human cardiomyocytes

TABLE 5. 1. | BINUCLEATE CELL TYPES IN INSECTS AND MAMMALS.

Organism	Tissue (cell type)	Reference
Drosophila melanogaster	Male accessory gland	BAIRATI (1968)
Hemiptera	Malpigian tubule	MADDRELL *et al.* (1985)
Mammalia	Endometrium	ANSELL *et al.* (1974)
	Heart (cardiomyocyte)	SOONPAA *et al.* (1996)
	Liver (hepatocyte)	BEAMS & KING (1942)
	Placenta (trophoblast)	WOODING (1982)

have been shown to undergo increased polynucleation after myocardial damage (HERGET *et al.*, 1997).

Increased ploidy amplifies the cellular DNA and gene content

Although already polyploid, binucleate cells can increase their gene copy number further still. Polyploidisation is a common feature of metabolically and synthetically active cells (ZHIMULEV & KORYAKOV, 2009; LEE, DAVIDSON & DURONIO, 2013). (See a selection in **TABLE 5. 2.**, below.) Well-known examples of polyploid cells in *D. melanogaster* include the biosynthetically active cells of the larval

TABLE 5. 2. | POLYPLOID CELL TYPES IN INSECTS AND MAMMALS.

Phylogenetic relationship	Organism	Tissue (cell type)	Ploidy (C)	Reference
	Drosophila melanogaster	Larval salivary gland	1024–2048	ZHIMULEV & KORYAKOV (2009)[a]
		Prothoracic gland	256–512	HOCHSTRASSER & SEDAT (1987)
		Ovary (nurse cell)	256–512	JACOB & SIRLIN (1959)
		Intestine (enterocyte)	4–16	CHOI, LUCCHETTA & OHLSTEIN (2011)
		Accessory gland (main cell and secondary cell)	8 (2 × 4)	TANIGUCHI *et al.* (2012)
	Caenorhabditis elegans	Intestine	32	HEDGHECOCK & WHITE (1985)
		Epidermis	8–10	FLEMMING *et al.* (2000)
	Rattus norvegicus	Blood (megakaryocyte)	4–32	ODELL *et al.* (1965)
	Mus musculus	Placenta (trophoblast)	32–850	BARLOW & SHERMAN (1972)
	Zea mays	Endosperm	16–178	KOWLES & PHILLIPS (1985)

[a] Despite my best scholarly efforts, I have failed to identify the article where the ploidy of the *D. melanogaster* salivary gland cells was first determined (although I have deduced it was sometime during the period 1951–1972). The missing reference has therefore been substituted with a contemporary review.

salivary gland, and the follicle cells of the female ovary—where there is preferential amplification of genes encoding products required for eggshell formation (CALVI *et al.*, 1998). In the male accessory gland, both main cells and secondary cells are believed to be octoploid (8C)[1]; containing two sets of nuclei, each with four copies of the haploid genome (4C) (TANIGUCHI *et al.,* 2012). Other, non-*Drosophilid* examples of metabolically active and polyploid cells include trophoblasts (an extra-embryonic cell type that contributes to the formation of the mammalian placenta) (BARLOW & SHERMAN, 1972), and plant endosperm (the tissue that nourishes the growing plant embryo) (KOWLES & PHILLIPS, 1985).

Increased cell growth and DNA content can be regulated by the cell cycle

Cells become polyploid as the result of successive rounds of DNA amplification, multiplying the number of individual gene copies in the cell. This DNA amplification occurs as cells progress through a variant of the cell cycle, known as the 'endocycle'. The standard (canonical) cell cycle consists of several phases: an initial growth phase ("**G_1**"), a synthetic phase ("**S**"), a second growth phase ("G_2"), and a mitotic phase ("**M**") (**FIGURE 5. 1.**) (ALBERTS *et al.,* 2002). When the cell is not actively cycling, it is said to be in the intercyclic phase ("**G_0**"). In contrast to the standard cell cycle, the endocycle consists of only two alternating phases: a synthetic DNA synthesis phase (S) and an intersynthetic growth phase ("**G**") (**FIGURE 5. 1.**) (ZHIMULEV & KORYAKOV, 2009). A cycle consisting of only alternating synthetic and intersynthetic growth phases allows a cell to multiply its DNA content,

[1] The haploid genome is typically denoted 'C'. A diploid cell/nucleus would therefore have two copies of the haploid genome and be denoted as '2C' (2 × C = 2C). The octaploid secondary cells are therefore '8C', as the result of having two tetraploid (4C) nuclei (2 × 4C = 8C).

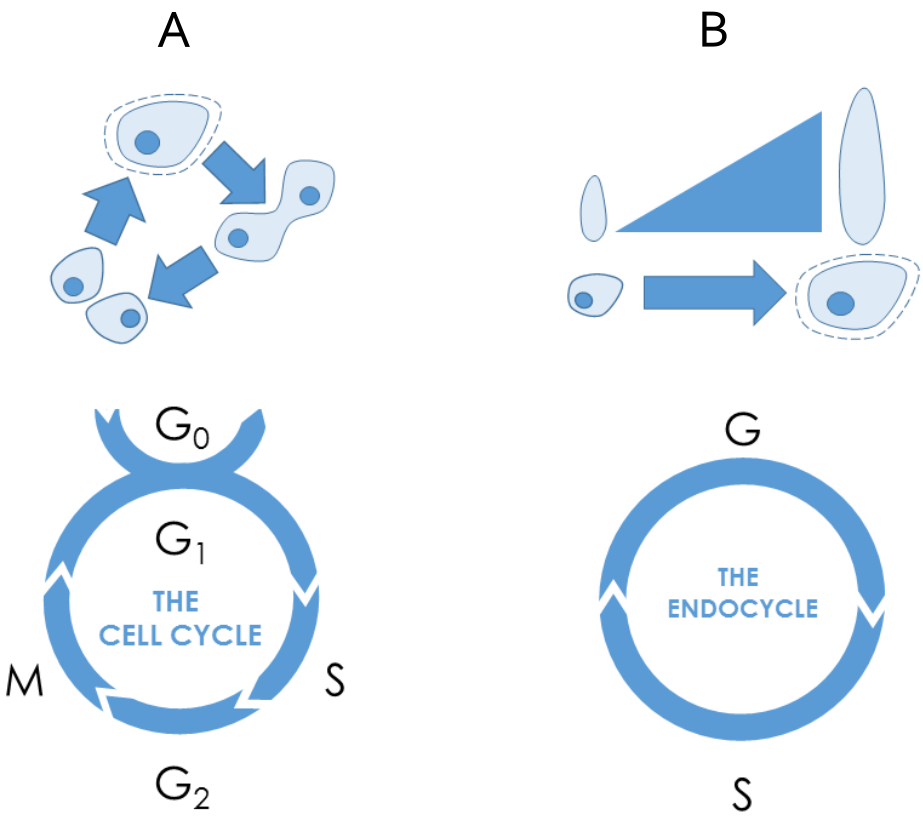


FIGURE 5. 1.| THE CELL CYCLE AND THE ENDOCYCLE.

A: The standard (canonical) cell cycle governs the mitotic (proliferative) growth of cells. The most distinctive phases of the cell cycle are the synthetic phase (S) and the mitotic phase (M). During the S-phase, the DNA content of the cell is replicated; to ensure that each of the two daughter cells contain the same amount of DNA as the parent cell. Cell division occurs during the M-phase; where the parent cell divides into two to give rise to two individual daughter cells. After division, each daughter cell is smaller than the original parent cell, and they each go through a further growth phase (G_1). Growth is an imperative part of the cell cycle, and continuous cell division in the absence of growth can cause the cell to undergo programmed cell death. There is a second growth phase (G_2) between S- and M-phase, that allows the cell further opportunity to grow after DNA replication, but before cell division. Cells can exit the cell cycle and enter a quiescent (gap) phase (G_0), where no cell cycle activity occurs—such as during terminal differentiation, or in response to cell damage.

B: An alternative, cell cycle is the endocycle, which is notable for its lack of an M-phase. Rather, the endocycle governs cell (and tissue) growth in the absence of cell division; allowing individual cells to grow larger and larger by cycling through successive synthetic (S) and growth (G) phases that increase both a cell's size and its DNA content.

as its genome is replicated over and over in the absence of cell division. As a result, endocycling cells can become highly polyploid (EDGAR & ORR-WEAVER, 2001)—such as in the *D. melanogaster* larval salivary gland, where epithelial cells can contain as many as 1024–2048 copies of the haploid genome (ZHIMULEV & KORYAKOV, 2009).

Different cell types utilise different types of endocycles, each generating a different configuration of chromosomes. In cells where the genome is fully replicated during each endocycle, the chromosome number is simply duplicated. During other types of endocycles, however, chromosomes are incompletely replicated; producing chromosomes with under- and over-replicated regions that can cause sister chromatids to stick together (ZHIMULEV & KORYAKOV, 2009) (**FIGURE 5. 2.**). These chromosomes are referred to as '*polytene*'.[2] In the *D. melanogaster* larval salivary gland, endocycling generates polytene chromosomes with under-replicated regions of heterochromatin. This causes the individual chromatids to stick together—attached by their centromeres—to form a common *chromocentre*, with the rest of the chromatids arranged into neat, parallel arrays (PAINTER & GRIFFEN, 1937; URATA & PARMLEE *et al.,* 1995; ZHIMULEV & KORYAKOV, 2009) (**FIGURE 5. 2. C**). These chromosome arrays can grow very large, and the polytene chromosomes of the larval salivary gland have traditionally been referred to as 'giant chromosomes' (*e.g.* PAINTER, 1934ab) (**FIGURE 5. 3.**). When these chromosomes are viewed using light microscopy, intercalating regions of hetero- and euchromatin form a distinctive banding pattern that made the first physical mapping of individual

[2] The term 'polytene' is derived from the pachytene stage of meiosis where sister chromatids separate to allow crossing-over during meiotic prophase. At this stage the chromatids appear thickened. 'Pachytene' derives from the Greek '*pachynema*' ('thick threads), and the term 'pachytene' was proposed by KOLLER (1935) and refers to the thick, cylindrical threads of chromatids that constitute the polytene chromosome.

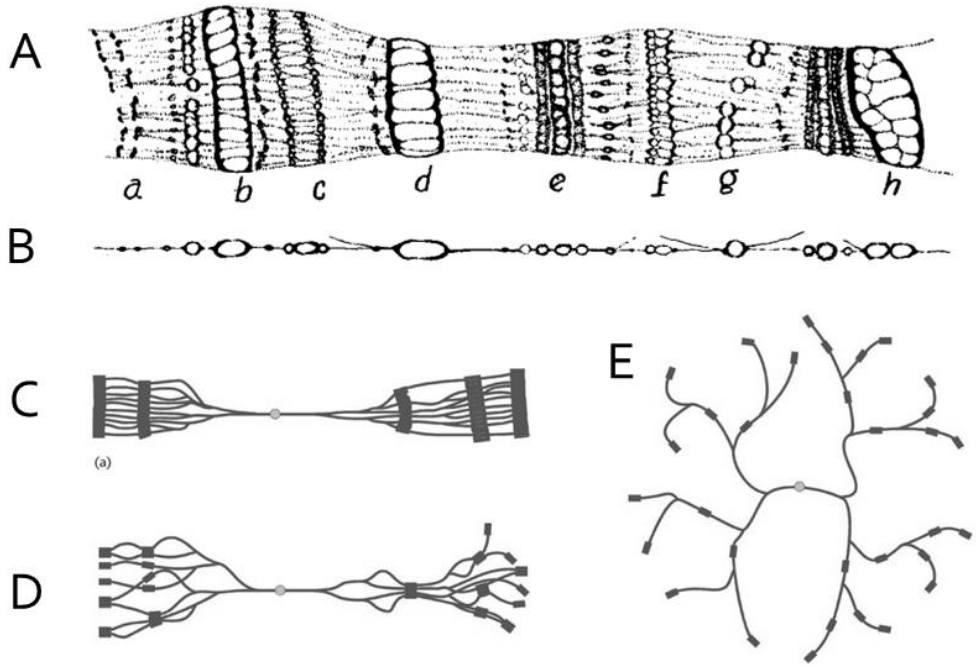


FIGURE 5. 2.| DIAGRAMMATIC REPRESENTATIONS OF DIFFERENT POLYTENE CHROMOSOME VARIANTS.

Different cell types undergo different endocycles, to produce different polytene chromosome configurations.

A: A *camera lucida* drawing showing the longitudinal chromatin fibres that constitute a polytene chromosome in the larval salivary gland of the black-fly, *Simulium virgatum*.

B: A semi-diagrammatic illustration of an individual chromomere fibre that, when bundled together, makes up the structure shown in panel **A.**

C: A diagrammatic representation of a 'classical' polytene chrosomosome, where there is good alignment between sister chromatids; forming a thick chromosome 'thread' with a characteristic banding pattern formed from alignment of chromomeres.

D: A diagrammatic representation of a 'cryptic' polytene chromosome—which is formed when sister chromatids are less well-aligned but with chromomeres still forming a discernible banding patten.

E: A diagrammatic representation of a 'lamp-brush' or 'pompom' polytene chromosome—where chromatid alignment is disrupted and the chromomere banding is diffuse and non-discernable.

The images in panels **A** and **B** are taken from FIGURE 1–1A in PAINTER & GRIFFEN (1937: *p.* 616), respectively. The images in panels **C–D** are derived from FIGURE 3 A–C in ZHIMULEV & KORYAKOV (2009: *p.* 3).

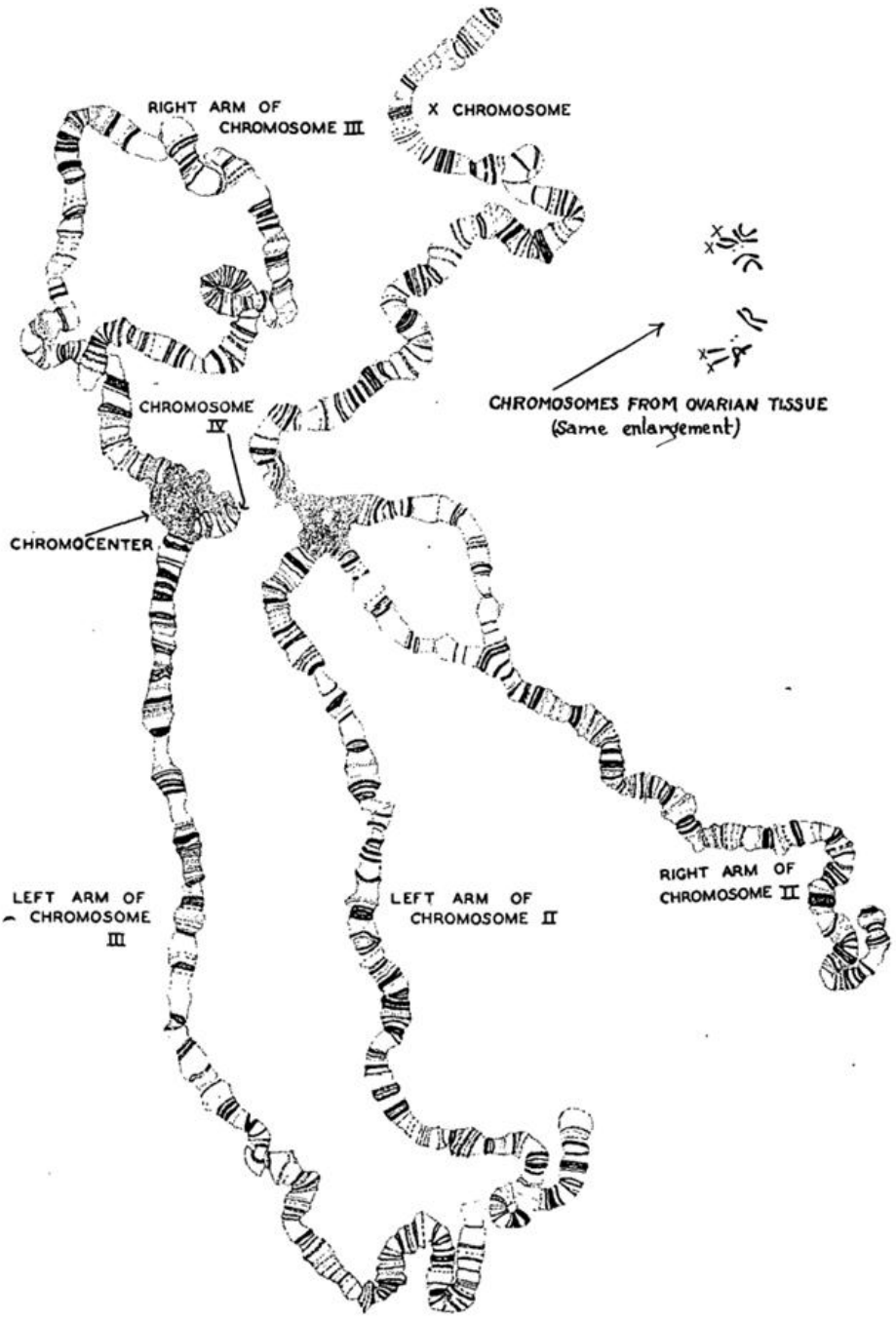


FIGURE 5. 3.| ENDOCYCLE-DRIVEN POLYPLOIDISATION GIVES RISE TO GIANT, POLYTENE CHROMOSOMES.

A *camera lucida* drawing of the nuclear contents of a cell in the larval salivary gland of a female *D. melanogaster* larva, showing the distinctive banding pattern of the giant chromosomes, as well as their large size—when compared to the 'normal' chromosomes of the adult ovary. The image in this figure is from PAINTER (1934a; FIGURE 7: *p.* 466–7).

gene *loci* along the chromosomes possible (KOLLER, 1935; BRIDGES, 1935; LEFEVRE, 1974; ZHIMULEV & KORYAKOV, 2009).

Endocycling causes cells to grow, and as a result, the size of a cell is generally associated with its ploidy (STOCKER & HAFEN, 2000). This relationship has been demonstrated across *Eukarya*; with plants, fungi, insects, and mammals all using endoreplication as a mechanism for cell growth (reviewed by EDGAR & ORR-WEAVER, 2001; ZHIMULEV & KORYAKOV, 2009; LEE, DAVIDSON & DURONIO, 2013). For example, in vertebrates there is a direct relationship between the total size of an animal's genome and the total volume of its blood cells—with increased *erythrocyte* (red blood cell) volume being a diagnostic feature of increased ploidy (reviewed in GREGORY, 2001). Indeed, this relationship between cell- and nuclear size has been known for well over a century (*e.g.* GULLIVER, 1875; DOBZHANSKY, 1929).

The endocycle is non-proliferative, and it has been suggested that endocycling permits growth in contexts where changes to cell morphology (as a result of mitosis) might otherwise destabilise or disrupt tissue (*e.g.* in organised epithelia) (EDGAR & ORR-WEAVER, 2001; LEE, DAVIDSON & DURONIO, 2013). For example, follicle cells in the *D. melanogaster* ovary can restore tissue homeostasis non-proliferatively in response to tissue damage through endocycle-driven growth (TAMORI & DENG, 2013). Another example of an organised epithelial tissue that grows as a result of endocycling is the *D. melanogaster* larval salivary gland, which remains fully differentiated throughout the ever-growing larval stages. Endocycling may therefore be considered an effective mechanism for non-proliferative growth in organised tissue (ZHIMULEV & KORYAKOV, 2009).

Endocycle-driven growth can also increase tissue volume, or even the size of the organism itself (LEE, DAVIDSON & DURONIO, 2009). In *D. melanogaster*, the larval body grows from its ecdysis from the egg up to its final larval stage that culminates in puparation, and this growth is the result of endoreplication of the cells in the epidermis (EDGAR & ORR-WEAVER, 2001). Similarly, *C. elegans* also grows as a result of endoreplication: showing a positive correlation between the nuclear ploidy of the hypodermis and the final body size of the nematode (LOZANO *et al.,* 2006). Although endoreplication-driven growth can be physiologically relevant in some tissues (*e.g.* in the *D. melanogaster* larval epidermis), the physiological relevance of such growth in other tissues (*e.g.* the adult *C. elegans* hypodermis) remains unknown. It is possible such growth is not adaptive, but that it represents a form of age-dependent 'hyperfunction' (BLAGOSKLONNY, 2013).

Molecular regulation of the cell cycle

Regardless of whether a cell cycles through a full or modified cell cycle, the process is initiated by extracellular stimuli in the form of 'mitogens'. Mitogens can take the form of proteins (like Hedgehog), or small molecules (like ecdysone), and what they all have in common is that they activate signalling cascades that cause the cell to enter the cell cycle—and proceed through it (**FIGURE 5. 4.**). One of the first genes to be expressed in response to mitogen signalling, is the growth-phase cyclin, *cyclin D* ("***cycD***") (BALDIN *et al.,* 1993). In *D. melanogaster*, the *cyclin D* gene is a direct target of Hedgehog signalling in the developing wing disc (DUMAN-SCHEEL *et al.*, 2003), and the gene is also up-regulated in response to ecdysone signalling in the proliferating midgut during metamorphosis (CRANNA & QUINN, 2009).

Cyclin D protein is turned over rapidly (its half-life can be as short as 10 minutes) and the gene therefore needs to be highly expressed for the protein to accumulate to sufficient levels for the cell cycle to begin (SHERR & ROBERTS, 1999). As a result, high Cyclin D levels can only result from sustained mitogen signalling—making the protein an effective intracellular mitogen 'sensor' that creates a link between the extracellular signal for the cell to grow/proliferate and the molecular machinery of the cell cycle itself (SHERR & ROBERTS, 1999). As a result, elevated Cyclin D levels mark the beginning of the cell cycle, as Cyclin D begins to interact with a type of serine-threonine kinase that requires a cyclin (activating subunit) to become active. These cyclin-dependent kinases ("**CDKs**") are present in the cell throughout the cell cycle (ALBERTS *et al.*, 2002); allowing the cell to respond rapidly to cyclin levels reaching their critical threshold. In *D. melanogaster*, Cyclin D interacts with the CDK, CDK4 (DATAR *et al.,* 2000). The formation of the CycD·CDK4 complex induces a conformational shift in the CDK4 subunit; exposing its active site to better allow the kinase to interact with its downstream protein targets (KATO *et al.,* 1993; ALBERTS *et al.*, 2002).

Inhibitors of the cell cycle normally keep cells in a quiescent or terminally differentiated state. Examples of such inhibitors are tumour suppressors like those from the retinoblastoma protein family (GIACINTI & GIORDANO, 2006). The *D. melanogaster* retinoblastoma family protein ("**RBF**"), acts as an inhibitor of the cell cycle by preventing the expression of the genes that regulate the cell cycle and its progression (DU *et al.,* 1996). Many of the proteins targeted by RBF are regulated by the E2-factor 1transcription factor ("**E2F1**" (dE2F1 in *D. melanogaster,* but 'E2F1' from hereon), which is itself an important target of RBF (CHELLAPPAN *et al.*, 1991). For example, the human orthologue of the EcR, the androgen receptor (AR),

can promote cell proliferation (ZEGARRA-MORO *et al.,* 2002), and it is positively regulated by E2F1 in a human prostate cancer cell line (SHARMA *et al.,* 2010). Although E2F1 is a transcriptional activator, interaction between E2F1 and RBF to form a RBF·E2F1 protein complex inhibits the E2F1 transactivation domain; preventing the transcription of its downstream-regulated genes (WEINTRAUB *et al.*, 1992; FLEMINGTON *et al.,* 1993). RBF can also recruit chromatin remodelling factors, such as the histone deacetylase 1 ("**HDAC1**")—modifying the epigenetic landscape around gene promoters to prevent them from becoming active (HARBOUR *et al.,* 1999). RBF-dependent repression of gene promoters can be inhibited by the CycD·CDK4 complex, which phosphorylates RBF to disrupt its interaction with HDAC1 (HARBOUR *et al.*, 1999). CDK4-dependent phosphorylation of RBF primes the protein for further phosphorylation events by other cyclin·CDK complexes, by exposing other phosphorylation sites within the protein (HARBOUR *et al.,* 1999).

Phosphorylated RBF can no longer silence E2F1-regulated gene promoters, and as E2F1 levels accumulate, it can directly regulate the expression of another cyclin, *cyclin E* ("**cycE**") (GENG *et al.,* 1996). Unlike Cyclin D, which is a growth-phase cyclin, Cyclin E is a synthetic-phase cyclin that is required for the cell to progress into S-phase (OHTSUBO *et al.,* 1995; COVERLY *et al.*, 2002). As Cyclin E levels accumulate in the cell, the protein complexes with another CDK, CDK2, to form a CycE·CDK2 complex (OHTSUBO *et al.*, 1995). The formation of this complex further inhibits RBF via further phosphorylation of the protein; disrupting its physical association with E2F1 (HARBOUR *et al.,* 1999). As the result of RBF hyper-phosphorylation, additional cell cycle genes become active. These include the

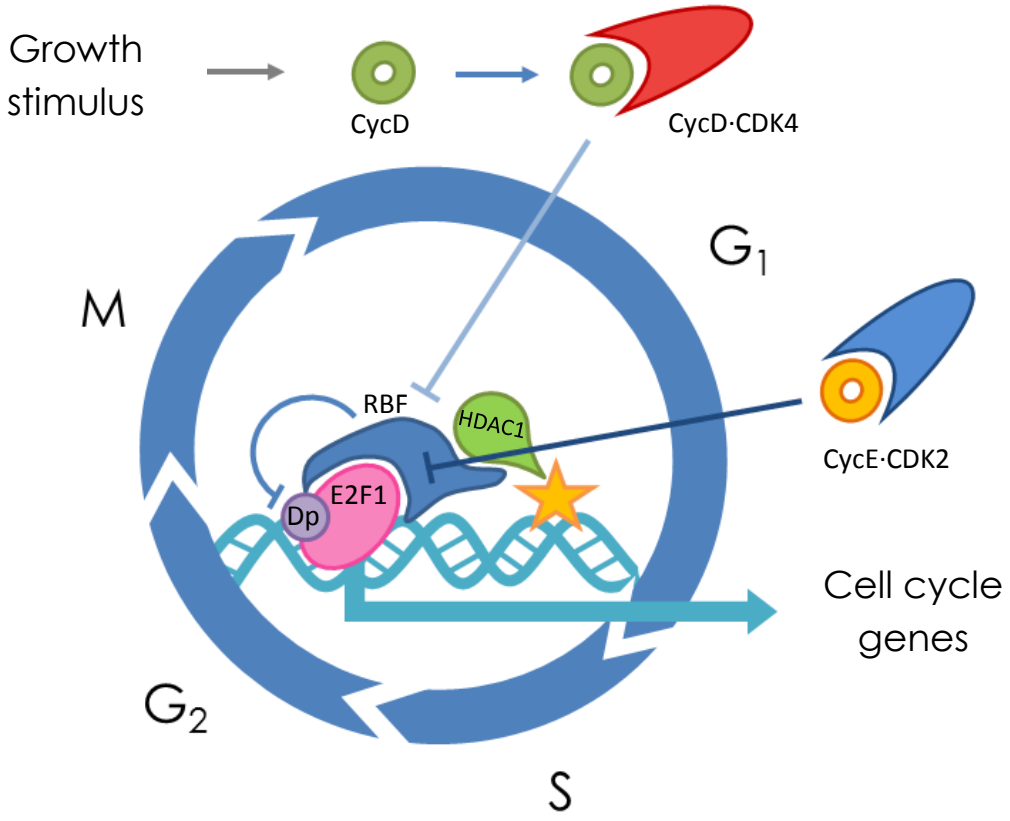


FIGURE 5. 4.| MOLECULAR REGULATION OF THE CELL CYCLE.

Multiple levels of control need to be overcome for the cell cycle to begin. Levels of the growth-phase cyclin, Cyclin D, are very labile and only increase in response to a growth (mitogen) stimulus; making the protein an efficient 'mitogen sensor'. Cyclin D is the regulatory subunit of the cyclin-dependent kinase (CDK), CDK4, which is present in the cytoplasm—allowing the cell to rapidly respond to elevated levels of Cyclin D. Cyclin D and CDK4 interact to form a functional CycD·CDK4 complex that translocates to the nucleus. In the nucleus, the transcription factor E2F1 and its co-activator, Dp, regulate the expression of genes required for the cell cycle to proceed. In the absence of mitogen signalling, E2F1 is however inhibited by the cell cycle inhibitor, RBF, which also recruits chromatin remodelling complexes like HDAC1 to further silence cell cycle gene activity. RBF itself is inhibited by phosphorylation mediated by the CycD·CDK4 complex, which allows expression of the synthetic-phase cylin, Cyclin E. Cyclin E complexes with another CDK, CDK2, to form a CycE·CDK2 complex—that is able to further phosphorylate RBF to inhibit its activity. Following successive rounds of phosphorylation of RBF, E2F1 can transcribe genes at high levels and enable the cell cycle to proceed.

genes responsible for assembling protein complexes involved in specifying the replication origins on the DNA (pre-replication complexes) (COVERLY *et al.,* 2002). The assembly of the pre-replication complexes promotes the G_1 → S-phase transition, and DNA synthesis can begin. The cell enters the S-phase proper as levels of the mitotic cyclin, Cyclin A increase, allowing Cyclin A to complex with CDK2 (COVERLY *et al.*, 2002). High levels of CDK2 activity promotes DNA replication from the assembled pre-replication complexes, and also inhibits the further assembly of these (EDGAR *et al.,* 2014COVERLY *et al.*, 2002). Once DNA synthesis is complete and the cell has transitioned into the second growth phase (G_2), another mitotic cyclin, Cyclin B, promotes the G_2 → M-phase transition to complete the cell cycle.

The main difference between the 'standard' cell cycle and the endocycle is that the ability of endocycling cells to segregate their chromosomes is suppressed through the inhibition of mitotic CDK activity—either via mitotic *cyclin* gene silencing or the selective degradation of M-type cyclins (ZIELKE *et al.,* 2007; MAQBOOL *et al.,* 2010). In the *D. melanogaster* larval salivary gland, the endocycle is regulated by E2F1-dependent Cyclin E activity, which triggers DNA replication via CDK2 to promote the G → S-phase transition (EDGAR *et al.,* 2014). The cycle transitions back to the G-phase in the salivary gland cells as DNA replication promotes the degradation of E2F1 protein via a replication fork-dependent ubiquitin ligase (EDGAR *et al.,* 2014). This causes a decrease in levels of Cyclin E protein. Overall, the speed of the endocycle is determined by factors that regulate the E2F1 translation rate, like the *D. melanogaster* target of rapamycin ("**dTOR**") (EDGAR *et al.,* 2014), which connects the progression of the endocycle to the nutritional state of the larva.

Aims of this Chapter

Some reports claim to have found evidence of cell proliferation in the adult *D. melanogaster* male accessory gland (*e.g.* REZAEI *et al.,* 2015). The general consensus, however, is that the cells within this tissue are not proliferating (WILSON *et al.,* 2016). So why would the regulation of the cell cycle be of interest to my work? As it happens, previous work in our laboratory has shown a role for both E2F1 and RBF in the regulation of secondary cell nuclear growth: demonstrating that overexpression of the E2F1 transcription factor or knock-down of the RBF cell cycle inhibitor are sufficient for large secondary cell nuclei to develop (LEIBLICH, 2015). In addition, these treatments have been shown to increase levels of EcR protein within these cells, and the EcR has previously been shown to promote the growth of the secondary cell nuclei (LEIBLICH, 2015). Therefore, I felt that any work trying to understand the processes of nuclear growth in secondary cells would be incomplete without exploring a role for the cell cycle in this process.

The work presented in this Chapter builds on my results in CHAPTER 4; where I show that Hedgehog signalling regulates the growth of secondary cell nuclei. Previous work in our laboratory has shown that the BMP signalling pathway is an important regulator of this process, and that mating can stimulate it (LEIBLICH *et al.*, 2012). However, exactly how these stimuli promote nuclear growth remains unclear. Therefore, to better understand how secondary cell nuclear growth is regulated, I set myself the following aims:

- To determine how secondary cell nuclear size changes after eclosion, during the period when the male fruit fly reaches full sexual competence, and how the secondary cell nuclei behave thereafter, and in response to mating;

- To determine the molecular mechanisms underlying the growth of the secondary cell nuclei (and if there is any difference between the growth before and after mating), and how these relate to previously characterised signalling inputs (*e.g.* BMP and EcR signalling), and regulators of the cell cycle proper.

In this Chapter, I describe how secondary cell nuclei behave over the first ten days of the male's adult life, and how this behaviour changes in response to mating. In addition, I show that epistasis between BMP signalling and the EcR promote the mating-dependent growth of secondary cell nuclei, and I provide some evidence to suggest that Hedgehog signalling might negatively regulate this process. Overall, I conclude that the mating-dependent growth specifically involves endoreplication, which is controlled by the BMP/EcR signalling axis. I also discuss how the different mechanisms regulating the growth of secondary cell nuclei might control the balance between secretory output and demand.

RESULTS

Epithelial cells in the male accessory gland undergo an early process of nuclear growth

It has previously been shown that the cross-sectional sum-area ratio of secondary cell to main cell nuclei increases over the first days of adult life (LEIBLICH *et al.,* 2012). However, such a ratio only describes the relationship between the two cell types and does not provide measurements of their absolute cross-sectional nuclear area—which might perhaps be a better metric for describing their growth. In addition, previous characterisations of this metric have not used wild-type flies for

their measurements. Therefore, in order to obtain absolute sum-area measurements of physiologically relevant nuclear growth in the accessory gland epithelium of wild-type males, I decided to quantify this using the genotype that has been considered 'wild-type' for the purpose of previous experiments in our laboratory: the mutant line w^{1118} (HAZELRIGG *et al.,* 1984)—from which many transgenic fly stocks are derived.[3] To this purpose, I reared male w^{1118} flies and dissected them at specific time-points (*viz.* day 0, day 1, day 3, day 6, and day 10 post-eclosion).

Using α-ANCE to visualise secondary cells in the accessory gland epithelium, I quantified the sum-area (size) of both secondary cell and main cell nuclei at my chosen time-points. These males were kept away from females (virgin condition).[4] Doing so, I found that secondary cell nuclei start out small, but increase in size already by day 1 (p {d0|d1} < 0.01)—and through to day 3 (p {d0|d3} < 0.0001) (**FIGURE 5. 5. A–C**; **FIGURE 5. 6. A**; **SUPPLEMENTARY TABLE 5. 1.**, *p.* 444). This early (day 1–3) growth is consistent with the growth reported previously by LEIBLICH *et al.* (2012). However, contrary to the continuous growth observed by LEIBLICH *et al.* (2012), where GFP-expressing secondary cell nuclei show a persistent increase in size over time, the early growth I observe in the w^{1118} background did not continue past day three. Instead, in these flies, secondary cell nuclei stopped growing by day six (p {d3|d6} > 0.01). In fact, they actually decreased in size beyond this point (p {d6|d10} < 0.01) (**FIGURE 5. 5. D, F**; **FIGURE 5. 6. A)**. This does, of course, not suggest that any of the measurements made by LEIBLICH *et al.* (2012) are inaccurate. Rather, what it does suggest is that the

[3] Including several of the lines used by LEIBLICH *et al.* (2012).

[4] LEIBLICH *et al.* (2012) show that secondary cell nuclei grow in response to mating.

genetic background of the flies is likely to have an effect on the behaviour of the secondary cell nuclei.

LEIBLICH *et al.* (2012) reported that main cells do not show any nuclear growth over the first ten days of adult life (when assayed as a secondary cell to main cell nuclear size ratios). To get a better idea of how the absolute sum area of main cell nuclei changes over the first ten days of a male's adult life, I decided to quantify this. In doing so, I found that main cell nuclei start out at similar size to those of the neighbouring secondary cells (**FIGURE 5. 5. A**; **FIGURE 5. 6.**; **SUPPLEMENTARY TABLE 5. 2.**, *p.* 444). Similar to secondary cells, main cell nuclei also increase in size over the first days after eclosion (p {d0|d3} < 0.0001) (**FIGURE 5. 5. B–C**; **FIGURE 5. 6. B**). However, whereas secondary cell nuclei decrease in size between day three and day six, main cell nuclei do not show this decrease, but rather appear to stay the same size over time (p {d3|d6} > 0.01; p {d6|d10} > 0.01) (**FIGURE 5. 5. D, F**; **FIGURE 5. 6. B**).

Mating has cell type-specific effects on the size of accessory gland epithelial cells and their nuclei

It has previously been reported that secondary cell nuclei grow in response to mating (LEIBLICH *et al.*, 2012). To confirm that this is true also in the w^{1118} background, I devised a multiple-mating protocol to reliably measure and assess mating-dependent secondary cell nuclear growth to answer the questions:

1) How much growth does each mating contribute to the nuclear size of secondary cells?, and,
2) How many mating events are required before a significant increase in secondary cell nuclear size can be observed?

FIGURE 5. 5. | THE SIZE OF SECONDARY CELLS AND THEIR NUCLEI CHANGE OVER TIME AND AFTER MATING.

A: Secondary cell nuclei (marked with pink arrows) are located extremely basally in the gland at eclosion, and appear the same size as main cell nuclei (yellow arrow). Main cell nuclei are stacked one on top of the other in the epithelium at this time-point, so only one main cell nucleus is highlighted in this panel.

B: The secondary cell nuclei (pink arrows) have not grown significantly by day one after eclosion.

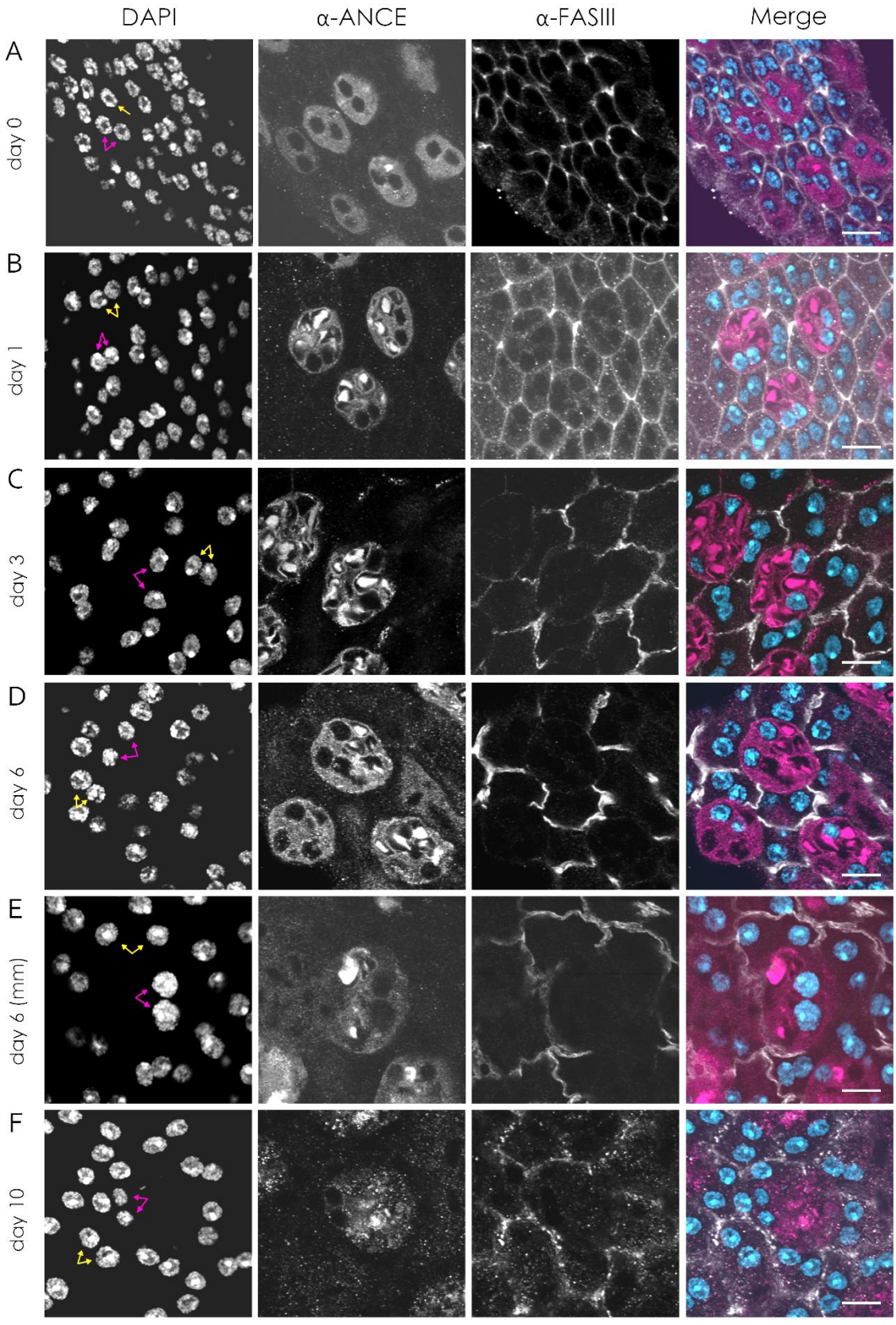
C: Secondary cell nuclei are somewhat larger by day three after eclosion, and the epithelium appears more stretched.

D: Secondary cell nuclei as they appear by day six, in the absence of mating.

E: Secondary cell nuclei appear enlarged, compared to those of neighbouring main cells, when the male has mated with females.

F: Secondary cell nuclei appear smaller than those of the surrounding main cells by day ten, if the male remains virgin.

In all panels, secondary cells are stained with α-ANCE, and α-FASIII (white) labels main cell junctions. DAPI stains DNA (cyan). Scale bar in all panels corresponds to 10 μm.


DAPI
α-ANCE
α-FASIII
Merge
A
day 0
B
day 1
C
day 3
D
day 6
E
day 6 (mm)
F
day 10

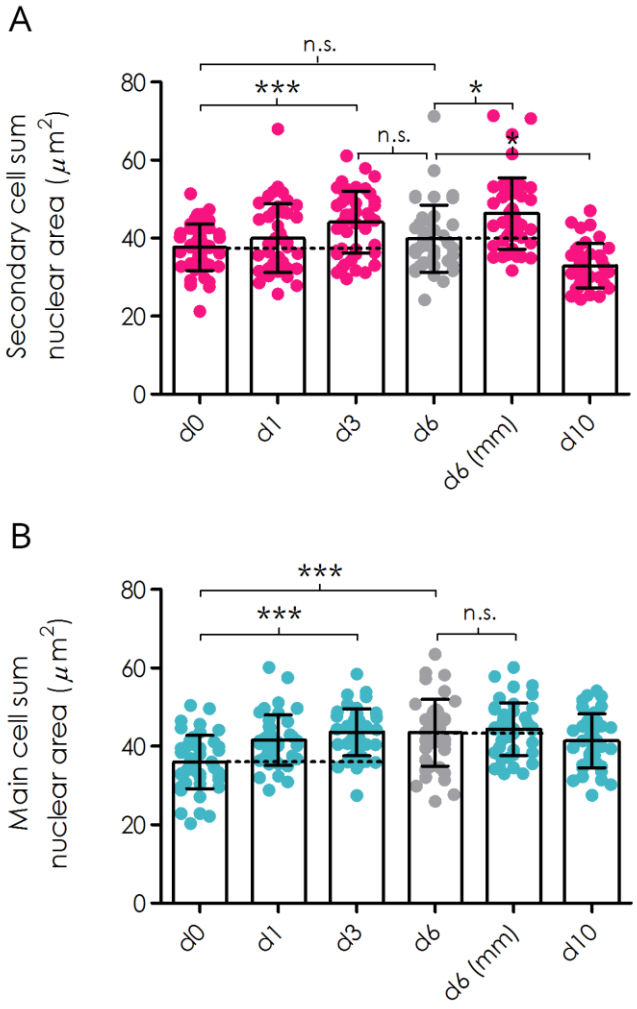


FIGURE 5. 6. | NUCLEI IN THE MALE ACCESSORY GLAND EPITHELIUM GROW OVER TIME AND SHOW CELL TYPE-SPECIFIC NUCLEAR GROWTH IN RESPONSE TO MATING.

Both accessory gland epithelial cell types—secondary cells and main cells—show age-dependent nuclear growth as the gland matures.

A: Scatterplot bar chart showing how the nuclear size (cross-sectional sum nuclear area) of secondary cells change over time, and after mating.

B: Scatterplot bar chart showing how the nuclear size of main cells change over time, and after mating.

In the scatterplots, each dot represents the value of an individual secondary cell, with three cells quantified per male (see **SUPPLEMENTARY TABLES 5. 1–5. 2.**, *p.* 444). Error bars show the standard deviation within the sample. In both panels, the number of stars represents the *p*-value obtained by one-way ANOVA with a Bonferroni correction for multiple comparisons (all columns): * = $p < 0.01$; *** = $p < 0.0001$; n.s. = $p > 0.01$.

LEIBLICH *et al.* (2012) describe a multiple-mating protocol, and I modified this protocol further: allowing male flies (from eclosion) access to either 3, 5, or 7 virgin females, over a duration of 6 days at the required temperature (in this case 29 °C). Quantifying secondary cell nuclear size under these conditions (and comparing these to the size observed in the secondary cells of virgin males) I found that increasing the number of matings also increases the size of the secondary cell nuclei: whereas the sum area of secondary cell nuclei did not change significantly when males were kept in the presence of three females, males kept together with five females showed a slight increase in the average secondary cell nuclear area—and this increase which was further accentuated by keeping the male together with seven females over the same time period (**FIGURE 5. 7.**). Altogether, these results show that secondary cell nuclei grow in response to mating in wild-type flies, consistent with previous reports in other genetic backgrounds. Interestingly, it seems as if secondary cell nuclei grow similar amounts with every two additional females added (**SUPPLEMENTARY TABLE 5. 3.,** *p.* 445). A one-way ANOVA trend-test detects a positive linear trend between the secondary cell sum nuclear area and the number of females given to each male ($p = 0.0005$). Since a significant difference in the size of the secondary cell nuclei was detected in males allowed access to seven females *versus* none, subsequent multiple mating experiments in this Dissertation will use a ratio of seven females to each male. Importantly, no size increase after mating was detected in main cell nuclei ($p\ \{♂_{0\ \text{females}} \mid ♂_{7\ \text{females}}\} > 0.1$) (**FIGURE 5. 5. B; SUPPLEMENTARY TABLE 5. 2.,** *p.* 444). This is consistent with previous reports (LEIBLICH *et al.,* 2012).

Quantifying how secondary cell nuclear size changes over time, I noticed that the secondary cells themselves seem to increase in size during this period (compare panels **A–B** with panels **C–D** in **FIGURE 5. 5.**). Hypothesising that the secondary cell

nuclear size correlates with the size of the secondary cell itself—as has been reported in other systems (*e.g.* SCHRADER & LEUCHTENBERGER, 1950; NEUMANN & NURSE, 2007)—I used confocal *z*-stacks of the accessory gland epithelium to approximate secondary cell volume. Doing so, I found that the approximate volume of the secondary cells remains relatively constant even as the gland matures over time (days 0–10) (p {d0|d3/d6} > 0.1) (**FIGURE 5. 8.**; **SUPPLEMENTARY TABLE 5. 4.**, *p.* 445). An increase was however evident when comparing the approximate volume of secondary cells in six-day old virgin males with the volume of age-matched males allowed access to multiple females (p {$\male_{virgin}$|$\male_{mated}$} < 0.0001). This suggests that mating might cause secondary cells to increase in volume—just as the size of the secondary cell nuclei grows. Altogether, this suggests that both secondary cell nuclear sum area and cell volume increase in response to mating in wild-type flies. Importantly, these data are also consistent with the lack of any apparent decrease in the size of the secondary cells (albeit measured as maximal cross-sectional area rather than approximate volume) when the nuclei decrease in size (as shown in **FIGURE 4. 22**. (*p.* 186) and **FIGURE 4. 23.** (*p.* 188).

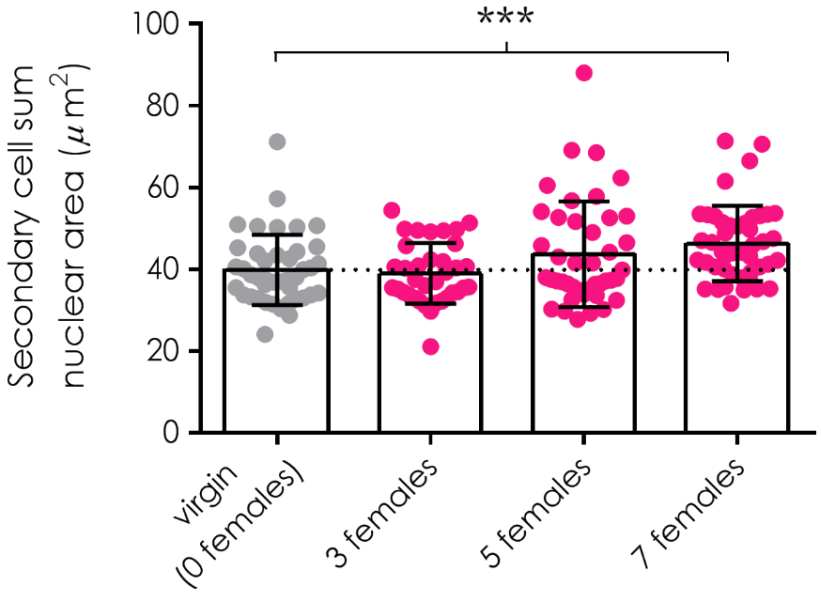


FIGURE 5. 7. | THE NUMBER OF MATING EVENTS HAS AN ADDITIVE EFFECT ON SECONDARY CELL NUCLEAR GROWTH.

Secondary cell nuclei grow in response to mating, and the number of matings affect the final size of secondary cell nuclei. In this scatterplot, each dot represents the value of an individual secondary cell, with three cells quantified per male (see **SUPPLEMENTARY TABLE 5. 3.**, *p.* 445). Error bars show the standard deviation within the sample. The number of stars represents the *p*-value obtained by one-way ANOVA (test for trend): *** $p = 0.0005$.

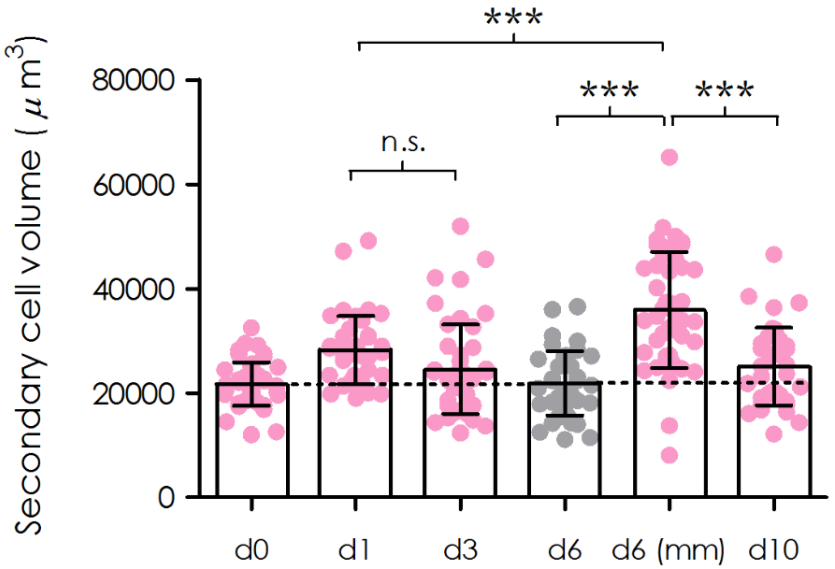


FIGURE 5. 8. | SECONDARY CELL VOLUME INCREASES IN RESPONSE TO MATING.

Like secondary cell nuclei, the secondary cells themselves grow in response to mating. In this scatterplot, each dot represents the value of an individual secondary cell, with three cells quantified per male (see **SUPPLEMENTARY TABLE 5. 4.**, *p.* 445). Error bars show the standard deviation within the sample. The number of stars represents the *p*-value obtained by one-way ANOVA with a Tukey correction for multiple comparisons (all columns): *** = $p < 0.001$.

Mating promotes BMP signalling activation in secondary cells to drive nuclear growth

Having established that mating causes secondary cell nuclei to grow in wild-type flies, I wanted to better understand *how* this mating-dependent phenotype develops. Previous work in our laboratory has shown that either mating or increased BMP signalling activity is sufficient to drive secondary cell nuclear growth (LEIBLICH *et al.,* 2012). Does this suggest that BMP signalling might be activated upon mating, and that this is the mechanism underlying the mating-dependent nuclear growth?

Working together with Siamak Redhai (a previous *D. Phil.* student in our laboratory), we sought to answer this question, and some of the results described in this section were published in REDHAI *et al.* (2016). Together (and as described in CHAPTER 3, *pp.* 84–87), Siamak and I have already shown that the BMP-dependent, phosphorylated, transcription factor, pMad, can undergo nuclear translocation in some secondary cells in the accessory glands of virgin males. This provides evidence that BMP signalling is active in these cells (see **FIGURE 3. 6.**, *p.* 87; and REDHAI *et al.*, 2016). However, we wondered, does *mating* promote BMP signalling activation in these cells? Again working with antiserum recognising phosphorylated Mad protein (pMad), Siamak and I compared the proportion of secondary cells positive for nuclear pMad in the glands of virgin and just-mated male flies (**FIGURE 5. 9.**). Quantifying these numbers (**SUPPLEMENTARY TABLE 5. 5.**, *p.* 445), we found that mating *does* cause an increase in the proportion of secondary cells positive for pMad antibody staining ($p\ \{\male_{\text{virgin}} | \male_{\text{mated}}\} < 0.001$) (REDHAI *et al.,* 2016). This suggests that BMP signalling is indeed activated by mating.

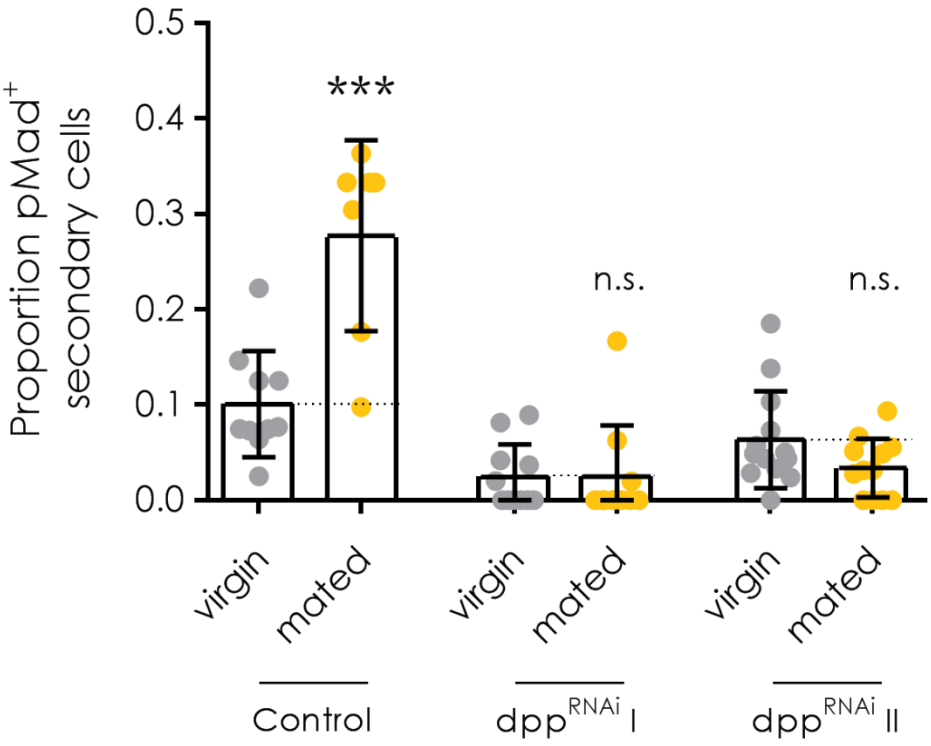


FIGURE 5. 9. | MATING ACTIVATES DPP-DEPENDENT BMP SIGNALLING IN SECONDARY CELLS

A scatterplot chart showing how the proportion of pMad+ secondary cells increases after mating in control glands, but not in glands where the secondary cells express either of two different hairpins targeting *dpp* for six days after eclosion.

The number of stars on the chart represent the *p*-value obtained by one-way ANOVA with Tukey's correction for multiple comparisons (all columns): *** = $p < 0.001$; 'n.s.' denotes $p > 0.1$. Each dot represents the value of an individual male (see **SUPPLEMENTARY TABLE 5. 5.,** *p.* 445). Error bars show the standard deviation within the sample. The work shown in this panel was carried out in collaboration with Siamak Redhai, and was published in REDHAI *et al.* (2016).

N. B. The virgin control in this chart is the same as shown for the day-six virgin in **FIGURE 3. 6.** (*p.* 87), since the experiments were performed at the same time. This has been taken into account for the statistical analysis.

Having already shown that Dpp is contained within the secondary cell secretory granules and that it is released into the seminal fluid (REDHAI *et al.,* 2016), Redhai suggested that we work together to determine if Dpp is the molecule driving the mating-dependent activation of BMP signalling in the secondary cells. To this effect, we used *esg F/O*^{ts} flies to express two different inverted repeats targeting *dpp* (*dpp*^{RNAi} I and II; NI *et al.*, 2009; 2008) in secondary cells (*esg F/O*^{ts} > *dpp*^{RNAi}). A small proportion of secondary cells are positive for nuclear pMad in the glands of virgin males when expressing a hairpin construct targeting *dpp*. However, this proportion does not increase after mating (p {♂$_{virgin}$|♂$_{mated}$} > 0.1) (**FIGURE 5. 9.**; **SUPPLEMENTARY TABLE 5. 5.**, *p.* 442). This suggests that *dpp* is needed in the secondary cells for the mating-dependent BMP signalling activation to occur (REDHAI *et al.,* 2016).

In addition to knocking down the BMP signalling ligand, *dpp*, BMP signalling can also be reduced through overexpression of the BMP signalling inhibitor, *Dad* (TSUNEIZUMI *et al.,* 1997). Using *esg F/O*^{ts} to express *UAS-Dad* in secondary cells (*esg F/O*^{ts} > *Dad*), Redhai and I found that the pMad nuclear translocation was even more dramatically reduced after mating: with no secondary cells showing evidence of nuclear pMad (p {control|*Dad*} < 0.001) (**FIGURE 5. 10.**; **SUPPLEMENTARY TABLE 5. 5.**, *p.* 446) (REDHAI *et al.,* 2016). In addition, when I quantified the size of these secondary cell nuclei, I found that they had failed to grow (**FIGURE 5. 11. C–E**). In fact, when *Dad* is overexpressed, mating seems to cause secondary cell nuclei to actually *decrease* in size (p {♂$_{virgin}$|♂$_{mated}$} < 0.0001) (**SUPPLEMENTARY TABLE 5. 6.**, *p.* 446). Following up on this work, Redhai showed that pMad nuclear translocation is promoted in the secondary cells of virgin flies after expression of the activated BMP signalling receptor, Tkv^{Q253D} (REDHAI *et al.,* 2016). Taken together, these

results suggest that mating activates BMP signalling in secondary cells, and that this BMP signalling activation seems to be required for the secondary cell mating-dependent nuclear growth.

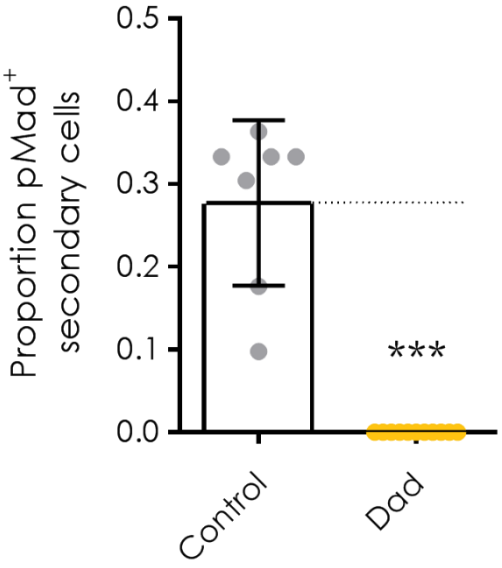


FIGURE 5. 10. | MATING ACTIVATES BMP SIGNALLING IN SECONDARY CELLS

A scatterplot chart showing that the proportion of pMad$^+$ secondary cells is much lower after mating in glands where the secondary cells overexpress the BMP signalling inhibitor, *Dad*.

The number of stars on the chart represents the *p*-value obtained by one-way ANOVA with Tukey's correction for multiple comparisons (all columns): *** = $p < 0.001$; 'n.s.' denotes $p > 0.1$. Each dot represents the value of an individual male, (see **SUPPLEMENTARY TABLE 5. 5.,** *p.* 445), and error bars show the standard deviation within the sample.

The work shown in this panel was carried out in collaboration with S. Redhai, and was published in REDHAI *et al.* (2016).

FIGURE 5. 11. | SECONDARY CELL NUCLEI FAIL TO GROW AFTER MATING IN THE ABSENCE OF BMP SIGNALLING.

BMP signalling is required for the mating-dependent growth of secondary cell nuclei.

A: Secondary cell nuclei (marked with pink arrows) are similar in size to those of the surrounding main cells (marked with yellow arrows) in the accessory gland of a virgin control male.

B: After mating, the secondary cell nuclei have increased in size, compared to the surrounding main cell nuclei.

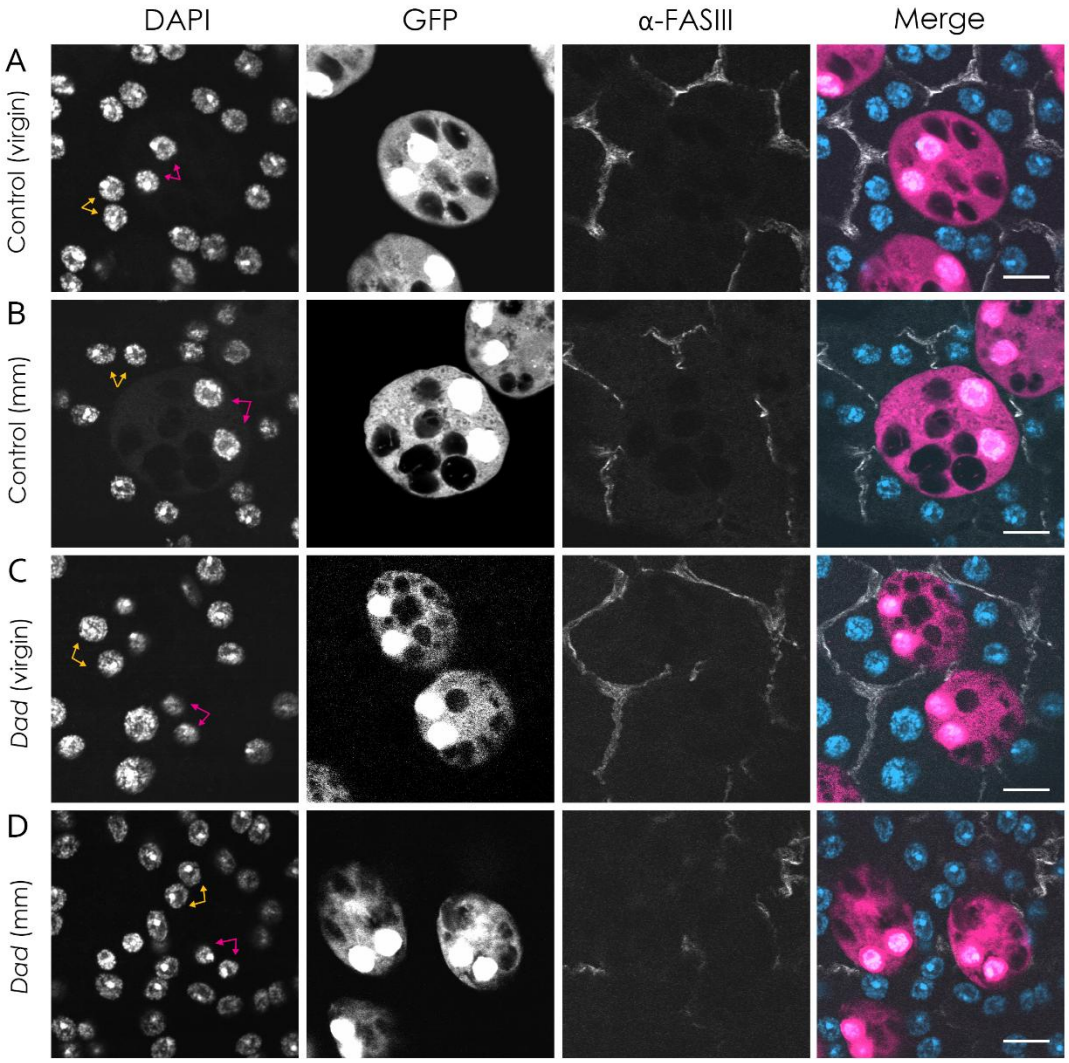
C: In secondary cells overexpressing the BMP signalling inhibitor, *Dad*, the secondary cell nuclei are smaller than those of the surrounding main cells in virgin males.

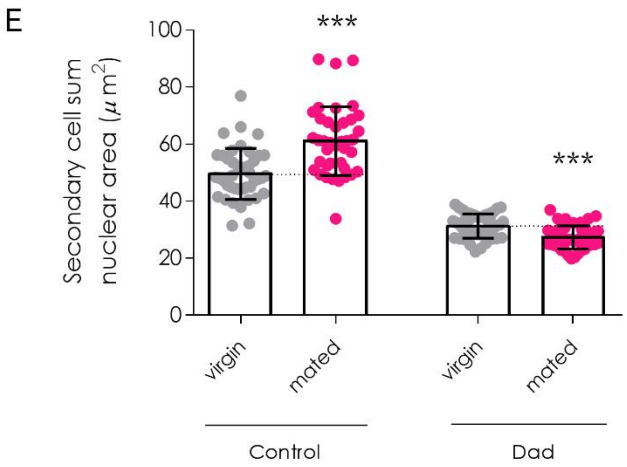
D: When BMP signalling is inhibited, through *Dad* overexpression, secondary cell nuclei do not grow after mating.

E: Quantifying the nuclear size of secondary cells before (virgin) and after mating (mated) shows that *Dad* overexpression prevents mating-dependent nuclear growth. Each dot in the chart corresponds to data obtained from one individual cell, with three cells quantified per male (see **SUPPLEMENTARY TABLE 5. 6.**, *p.* 446).

Scale bars in panels **A–D** correspond to 10 μm and α-FASIII labels main cell junctions (white). Secondary cells are labelled with GFP (magenta), and DAPI labels DNA (cyan).

In panel **E**, each dot represents the value of an individual secondary cell, and error bars show the standard deviation within the sample. The number of stars represent the *p*-value obtained by a two-tailed Student's *t*-test: *** = $p < 0.0001$.


DAPI
GFP
α-FASIII
Merge
A
Control (virgin)
B
Control (mm)
C
Dad (virgin)
D
Dad (mm)


E
Secondary cell sum nuclear area (μm²)
100
80
60
40
20
0
***
***
virgin
mated
virgin
mated
Control
Dad

Multiple mating events act synergistically with activated BMP signalling and EcR activity to drive secondary cell nuclear growth

Having established that BMP signalling is activated in response to mating, I wondered: is BMP signalling the main signalling pathway that is involved in regulating mating-dependent nuclear growth in secondary cells? To investigate, I wanted to see what would happen to the size of secondary cell nuclei after mating if BMP signalling was already active. To this effect, I used *esg F/O*^{ts} to drive the expression of *UAS-tkv*^{Q253D} in secondary cells (*esg F/O*^{ts} > *UAS-tkv*^{Q253D}), and then mated some of the males. Quantifying secondary cell nuclear size after this treatment, I found that although *tkv*^{Q253D} nuclei are larger than control nuclei in virgins, they grew still further after mating ($p\ \{♂_{virgin} | ♂_{mated}\} < 0.0001$) (**FIGURE 5. 12.**; **SUPPLEMENTARY TABLE 5. 7.**, *p.* 446). This suggests that the nuclear growth resulting from BMP signalling activation can be further supplemented through mating, implying that Tkv^{Q253D} either does not 'max-out' BMP signalling, or that other signalling inputs also contribute.

BMP signalling interacts with the steroid hormone receptor, EcR, in secondary cells; stabilising the receptor and allowing EcR protein levels to accumulate, resulting in an increase in secondary cell nuclear size (LEIBLICH, 2015). If BMP signalling can be further supplemented by mating, I wondered: does the EcR contribute to this process? To figure this out, I turned to the two EcR constructs which we came across back in CHAPTER 3 (*p.* 79): the inverted repeat that targets the *EcR* transcript, and the synthetic EcR isoform, EcR-C. Again using *esg F/O*^{ts} to drive the expression of these (either alone, or in combination with *tkv*^{Q253D}), and by mating the flies, I hoped to dissect out the role of the EcR in the BMP-dependent nuclear

growth after mating. What I found is that secondary cell nuclei grow after mating when expressing either the EcR-C alone (p {♂virgin | ♂mated} < 0.0001), or in combination with tkv^{Q253D} (p {♂virgin | ♂mated} < 0.0001) (**FIGURE 5. 13**; **FIGURE 5. 14.; SUPPLEMENTARY TABLE 5. 8.**, *p.* 446). However, the mating-dependent growth seems to be reduced when *EcR* is knocked-down (p {♂virgin | ♂mated} = 0.008), or when co-expressed with tkv^{Q253D} (p = 0.01)—although these effects are much weaker (**FIGURE 5. 15**; **FIGURE 5. 16.**; **SUPPLEMENTARY TABLE 5. 9.**, *p.* 447). Altogether, these results imply that although BMP signalling can be further supplemented by the EcR to drive secondary cell nuclear growth after mating, this growth ultimately seems to depend on the EcR. This suggests that BMP signalling might mediate the mating-dependent nuclear growth via the EcR.

Hedgehog signalling does not appear to promote mating-dependent nuclear growth

Having shown that mating-dependent nuclear growth further supplements the BMP/EcR axis in secondary cells, I still wondered: are other signalling pathways involved in this process? In the previous Chapter, I showed that Hedgehog not only regulates secondary cell nuclear growth (**FIGURES 4. 22.–4. 25.**, *pp.*186–191), but also that a GFP-tagged form of the molecule seems to be released into the accessory gland lumen (**FIGURE 4. 9.**, *p.* 163), where it has the potential to signal back to the secondary cell. Incidentally, Siamak found that the BMP pathway ligand, Dpp, behaves similarly: being carried in secondary cell secretory granules and being released into the seminal fluid, where it can act in an autocrine fashion to regulate secondary cell nuclear size (REDHAI *et al.*, 2016). Based on

FIGURE 5. 12. | SECONDARY CELL NUCLEI GROW AFTER MATING—EVEN WHEN BMP SIGNALLING IS ACTIVE.

Nuclear growth resulting from activated BMP signalling can be further supplemented by mating.

A: Secondary cell nuclei (marked with pink arrows) are similar in size to those of the surrounding main cells (marked with yellow arrows) in the accessory gland of a virgin control male.

B: After mating, the secondary cell nuclei have increased in size, compared to the surrounding main cell nuclei.

C: In secondary cells expressing the activated BMP signalling receptor, tkv^{Q253D}, the secondary cell nuclei are larger than those of the surrounding main cells.

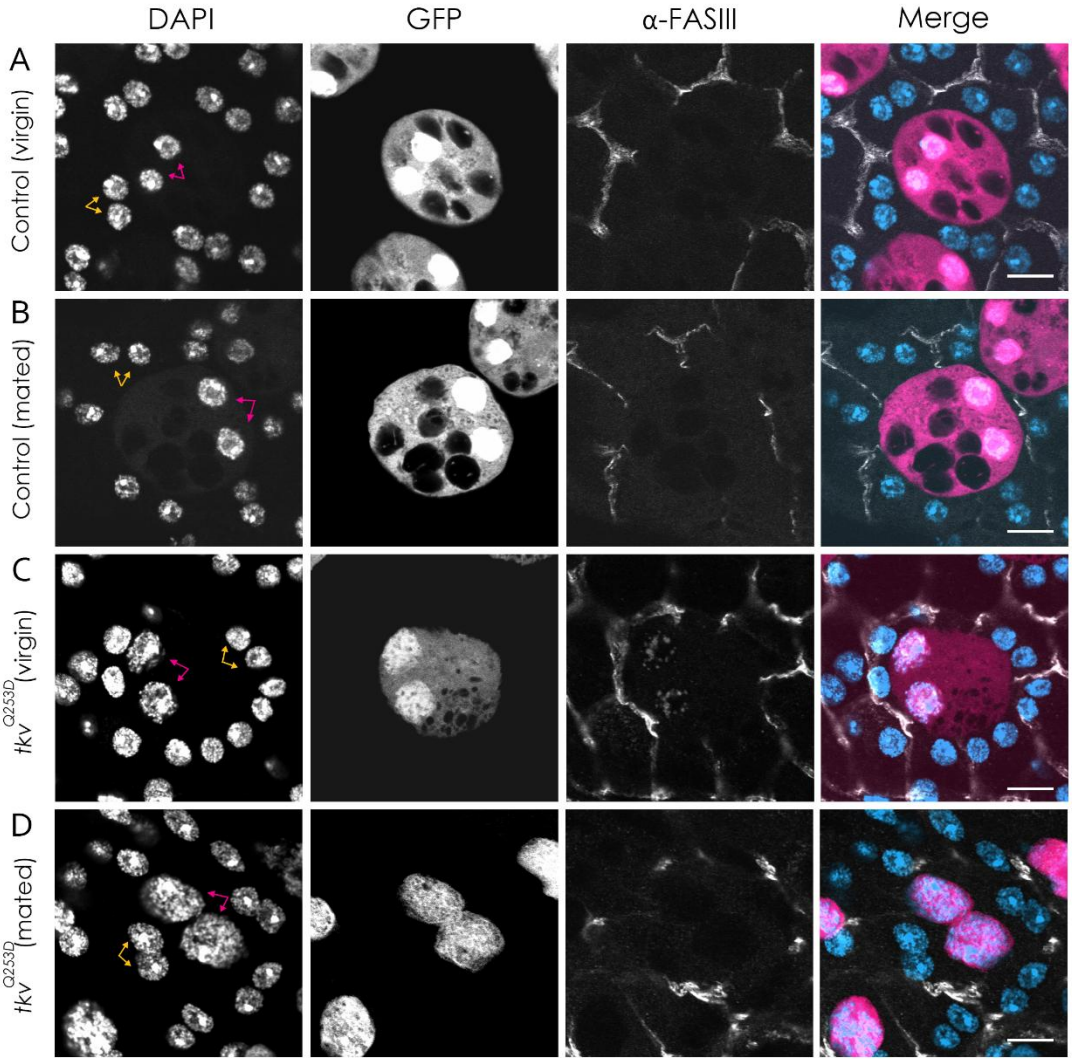
D: When BMP signalling is activated, via tkv^{Q253D} expression, secondary cell nuclei grow after mating.

E: Quantifying the nuclear size of secondary cells before (virgin) and after mating (mated) shows that mating drives further nuclear growth in secondary cells expressing tkv^{Q253D}. Each dot in the chart corresponds to data obtained from one individual cell, with three cells quantified per male (see **SUPPLEMENTARY TABLE 5. 7.**, *p.* 446).

Scale bars in panels **A–D** correspond to 10 μm and α-FASIII labels main cell junctions (white). Secondary cells are labelled with GFP (magenta), and DAPI stains DNA (cyan).

In panel **E**, each dot represents the value of an individual secondary cell, and error bars show the standard deviation within the sample. The number of stars represent the *p*-value obtained by a two-tailed Student's *t*-test: *** = $p < 0.0001$.

N.B. The controls in panels **A–B** and **E** are the same as shown in **FIGURE 5. 11.**, since the experiments were performed at the same time. This has not affected the statistical analysis.


DAPI
GFP
α-FASIII
Merge
A
Control (virgin)
B
Control (mated)
C
tkvQ253D (virgin)
D
tkvQ253D (mated)

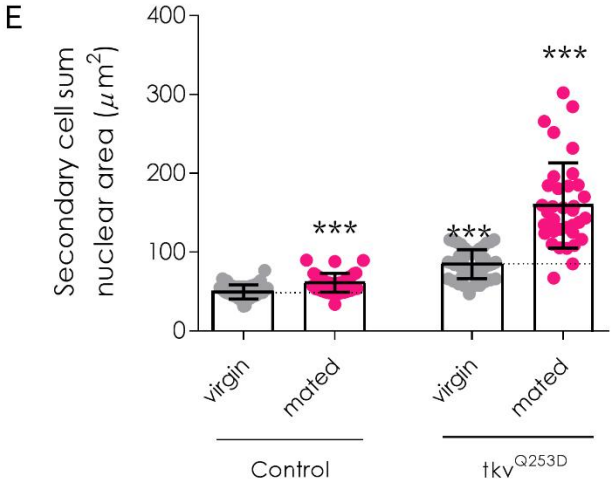

E
Secondary cell sum nuclear area (μm2)
400
300
200
100
0
***
***
***
virgin
mated
virgin
mated
Control
tkvQ253D

FIGURE 5. 13. | SECONDARY CELL NUCLEI GROW AFTER MATING—EVEN WHEN THE BMP/ECR SIGNALLING AXIS IS ACTIVE.

Mating can further supplement the BMP- and EcR-dependent growth of secondary cell nuclei.

A: Secondary cell nuclei (marked with pink arrows) are similar in size to those of the surrounding main cells (marked with yellow arrows) in the accessory gland of a virgin control male.

B: After mating, the secondary cell nuclei have increased in size, compared to the surrounding main cell nuclei.

C: In secondary cells overexpressing the synthetic EcR isoform, EcR-C, the secondary cell nuclei are larger than those of the surrounding main cells.

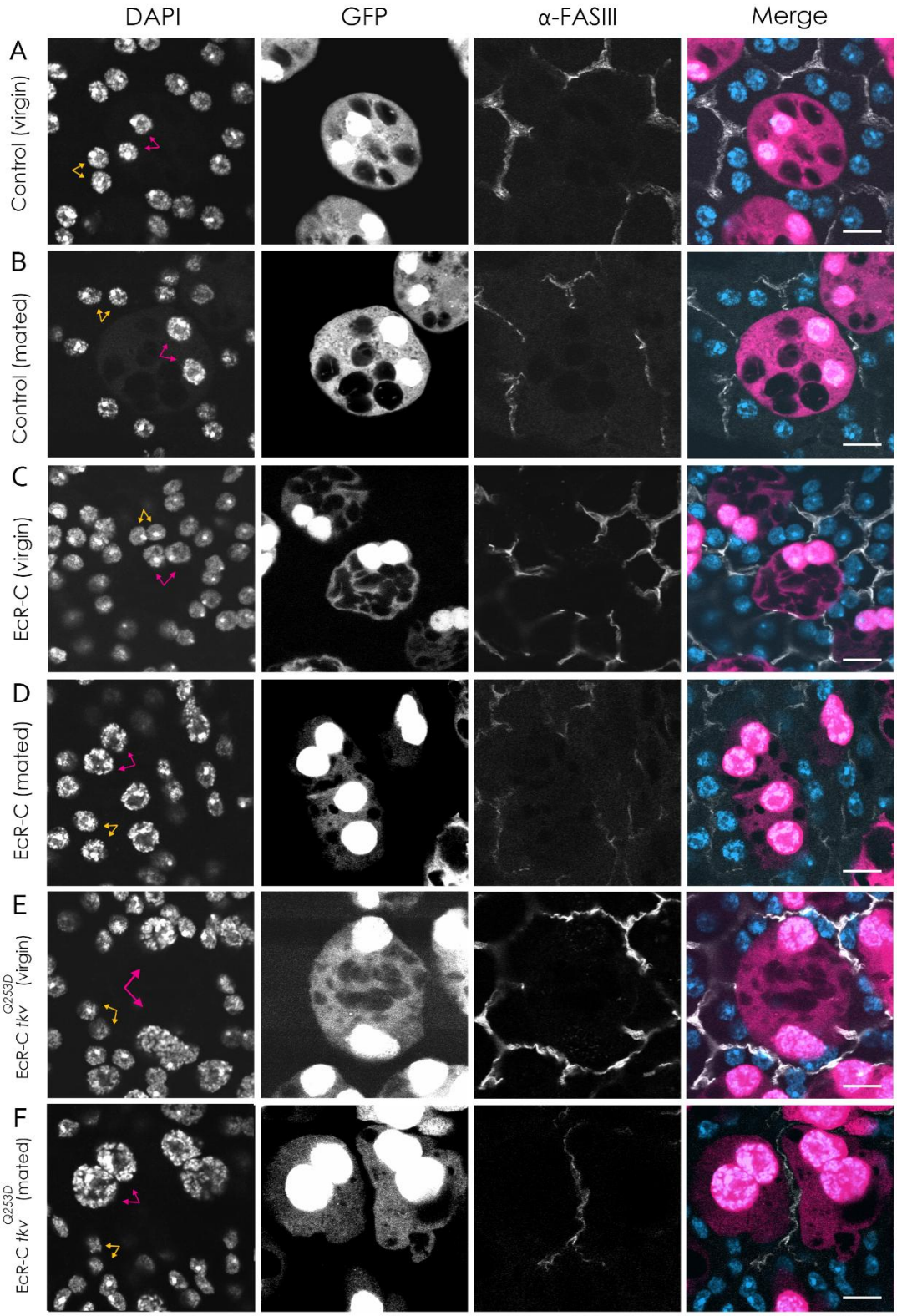
D: When the EcR-C is expressed, secondary cell nuclei grow after mating.

E: Secondary cells overexpressing the EcR-C in combination with *tkv*^{Q253D} have much larger nuclei than those of the surrounding main cells.

F: When overexpressing EcR-C and *tkv*^{Q253D}, mating can further supplement the secondary cell nuclear growth.

Scale bars in panels **A–F** correspond to 10 μm and α-FASIII labels main cell junctions (white). Secondary cells are labelled with GFP (magenta), and DAPI stains DNA (cyan).

N.B. The controls in panels **A–B** and **E** are the same as shown in **FIGURE 5. 11.**, since the experiments were performed at the same time.


DAPI
GFP
α-FASIII
Merge
A
Control (virgin)
B
Control (mated)
C
EcR-C (virgin)
D
EcR-C (mated)
E
EcR-C tkv Q253D (virgin)
F
EcR-C tkv Q253D (mated)

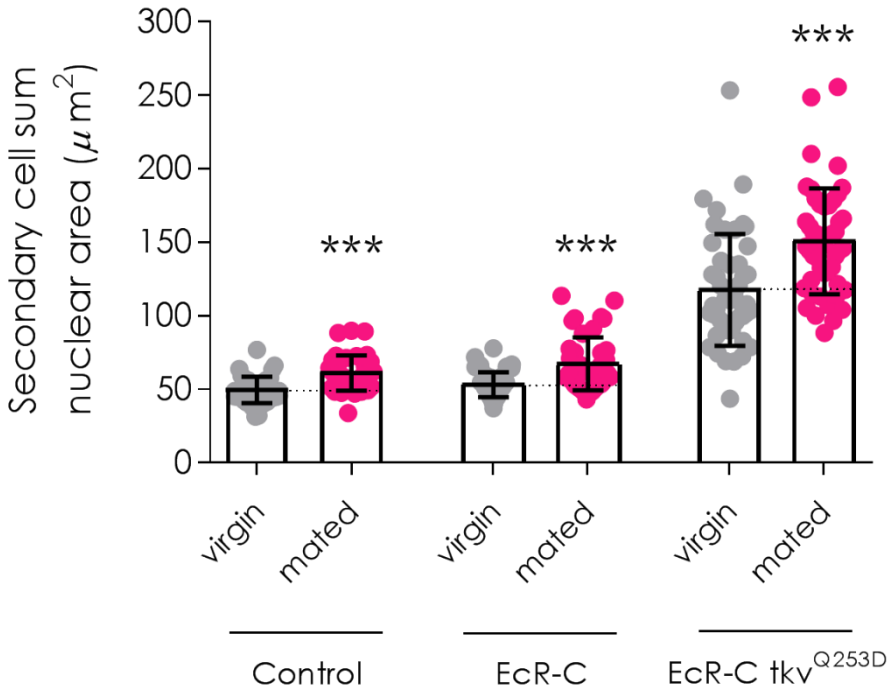


FIGURE 5. 14. | SECONDARY CELL NUCLEI GROW AFTER MATING—EVEN WHEN THE BMP/ECR SIGNALLING AXIS IS ACTIVATED.

Quantifying the nuclear size of secondary cells before (virgin) and after mating (mated) shows that growth resulting from expression of the EcR-C, either alone or in combination with *tkv*^{Q253D}, can be further supplemented by mating.

Each dot on the plot represents the value of an individual secondary cell, with three cells quantified per male (see **SUPPLEMENTARY TABLE 5. 8.**, *p.* 446). Error bars show the standard deviation within the sample. The number of stars represent the *p*-value obtained by a two-tailed Student's *t*-test: *** = $p < 0.0001$.

N.B. The controls are the same as shown in **FIGURE 5. 11.**, since the experiments were performed at the same time. This has not affected the statistical analysis.

these observations, I became curious if Hedgehog signalling might participate in mating-dependent secondary cell nuclear growth?

To investigate this, I used *esg F/O*^{ts} to express either the most effective inverted repeat targeting *hedgehog* (*hh*^{RNAi}), or the *Ci::VP16* construct, and then mating the males. Interestingly, I found that in glands expressing *esg F/O*^{ts} > *hh*^{RNAi}, secondary cell nuclei still grow after mating (p {♂$_{virgin}$|♂$_{mated}$} < 0.001) (**FIGURE 5. 17. C–D**), and reach a similar final size as that normally observed in controls (p {control|*hh*^{RNAi}} > 0.1) (**FIGURE 5. 18.**; **SUPPLEMENTARY TABLE 5. 10.**, *p.* 447). This is the opposite effect to when the construct is expressed in virgin males, and suggests that Hedgehog signalling might not actually be required for the mating-dependent growth to occur. However, when *esg F/O*^{ts} drove expression of *Ci::VP16*, although the secondary cell nuclei were larger than those of controls in virgin males, they fail to grow further after mating (p {♂$_{virgin}$|♂$_{mated}$} > 0.1) (**FIGURE 5. 17. E–F; FIGURE 5. 18.**; **SUPPLEMENTARY TABLE 5. 10.**, *p.* 447). This suggests that while overexpression of Hedgehog target genes (via Ci::VP16) does not promote mating-dependent nuclear growth, the loss of *hedgehog* also does not inhibit it. This implies that Hedgehog signalling either does not participate in the mating-dependent growth or that it might be a negative regulator of this growth in secondary cells.

FIGURE 5. 15. | MATING-DEPENDENT SECONDARY CELL NUCLEAR GROWTH REQUIRES THE EcR.

A: Secondary cell nuclei (marked with pink arrows) are similar in size to those of the surrounding main cells (marked with yellow arrows) in the accessory gland of a virgin control male.

B: After mating, the secondary cell nuclei have increased in size, compared to the surrounding main cell nuclei.

C: In secondary cells expressing an inverted repeat targeting *Ec*R, $Ec R^{RNAi}$, the secondary cell nuclei are smaller than those of the surrounding main cells.

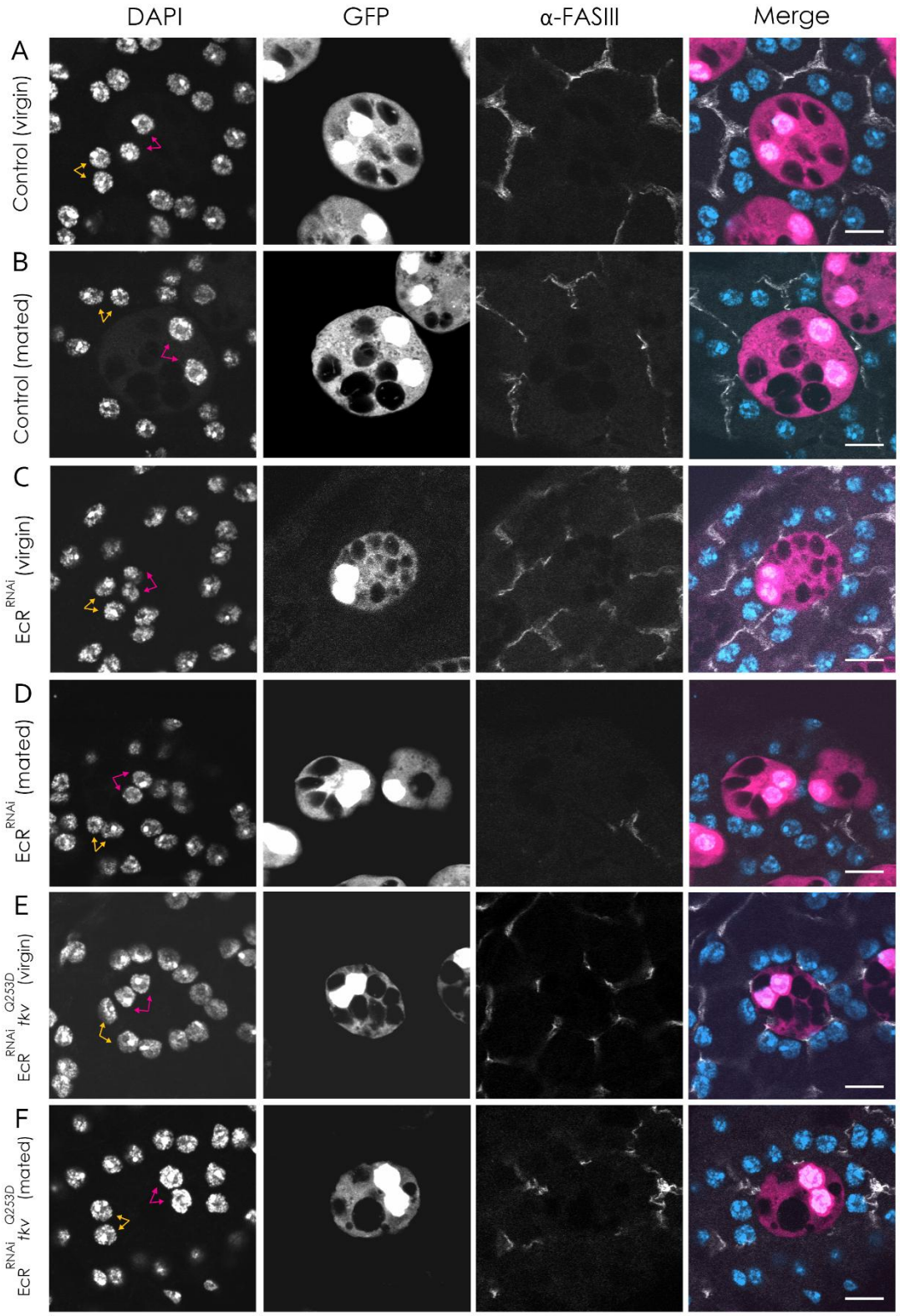
D: When *Ec*R is knocked down, secondary cell nuclei show limited growth after mating.

E: Expressing the *Ec*R-targeting hairpin in combination with tkv^{Q253D} does not promote secondary cell nuclear growth.

F: Secondary cells expressing both the *Ec*R inverted repeat and the activated BMP receptor, tkv^{Q253D}, show limited nuclear growth in response to mating.

Scale bars in panels **A–F** correspond to 10 μm and α-FASIII labels main cell junctions (white). Secondary cells are labelled with GFP (magenta), and DAPI stains DNA (cyan).

N.B. The controls in panels **A–B** are the same as shown in **FIGURE 5. 11.**. since the experiments were performed at the same time. This has not affected the statistical analysis.


DAPI
GFP
α-FASIII
Merge
A
Control (virgin)
B
Control (mated)
C
EcR RNAi (virgin)
D
EcR RNAi (mated)
E
EcR RNAi tkv Q253D (virgin)
F
EcR RNAi tkv Q253D (mated)

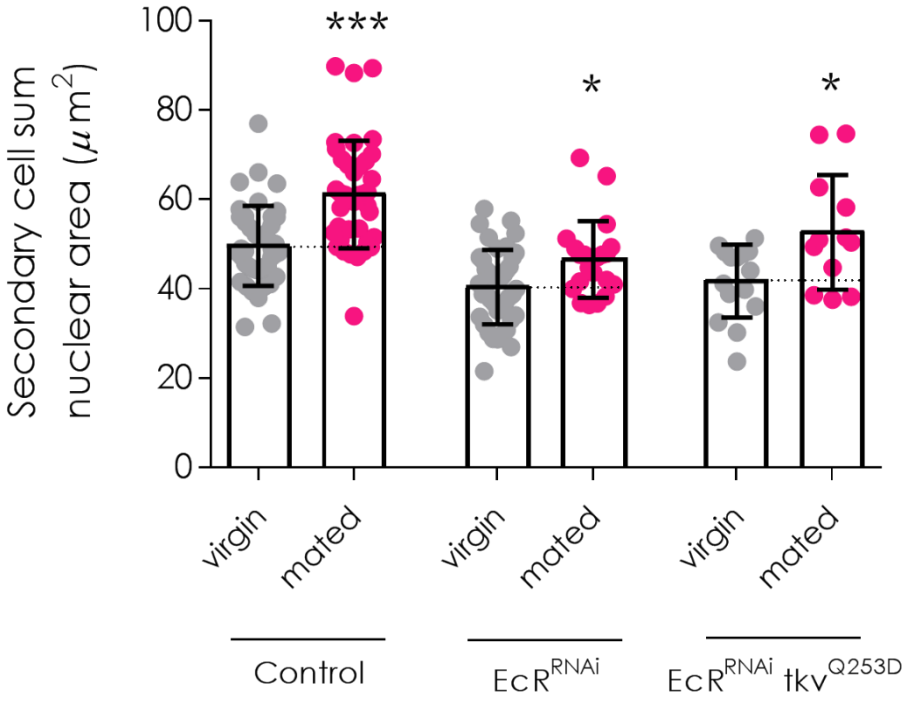


FIGURE 5. 16. | KNOCKING-DOWN THE EcR SEEMS TO RESTRICT MATING-DEPENDENT SECONDARY CELL NUCLEAR GROWTH.

The EcR seems to be required for the mating-dependent nuclear growth in secondary cells, since its loss via RNAi-mediated knockdown reduces secondary cell nuclear growth after mating—even when co-expressed with the activated BMP signalling receptor, *tkv*^{Q253D}.

Each dot represents the value of an individual secondary cell, with three cells quantified per male (see **SUPPLEMENTARY TABLE 5. 9.**, *p.* 447). Error bars show the standard deviation within the sample. The number of stars represent the *p*-value obtained by a two-tailed Student's *t*-test: * = $p < 0.01$.

N.B. The controls are the same as shown in **FIGURE 5. 11.**. since the experiments were performed at the same time. This has not affected the statistical analysis.

Cyclins D and E participate in the regulation of mating-dependent and -independent secondary cell nuclear growth

Although Hedgehog signalling and the EcR appear to have very different effects on mating-dependent growth in secondary cells, it is notable that both can be involved in regulating the cell cycle regulators, Cyclin D and Cyclin E. For example, Hedgehog signalling can regulate *cyclin D* directly (via Ci) in the developing *D. melanogaster* eye, although its regulation of *cyclin E* is indirect (DUMAN-SCHEEL *et al.,* 2002). Similarly, ecdysone has been shown to regulate the expression of both *cyclin D* and *cyclin E* in several tissues (*e. g.* midgut, ovary), during development, metamorphosis, and adulthood (CRANNA & QUINN, 2009). Because both Hedgehog signalling and the EcR have roles regulating the growth of secondary cell nuclei, I was curious to determine what role Cyclin D and Cyclin E have in this process.

First, I wanted to determine what effect both knock-down and overexpression of *cyclin D* would have on the growth of secondary cell nuclei after mating. Using *esg F/O*^{ts}, I expressed an inverted repeat targeting *cyclin D* (*cycD*^{RNAi}; NI *et al.*, 2011) (*esg F/O*^{ts} > *cycD*^{RNAi}), and found that expression of the *cycD* hairpin permits mating-dependent nuclear growth ($p\ \{♂_{virgin} | ♂_{mated}\} < 0.0001$) (**FIGURE 5. 19. C–D; FIGURE 5. 20.**; **SUPPLEMENTARY TABLE 5. 11.**, *p.* 448). This suggests that Cyclin D might be dispensable for this growth. Overexpression of *cyclin D* requires simultaneous overexpression of its cyclin-dependent kinase (CDK), *CDK4* (DATAR *et al.*, 2000). Using *esg F/O*^{ts}, to express *cycD* in combination with *CDK4* (*cycD·CDK4*; DATAR *et al.,* 2000) (*esg F/O*^{ts} > *cycD·CDK4*), I found that this treatment causes the secondary cell nuclei of virgin glands to increase in size—and grow still larger after mating

FIGURE 5. 17. | HEDGEHOG SIGNALLING DOES NOT AFFECT MATING-DEPENDENT SECONDARY CELL NUCLEAR GROWTH.

A: Secondary cell nuclei (marked with pink arrows) are similar in size to those of the surrounding main cells (marked with yellow arrows) in the accessory gland of a virgin control male.

B: After mating, the secondary cell nuclei have increased in size, compared to the surrounding main cell nuclei.

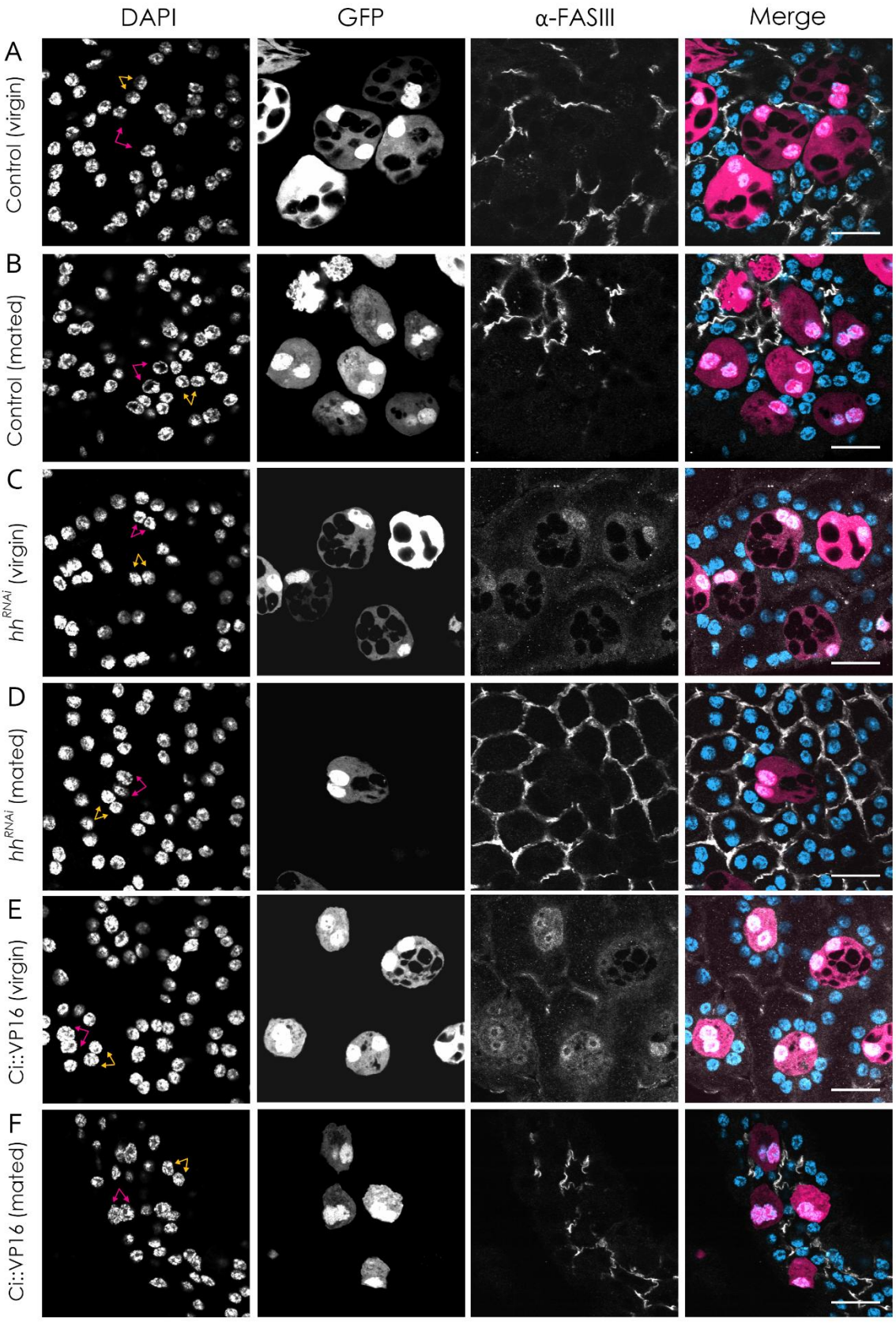
C: In secondary cells expressing an inverted repeat targeting *hedgehog*, hh^{RNAi}, the secondary cell nuclei are smaller than those of the surrounding main cells in virgin males.

D: When secondary cells expressing the *hedgehog*-targeting hairpin are mated, the secondary cells still grow after mating.

E: Secondary cells expressing the Ci::VP16 fusion protein contain nuclei that are larger than those of the surrounding main cells in virgin males.

F: When expressing the Ci::VP16 fusion protein, secondary cell nuclei fail to grow further in response to mating.

Scale bars in panels **A–F** correspond to 10 μm and α-FASIII labels main cell junctions (white). Secondary cells are labelled with GFP (magenta) and DAPI stains DNA (cyan). Please note that the FASIII channel in panels **C** and **E** show bleed-through of another antibody. This has not affected the results.


DAPI
GFP
α-FASIII
Merge
A
Control (virgin)
B
Control (mated)
C
hh^RNAi (virgin)
D
hh^RNAi (mated)
E
Ci::VP16 (virgin)
F
Ci::VP16 (mated)

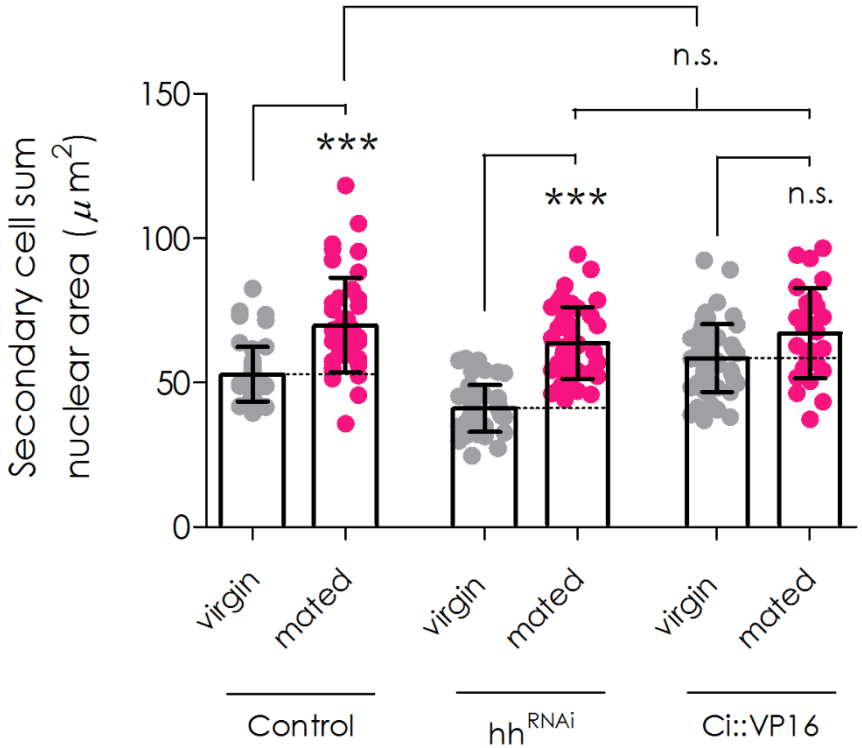


FIGURE 5. 18. | HEDGEHOG SIGNALLING DOES NOT APPEAR TO PROMOTE MATING-DEPENDENT GROWTH OF SECONDARY CELL NUCLEI.

Scatterplot bar chart showing how mating affects the size of secondary cell nuclei when Hedgehog signalling is manipulated. Each dot represents the value of an individual secondary cell, with three cells quantified per male (see **SUPPLEMENTARY TABLE 5. 10.**, *p.* 447). Error bars show the standard deviation within the sample. The number of stars in the panel represent the *p*-value obtained by a two-tailed Student's *t*-test: *** = $p < 0.0001$; 'n.s.' denotes $p > 0.1$.

(p {♂$_{virgin}$ | ♂$_{mated}$} = 0.0013) (**FIGURE 5. 19. E–F; FIGURE 5. 20.**; **SUPPLEMENTARY TABLE 5. 11.**, *p.* 448). This suggests that Cyclin D might sensitise secondary cell nuclei to grow in response to mating. In addition, when imaging secondary cells in mated accessory glands overexpressing *cycD·CDK4*, I observed something interesting: that the secondary cell nuclei in this background are enlarged to such an extent that the confocal microscope could resolve the structure of the chromosomes themselves; with the secondary cell nuclei adopting the striking, banded pattern of giant, polytene chromosomes (compare **FIGURE 5. 21.** with **FIGURE 5. 3.** (*p.* 231)). This suggests that *cycD·CDK4* overexpression in combination with mating might increase the secondary cell DNA content. It also implies that secondary cell nuclei might grow partly as the result of new DNA being synthesised. Indeed, this may not be too surprising given the already-polyploid nature of the secondary cell nuclei (which is generated during puparation) (TANIGUCHI *et al.,* 2012), and the fact that Cyclin D is an upstream activator of E2F1 (which is known to promote polyploidy when overexpressed in the cells of the larval salivary gland) (DATAR *et al.,* 2000).

Cyclin E is a synthetic-phase cyclin, with a role in DNA synthesis (OHTSUBO *et al.*, 1995). Therefore, to investigate if DNA synthesis (via Cyclin E) has a role regulating secondary cell mating-dependent nuclear growth, I used *esg F/O^{ts}* to express two inverted repeats targeting *cyclin E* (*esg F/O^{ts} > cycE^{RNAi}* I and II; NI *et al.,* 2011). Like when *cyclin D* is knocked down, the secondary cell nuclei still grow after mating in this background (p {*cycE^{RNAi}* I$_{virgin}$ | *cycE^{RNAi}* I$_{mated}$} = 0.0052; p {*cycE^{RNAi}* II$_{virgin}$ | *cycE^{RNAi}* II$_{mated}$} < 0.0001) (**FIGURE 5. 22. C–F; FIGURE 5. 23.**; **SUPPLEMENT-**

FIGURE 5. 19. | CYCLIN D REGULATES MATING-DEPENDENT SECONDARY CELL NUCLEAR GROWTH.

The growth-phase cyclin, Cyclin D, appears to have a role regulating secondary cell nuclear growth after mating.

A: Secondary cell nuclei (marked with pink arrows) are similar in size to those of the surrounding main cells (marked with yellow arrows) in the accessory gland of a virgin control male.

B: After mating, the secondary cell nuclei have increased in size, compared to the surrounding main cell nuclei.

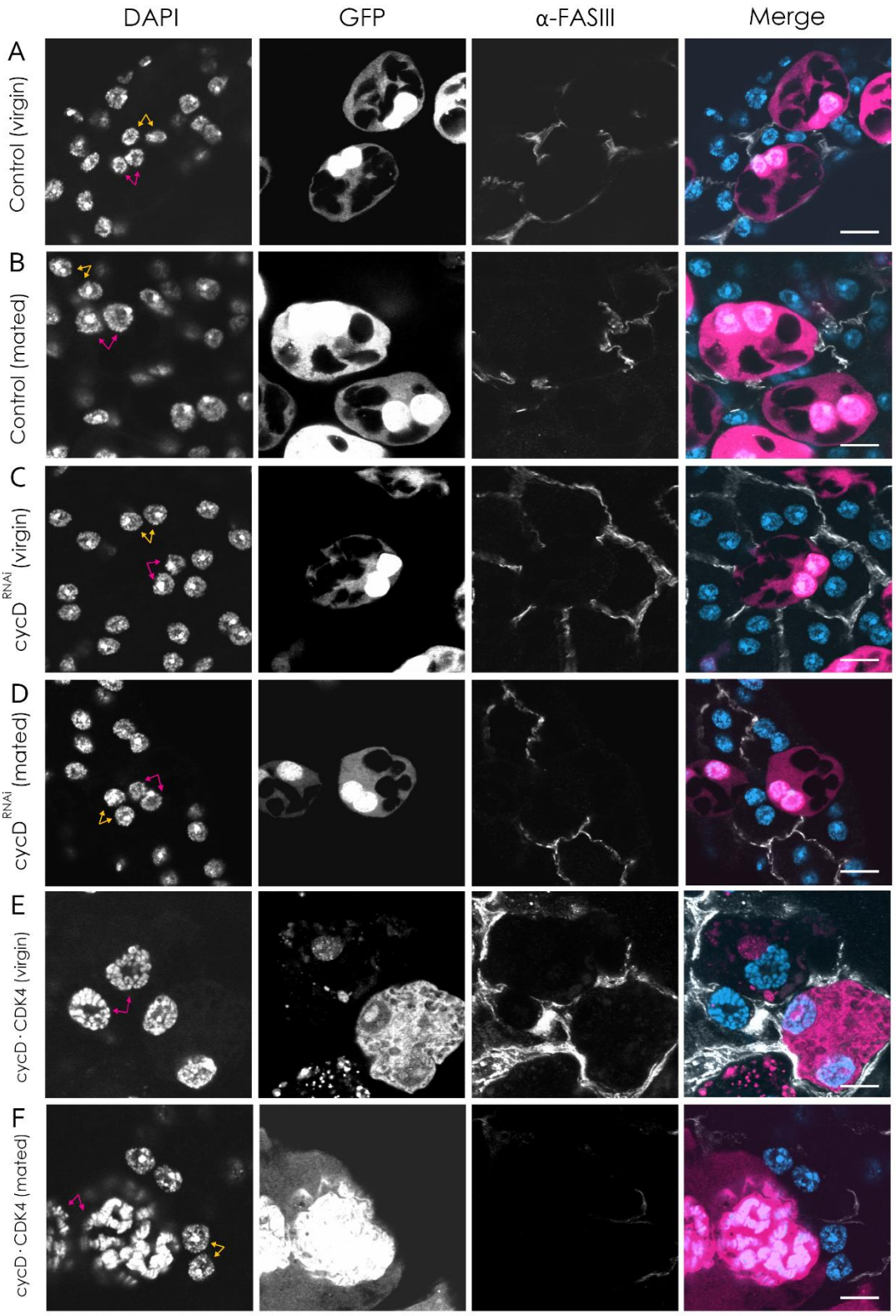
C: In secondary cells expressing an inverted repeat targeting *cycD*, $cycD^{RNAi}$, the secondary cell nuclei remain similar in size to those of the surrounding main cells.

D: When *cycD* is knocked down, secondary cell nuclei do not grow as much as those in controls in response to mating.

E: Overexpressing *cyclin D* together with its cyclin-dependent kinase, *CDK4*, causes secondary cell nuclei to grow. Note that no main cell nuclei are shown in this panel, due to the plane of section.

F: Secondary cells overexpressing *cycD·CDK4* experience further nuclear growth in response to mating—causing the secondary cell nuclei to grow very large and revealing a characteristic banding pattern on the chromosomes.

Scale bars in panels **A–F** correspond to 10 μm and α-FASIII labels main cell junctions (white). Secondary cells are labelled with GFP (magenta) and DAPI stains DNA (cyan)


DAPI
GFP
α-FASIII
Merge
A
Control (virgin)
B
Control (mated)
C
cycD^RNAi (virgin)
D
cycD^RNAi (mated)
E
cycD·CDK4 (virgin)
F
cycD·CDK4 (mated)

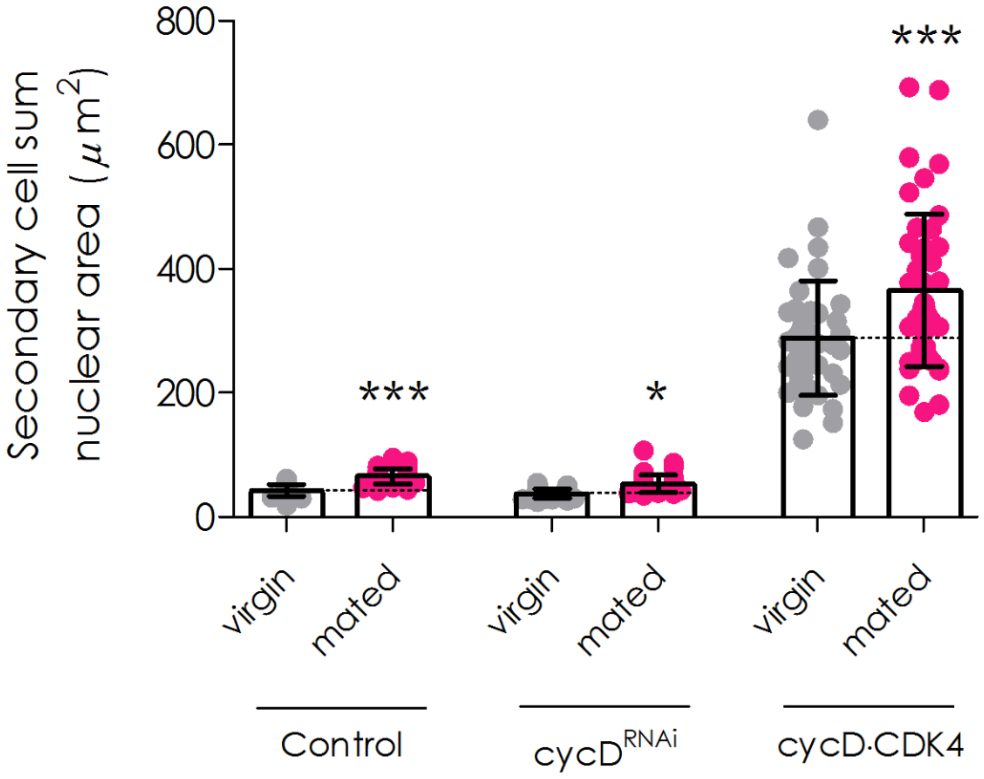


FIGURE 5. 20. | MATING-DEPENDENT SECONDARY CELL NUCLEAR GROWTH REQUIRES CYCLIN D.

Scatterplot bar chart showing how Cyclin D affects the mating-dependent growth of secondary cell nuclei. Each dot represents the value of an individual secondary cell, with three cells quantified per male (see **SUPPLEMENTARY TABLE 5. 11.**, *p.* 448). Error bars show the standard deviation within the sample. The number of stars in the figure represent the *p*-value obtained by a two-tailed Student's *t*-test: * $p < 0.01$; *** = $p < 0.0001$.

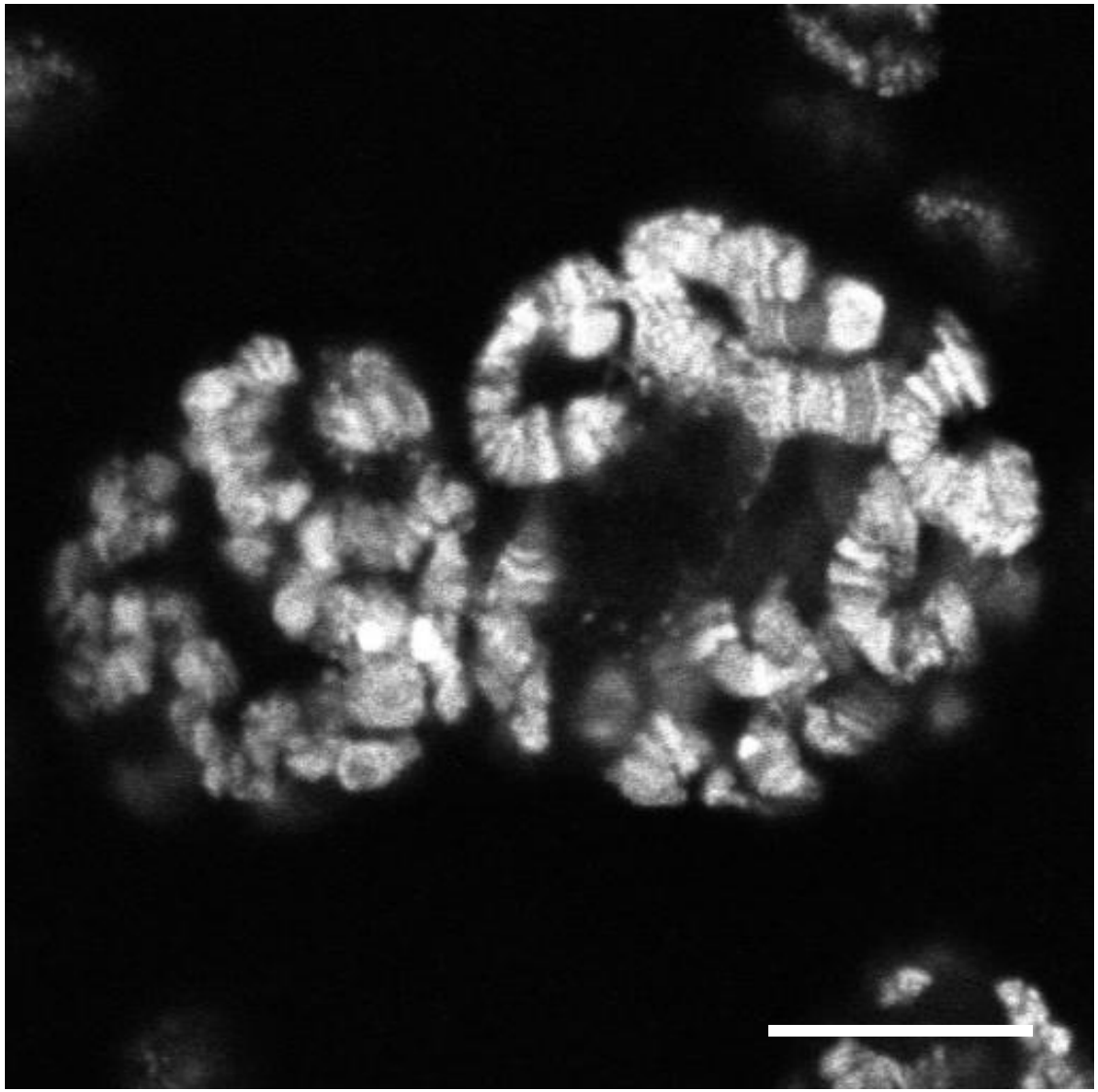


FIGURE 5. 21. | MATING, IN COMBINATION WITH OVEREXPRESSION OF *CYCLIN D·CDK4*, PRODUCES GIANT SECONDARY CELL CHROMOSOMES.

Mating secondary cells overexpressing *cyclin D*, in combination with *CDK4*, causes the secondary cell nuclei to grow very large. This enlargement of the secondary cell nuclei of the cell also shown in **FIGURE 5. 19. F**, reveals the characteristic banding pattern of giant, polytene chromosomes folded within the confines of the nuclei. Compare the density of the DAPI stain of the giant secondary cell chromosomes in this figure with the much fainter DAPI staining of the main cell nuclei near the upper right-hand corner of the figure.

FIGURE 5. 22. | CYCLIN E REGULATES MATING-DEPENDENT SECONDARY CELL NUCLEAR GROWTH.

The S-phase cyclin, Cyclin E, might have a role regulating the mating-dependent nuclear growth of secondary cells.

A: Secondary cell nuclei (marked with pink arrows) are similar in size to those of the surrounding main cells (marked with yellow arrows) in the accessory gland of a virgin control male.

B: After mating, the secondary cell nuclei have increased in size, compared to the surrounding main cell nuclei.

C: In secondary cells expressing an inverted repeat targeting *cyclin E*, *cycE*^{RNAi} I, the secondary cell nuclei remain similar to those of the surrounding main cells.

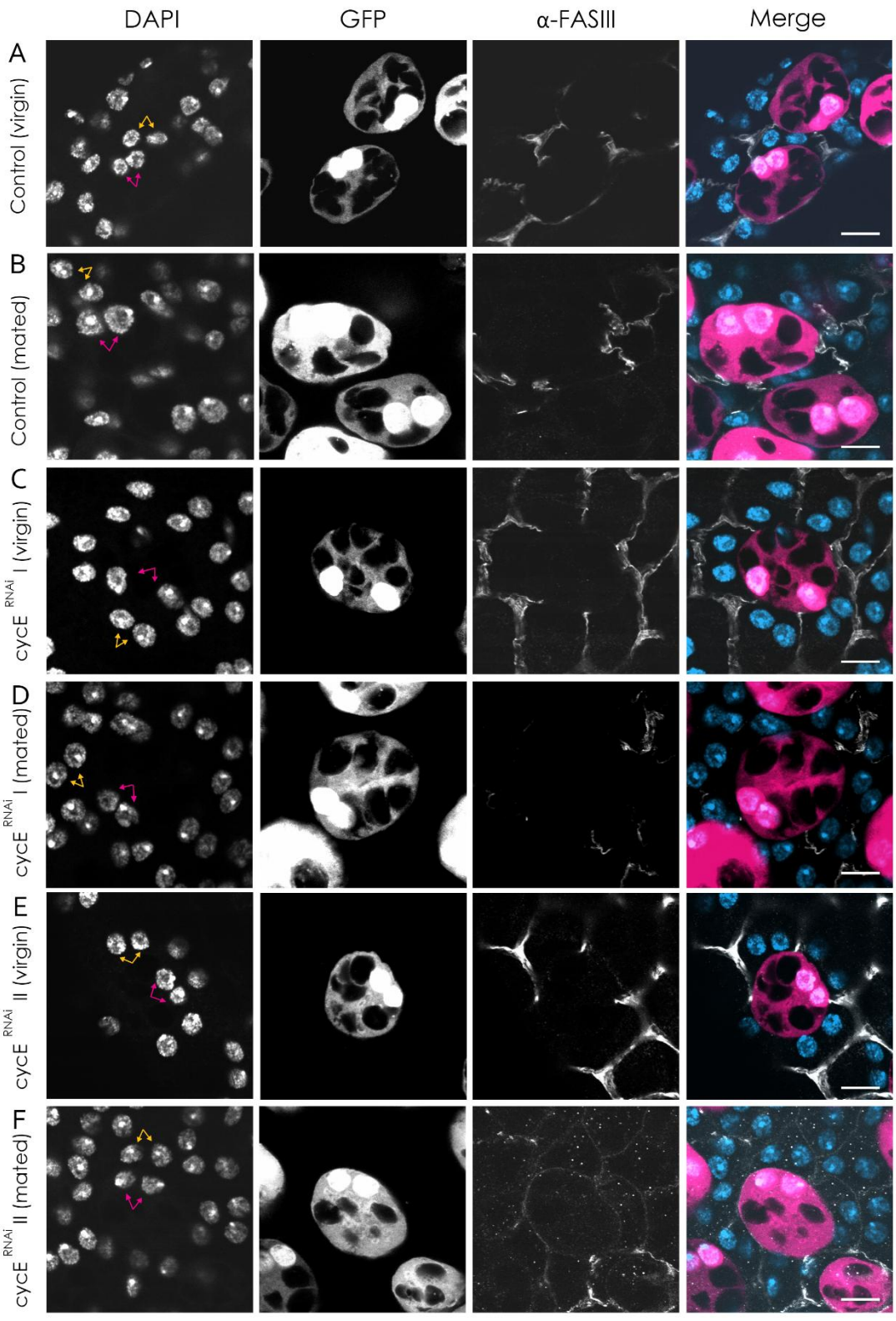
D: When expressing the *cycE*-targeting hairpin, secondary cell nuclei grow significantly less after mating.

E: In secondary cells expressing another inverted repeat targeting *cyclin E*, *cycE*^{RNAi} II, the secondary cell nuclei are slightly smaller than those of the surrounding main cells

F: When secondary cells express *cycE*^{RNAi} II, the secondary cell nuclei grow significantly less after mating.

Scale bars in panels **A–F** correspond to 10 μm and α-FASIII labels main cell junctions (white). Secondary cells are labelled with GFP (magenta) and DAPI stains DNA (cyan).

N.B. The controls in panels **A–B** are the same as shown in **FIGURE 5. 19.** since the experiments were performed at the same time.


DAPI
GFP
α-FASIII
Merge
A
Control (virgin)
B
Control (mated)
C
cycE RNAi I (virgin)
D
cycE RNAi I (mated)
E
cycE RNAi II (virgin)
F
cycE RNAi II (mated)

ARY TABLE 5. 12., *p.* 445). However, it is possible that the RNAi is not 100 % effective at knocking down the expression of *cycE*. Work in the larval salivary gland (a polytene tissue) has shown that whereas overexpression of *cyclin E* is sufficient to induce one round of DNA synthesis, subsequent synthetic cycles are inhibited; causing Cyclin E to act as an effective inhibitor of further DNA synthesis (WEISS *et al.,* 1998). Therefore, to further address the question whether *cyclin E* (and DNA synthesis) can affect secondary cell mating-dependent nuclear growth, I turned to two *cyclin E* overexpression constructs: *cycE* I and *cycE* II (JOHNSTON & EDGAR, 1998; RICHARDSON *et al.*, 1995). I reasoned that, if secondary cell nuclei grow after mating as the result of new DNA being synthesised, this growth should be inhibited if *cycE* is overexpressed and DNA synthesis cannot proceed. Consistent with this expectation, I found that overexpression of *cyclin E* did cause secondary cell nuclei to increase in size—but that this size did not change after mating (p {*cycE* I_{virgin} | *cycE* I_{mated}} = 0.65; p {*cycE* II_{virgin} | *cycE* II_{mated}} = 0.051) (**FIGURE 5. 24. C–F**; **FIGURE 5. 25.**; **SUPPLEMENTARY TABLE 5. 12.,** *p.* 448). This suggests that Cyclin E can induce secondary cell nuclear growth and that fluctuations in levels of Cyclin E protein are essential for further mating-dependent nuclear growth. It also implicates new DNA synthesis in the growth process.

Some manipulations that increase secondary cell nuclear growth drive the synthesis of new DNA

Having implicated a potential role for DNA content increases in the mating-dependent nuclear growth of secondary cells, I wanted to determine if other manipulations that alter secondary cell nuclear growth also correlate with DNA synthesis. To assay DNA synthesis in secondary cells, I made use of the thymidine

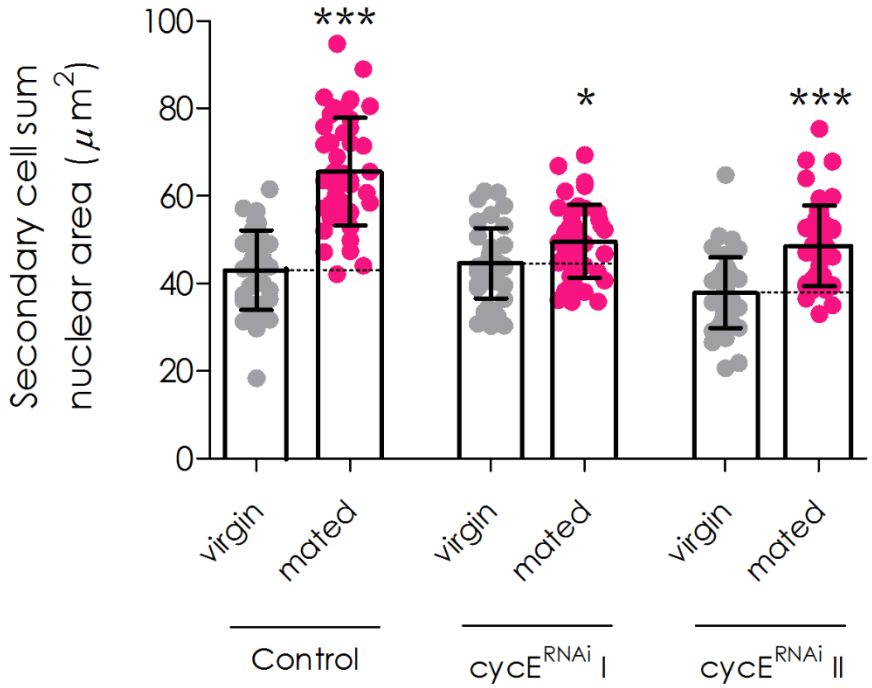


FIGURE 5. 23. | THE MATING-DEPENDENT NUCLEAR GROWTH OF SECONDARY CELLS IS REGULATED BY CYCLIN E.

Scatterplot bar chart showing how Cyclin E affects the mating-dependent secondary cell nuclear growth. Each dot represents the value of an individual secondary cell, with three cells quantified per male (see **SUPPLEMENTARY TABLE 5. 12.**, *p.* 447). Error bars show the standard deviation within the sample. The number of stars represent the *p*-value obtained by a two-tailed Student's *t*-test: * = $p < 0.01$; *** = $p < 0.0001$. *N.B.* The controls are the same as shown **in FIGURE 5. 19.,** since the experiments were performed at the same time. This has not affected the statistical analysis.

FIGURE 5. 24. | CYCLIN E OVEREXPRESSION AFFECTS SECONDARY CELL NUCLEAR GROWTH.

Overexpression of *cyclin E* seems to prevent further secondary cell nuclear growth after mating.

A: Secondary cell nuclei (marked with pink arrows) are similar in size to those of the surrounding main cells (marked with yellow arrows) in the accessory gland of a virgin control male.

B: After mating, the secondary cell nuclei have increased in size, compared to the surrounding main cell nuclei.

C: In secondary cells overexpressing the synthetic cyclin, *cyclin E*, the secondary cell nuclei are much larger than those of the surrounding main cells in virgin males.

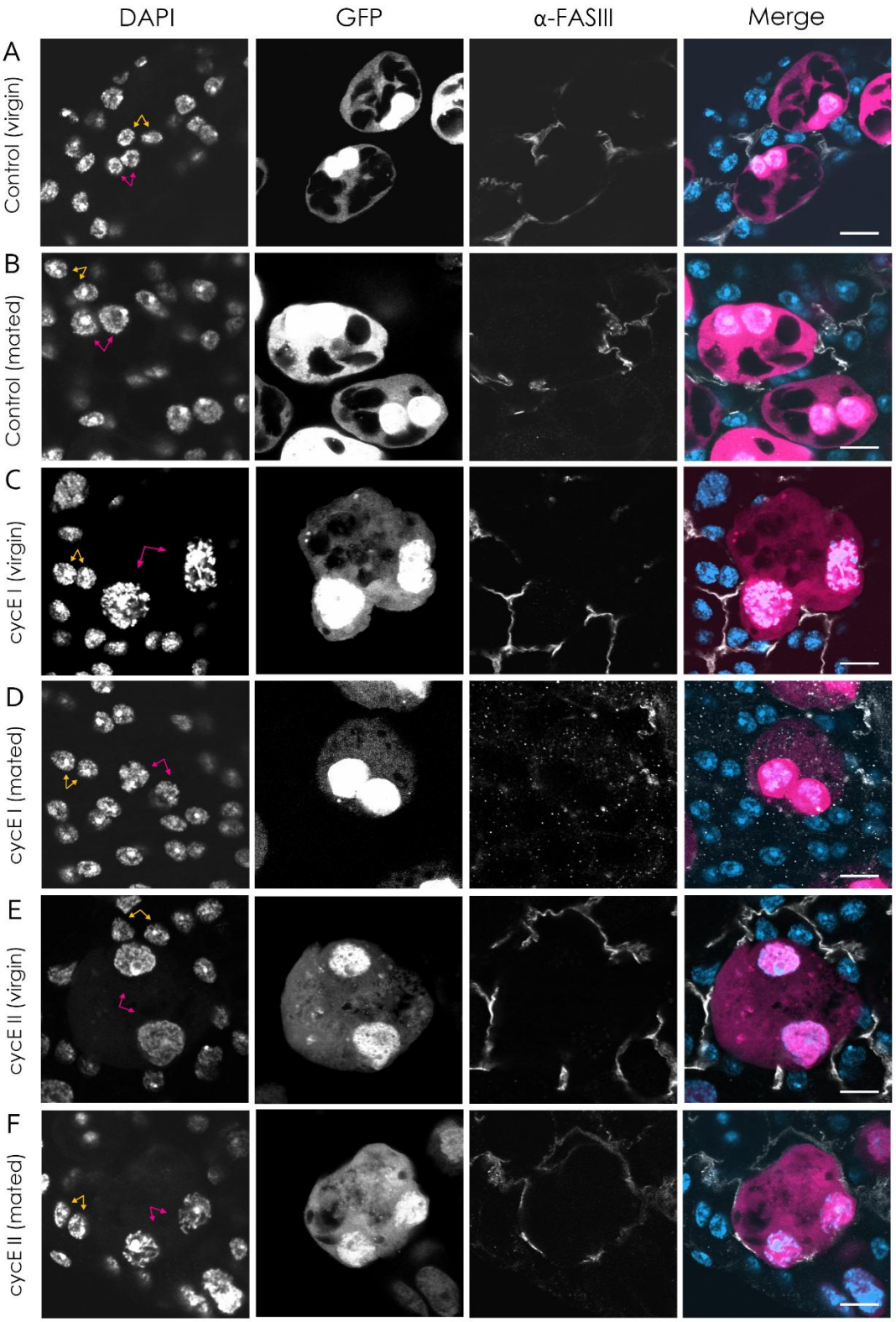
D: When *cycE* is overexpressed, secondary cell nuclei fail to grow further after mating.

E: Overexpressing *cycE* using another construct causes secondary cell nuclei to grow in virgin males.

F: Again, when *cycE* is overexpressed, the enlarged secondary cell nuclei fail to grow further after mating.

Scale bars in panels **A–F** correspond to 10 μm and α-FASIII labels main cell junctions (white). Secondary cells are labelled with GFP (magenta) and DAPI stains DNA (cyan).

N.B. The controls in panels **A–B** are the same as shown in **FIGURE 5. 19.** since the experiments were performed at the same time.


DAPI
GFP
α-FASIII
Merge
A
Control (virgin)
B
Control (mated)
C
cycE I (virgin)
D
cycE I (mated)
E
cycE II (virgin)
F
cycE II (mated)

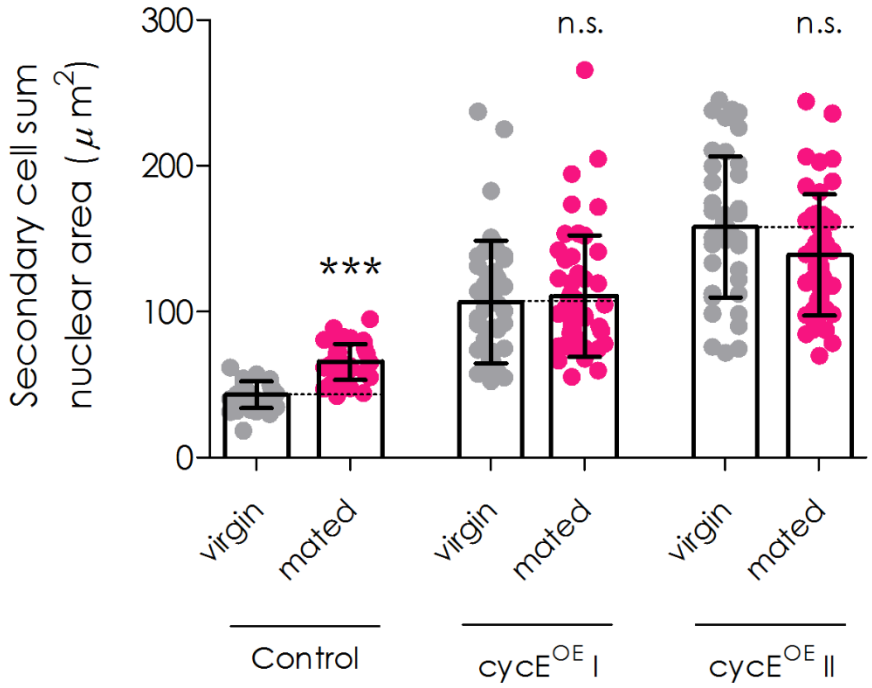


FIGURE 5. 25. | SECONDARY CELL NUCLEAR GROWTH IS AFFECTED BY *cyclin E* OVEREXPRESSION.

Scatterplot bar chart showing how Cyclin E affects the mating-dependent nuclear growth. Each dot represents the value of an individual secondary cell, with three cells quantified per male (see **SUPPLEMENTARY TABLE 5. 12.**, *p.* 447). Error bars show the standard deviation within the sample. The number of stars represent the *p*-value obtained by a two-tailed Student's *t*-test: *** = $p < 0.0001$; 'n.s.' denotes $p > 0.1$.

N.B. The controls are the same as shown in **FIGURE 5. 19**. since the experiments were performed at the same time.

analogue, 5-ethynyl-2'-deoxyuridine (EdU) (SALIC & MITCHISON, 2008). Maintaining flies on medium containing EdU allows EdU to incorporate into newly-synthesised DNA; providing a reliable indicator of where DNA synthesis has occurred (DAUL *et al.,* 2010). Wild-type virgin males (w^{1118}) maintained on medium containing 0.2 mM EdU for six days show no evidence of EdU incorporation anywhere in their accessory glands (**FIGURE 5. 26. A**). However, both the distal tip of the testis and cells in the alimentary tract (midgut) show evidence of EdU incorporation (**FIGURE 5. 26. C–D**). This shows that the EdU labelling protocol is effective. Interestingly, a subset of cells in the ejaculatory duct showed evidence of EdU incorporation (**FIGURE 5. 26. B**). This suggests that the epithelial cells in this tissue routinely undergo DNA content increases during the first six days of adult life. Similarly, when I examined male progeny from *esg F/O*^{ts} flies crossed to the wild-type, w^{1118}, very few secondary cells are positive for EdU incorporation in virgin males (**FIGURE 5. 27. A**). This suggests that DNA synthesis does not normally occur in the secondary cells of virgin males (**SUPPLEMENTARY TABLE 5. 13.**, *p.* 449).

Secondary cell nuclei grow after expressing either the activated BMP signalling receptor, tkv^{Q253D}, or the synthetic EcR isoform, EcR-C (*i.e.* LEIBLICH *et al.*, 2012; LEIBLICH, 2015). Using *esg F/O*^{ts} to express either of these in the secondary cells of flies kept on the EdU medium, revealed that new DNA is synthesised in response to either treatment (**FIGURE 5. 27. B–C**). This suggests that both activated BMP signalling and the EcR-C can increase the secondary cell DNA content. However, activated BMP signalling cannot promote nuclear growth in secondary cells in the absence of the EcR (LEIBLICH, 2015). Indeed, when I co-expressed tkv^{Q253D} with the inverted repeat targeting the EcR (EcR^{RNAi}), the proportion of secondary cells

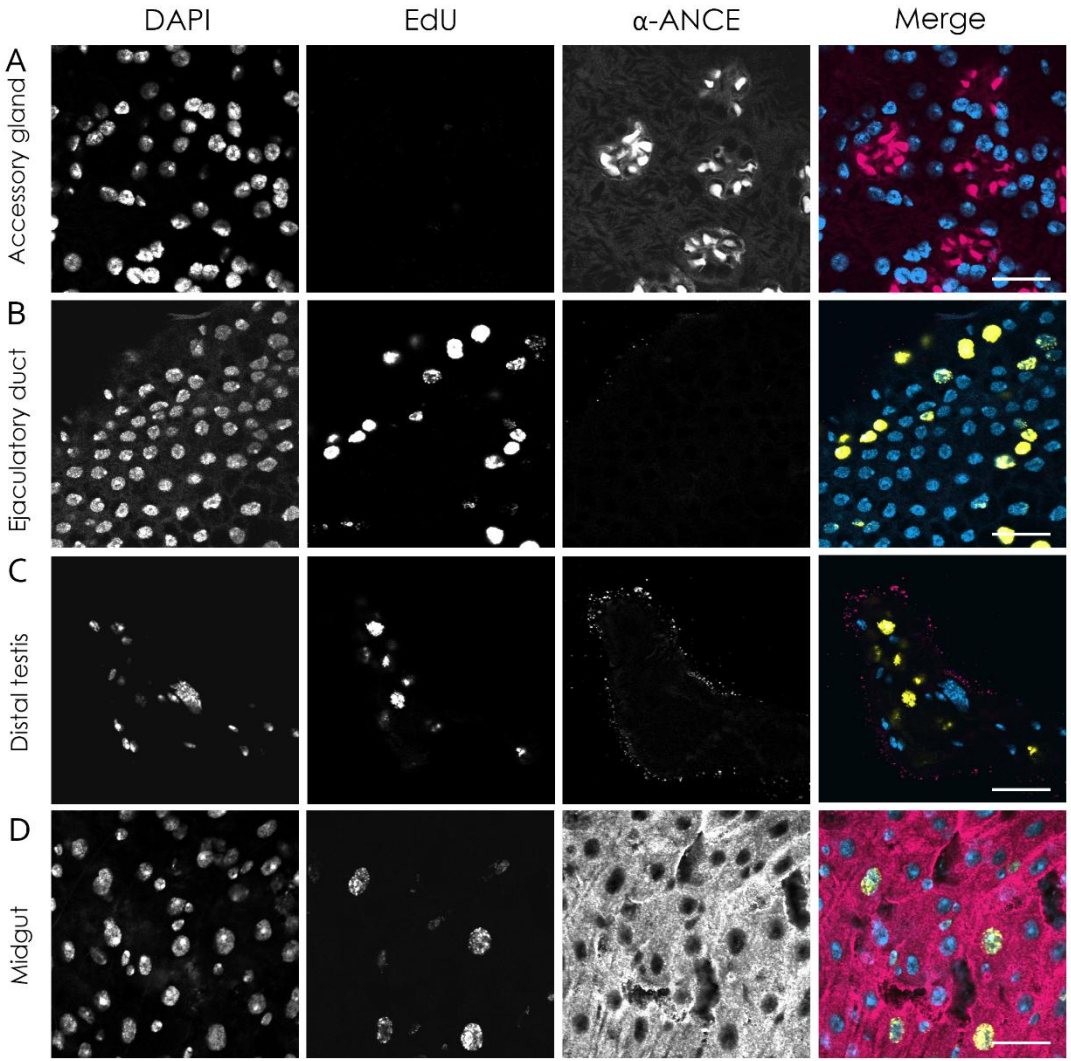


FIGURE 5. 26. | EdU LABELLING REVEALS SITES OF DNA SYNTHESIS IN ADULT VIRGIN MALE FLIES.

The thymidine analogue, EdU, was incorporated into newly synthesised DNA, revealing sites of DNA synthesis in w^{1118} virgin male flies fed 0.2 mM EdU for six days.

A: The accessory gland epithelium of the virgin male does not contain any nuclei showing evidence of EdU incorporation; suggesting that neither secondary cells (stained with α-ANCE) nor main cells are a site of DNA synthesis.

B: A subset of cells in the ejaculatory duct epithelium show evidence of EdU incorporation; suggesting that this tissue is a site of DNA synthesis.

C: The distal testis, which is an established site of DNA synthesis, shows evidence of EdU incorporation. Note that the speckles in the ANCE channel probably do not correspond to staining in this tissue, but are most likely lumps of secondary antibody that have not been properly washed off during the preparation of the tissue

D: The cells in the *D. melanogaster* alimentary tract, including the midgut, represent another established site of DNA synthesis, and the cells in this tissue also show evidence of EdU incorporation.

Scale bars in panels **A–D** correspond to 20 μm. In all panels, EdU labelling is shown in yellow, α-ANCE staining is shown in magenta, and DAPI staining of DNA is shown in cyan.

positive for EdU incorporation was much decreased (**FIGURE 5. 28.**). This suggests that the EcR is required for the BMP-dependent DNA content increases in secondary cells. A cursory examination also revealed that overexpression of both *cycD·CDK* or *cyclin E* (*cycE* II) is associated with EdU incorporation into the secondary cells (**FIGURE 5. 27. D–E**). Altogether, these results suggest that manipulations that we know to increase the size of secondary cell nuclei correlate with increases in the secondary cell DNA content. Importantly, since none of these manipulations is known to increase the number of secondary cells in the male accessory gland, it is likely that these DNA content increases are the result of endocycling, rather than the cell cycle proper.

Mating increases the secondary cell DNA content

Elevated BMP signalling can increase secondary cell nuclear size, and, as shown above, activated BMP signalling can also drive the synthesis of new DNA. Based on these observations, I became curious to what extent mating (which activates BMP signalling, and promotes secondary cell nuclear growth) can drive DNA synthesis in secondary cells. Using control flies (*esg F/O*^{ts} crossed to w^{1118}) and keeping them on the EdU medium, either in the absence or presence of females (virgin or mated treatments) for six days, revealed that whereas very few secondary cells incorporate EdU in virgin males, this proportion increases when the males have been kept together with females ($p < 0.01$) (**FIGURE 5. 29. A–B; D**)—reaching a final proportion of around 30 % EdU-positive secondary cells per gland (**SUPPLEMENTARY TABLE 5. 14.**, *p.* 449). This suggests that mating can drive the synthesis of new DNA in secondary cells—and, importantly, that the synthesis of new DNA does not occur in

the absence of mating. To confirm that the mating-dependent DNA synthesis is physiologically relevant, I also performed this experiment in wild-type (w^{1118}) flies, and found a similar relationship (p {virgin|mated} = 0.002) (**FIGURE 5. 30.**; **SUPPLEMENTARY TABLE 5. 14.**, *p.* 449). Interestingly, after mating the proportion of EdU-positive cells in the ejaculatory duct epithelium seemed to show a strong increase, although this was not quantified (not shown).

FIGURE 5. 27. | EdU LABELLING REVEALS A CORRELATION BETWEEN DNA SYNTHESIS AND GENETICALLY-INDUCED CHANGES IN SECONDARY CELL NUCLEAR SIZE.

The thymidine analogue, EdU, was incorporated into newly synthesised DNA, revealing sites of DNA synthesis in *esg F/O*^{ts} virgin male flies kept on 0.2 mM EdU for six days.

A: The accessory gland epithelium of the virgin male contains very few nuclei showing evidence of EdU incorporation.

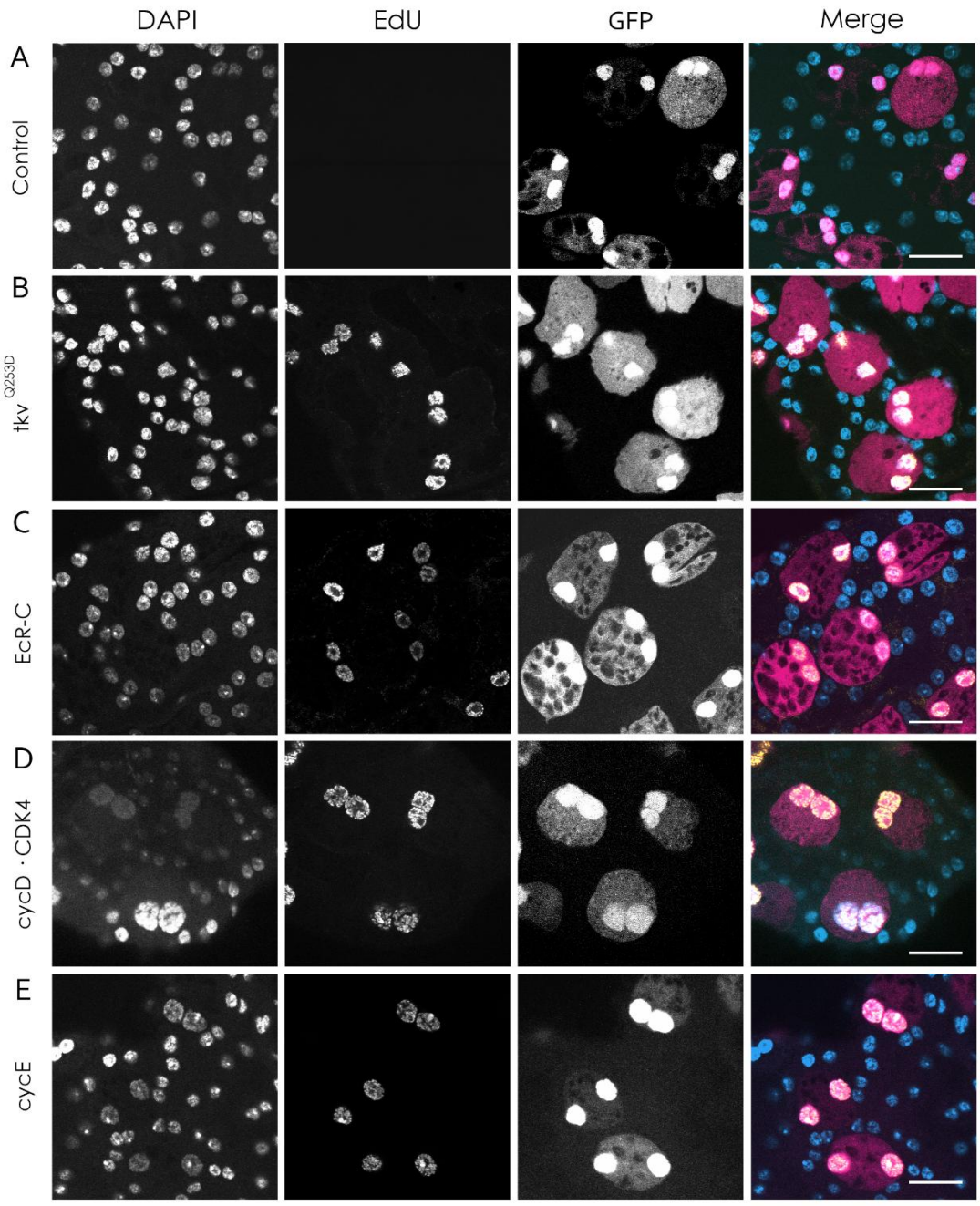
B: When BMP signalling is activated in all secondary cells via expression of *tkv*^{Q253D}, many of their nucleu show evidence of EdU incorporation.

C: Expression of the synthetic EcR isoform, EcR-C, is correlated with secondary cell EdU incorporation in almost all nuclei. Note how the number of intracellular compartments appears to be increased in this genotype.

D: When expressing the growth cyclin, *cyclin D*, together with *CDK4*, secondary cells incorporate EdU.

E: Secondary cells incorporate EdU also when overexpressing the synthetic cyclin, *cyclin E*.

Scale bars in panels **A–E** correspond to 20 μm. In all panels, EdU labelling is shown in yellow, α-ANCE staining is shown in magenta, and DAPI staining of DNA is shown in cyan.


DAPI
EdU
GFP
Merge
A
Control
B
tkv Q253D
C
EcR-C
D
cycD · CDK4
E
cycE

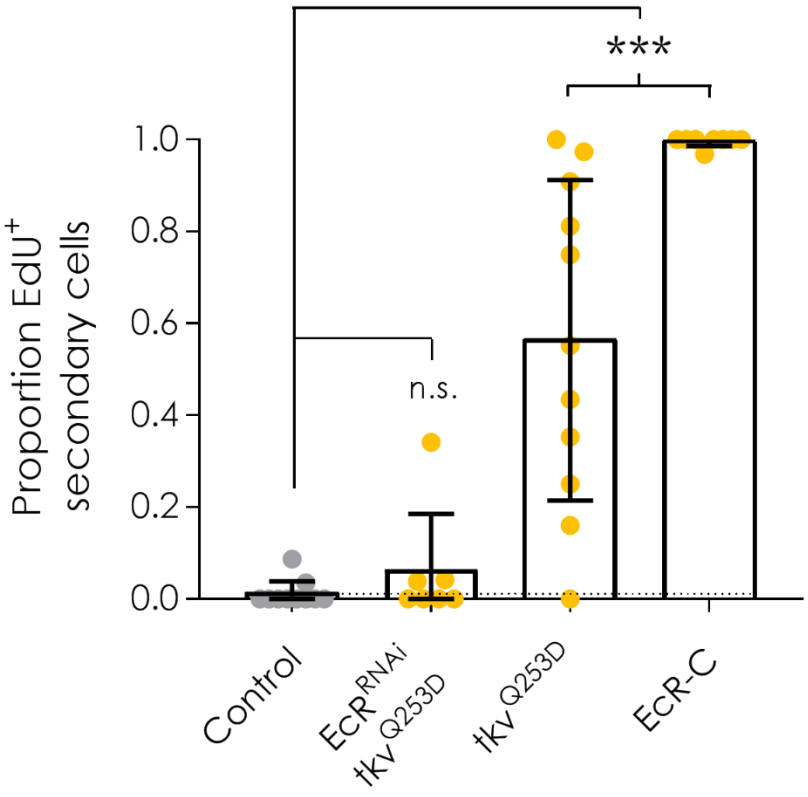


FIGURE 5. 28. | SECONDARY CELL EdU INCORPORATION IN VIRGIN MALES IS INDUCED BY BMP AND EcR SIGNALLING.

Scatterplot bar chart showing how the proportion of EdU-positive secondary cells change in response to different genetic treatments. Each dot represents the value of an individual male (see **SUPPLEMENTARY TABLE 5. 13.**, *p.* 449), and error bars show the standard deviation within the sample. The number of stars represent the *p*-value obtained by one-way ANOVA with Tukey's correction for multiple comparisons (all columns): *** = $p < 0.0001$; 'n.s.' denotes $p > 0.1$.

Because the accessory glands of mated males kept on the EdU-containing medium contain a mixture of EdU-positive and EdU-negative secondary cells, I wanted to test whether the nuclear size differs between these two different populations of secondary cells. Quantifying the nuclear size of EdU-negative secondary cells in mated *esg F/O*^{ts} glands, and comparing these to the size of the secondary cell nuclei of virgin glands of sibling flies kept on the same medium, revealed that although the size of the EdU-negative secondary cell nuclei in mated glands are larger than those in virgins (p {virgin|mated$_{EdU-}$} < 0.001), the nuclei of secondary cells incorporating EdU are larger still (p {mated$_{EdU-}$|mated$_{EdU+}$} < 0.01) (**FIGURE 5. 29. E; SUPPLEMENTARY TABLE 5. 15**, *p.* 449). This suggests that the presence of new DNA correlates partially with the mating-dependent growth of secondary cell nuclei.

Increases in DNA content can be detected through another (more approximate) method through quantification of the DAPI staining intensity of nuclei, as captured through the confocal DAPI channel in multiple z-planes through each nucleus. This is because larger quantities of DNA will take up more DAPI, and therefore stain more strongly (McCOY *et al.*, 2012). Employing this method on the data collected for **FIGURE 5. 6.** (*p.* 244) revealed that whereas the size of the nuclei changes throughout the first ten days of adult life, the total DAPI staining intensity (expressed as a SC:MC ratio of secondary cell nuclear staining intensity relative to main cell nuclear staining intensity) remains relatively constant, with the only significant increase occurring after mating (p day 6{♂$_{virgin}$|♂$_{mated}$} < 0.05) (**FIGURE 5. 30.**). Taking the obtained DAPI intensity measurements on day six at face-value (1.13 ± 0.35 for virgin glands, and 1.41 ± 0.67 for mated glands) (**SUPPLEMENTARY**

FIGURE 5. 29. | EdU LABELLING REVEALS THAT MATING CAN DRIVE BMP AND ECR-DEPENDENT DNA SYNTHESIS IN SECONDARY CELLS.

The thymidine analogue, EdU, is incorporated into newly synthesised DNA, revealing that mating can drive DNA synthesis in mated *esg F/O*^{ts} male flies kept on medium containing 0.2 mM EdU for six days.

A: The accessory gland epithelium of the virgin male contains very few nuclei showing evidence of EdU incorporation, and the secondary cell nuclei are similar in size to those of the surrounding main cells.

B: When the male is mated, the proportion of secondary cells showing evidence of EdU incorporation increases.

C: If the male is mated when overexpressing the BMP signalling inhibitor, Dad, very little EdU uptake is observed in the gland.

D: Quantifying the proportion of EdU-positive secondary cell nuclei in virgin and mated accessory glands shows a significant increase in EdU labelling after mating that seems to depend on both BMP signalling activity and the EcR.

E: Quantifying the secondary cell sum nuclear area before and after mating, and in cells that have and have not incorporated EdU, reveals that although secondary cell nuclei do grow after mating in the absence of EdU incorporation, the incorporation of EdU seems to correlate with further nuclear size increases.

Scale bars in panels **A–C** correspond to 20 μm. Secondary cells are labelled with GFP (magenta), and EdU is shown in yellow. DAPI stains DNA (cyan).

In panel **D**, each dot represents the value of an individual male (see **SUPPLEMENTARY TABLE 5. 14.**, *p.* 449). In panel **E**, each dot represents the value of an individual secondary cell, with three cells quantified per male (see **SUPPLEMENTARY TABLE 5. 15.**, *p.* 449). Error bars show the standard deviation within the sample. The number of stars in panel **D** represents *p*-values obtained by one-way ANOVA with Dunnett's correction for multiple comparisons (all columns compared to the control), whereas the stars in panel **E** represent the *p*-value obtained by one-way ANOVA with Tukey's correction for multiple comparisons (all columns): * = $p < 0.01$; ** = $p < 0.001$; *** = $p < 0.0001$; 'n.s.' denotes $p > 0.1$.

N.B. The controls in panels **A–C** are the same as shown in **FIGURE 5. 27.**, since the experiments were performed at the same time. This has been taken into account for the statistical analysis.

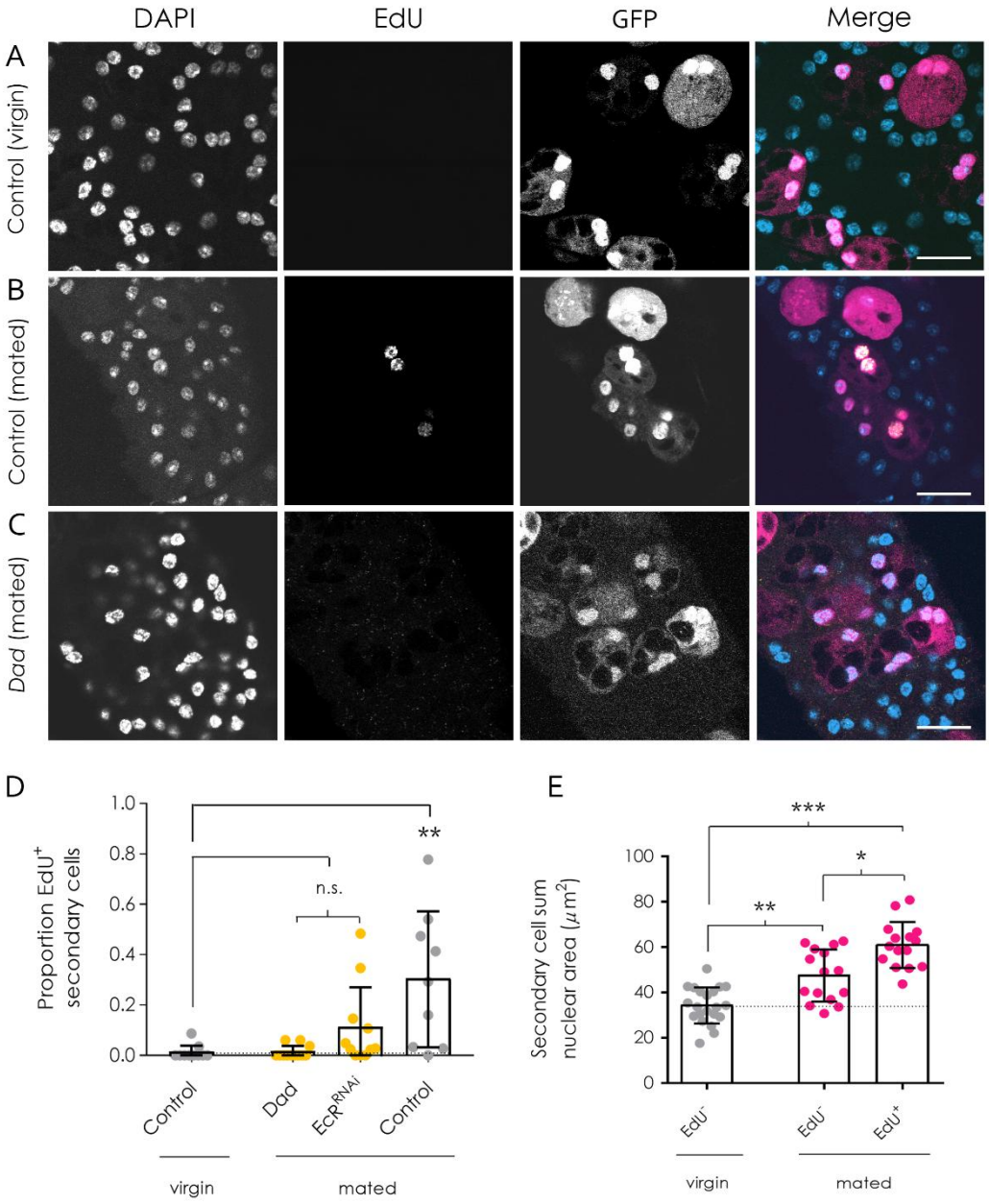

DAPI
EdU
GFP
Merge
A
Control (virgin)
B
Control (mated)
C
Dad (mated)
D
Proportion EdU+ secondary cells
1.0
0.8
0.6
0.4
0.2
0.0
**
n.s.
Control
Dad
EcRRNAi
Control
virgin
mated
E
Secondary cell sum nuclear area (μm2)
100
80
60
40
20
0
***
*
**
EdU-
EdU-
EdU+
virgin
mated

TABLE 5. 16., *p.* 450) suggests that mating increases the secondary cell (relative to main cell) DAPI staining intensity by a factor of 1.25 (1.41 ÷ 1.13 = 1.25). This would represent an approximate DNA content increase of about 25 %, on average.[5]

Having found that BMP signalling and the EcR-C promote secondary cell EdU uptake, and that BMP signalling is activated in response to mating, I wanted to further investigate if the BMP/EcR axis regulates the mating-dependent EdU uptake. Already, as I have shown, secondary cell EdU uptake is reduced when co-expressing EcR^{RNAi} and tkv^{Q253D} (**FIGURE 5. 28.**), suggesting that the EcR is required for the BMP-dependent uptake of EdU. However, this tells us nothing about EdU uptake after the mating-dependent activation of BMP signalling. To learn more about the behaviour of secondary cells in response to mating, I wanted to determine what proportion of secondary cells show evidence of EdU uptake after mating—either when BMP signalling is inhibited (*Dad* overexpression) or when the EcR is knocked down (EcR^{RNAi}). Using *esg F/O*^{ts} to express these constructs in secondary cells, I found that both treatments greatly reduced the mating-dependent EdU uptake compared to controls, so that EdU incorporation was not significantly higher than in virgins (**FIGURE 5. 29. C–D; SUPPLEMENTARY TABLE 5. 14.**, *p.* 449). This suggests that the BMP/EcR axis is indeed required for mating to induce DNA synthesis is secondary cells.

[5] An SC:MC DAPI staining ratio of 1.25 implies that the secondary cell genome is about 25 % larger than the main cell genome, on average, in mated male accessory glands. This size difference does not correspond to any genome doublings, and might be the result of measurement error. However, I think this is unlikely. For example, FLEMING *et al.* (2000) argue against measurement error being the cause of DAPI intensity measurements not falling on a linear series of genome doublings (*e.g.* 2C → 4C → 8C). Rather, it is possible that the nuclear DNA content is incompletely replicated or that only a subset of secondary cells undergo a doubling of the DNA content.

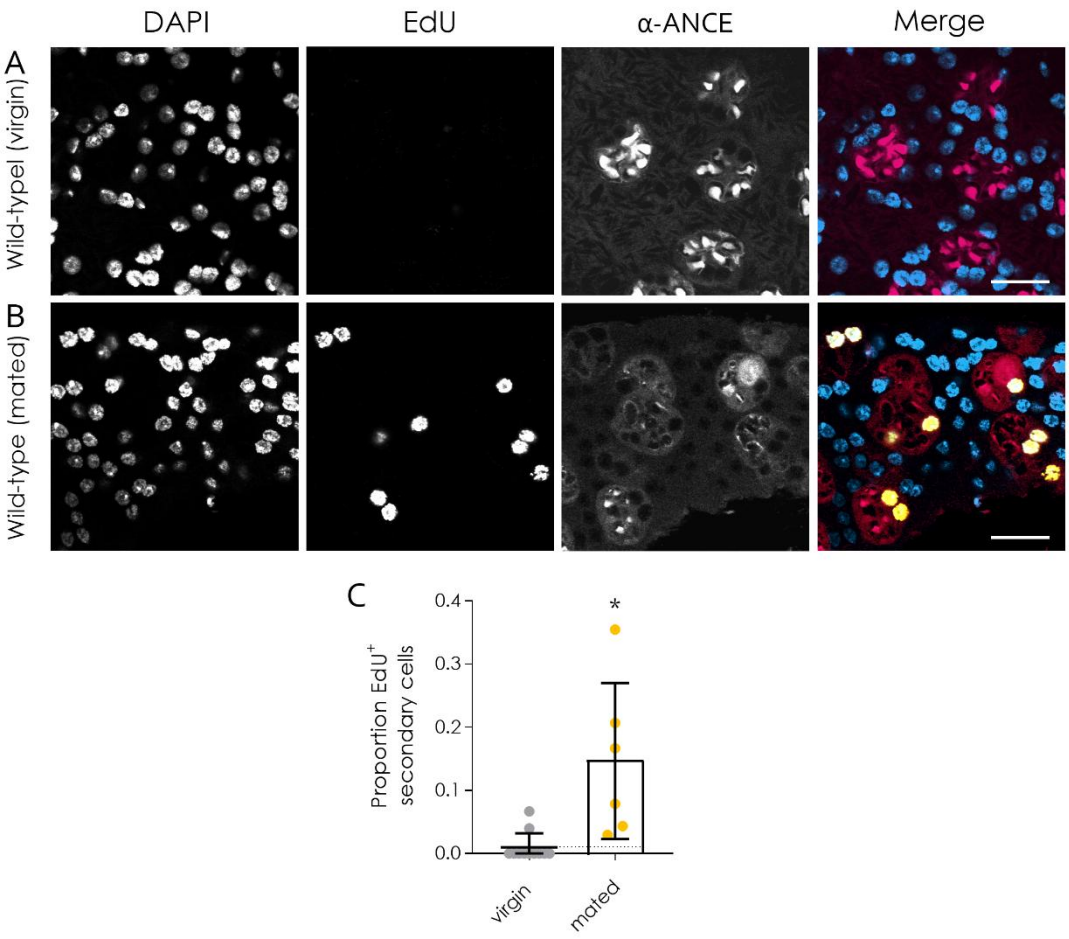


FIGURE 5. 30. | MATING CAN DRIVE DNA SYNTHESIS ALSO IN WILD-TYPE SECONDARY CELLS.

The thymidine analogue, EdU, is incorporated into newly synthesised DNA, revealing that mating can drive DNA synthesis in mated, wild-type male flies kept on 0.2 mM EdU for six days.

A: The accessory gland epithelium of the virgin male contains few nuclei showing evidence of EdU incorporation.

B: Secondary cells incorporate EdU in response to mating.

C: Quantifying the proportion of secondary cells positive for incorporated EdU shows a significant increase after mating. Each dot corresponds to an individual male (see **SUPPLEMENTARY TABLE 5. 14.**, *p.* 449).

Scale bars in panels **A–B** correspond to 20 μm. Secondary cells are labelled with GFP (magenta), and EdU is shown in yellow. DAPI stains DNA (cyan).

The number of stars in panel **C** represent the *p*-value obtained by a two-tailed Student's *t*-test: '*' denotes $p = 0.002$. Each dot represents the value of an individual accessory gland, and error bars show the standard deviation within the sample.

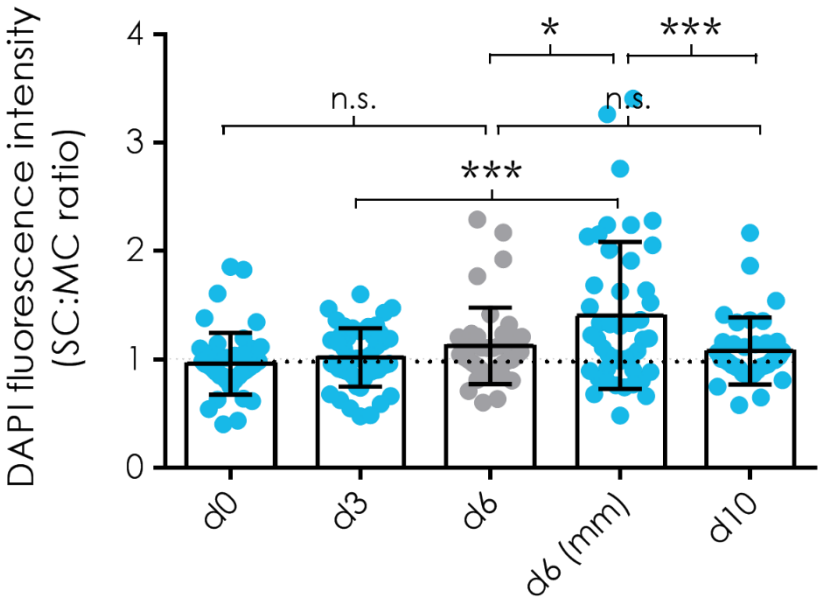


FIGURE 5. 31. | MATING INCREASES THE DAPI STAINING INTENSITY OF SECONDARY CELL NUCLEI RELATIVE TO MAIN CELL NUCLEI.

Scatterplot bar chart showing how the total DAPI staining intensity changes over time, and after mating. The staining intensity was measured in 20 z-planes, 1 μm apart, including the entire nuclear volume.Each dot represents the value of an individual SC:MC ratio, with three ratios determined per male (see **SUPPLEMENTARY TABLE 5. 16.**, *p.* 450). Error bars show the standard deviation within the sample. The number of stars represent the p-value obtained by one-way ANOVA with Tukey's correction for multiple comparisons (all columns): * = $p < 0.01$; ** = $p < 0.001$; *** = $p < 0.0001$; 'n.s.' denotes $p > 0.1$.

Secondary cells can grow after mating even in the absence of E2F1-dependent DNA synthesis

The transcription factor, E2F1, has a role in DNA amplification in polyploid tissues in *D. melanogaster* (*e.g.* ovarian follicle cells: CAYIRLIOGLU *et al.,* 2003), and its overexpression is sufficient to induce a hyper-polyploid phenotype (EDGAR *et al.,* 2014). Furthermore, overexpression of *E2F1* and its binding partner, *Dp*, has been shown to increase the nuclear size of secondary cells in our laboratory—as has knock-down of the cell cycle inhibitor, *RBF* (LEIBLICH, 2015). Having established that there is a relationship between the cell cycle and the mating-dependent growth of secondary cells, I wanted to determine to what extent E2F1 and RBF participate in this process. To investigate, I used *esg F/O*^{ts} to express both an inverted repeat targeting *E2F1* (*E2F1*^{RNAi}; NI *et al.,* 2008) and a constitutively active form of RBF (RBF^{CA}; XIN & WENG *et al.,* 2002). The aim of this experiment was to determine how these cell cycle regulators affect the growth of secondary cell nuclei after mating. Based on my previous results, and the fact that both these constructs have previously been shown to inhibit secondary cell nuclear growth (LEIBLICH, 2015), I predicted that their expression would also inhibit the mating-dependent growth. However, assaying mated glands expressing either construct, I found that neither *E2F1*^{RNAi} nor RBF^{CA} were capable of inhibiting the mating-dependent growth (p {♂$_{virgin}$|♂$_{mated}$} < 0.0001) (**FIGURE 5. 31.**; **FIGURE 5. 32.**; **SUPPLEMENTARY TABLE 5. 17.**, *p.* 450). In addition, although the nuclei grow after mating when *E2F1* is knocked down, quantification of the DAPI staining intensity in this background does not reveal a significant increase after mating (virgin: 1.13 ± 0.90; mated: 1.41 ± 0.78; p = 0.09; $n_{mated}|n_{virgin}$ = 10; 2-tailed Student's *t*-test). Altogether, this suggests that secondary cell nuclei can grow after mating even when their DNA content does not increase.

FIGURE 5. 32. | SECONDARY CELL NUCLEI GROW AFTER MATING EVEN WHEN *E2F1* IS KNOCKED DOWN OR WHEN RBF IS CONSTITUTIVELY ACTIVE.

A: Secondary cell nuclei (marked with pink arrows) are similar in size to those of the surrounding main cells (marked with yellow arrows) in the accessory gland of a virgin control male.

B: After mating, the secondary cell nuclei have increased in size, compared to the surrounding main cell nuclei.

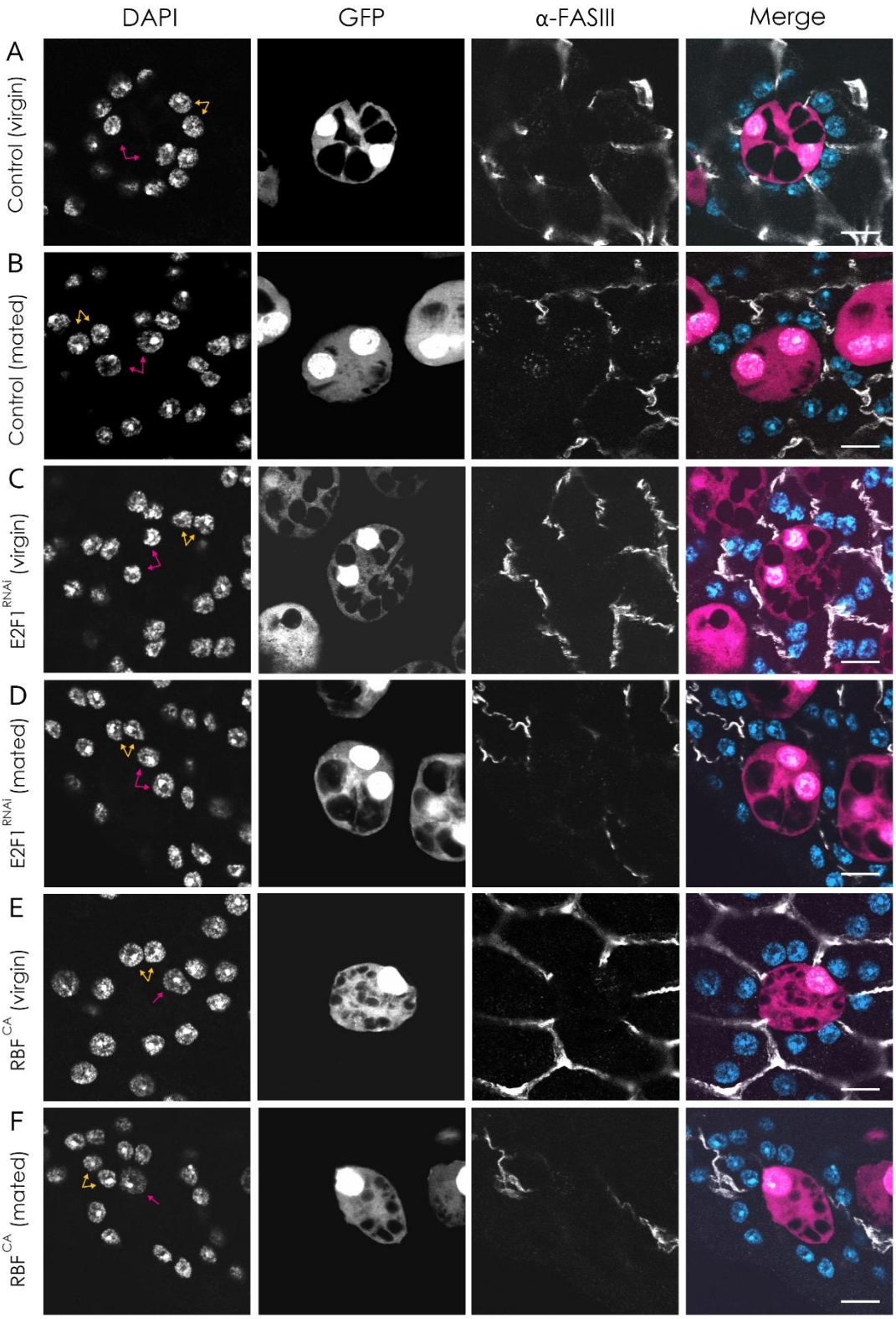
C: In secondary cells expressing an inverted repeat targeting *E2F1*, the secondary cell nuclei remain similar to those of the surrounding main cells.

D: When *E2F1* is knocked down, secondary cell nuclei retain the capacity to grow after mating.

E: Expressing a constitutively active form of the negative cell cycle regulator, *RBF* (RBF^{CA}) causes secondary cells to become largely mononucleate. These nuclei are similar in size (if not slightly larger) to the individual nuclei of the surrounding main cells.

F: When expressing RBF^{CA}, the single secondary cell nucleus seems to grow after mating to a similar size to controls.

Scale bars in panels **A–F** correspond to 10 μm and α-FASIII labels main cell junctions (white). Secondary cells are labelled with GFP (magenta) and DAPI stains DNA (cyan).


DAPI
GFP
α-FASIII
Merge
A
Control (virgin)
B
Control (mated)
C
E2F1RNAi (virgin)
D
E2F1RNAi (mated)
E
RBFCA (virgin)
F
RBFCA (mated)

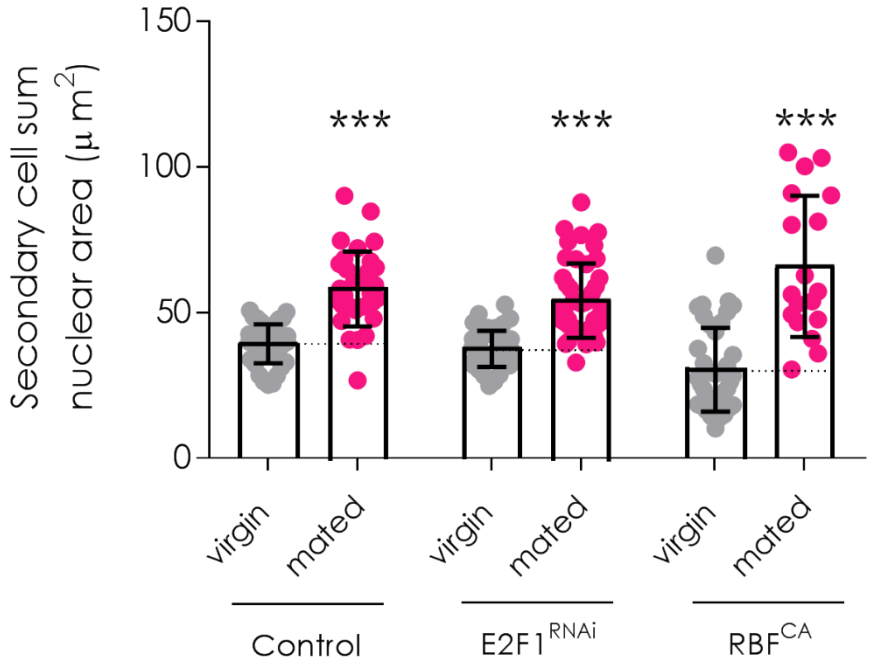


FIGURE 5. 33. | THE MATING-DEPENDENT NUCLEAR GROWTH OF SECONDARY CELLS DOES NOT REQUIRE E2F1 OR RBF.

Scatterplot bar chart showing how secondary cell nuclei respond after mating in the presence of an inverted repeat targeting *E2F1*, or a constitutively active form of RBF. Each dot represents the value of an individual secondary cell, with three cells quantified per male (see **SUPPLEMENTARY TABLE 5. 17.**, *p.* 450). and error bars show the standard deviation within the sample. (Note the two populations that emerge in the RBF^{CA} background—representing the values obtained for either the mononucleate or binucleate secondary cells. In retrospect, I should have treated the mono- and binucleate populations independently.) The number of stars represent the *p*-value obtained by a two-tailed Student's *t*-test: *** = $p < 0.0001$.

Cell cycle manipulations that alter secondary cell nuclear growth can also drive the growth of main cell nuclei

So far, I have focused almost exclusively on the behaviour of the secondary cells. As I show in **FIGURE 5. 6.** (*p.* 244), this is because within the male accessory gland epithelium, only the secondary cells nuclei grow in response to mating. However, this is, in itself, an interesting observation, and it inspires the question: why do only the secondary cell nuclei grow in response to mating? Previous work in our laboratory has examined this question to some extent—showing that activating BMP/EcR signalling in main cells does not affect the size of the main cell nuclei (LEIBLICH, 2015). However, as suggested by the work presented in this Chapter, it is likely that BMP/EcR signalling increases the secondary cell nuclear size by promoting activation of the cell cycle. Based on this, I decided to determine how main cells respond to ectopic activation of the cell cycle: do their nuclei behave similarly to those of secondary cells?

The accessory gland protein, Ovulin (Acp26Aa), is expressed in main cells (MONSMA *et al.*, 1990). Using the A*cp26Aa* promoter to drive expression of GAL4 (A*cp26Aa* > GAL4; CHAPMAN & BANGHAM *et al.,* 2003), therefore allows *UAS*-driven transgenes to be expressed in main cells (LEIBLICH, 2015)[6]. Combining *Acp26Aa* > GAL4 together with nuclear GFP (*Acp26Aa* > GFP; CORRIGAN, 2014) also allows the visualisation of main cells in the accessory gland epithelium. Using this combined marker-driver, I overexpressed the cell cycle regulators: *cyclin D*

[6] The *Acp26Aa* promoter does not become active until very late in pupal development (the protein is first detectable during the P13 stage, or about 12 hours prior to eclosion at 25 °C), whereas *Acp26Aa* RNA accumulates during the first 24 hours after eclosion (MONSMA & WOLFNER, 1988; MONSMA *et al.*, 1990). GAL80^{ts} is therefore not typically needed in this background to ensure that transgenes are active only in the adult (M. WAINWRIGHT, personal comments).

FIGURE 5. 34. | MAIN CELL NUCLEAR SIZE RESPONDS TO POSITIVE STIMULATION OF THE CELL CYCLE.

Main cell nuclei respond differently to manipulations that alter the nuclear size of secondary cells, but they seem to respond to up-regulation of the cell cycle.

A: Secondary cell nuclei (marked with pink arrows) are similar in size to those of the surrounding main cells (marked with yellow arrows) in control males.

B: When expressing the growth cyclin, *cyclin D*, and its kinase, *CDK4*, main cell nuclei increase in size.

C: Main cells expressing the transcription factor, *E2F1*, and its binding partner, *Dp*, show greatly enlarged nuclei.

D: When expressing a constitutively active form of the negative cell cycle regulator, RBF, the absolute size of main cell nuclei remain similar to the nuclei of the neighbouring secondary cells. However, this treatment also causes the secondary cells to become mononucleate.

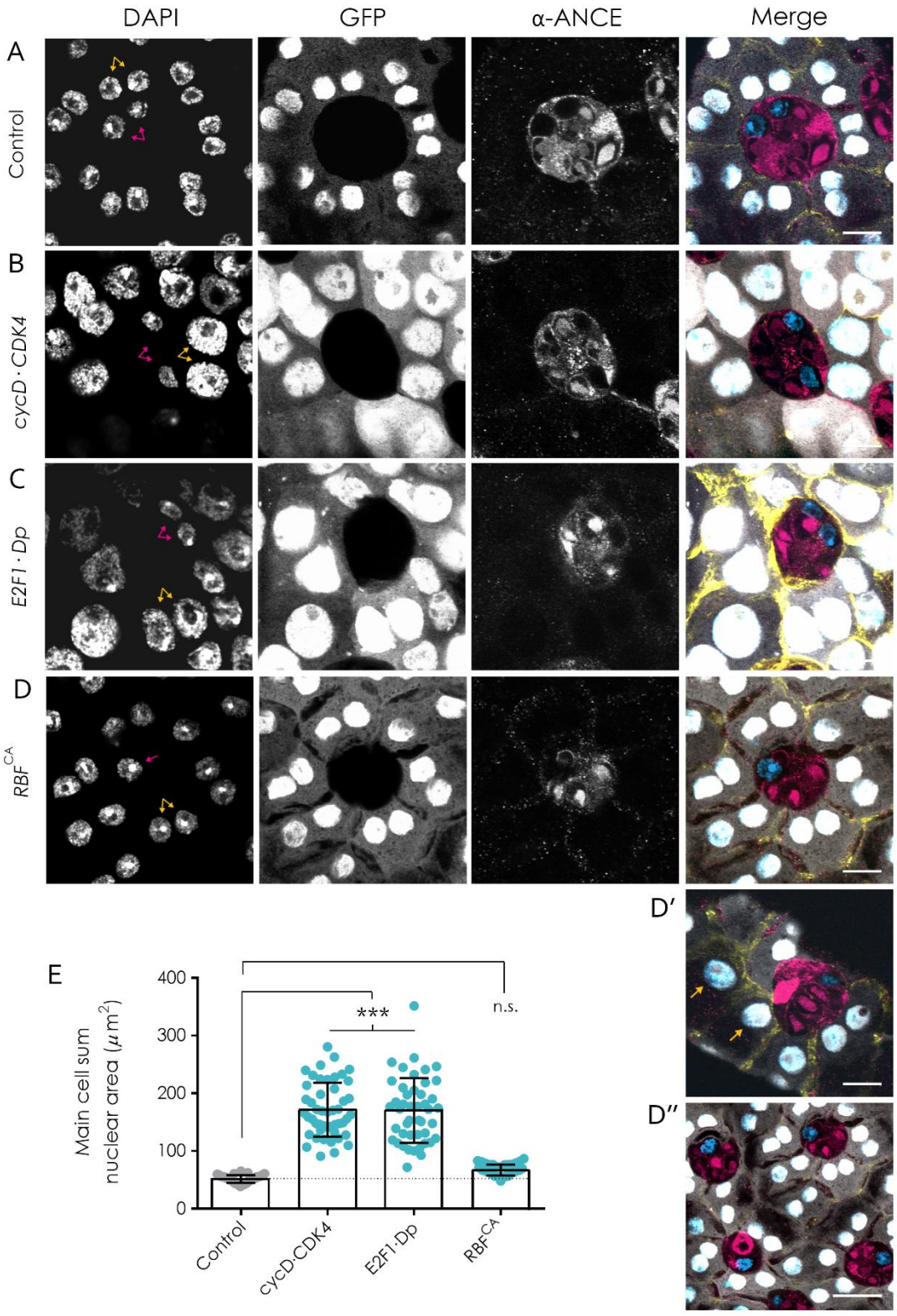
D': Main cells become only occasionally mononucleate following RBF^{CA} expression (yellow arrows).

D": When main cells express RBF^{CA}, all the neighbouring secondary cells become mononucleate—and at rates much higher than what has previously been seen when they themselves express the construct.

E: Quantifying the size of main cell nuclei shows that main cell nuclei are capable of growth following positive stimulation of the cell cycle.

Scale bars in panels **A–D'** correspond to 10 μm, whereas the scale bar in panel **D"** correspond to 20 μm. Epithelial cell junctions are labelled with α-FASIII (white). Secondary cells are stained with α-ANCE (magenta) and DAPI stains DNA (cyan).

The number of stars in panel **E** represent the *p*-value obtained by one-way ANOVA with Dunnett's correction for multiple comparisons (all columns compared to the relevant control): *** = $p < 0.0001$, whereas 'n.s.' denotes $p > 0.1$. Each dot represents the value of an individual MC:SC ratio, with three ratios determined per male (see **SUPPLEMENTARY TABLE 5. 18.**, *p.* 450). Error bars show the standard deviation within the sample.


DAPI
GFP
α-ANCE
Merge
A
Control
B
cycD · CDK4
C
E2F1 · Dp
D
RBF^CA
D′
D″
E
Main cell sum nuclear area (μm²)
400
300
200
100
0
***
n.s.
Control
cycD-CDK4
E2F1-Dp
RBF^CA

(*cycD·CDK4*); *E2F1* (*E2F1·Dp*); and *RBF* (RBF^{CA}). Both c*yclin D* and *E2F1* overexpression increased the size of main cell nuclei ($p < 0.0001$); suggesting that main cells, like secondary cells, are capable of responding to positive cell cycle stimuli (**FIGURE 5. 34**. **B–C, E**; **SUPPLEMENTARY TABLE 5. 18.**, *p.* 450). The DAPI intensity of the main cell nuclei also showed a significant increase in these backgrounds (data not shown); suggesting that the nuclei in main cells grow as a result of endoduplication—just like those of secondary cells expressing the same constructs.

Expressing *Acp26Aa* GFP > RBF^{CA} had no effect on the size of main cell nuclei ($p > 0.1$) (**FIGURE 5. 34**. **D**; **SUPPLEMENTARY TABLE 5. 18.**, *p.* 450), although it did cause the occasional main cell to become mononucleate (**FIGURE 5. 34**. **D'**). That expression of RBF^{CA} can cause mononucleation has been shown previously in secondary cells (LEIBLICH, 2015)—but at a higher frequency than observed in the main cells. (I have not, however, properly quantified the proportion of mononucleate main or secondary cells in my experiments). This might suggest that main cells are more resistant to RBF^{CA}-induced mononucleation than are secondary cells. However, when expressing A*cp26Aa* GFP > RBF^{CA}, I observed something very interesting: although the RBF^{CA} construct was expressed exclusively in the main cells, the *secondary cells* responded by becoming mononucleate (**FIGURE 5. 34**. **D"**).[7] This suggests that main cells might be able to share transcripts with secondary cells. This has been proposed previously by SITNIK & GLIGOROV *et al.* (2016) to account for abnormal processing of Ovulin protein in the absence of functional secondary cells in the male accessory gland. Indeed, Ovulin protein can be detected in the

[7] After two and a half years of near-daily confocal imaging of accessory gland material, I have not seen a mononucleated secondary cell in a non-RBF^{CA} background more than once. Trinucleate secondary cells also occur, but are equally rare.

secondary cells at high levels, especially in unmated males (MONSMA & WOLFNER, 1988).

DISCUSSION

Secondary cell nuclear size is dynamic, and changes over time and after mating. Previous work in our laboratory has identified BMP signalling as a key pathway regulating secondary cell nuclear size, but the mechanisms underlying this growth have remained unknown. In this Chapter, I have characterised how the size of the secondary cell nuclei changes over time in wild-type flies, and I have further examined how the nuclei behave in response to mating. Together with Siamak Redhai, I have shown that mating activates BMP signalling, and that this signalling is required for the mating-dependent growth to occur. Building on Aaron Leiblich's work showing an important role for the EcR and its interaction with BMP signalling in the secondary cells, I have further shown that the EcR is required for the mating-dependent nuclear growth to occur; implicating the BMP/EcR signalling axis in this process.

In addition, results in this Chapter also show that regulators of the cell cycle can promote secondary cell nuclear growth, and that enlarged secondary cell nuclei contain giant chromosomes. Finding that continued overexpression of the S-phase cyclin, Cyclin E, effectively prevents the growth of secondary cell nuclei in response to mating suggests that there is a causal relationship between secondary cell nuclear growth and increases in the secondary cell DNA content. Using EdU labelling to detect newly-synthesised DNA, I confirmed this relationship by showing that molecular treatments that alter secondary cell nuclear size are associated with DNA synthesis. DNA synthesis is also exclusively promoted by mating; suggesting that

mating-dependent DNA content increases represents a physiologically-relevant response. Indeed, when the E2F1 transcription factor is knocked down, although the mating-dependent increase in DNA synthesis is reduced, secondary cell nuclei still grow in response to mating. This suggests that there might be two different mechanisms by which secondary cell nuclei grow after mating: one which requires DNA content increases, and one that does not. Altogether, the results presented in this Chapter demonstrate that the secondary cells of the *D. melanogaster* male accessory gland undergo DNA content increases in response to mating, and that this process is regulated by the cell cycle and the BMP/EcR signalling axis.

Two mechanisms of nuclear growth in secondary cells: mating-independent and mating-dependent

As shown in this Chapter, the nuclei of both main cells and secondary cells increase in size over the first three days after eclosion. Both cell types help to synthesise the proteins that make up the accessory gland secretions, and I think it plausible that there is a relationship between the synthetic activity of the accessory gland epithelium and the accumulation of proteins in the gland lumen. However, does the size of cell nuclei correlate with the synthetic activity of the cell(s)?

In the spermatocytes of the Hemipteran, *A. albopunctatus*, there is a relationship between the size of the nuclei and the amount of water and protein they contain (SCHRADER & LEUCHTENBERGER, 1950). A similar relationship has been observed in rat hepatocytes (LEUCHTENBERGER & SCHRADER, 1951), where circadian increases in the cell size has been shown to correlate with higher levels of ribosomal RNA and protein synthesis (SINTUREL *et al.,* 2017). This suggests that nuclei can grow vas the result of increasing biosynthetic activity and/or an increasing

nuclear protein or RNA content. That the size of both main cell and secondary cell nuclei increase during the first days of adult life, when the male accessory gland fills up with secretions is interesting to consider in this context. In addition, that mating also increases secondary cell nuclear size—and the rate of protein translation (SCHMIDT *et al.*, 1985)—fits with this model. For example, MONSMA *et al.* (1990) found that mating two week-old males increases the expression of *Acp26Aa* to levels even higher than those observed in young adult males.

The size of a cell or its nuclei does not necessarily reflect its biosynthetic activity. For example, LEUCHTENBERGER & SCHRADER (1951) found no relationship between nuclear size and nuclear protein contents in the chief and parietal cells of the rat gastric tissues. Yet, I still believe it very possible that the size of the secondary cell nuclei reflects their biosynthetic potential. First, consider the difference in the nuclear sum area between the secondary cells of individual six-day old virgin males of either the wild-type (w^{1118}), or the *esg F/O*^{ts} genotype: in the wild-type, the secondary cell nuclei average 39.9 ± 8.5 μm^2 (**FIGURE 5. 6.**, *p.* 244)—and 49.6 ± 8.9 μm^2 (**FIGURE 5. 11.**, *p.* 252) in *esg F/O*^{ts} males. In the latter genotype, the secondary cell nuclei contain a nuclear-localised GFP that is expressed at high enough levels to flood the cytoplasm—and the average nuclear sum area is more than one standard deviation larger. Is it possible that expressing a nuclear protein at high levels is sufficient to increase the size of the nucleus (possibly also by affecting the size of the late endosomes and lysosomes in the cell)? I would be eager to investigate this in more detail. For example, it would be interesting to use *esg F/O*^{ts} flies to create 'flipped' mosaics in the accessory gland (as described in LEIBLICH *et al.*, 2012). This would allow the comparison of the nuclear size of GFP-positive and GFP-negative secondary cells to determine how much the presence of the nuclear GFP contributes

to secondary cell nuclear size. To determine how much the GAL4 protein itself (also a nuclear protein) contributes to secondary cell nuclear growth would also be interesting. In the absence of such data, we already know that secondary cell nuclei grow after mating—even in the absence of DNA synthesis. (Recall that **FIGURE 5. 29. E** (*p.* 289) shows that mating increases the size of the secondary cell nucleus—even when it does not incorporate any EdU.) It is possible this is because the amount of protein in the nucleus has increased, or because of de-condensation of the resident DNA. A similar result is obtained when mating male flies lacking *E2F1* in their secondary cells (**FIGURE 5. 32.**, *p.* 295): where (based on the DAPI staining intensity)the DNA content does not appear to increase—despite the nuclei growing larger.

Although an increase in the nuclear protein content after eclosion and after mating needs to be confirmed, I think these observations suggest that there might be two different mechanisms underlying the growth of secondary cell nuclei: one that is driven by increases of the nuclear protein content (and other macromolecules, DNA de-condensation, *etc.*); and one that depends on increases of the nuclear DNA content. Whereas the former might correlate with the biosynthetic activity of the cell in both virgin and mated males, the latter seems to be entirely dependent on mating, in the absence of genetic manipulation. Therefore, I would like to refer to these different mechanisms as 'mating-*independent*' and 'mating-*dependent*' mechanisms of nuclear growth, respectively. Both mechanisms might involve increases in the nuclear protein content, but only the mating-dependent growth involves an increase of the nuclear DNA content.

BMP signalling regulates the mating-dependent nuclear growth via EcR-dependent endoreplication

A key aim of my *D. Phil.* was to better understand the mechanisms underlying the mating-dependent growth of secondary cell nuclei. In this Chapter, I have shown (in collaboration with Siamak Redhai) that mating promotes BMP signalling activation. In addition, building on Aaron Leiblich's *D. Phil.* work showing that BMP signalling interacts with the EcR to drive secondary cell nuclear growth (LEIBLICH, 2015), I have shown that the BMP/EcR signalling axis is required for the mating-dependent nuclear growth. Altogether, these results allow us to construct a model of how BMP signalling interacts with the EcR to regulate the growth of secondary cell nuclei (see **FIGURE 5. 35.**). In this model, Dpp is carried on the secondary cell secretory granules (REDHAI *et al.*, 2016). Mating mobilises secretory compartments, releasing the content of the secretory granules into the accessory gland lumen (REDHAI *et al.*, 2016)—to replenish the contents depleted by mating. In the gland lumen, Dpp can interact with its heterodimeric receptors (including Thickveins) that are present on the secondary cell plasma membrane; forming an autocrine BMP signalling loop. This promotes the phosphorylation of the transcription factor, Mad, in secondary cells. Phosphorylated Mad (pMad) complexes with its co-factor, Medea, and the resultant pMad·Medea complex translocates to the nucleus—where it can regulate BMP-dependent gene transcription.

Activated BMP signalling also stabilises EcR protein levels via an unknown mechanism. Results from Leiblich's work suggest part of this mechanism is post-transcriptional and involves the EcR N-terminal domain. It is possibly dependent on silencing of the EcR transcript or, more likely, degradation of the EcR protein when BMP signalling is reduced (LEIBLICH, 2015). Stabilisation of the EcR protein

results in elevated EcR protein levels, allowing the EcR to regulate the transcription of its target genes. Further work will confirm that the EcR (either directly or indirectly) regulates the genes involved in the initiation of the cell cycle. The failure of the secondary cell nuclei to grow in the absence of EcR protein suggests a simple model of secondary cell nuclear growth, where the EcR regulates the expression of genes like *cyclin D* and *cyclin E* to initiate an endocycle that increases the DNA content, which promotes secondary cell nuclear growth. Increases in the cellular DNA content will also increase the number of gene copies available to the cell—and therefore (theoretically) its biosyntethic potential. Importantly, BMP signalling has been shown to promote endoreplication in *C. elegans* (FLEMMING *et al.,* 2000), and the EcR regulates endocycle-dependent gene amplification in the *D. melanogaster* ovary (TZOLOVSKY & DENG *et al.,* 1999). This suggests that although the BMP/EcR axis might (so far) be unique to secondary cells, the individual signalling axis components parrticipate in the regulation of endoduplication in other tissues and organisms.

Does Hedgehog signalling negatively regulate BMP signalling and/or BMP-dependent endocycling in secondary cells?

Cyclin D and Cyclin E are important regulators of the mating-dependent nuclear growth in secondary cells, and this might explain why Hedgehog signalling appears to be a negative regulator of the BMP/EcR-dependent nuclear growth. As we saw in CHAPTER 4, knocking down *hedgehog* can reduce the size of the secondary cell nuclei, whereas forced overexpression of Hedgehog target genes can increase it. However, as shown in this Chapter, the nuclei of secondary cells expressing hh^{RNAi} can still grow after mating, whereas the nuclei of cells expressing *Ci::VP16* (which are

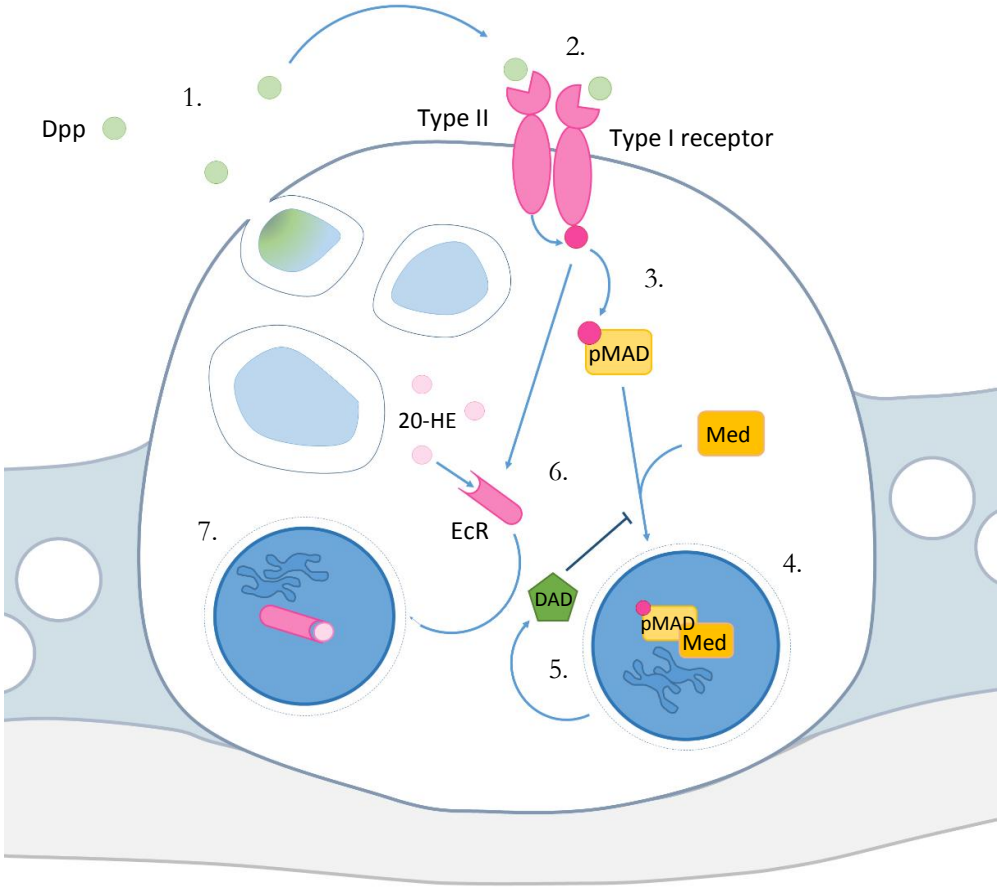


FIGURE 5. 35. | THE BMP AND ECR SIGNALLING PATHWAYS INTERACT TO REGULATE SECONDARY CELL BIOLOGY.

Secondary cells produce the BMP pathway ligand, Dpp (light green), which is packed into exocytic compartment secretory granules, to be released into the accessory gland lumen (1). Luminal Dpp is able to interact with BMP receptor molecules (2; pink), which are present on the secondary cell membrane. Dpp binding allows receptor dimerisation and phosphorylation of a type I receptor by its type II dimerisation partner, activating the pathway. BMP pathway activation leads to the phosphorylation (dark pink) of the intracellular signalling component, Mad (light yellow) (forming pMad) (3), which complexes with Med (dark yellow). The pMad·Med complex translocates to the nucleus, where it can function as a transcription factor to regulate the expression of BMP signalling target genes; some of which cause the size of secondary cell nuclei to increase (4). One target gene of the BMP signalling pathway is the pathway suppressor, Dad (dark green), which completes the pathway through the formation of a negative feedback loop (5). BMP signalling also regulates the activity of the steroid-sensitive nuclear receptor, EcR (pink), allowing levels of EcR protein to accumulate in the cell (6). The EcR also has transcription factor activity, and regulates the expression of genes involved in secondary cell nuclear growth (7). The steroid hormone, 20-hydroxyecdysone (20-HE), is shown in light pink.

already enlarged in virgin males) do not grow further after mating. Does this indicate that Hedgehog signalling is a negative regulator of the mating-dependent nuclear growth? During development in *D. melanogaster,* Hedgehog signalling regulates the expression of *dpp* in diverse tissues (STRIGINI & COHEN, 1997; SÁNCHEZ & GUERRERO, 2001). Therefore, Hedgehog signalling might regulate the expression of *dpp* also in secondary cells—although further work will be required to determine if this is the case (as discussed previously in CHAPTER 4).

Hedgehog signalling can regulate the expression of *cyclin D* (directly) and *cyclin E* (indirectly) in *D. melanogaster* (DUMAN-SCHEEL *et al.,* 2003). It is possible that these genes are regulated by Hedgehog signalling also in secondary cells (although, again, further work (*e.g.* using reporters, or qRT-PCRs) will be needed to confirm this). Nevertheless, my work suggests that this might be the case. For example, Ci::VP16 promotes the synthesis of new DNA in secondary cells (preliminary data not shown), and the nuclei of cells expressing *Ci::VP16* do not grow after mating. Both of these phenocopy the phenotypes of cells overexpressing *cyclin E*. However, this cannot be the complete story, since nuclear localisation of pMad is increased in secondary cells in the absence of Hedgehog signalling, and nuclear pMad is appears to be completely absent in cells expressing *Ci::VP16* (preliminary data not shown). These results suggest that BMP signalling might be negatively regulated by Hedgehog signalling.

Further work will be required to understand the exact relationship between Hedgehog signalling, BMP signalling, and the growth of secondary cell nuclei—as well as to determine whether the BMP/EcR signalling axis might have a role regulating Hedgehog signalling, in turn. As discussed in CHAPTER 4, informative experiments would involve qRT-PCR to determine patterns of gene expression in different genetic backgrounds (*e.g dpp* expression in the absence of *hedgehog*; *hedgehog*

expression in the absence of *TRR* or *EcR*, *etc.*). In addition, it might be interesting to evaluate the role of Notch ("**N**") signalling in secondary cells, since Notch can regulate endoduplication in combination with Hedgehog signalling in other cell types in *D. melanogaster* (*e.g.* SUN & DENG, 2007). Such work is likely to be informative, since I have found that Notch might be present in the secondary cells (APPENDIX 8: *p.* 451). In addition, Notch interacts with another protein, Numb (knock-down of which creates an intracellular trafficking phenotype in secondary cells; data not shown), whose activity can be modulated by non-apoptotic DRONC in *D. melanogaster* neural stem cells (OUYANG *et al.,* 2011).

Is there a threshold governing BMP signalling activation and endoreplication after mating?

Only some secondary cells show nuclear translocation of pMad and BMP/EcR-dependent endocycling after mating. It would be interesting to explore if this is because a threshold level of BMP signalling activation is required for these processes to result. In experiments in our laboratory, we find that only 30 % of secondary cells show evidence of BMP signalling activation and DNA synthesis after mating. This is intriguing. Is it possible that only 30 % of secondary cells are normally capable of activating BMP signalling at any given time, and if so, why? Our experiments have shown that when BMP signalling is activated ectopically using the activated BMP receptor, Tkv^{Q253D}, the majority of secondary cells show evidence of pMad nuclear translocation (REDHAI *et al.,* 2016) and DNA synthesis. This suggests that the majority of secondary cells are able to respond to BMP signalling when forced to, but that they do not do so normally. Do levels of Thickveins receptor protein vary across the secondary cell population at any given time, for example? Secondary cells, with

their large intracellular compartments, would make the detailed study of intracellular compartment dynamics and protein localisation simple, and they would therefore make more detailed examination of BMP receptor localisation easy to perform. In addition, such work in combination with more molecular approaches, such as single-cell and population-level transcriptomics might also shed light on the differences that exist among these cells. Such differences have been shown to have important functional consequences in other cell types during both development and different disease processes—such as in cancer and diabetes (*e.g.* DUECK *et al.,* 2016; DENG & RAMSKÖLD *et al.*, 2014; BURRELL & MCGRANAHAN *et al.*, 2013; PIPELEERS, 1992). Another interesting question to ask is: is it the same 30 % of secondary cells that activate BMP signalling and undergo endoreplication after multiple matings? This would be simple to determine by combining EdU labelling with pMad antibody staining on mated accessory gland material.

Endocycle-driven genome duplication and gene amplification in secondary cells

As shown in this Chapter, mating causes the secondary cell DNA content to increase. When I measured the DAPI staining intensity of secondary cell nuclei in six day-old males before and after mating, I found that mating increases the secondary cell DNA content by approximately 25 % (*p.* 290). If TANIGUCHI *et al.* (2012) are correct that secondary cells have 8 copies of the haploid genome (8C), a 25 % increase would yield a C-value of 10. However, 10C does not correspond to a doubling of the virgin 8C value, suggesting that mating does not cause a complete duplication of the secondary cell genome. However, only a subset of secondary cells (about 30 %) seems to undergo DNA content increases in response to mating. That

only about 30 % of secondary cells incorporate EdU after mating corresponds (reasonably) well to the approximate 25 % increase in the average DNA content after mating. This suggests that if mating induces a complete duplication of the secondary cell genome, this happens in only 1 out of every 3 secondary cells. My data therefore suggest that the secondary cells are not a homogenous population, and that they respond differently to the stimulus of mating. Interestingly, when I performed the α-pMAD staining experiments with Siamak Redhai, we again found that only about 30 % of secondary cells showed nuclear translocation of pMAD in response to mating (**FIGURE 5. 10.**, *p.* 251). Altogether, these data suggest that only about one-third of secondary cells is competent to activate BMP signalling and to increase the DNA content in response to mating. It is tempting to speculate that these are the same 30 % of cells. The matter is all the more curious, because 30 % of secondary cells are positive for pMad following two different mating regimes (immediately after mating, or after six days of co-culture with seven females to each male): does this mean that only one in every three secondary cells is 'functional' and able to respond to mating-dependent cues?; or does this suggest that there is a threshold level of some molecular competence that is required for a secondary cell to respond to these mating-dependent cues, and that only one in every three cells can reach this competence threshold at any one time?

An additional explanation for why the secondary cell DNA content only increases by 25 % after mating is that the DNA is incompletely replicated. This is the case in the larval salivary gland, or in the *C. elegans* epidermis (LOZANO *et al.*, 2006). Indeed, the giant chromosomes that I have observed in the secondary cells very much resemble those of the larval salivary gland. This might suggest that the secondary cell DNA is incompletely replicated, causing sister chromatids to stick

together to produce the rope-like structures. However, this scenario does not explain why only 30 % of secondary cells incorporate EdU even after several mating events. Indeed, if only 30 % of secondary cells undergo incomplete DNA replication, the average DNA content per cell would be much lower than 25 %. The similarity between the giant chromosomes in the secondary cells and those of the salivary gland cells, also raises the question: can anything like the salivary chromosome 'puffs' be observed on the secondary cell chromosomes, and if so, can their appearance be stimulated by mating? (Originally described by BRIDGES (1935), the puffs that can be observed along the salivary gland chromosomes have since been shown to be regions of active gene activity, and their appearance can be induced by ecdysone (ASHBURNER, 1971).) It would be interesting to probe secondary cells with EcR antiserum, to see if any interaction between the EcR and the DNA can be detected. Given the small size of the secondary cell chromosomes in unmated glands, high-resolution microscopy would be useful to address this question.

Having established that the secondary cell DNA content increases after mating, one wonders: What is the physiological relevance of this? As mentioned, each secondary cell is polyploid: possessing eight haploid genome copies per cell. Polyploidy is a common feature of metabolically active cells, since each additional genome copy increases the cell's synthetic potential. Famous examples of metabolically active and polyploid cells in the fruit fly are the cells of the larval salivary gland, and the nurse cells of the female ovary (ZHIMULEV & KORYAKOV, 2009; CALVI *et al.*, 1998)—where increased ploidy promotes the synthesis of more salivary glue proteins and proteins required for egg-shell formation, respectively. However, polyploid cells can also be of economic importance, since the plant endosperm (which nourishes the angiosperm plant

embryo within its seed, and which forms much of the edible portion of many seeds and grains) can be highly polyploid (KOWLES & PHILLIPS, 1985; DANTE *et al.,* 2014).

Polyploidy can be systemically important in animals. In addition to the larval salivary gland, the prothoracic gland of the *D. melanogaster* larva is also highly polyploid, and it has an important role in the timing of larval development and metamorphosis (MIRTH *et al.*, 2005). The prothoracic gland is a source of the steroid hormone, ecdysone, which promotes the progression of the larva through its developmental stages and determines the point when the larva undergoes puparation and metamorphosis. To do so, the cells in the prothoracic gland undergo nutrient-dependent endocycling that is mediated by the insulin receptor, and the nutrient-sensor, dTOR (OHHARA *et al.,* 2017). As a result of this endocycling, the gland acts like a nutrient-dependent 'molecular clock' that effectively 'times' the development of the larva. This illustrates how tissue-specific endocycling can be physiologically (and developmentally) relevant. Nutrient-regulated endocycling has also been observed in other cell types in *D. melanogaster* (*e.g.* enterocytes (CHOI, LUCCHETTA & OHLSTEIN, 2011)). Interestingly, nutrient availability has been shown to affect the male's ability to induce the female post-mating response in *D. melanogaster* (FRICKE *et al.,* 2008). It would therefore be interesting to determine if secondary cell endoduplication is also nutrient-dependent—and if this somehow affects the fertility of the male. Happily, such experiments are already underway in our laboratory, and promise to yield some interesting results (P. MARIE & C. WILSON, personal comments).

A role for *patched*-positive neurons and mechano-sensing in male and female reproductive tracts and post-mating physiology?

Although we have an idea of what effects mating has on the post-mating physiology of the male accessory gland (*i.e.* increased biosynthetic activity), many questions remain. For example: how does mating induce the increased biosynthesis? Few direct experiments in either our laboratory or the literature at large have investigated the purely mechanical relationship between mating (gland emptying) and protein synthesis (gland content recovery/replenishment). As shown in the previous Chapter, and as examined in more detail by RUHMANN *et al.* (2016), increases in the male accessory gland secretory content causes the accessory gland to increase in size. The total protein content contained within the glands of both young and very old males however seems to be relatively similar (SANTHOSH & KRISHNA, 2013; REZAEI *et al.*, 2015). This might be the result of decreased secretory activity over time, and does suggest that there is an upper limit to the amount of secretions the lumen can contain without forcing a rupture of the epithelium.[8] Therefore, accepting the propositions…

a) that there is an upper limit to the volume possible to contain in the accessory gland; and,

b) that mating opportunities may not materialise for an individual male; and,

c) that the male does not empty accessory gland contents in the absence of mating (in virgin males, no GFP-labelled accessory gland lumen proteins are ever seen outside of the gland);

[8] Dissection of the ventral nerve cord in the mosquito, *Aedes aegypti,* causes the female mosquito to fill up with blood up to, and beyond, the point of rupture (GWADZ, 1969). This suggests that there are feedback mechanisms that prevent the over-distension of the internal organs.

… there are two solutions to the problem of excessive accessory gland distension:

1) that accessory gland contents can be re-absorbed in the absence of mating; or,
2) that the secretory activity of the accessory gland epithelium is somehow responsive to the amount of secretions already contained within the gland.

Supporting evidence for solution 1) is provided by electron micrographs of the accessory gland epithelial surface (*e.g.* CORRIGAN, 2014; B. KROEGER, personal comments), clearly showing microvilli-like projections on the surface of main cells. Microvilli are specialised for absorption, and if these structures are indeed microvilli, it is possible that they are involved in main cell absorption of accessory gland lumen contents. There is also evidence to suggest that the seminal fluid protein content becomes increasingly concentrated over time in the absence of mating (M. WAINWRIGHT & C. WILSON, personal comments). Another, possible scenario is that the secretory activity of the accessory gland epithelium is sensitive to the amount of secretions already contained within the gland lumen.

Physiological responses to stretching have been described for other extendable structures in insects—like the growing mosquito nymph and the mosquito stomach following a blood meal. In the mosquito, *Rhodnius prolixus*, distension of the cuticle of the growing nymph provides a mechano-sensory stimulus that induces the secretion of juvenile hormone, (JH; also known as 'moulting hormone') from the nymph's *corpora allata*[9] (WIGGLESWORTH, 1936). Similarly, in *A. aegypti*, abdominal distension during a blood feeding session provides a mechano-sensory stimulus that induces the secretion of JH (KLOWDEN, 1987)—a hormone that is required for insect oocyte development (WYATT & DAVEY, 1996). *T*here is a role for JH also

[9] The *corpora allata* is a gland located in the insect head, and which is the source of juvenile hormone. Interestingly, the cells in this gland grow (by undergoing endoreplication to cause an increase of their cytoplasmic volume) when actively secreting the hormone (WIGGLESWORTH, 1936).

in the male accessory gland: where the topical application of JH or ecdysone onto the abdominal cuticle can mimic the mating-dependent increase in protein synthesis in the male accessory gland (YAMAMOTO *et al.*, 1988; HERNDON *et al.,* 1997). Interestingly, HERNDON *et al.* (1997) speculate that:

> [T]he physical process of emptying the accessory gland of its secretions triggers the release of a hormone, such as JH, and causes an increase in protein synthesis in the accessory gland following mating. [*p.* 1121]

HERNDON *et al.* (1997) also refer to work by ROTH & STAY (1961) who, using the cockroach (*Diploptera punctata*) showed that mating can stimulate the female *corpora allata*. This mating-dependent stimulation is however not through the act of copulation *per se*, but is rather induced through receipt of the male ejaculate into the female copulatory pouch (the '*bursa copulatrix*'). When the ejaculate is applied directly to the *bursa,* it is insufficient to induce ovulation. This rules out a biochemical role for the ejaculate in the regulation of female ovulation (ROTH & STAY, 1961). Rather, the ejaculate seems to compose a purely mechanical stimulus, and for two reasons:

1) mating does not stimulate the *corpora allata* in females whose nerve cord was severed prior to mating; and,
2) the insertion of small objects (*e.g.* glass beads) into to the female *bursa* is sufficient to induce ovulation (ROTH & STAY, 1961).

Altogether, it therefore appears as if mating in *D. punctata* relies on mechano-sensory cues to stimulate JH release by the *corpora allata,* and thus oocyte development, and that these cues are provided by the male as he fills the female *bursa* with his ejaculate during the copulation. A mechanistic role for the male ejaculate in the hormone-dependent regulation of oocyte development therefore suggests the existence of sensory receptors in the female reproductive tract that are capable of sensing tissue stretching and relaying this information to the endocrine system.

Mating induces an increase in both JH and ecdysone also in the *D. melanogaster* female (MOSHITZKY *et al.,* 1996; HARSHMAN *et al.*, 1999), and this increase forms part of the female post-mating response. The *D. melanogaster* female reproductive tract contains two anatomical structures specialised for sperm storage and release: the seminal receptacle and the *spermathecae* (LEFEVRE & JONSSON, 1962). Interestingly, and as we saw in CHAPTER 4, both of these structures seem to be innervated with neurons that can be selectively labelled with *ptc* > GFP. That the apparent cell body of these neurons is visible in close association with the innervated tissue suggests that they might be sensory neurons, and their location suggests that they might be specialised stretch-sensory neurons; perhaps responsible for sensing the distension of the female sperm storage organs after mating (S. GOODWIN, personal communication). It is very possible that these neurons have been identified previously through the use of other neuronal markers, and it would be interesting to determine their exact identity through co-labelling experiments and determine if these neurons are capable of responding to Hedgehog or seminal fluid-induced distension of the female reproductive organs.

Also described in CHAPTER 4 is the finding of neurons innervating the male accessory gland, which are similarly positive for *ptc* > GFP. Given the speculation above, that there might be a mechano-sensory role in monitoring the extent of accessory gland epithelial stretching and lumen content, these neurons provide a very attractive starting-point for more detailed description and investigation of how the biosynthetic dynamics of the *D. melanogaster* male accessory gland are regulated whole-scale in response to sexual activity levels. Indeed, it is tempting to speculate that mating induces signalling through, not only the BMP signalling axis described by LEIBLICH *et al.* (2012) and REDHAI *et al.* (2016), but also through a mechano-

sensory axis (relayed via *patched*-positive neurons); both of which are involved in regulating the rate of accessory gland secretory activity. Furthermore, the existence of these *patched*-positive neurons in the male reproductive tract (if they indeed are stretch-sensory) provides the possibility of a link both between accessory gland filling (after eclosion and after mating) and emptying (during mating) and the JH-mediated increase in accessory gland epithelial cell protein production that has been previously described.

Replenishment of gland contents in response to an external stimulus

The amount of secretions contained within the male accessory gland is crucially important for the male fly's reproductive success: males lacking accessory gland secretions are sterile (XUE & NOLL, 2000), and immature males with few secretions are less able to induce the full post-mating response in their mates (RUHMANN *et al.*, 2016). Mating depletes the accessory gland secretory content, thus creating a need for the secretions to be replenished if consecutive mating events are to be successful. Might mating-dependent endoduplication be important for this process?

Endoduplication increases the DNA content, making the cell increasingly polyploid, and increasing the number of gene copies available to the cell. Therefore, it is possible that the BMP activation that happens in response to mating might 'push' the secondary cell over a cell cycle threshold (STERN & NURSE, 1996) and thus act as a 'switch' to increase the secondary cell DNA content (and gene copy number). To investigate this is more detail, it would be interesting to determine how the male accessory gland protein content changes after mating (and recovery) when BMP/EcR signalling or endoreplication have been inhibited: will those glands

produce less protein after mating than those of control males? This could be tested by Western blotting, and probing for secondary cell-specific candidate proteins (like Dpp, Hedgehog, or ANCE), or by a proteomic analysis. WIGBY & SIROT *et al.* (2009) have also used enzyme-linked immunosorbent assays ("**ELISAs**") to good effect when quantifying accessory gland protein levels. In addition, it would be interesting to determine if levels of EcR protein change after mating and whether this correlates with activation of BMP signalling in specific cells.

In the wild, *D. melanogaster* gather to mate on fermenting fruit and vegetable matter (MARKOW, 2015), and sexual activity occurs when male flies find themselves in the proximity of females (SPIETH, 1974). A successful mating might therefore often be associated with the presence of additional females; increasing the utility of elevated production of male accessory gland proteins in preparation for future mating opportunities. Each mating might therefore promote secondary cell DNA content increases—to increase the specialisation (and terminal differentiation) of the cells.[10] However, this might need the percentage of cells positive for EdU incorporation to increase in line with the number of matings, which is something that should be determined. Yet, it is interesting that the biosynthetic potential of secondary cells might increase after mating and that this might be facilitated by endoreplication-driven gene amplification—since this suggests that successful mating events might increase the success of future mating events. In effect, this means that mating (which depletes the accessory gland contents) might also facilitate their replenishment (by increasing protein synthesis by increasing the number of available

[10] Endoreplication-driven increases of the DNA content do not appear to be reversible in the absence of mitosis (EDGAR *et al.,* 2014). In addition, I do not see any decreases in the nuclear DNA content (measured as DAPI intensity) in treatments that reduce the size of the secondary cell nuclei, like overexpression of *Dad*, or knocking-down *hedgehog* or *E2F1* (data not shown).

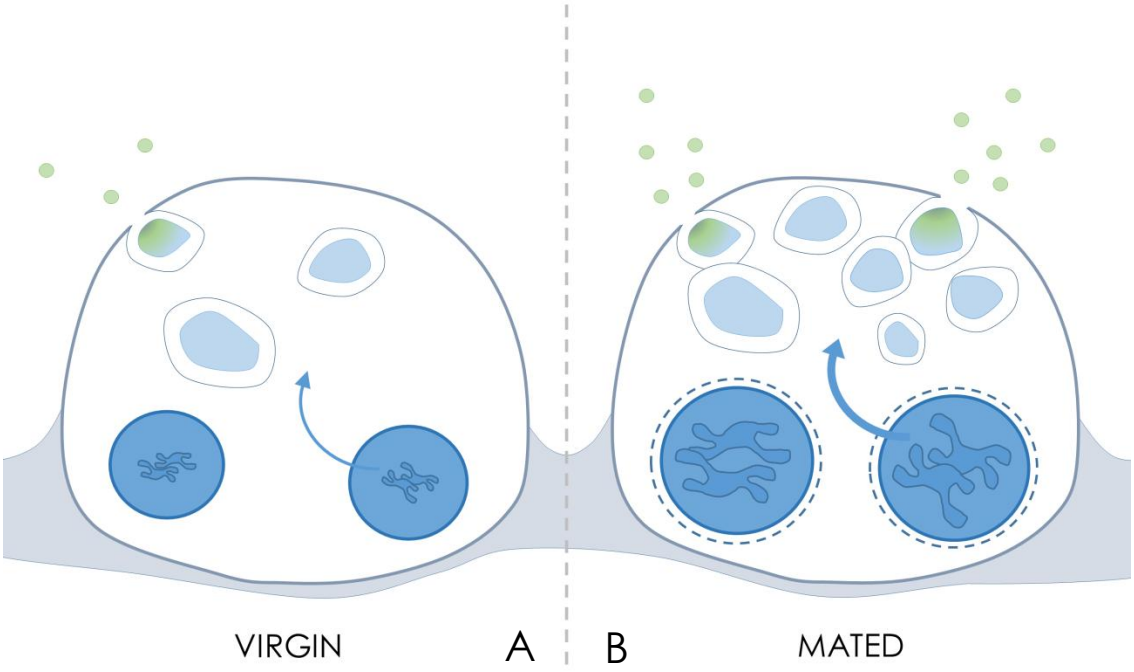


FIGURE 5. 36. | MODEL SHOWING HOW ENDOREPLICATION-DRIVEN GENE AMPLIFICATION MIGHT PROMOTE THE REPLENISHMENT OF MALE ACCESSORY GLAND CONTENTS AFTER MATING.

A: Secondary cells synthesise protein products, which are packaged onto secretory compartments and released into the gland lumen. In the glands of mature, but virgin, male flies this occurs at low levels.

B: BMP/EcR signalling axis activation after mating promotes mating-dependent endoreplication—increasing the secondary cell DNA content. Amplified gene numbers permit higher synthetic activity; increasing the amount of protein synthesised by the secondary cell and secreted into the gland lumen, to replenish accessory gland contents.

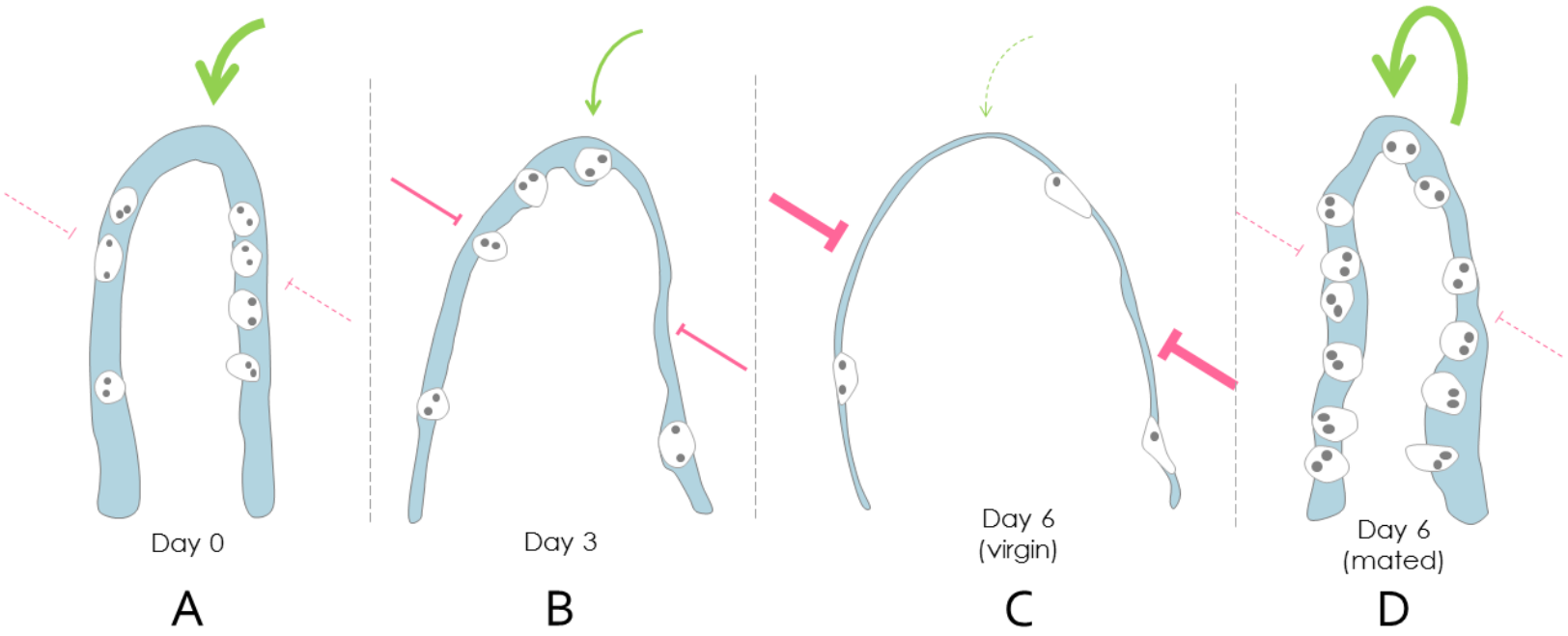


FIGURE 5. 37. | MODEL SHOWING HOW DIFFERENT REGULATORY INPUTS MIGHT BALANCE THE AMOUNT OF SECRETORY CONTENTS IN THE MALE ACCESSORY GLAND.

A: After eclosion, the gland contains few secretions. There is a strong regulation to promote the gland secretory output (green arrow).

B: In the gland of young males, containing sufficient secretions to support his reproductive functions, but too few to distend the gland fully, there is an increasing balance between the regulatory inputs to promote gland filling (green arrow) and to prevent gland overfilling (pink blunt arrows).

C: In the gland of older males, containing sufficient secretions to completely distend the accessory gland, there is very weak regulation promoting the production of further secretions (green arrow), and very strong regulation preventing this process (pink blunt arrows).

D: In the gland of mated males, the secretory content is partially depleted. Again, there is strong regulation to promote the gland secretory output (green arrow), and the regulation to prevent gland overfilling is very weak (pink blunt arrows).

gene copies) (**FIGURE 5. 36.**). This mechanism would work alongside other mechanisms that prevent the gland from overfilling, to ensure the accessory gland secretory content is optimised—both after mating, and in the absence thereof (**FIGURE 5. 37.**).

Does mating promote the secretion of seminal fluid in other organisms? Tantalising lines of evidence are available. For example, in rats, copulation is associated with increased secretory activity of the cells in the anterior prostate (HAWKINS & GEUZE, 1977). Ejaculation has also been associated with elevated levels of serum testosterone and elevated levels of *AR* mRNA in the ventral prostate of the rat (HERNANDEZ *et al.,* 2007). Because these effects developed soon after ejaculation, they were thought to be due to nerve signalling activity (HERNANDEZ *et al.,* 2007)—but is it possible that depletion of the seminal fluid might have played a role? This would be interesting to study further. Studies of human volunteers from fertility clinics have also helped improve our understanding how seminal fluid volumes are regulated. For example, it has been shown that the volume of the ejaculate increases (up to a point) over the time spent abstinent, and that it thereafter declines (COOPER *et al.,* 1993; SCHWARTZ *et al.,* 1979). This suggests that the seminal fluid content of the male rat or human might be regulated similar to the fruit fly, where the male accessory glands seem to produce secretions up to the point they can be comfortably accommodated by the gland(s), and effectively not produced thereafter in the absence of gland emptying. Whether this regulation in fruit flies and mammals makes use of similar regulatory outputs, however, remains to be established.

If seminal fluid is produced in 'bursts' after ejaculation, this process might be locally regulated (rather that systemically), and it might be regulated via either tissue

stretching or via an inhibitory factor that is present in the secretions themselves. Consider, for example, the case in mammals (including humans), where milk production has been shown to scale with demand (HARTMANN *et al.,* 1994) (presumably to keep up with the increasing appetite of a growing infant). In goats, selective milking of one udder has been shown to increase the lactation volume in that udder only (HENDERSON *et al.,* 1983), and replacing the milk with equal volumes of isometric fluids does not prevent further secretion (HENDERSON & PEAKER, 1984). This is because milk has been shown to contain a protein that acts as a local, autocrine inhibitor of lactation (WILDE *et al.,* 1995). We do not know if the seminal fluid of flies or mammals contain similar regulatory factors, or if such regulation might be mediated via a stimulus like the volume of the secretions themselves. Indeed, I do not think that we fully understand the dynamics of gland emptying and re-filling in the male reproductive system, and I think this would represent an interesting venue of future research. As my work has shown, the *D. melanogaster* male accessory gland would make an attractive model for such questions to be further explored.

Endoreplication might depend on the EcR, but also be steroid-independent, and affect the production of steroids by the secondary cells

An additional consequence of secondary cell endoreplication might be to increase the male fly's ecdysone titre. Ecdysone is important for male courtship behaviour: specifying the identity of neurons involved in preventing male-male courtship (DALTON & LEBO *et al.,* 2009), and regulating the formation of courtship memories (ISHIMOTO *et al.,* 2009). Ecdysone has also been shown to promote the biosynthetic potential of the male accessory gland (HERNDON *et al.,* 1997). The

secondary cells of the male accessory gland are a potential site of ecdysone biosynthesis (HENTZE *et al.,* 2013), and it has been shown that mating increases the ecdysone titre of mated males (A. LEIBLICH & C. WILSON, unpublished results). Therefore, it is possible that endoreplication in secondary cells might promote an increase in ecdysone biosynthesis. (LEIBLICH (2015) also suggested an autocrine role for some ecdysone biosynthesis genes in secondary cell growth.) It is also possible that most of the hormone is derived from a separate tissue in the adult male, and that this tissue also can respond to mating-dependent cues (A. LEIBLICH, personal comments). Perhaps there is a role for juvenile hormone in this process as well, as discussed above?

Intriguingly, ecdysone might not actually be required for the mating-dependent growth of secondary cell nuclei, since the otherwise-small secondary cell nuclei of *ecdysoneless* males (*ecd*^{1}; GAREN *et al.*, 1977) grow after mating (APPENDIX 9: *p.* 453). These secondary cells still show evidence of DNA synthesis in response to mating. This suggests that the EcR *might* work in a ligand-independent manner in this cell type, specifically after mating. Previously, the EcR-dependent cell survival in the prothoracic gland has been shown to be ligand-independent (MANSILLA & MARTÍN *et al.,* 2016); indicating that the EcR indeed can function in a ligand-independent manner. Further work will however be required to better understand how mating influences the production of ecdysone by the male accessory glands—for example by measuring the ecdysone titres of males lacking BMP signalling and/or EcR protein in their secondary cells before and after mating.

It is unknown whether main cells are able to respond to ecdysone, since they do not contain any detectable EcR protein—at least in the adult (LEIBLICH, 2015). SHARMA *et al.* (2017) do however find that the EcR might be required in main cells

during their development in the *puparium*. Yet, as my experiments have shown, main cells are able to grow their nuclei in response to ectopic stimulation of the cell cycle; suggesting that although they are competent to undergo endoreplication upon *cyclin* overexpression, it is likely that they lack the upstream molecular machinery that allows them to respond to the same signalling cues as do the secondary cells. Endoreplication in main cells appears to be a normally rare event: although my EdU labelling assays have shown that main cells do incorporate EdU on rare occasions (not shown), it is unclear if this is a physiologically relevant event. Still, all this together suggests that adult main cell biology is regulated independently of steroids. Perhaps this indicates that the two epithelial cell types are distinct because they respond to different upstream signals. Interestingly, BAUMANN *et al.* (2017), show that the mutually-redundant juvenile hormone receptors, Germ-cells expressed ("**GCE**") and Methoprene-tolerant ("**Met**") are expressed in the male accessory gland. Preliminary work in our laboratory using a GCE protein trap has suggested that GCE seems to be present in the nuclei of main cells but not secondary cells (J. HELLBERG & C. WILSON, preliminary observations). Further work will be required to confirm these observations and to put them into context.

Concluding remarks

In this Chapter I have built upon previous work in our laboratory, which had shown that secondary cell nuclei grow in response to mating, and that BMP signalling and the EcR also regulate the size of secondary cell nuclei. My work has shown that the age-dependent, mating-independent, growth of secondary cell nuclei is likely to reflect the biosynthetic activity of the nucleus; since it seems to reflect the mating status and length of the virgin period. In addition, I have shown that both BMP

signalling and the EcR are required for the mating-dependent growth of secondary cell nuclei, and I have provided evidence to suggest that the mating-dependent growth is partly driven by endoreplication that is regulated by the BMP/EcR signalling axis. I have shown that genes involved in the regulation of the cell cycle can promote mating-dependent endocycling to increase the secondary cell DNA content. Furthermore, it seems that although main cell nuclei can be induced to grow when regulators of the cell cycle are overexpressed, they fail to do so during normal conditions because they lack the upstream machinery to respond to the BMP and EcR signals. That secondary cell nuclei grow in response to mating as a result of BMP/EcR-dependent endoduplication suggests that the nuclear growth might represent a physiologically-relevant response that increases the biosynthetic potential of the secondary cells—to promote the replenishment of gland contents and prepare male *D. melanogaster* for future mating opportunities. Such regulatory feedback might also help us better understand the regulated secretion in other tissues that need to balance their secretory output with demand—as in the reproductive glands of male mammals.





CHAPTER 6

PREVIOUS PAGE:

View of the University of Oxford New Biochemistry Building (left) and the Le Gros Clark Building (right); the previous site of the University's Medical School and home of Professor Wilson's Laboratory.



CHAPTER 6

FINAL DISCUSSION AND CONCLUDING REMARKS

All models are wrong; some models are useful.

– George E. P. Box
(in BOX & HUNTER, 2005: *p.* 440)

This Dissertation has detailed my *D. Phil.* work on the *D. melanogaster* male accessory gland, and each Chapter has concerned itself with a different aspect of secondary cell biology. CHAPTER 3 built on a collaborator's work showing that the *D. melanogaster* caspase, DRONC, is expressed in the secondary cells of the male accessory gland, and as described therein, I have found that DRONC is regulated by both EcR and BMP signalling in these cells. In addition, I have found that caspases can act non-apoptotically to regulate the numbers of intracellular compartments in secondary cells. In CHAPTER 4, we explored a role for Hedgehog signalling in secondary cells; finding that the Hedgehog molecule appears to be expressed by these cells, from where it is released into the *D. melanogaster* seminal fluid. Hedgehog signalling also seems to regulate secondary cell nuclear growth. Finally, CHAPTER 5 explored the mechanisms regulating the growth of the secondary cell nuclei in more detail, and suggested that the growth of secondary cell nuclei can be an indicator of biosynthetic activity. In addition, we found that the mating-dependent growth of

secondary cell nuclei can be driven by EcR- and BMP-dependent endoreplication to increase the secondary cell DNA content. Altogether, despite their focus on three very different aspects of secondary cell biology, the three Chapters of this Dissertation come together to tell a greater story about how the biology and function of the secondary cells is regulated by *sex* (mating), *signalling* (BMP and Hedgehog signalling) and *steroids* (ecdysone and the EcR).

The male accessory gland matures after eclosion

In this Dissertation we have seen that the male accessory gland undergoes characteristic changes as the male matures sexually after eclosion. During this time, his accessory glands fill up with secretions and also increase in size (RUHMANN *et al.,* 2016). At the same time, changes occur on the cellular level. For example, the nuclei of both main cells and secondary cells grow during the first three days after eclosion. It is likely that this early growth reflects an increase in the cells' biosynthetic activity as they begin their adult function and secrete the proteins that go on to fill the gland lumen. Hedgehog signalling also becomes active during this time, and (although further work will be required to ascertain this) it is possible that steroid signalling during metamorphosis has a role in inducing the onset of Hedgehog signalling in the secondary cells. For example, qRT-PCR could be used to determine if the EcR regulates the expression of *hedgehog* in secondary cells?; and if the EcR co-activator, TRR, participates in this regulation? In addition, it would be very interesting to determine how the dynamics of *EcR* expression in the male accessory glad changes from late puparation through the first week of adult life.

As we saw in CHAPTER 4, the Hedgehog molecule appears to be released from secondary cells into the seminal fluid, where it likely signals back to these cells

to regulate their biology. (For example, Hedgehog signalling can regulate the size of the secondary cell nuclei.) Hedgehog signalling interacts with BMP signalling in other tissues in *D. melanogaster* (for example the developing wing disc; TANIMOTO *et al.,* 2000), and (although I have not been able to show it directly because of time constraints), it would be interesting if future work explored the relationship between Hedgehog and BMP signalling in the accessory gland in more detail—for example by determining the exact onset of *dpp* expression, and if mutation of *hedgehog* affects this. Please see **FIGURE 6. 1.** for a model explaining the interaction between Hedgehog and BMP signalling in the secondary cells.

Caspases might function non-apoptotically to regulate secretion

As we saw in CHAPTER 3, the BMP/EcR signalling axis can regulate the expression of the *D. melanogaster* initiation caspase, DRONC, in secondary cells. Intracellular compartments are trafficked along the cell cytoskeleton, and the actin cytoskeleton has an important role keeping secretory compartments from fusing prematurely with the cell plasma membrane (SEGAWA & YAMASHIMA, 1989; RUOSSO *et al.*, 2016). Caspases have been shown to regulate secretion by acting on the actin cytoskeleton to liberate secretory compartments (MASHIMA *et al.*, 1997). I hypothesise that caspases control this process also in secondary cells. One initial avenue of investigating this might involve characterising the role of the Rho1 GTPase in secondary cell intracellular trafficking dynamics—since *Rho1* has been shown to regulate the size of the lumen in the *D. melanogaster* larval salivary gland (XU *et al.,* 2011).

BMP/EcR signalling is activated in response to mating, and this signalling can

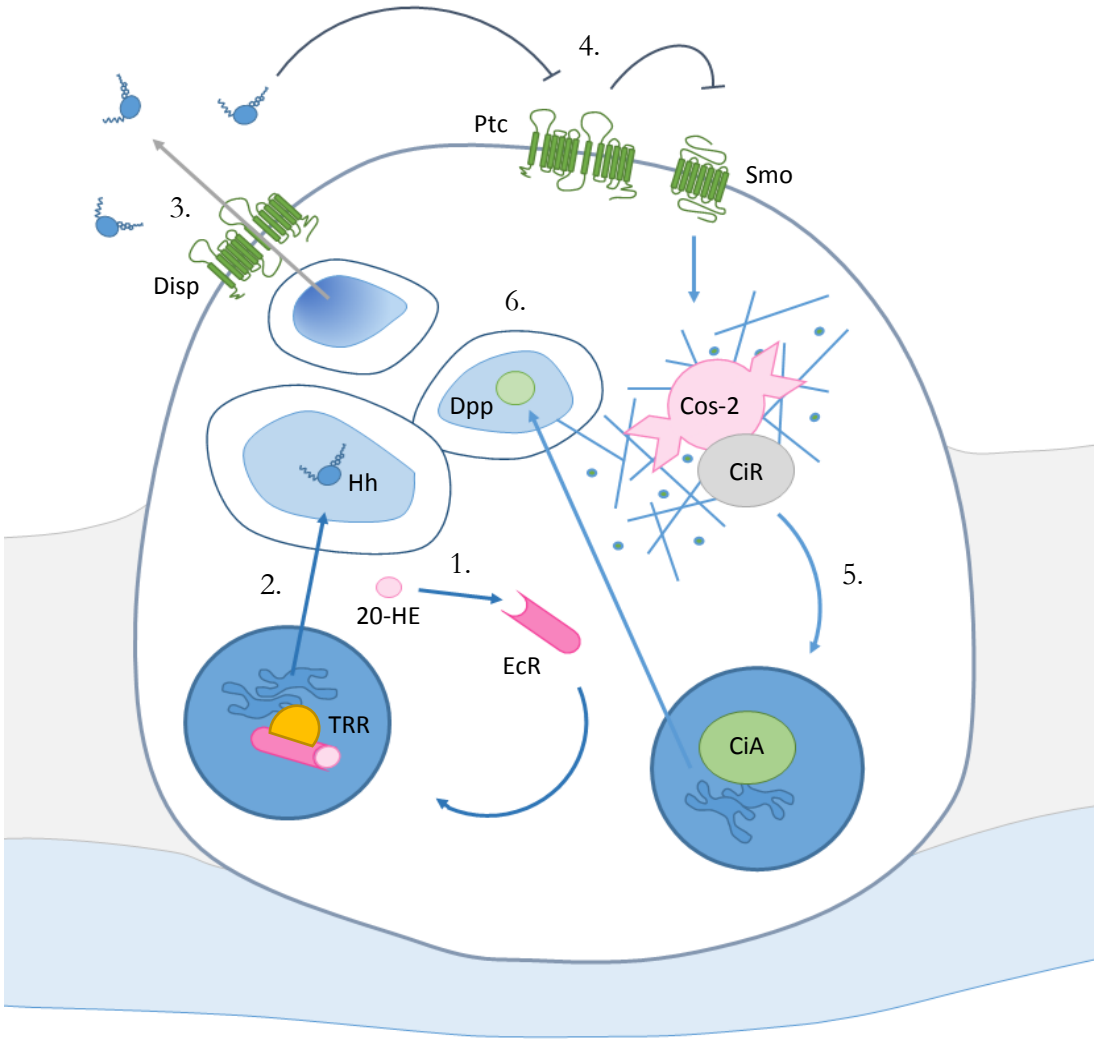


FIGURE 6. 1. | MODEL SHOWING HOW STEROID-DEPENDENT HEDGEHOG SIGNALLING MIGHT REGULATE THE PRODUCTION OF BMP LIGAND IN SECONDARY CELLS.

Ecdysone (20-HE) regulates metamorphosis, and might activate the EcR and its co-activator, TRR (1.). The EcR·TRR complex might regulate the expression of *hedgehog*, which, once expressed, is either loaded onto the secondary cell secretory granule (2.) and released from the cell (or is free to diffuse through the membrane via the transporter, Dispatched (3.)). Once released into the seminal fluid, Hedgehog signals back to the secondary cell (4.) and promotes the translocation of the transcription factor, Ci, into the secondary cell nucleus (5.). There, Ci might regulate the expression of *dpp*, and, once translated, is loaded onto the secondary cell secretory granules to be released from the cell (6.).

promote endoduplication in secondary cells. BMP/EcR signalling can however also regulate DRONC, and it is possible that mating might therefore also promote the mobilisation of secretory compartments in secondary cells; to help replenish the accessory gland's luminal contents. If so, it is possible that mating might serve as a signal to 'reset' (or replenish) the gland by activating BMP/EcR signalling to regulate both the continued (and increased?) production and release of secondary cell-derived secreted proteins. For example, REDHAI *et al.* (2016) showed, via pulse-chasing, that mating increases the turnover of intracellular compartments from secondary cells. Furthermore, if BMP signalling is autocrine in secondary cells (as suggested by REDHAI *et al.* (2016)), and secretory granules carry the BMP signalling ligand (and activated BMP signalling helps regulate DRONC—which might act to liberate these granules), mating might serve as an external input to complete the feedback loop that is acting to replenish the accessory gland contents. (Please see **FIGURE 3. 15.** in CHAPTER 3 (*p.* 128) for a model of the form this feedback loop might take.) However, it should be noted that physical factors like epithelial stretching (as discussed in CHAPTER 5: *pp.* 314–19) or the removal of an inhibitor (as discussed in CHAPTER 5: *p.* 319–24) also might regulate the replenishment of the accessory gland contents. This highlights the need for further work on this topic. Although methodologically difficult, it would be interesting to determine the effect of mating on secondary cell nuclear growth in the absence of ejaculation. Conversely, the use of flies where ejaculation can be induced in the absence of mating (for example via activation of neurons expressing the male isoform of the *fruitless* gene, fru^{M}: PAN *et al.,* 2011) might inform us of the role of gland emptying *versus* the need for females and/or ecdysone in the male socio-sexual environment for the mating-dependent growth to occur.

The BMP/EcR signalling axis regulates secondary cell biology and might increase the cells' biosynthetic potential

A recurrent theme of my work has been the ways in which BMP signalling regulates the biology of secondary cells—since it regulates the growth of the secondary cell nuclei (LEIBLICH *et al.,* 2012). One implication of my work, and that of my colleagues, is that the BMP signalling pathway has a role regulating the biosynthetic activity of the secondary cells (although this needs to be confirmed through more specific measurements, for example by measuring the exact levels of protein produced by virgin and mated glands where BMP and/or EcR signalling has been inhibited). Like Hedgehog, the BMP signalling ligand, Dpp, is carried on the secondary cells' secretory granules to be released into the seminal fluid (REDHAI *et al.,* 2016). Working together with Siamak Redhai during the final year of his *D. Phil.*, he and I showed that secondary cell-derived Dpp can signal back into the secondary cell to induce the nuclear translocation of the BMP-dependent transcription factor, pMad (a process that is stimulated by mating).

Importantly, as I showed in CHAPTER 5, the BMP/EcR signalling axis is required for the mating-dependent growth of the secondary cell nuclei, and this growth seems to be (at least partly) driven by increases in the secondary cell DNA content. It is likely that the BMP/EcR signalling axis regulates secondary cell nuclear size via E2F1-dependent regulation of the cell cycle. For example, knocking down *E2F1* inhibits the increase in the secondary cell DNA content after mating. Furthermore, E2F1 regulates the expression of positive regulators of the cell cycle in other tissues (like *cyclin D and cyclin E*), and the ectopic expression of these in secondary cells can increase the total DNA content. Ectopic expression of *cyclin E* has also been shown to inhibit multiple rounds of endoreplication (*e.g.* WEISS *et al.,*

1998). As we saw in CHAPTER 5, this treatment stimulates nuclear growth and endoreplication in secondary cells of virgin males, but prevents further mating-dependent growth. Altogether, this suggests that mating activates a BMP/EcR-dependent endocycle in the secondary cells, and that this mechanism underlies a substantial proportion of the mating-dependent growth of the secondary cell nuclei. If you recall from CHAPTER 5, RNAi of *cyclin E* does, however, not fully inhibit nuclear growth after mating, suggesting that other mechanisms also contribute to this process. Again, it would be interesting to determine if the amount of protein produced by mated glands is reduced when *cycE* has been knocked down.

Polyploidy is a common feature of metabolically active cell types because increases in the cellular DNA content also increases the number of gene copies available to the cell. In virgin *D. melanogaster,* the secondary cell nuclei are already polyploid; containing an estimated eight copies of the haploid genome (TANIGUCHI *et al.,* 2012). As we saw in CHAPTER 5, activated BMP signalling (induced ectopically, or by mating) increases the secondary cell DNA content in many, but not all, cells (even when BMP signalling is activated through expression of tkv^{Q253D}). Perhaps this suggests that the EcR might be the key signalling component that needs to be active (or present at high enough levels) for the BMP-driven nuclear growth to occur? EcR antibody staining in combination with α-pMad staining and/or EdU-labelling might provide insight into how the cells that undergo endoreplication after mating differ from those that do not. Regardless, as the result of mating-dependent endoreplication, the number of gene copies (and therefore the cells' biosynthetic potential) is increased. It is likely that endoreplication is not the only mechanism by which biosynthesis can be elevated in the secondary cells, however.

As we saw in CHAPTER 5, although knock-down of *E2F1* prevents increases in the secondary cell DNA content after mating, the nuclei still grow after mating in this background. To date, I have shown that only blockade of BMP signalling via *Dad* overexpression and knockdown of *EcR* completely blocks the post-mating growth, suggesting that the residual growth might also be dependent on BMP/EcR signalling. Overall, it is likely that the biosynthetic potential of the secondary cell does increase after mating—possibly through separate endoreplication-dependent and endoreplication-independent mechanisms. These mechanisms might involve endoreplication-dependent processes such as DNA synthesis and gene amplification, and endoreplication-independent mechanisms such as increased protein translation, via the increased assembly of ribosomes and/or increased chromatin decondensation (as has previously been observed after mating in the male accessory gland (SCHMIDT *et al.,* 1985)). How these mechanisms depend on, and interact with, BMP and EcR signalling, will form an interesting focus of future work. For example, it would be interesting to determine (perhaps via electron microscopy) how (and if) the number of ribosomes change before and after mating in these genetic backgrounds.

Function and hyperfunction in the male accessory gland

Regardless of the exact mechanism by which mating leads to the replacement of male accessory gland contents, accepting the premise that: mating → luminal replenishment, raises the question: what is the role of the secondary cell nuclear growth in this process? The most attractive/appealing answer is that the nuclear growth, partly driven by endoreplication, promotes the cell's biosynthetic potential through gene amplification. However, although this is the explanation that I have

spent the most time exploring, I think it is important to highlight that it is an *adaptionist* explanation, and that it therefore comes with certain caveats that are important to acknowledge. In a 1979 article, '*The Spandrels of San Marco and the Panglossian Paradigm*' (which ought to be more widely read), Stephen Gould and Richard Lewontin critique the 'adaptionist programme' that they saw permeating biology and the life sciences overall, faulting it

> for its unwillingness to consider alternatives to adaptive stories; for its reliance upon plausibility alone as a criterion for accepting speculative tales; and for its failure to consider adequately such competing themes as the random fixation of alleles, production of non-adaptive structures by developmental correlation with selected features (allometry, pleiotropy, material compensation, mechanically forced correlation), the separability of adaptation and selection multiple adaptive peaks, and current utility as an epiphenomenon of non-adaptive structures (GOULD & LEWONTIN, 1979: *p.* 581)

For this reason, I think it important to entertain alternative explanations for the phenotypes that I have observed through my work, and which are described in this Dissertation. When I initially started my *D. Phil.*, I was intrigued by the possibility that the nuclear growth of the secondary cells might represent a form of 'hyperfunction'. Because my results do not refute this hypothesis, I will discuss its implications here.

The changes that organisms undergo as they age can be separated into two categories: maturation and senescence. Whereas the process of maturation is adaptive (preparing the organism for its adult functions), there is no adaptive advantage for the organism to grow increasingly frail with age (senescence). Yet, senescence is pervasive throughout the animal kingdom (SHEFFERSON, JONES & SALGUERO-GÓMEZ, 2017), and several theories have been proposed to explain this phenomenon. Famous among these are Thomas Kirkwood's theory of the 'disposable soma' and George William's theory of 'antagonistic pleiotropy'. The disposable soma theory suggests that an organism has a fixed amount of energy

available to it, and that there is a trade-off in how this energy is distributed to support the organism's reproductive output or to maintain its body and its tissues (the *soma*) (KIRKWOOD & HOLLIDAY, 1979). According to this interpretation, ageing is the result of differential allocation of resources towards either reproduction or somatic maintenance, with the soma being the 'disposable' component that suffers detrimental consequences when the organism's energies are channelled towards reproduction. William's theory of antagonistic pleiotropy interprets the issue slightly differently, suggesting that the genetic variation that supports the organism's function in early life (maturation and reproduction) can have deleterious (antagonistic) consequences in later life (WILLIAMS, 1957). As a result, ageing would evolve as the pleiotropic response to selection of genetic variants that promote the organism's fitness in early life—since the strength of natural selection decreases with age as the organism's future reproductive output decreases (HAMILTON, 1966). Young organisms (with a high future reproductive output) are more 'visible' to natural selection; facilitating the fixation of traits and alleles that promote reproduction and increase the reproductive output (even if these might have deleterious consequences in later life). Conversely, older organisms (with a low future reproductive output) are less visible to natural selection, allowing late-acting, deleterious variation to accumulate.

An implication of ageing being the result of the accumulation of deleterious genetic variation is the controversial (but attractive) suggestion that ageing might be an 'age-dependent genetic disease' (GEMS, 2015). Recently, Mikhail Blagosklonny has built upon William's theory of antagonistic pleiotropy to propose a 'hyperfunction theory' of ageing (BLAGOSKLONNY, 2013). According to the hyperfunction theory, processes that support the organism's *normal* functions

(maturation and reproduction)—such as insulin signalling, and the target of rapamycin (TOR)—continue to function beyond the point when they are no longer needed (*e.g.* after maturation), resulting in *hyper*-function and a 'bloated soma'. In this view, senescence is a consequence of youth in the same way as adulthood is a consequence of maturation. GEMS and PARTRIDGE (2013) discuss the implications of each of the three theories of ageing in more detail. Different lines of evidence support each theory, and although they have heuristic value, sentiment in the field of biogerontology (the study of the biology of ageing) remains divided (GEMS, 2015).[1]

Nuclear growth (and epithelial delamination) after mating in the secondary cells of the *D. melanogaster* male accessory gland has some of the features of a 'spandrel' phenotype that results from reproductive hyperfunction. Although adaptionist explanations are satisfying, that does not guarantee them to be true. As we have seen throughout this Dissertation (and other publications from the Wilson laboratory), BMP signalling is a crucial regulator of secondary cell biology. For example, LEIBLICH *et al.* (2012) show that BMP signalling is required for the normal reproductive function of the male fruit fly, with reduced BMP signalling (as a result of *Dad* overexpression) reducing the male's ability to induce the full post-mating response in his mate. *Dad* overexpression also reduces the number of secondary cell intracellular compartments, whereas expression of the activated Thickveins receptor, tkv^{Q253D}, increases the number of these (CORRIGAN *et al.*, 2014). Similar effects are observed with *Ec*R knock-down and expression of the *Ec*R-*C*, respectively

[1] I should note that I spent the better part of the 2012–2013 academic year working in the laboratory of Professor David Gems at the Institute of Healthy Ageing at University College London, where I worked on testing the predictions of the hyperfunction theory of ageing. The results of this work contributed to my *M. Sci.* dissertation (HELLBERG, 2014), two published manuscripts (RIESEN *et al.*, 2014; DE LA GUARDIA *et al.*, 2016), and one manuscript currently uploaded onto the preprint server for biology (*BioRxiv*) while awaiting publication in a scientific journal (WANG *et al.,* 2017).

(LEIBLICH, HELLBERG & WILSON, unpublished observations). It is reasonable, therefore, to think of both BMP signalling and the EcR as important to the normal reproductive function of the male accessory gland—presumably because BMP signalling and the EcR are key regulators of secondary cell biology, and because the secondary cells are important for the function of the gland itself (GLIGOROV & SITNIK *et al.,* 2016).

When we speak of the male accessory gland, what constitutes 'function' in this context? To me, 'function' would be the mechanism by which the gland 1) fills up with secretions after eclosion; and, 2) fills up with further secretions after mating. Assuming that this filling and re-filling is the main tissue-level function of the accessory gland, the growth of the secondary nuclei after mating (after the gland has emptied and become replenished) might be 'hyperfunctional'; representing a cellular-level response that constitutes no adaptive advantage to the tissue itself. (This, for example, might explain why only a subset of secondary cells undergoes endoreplication after mating.) There is no reason to suppose any fundamental difference in how processes 1) and 2) are regulated (since the outcomes are similar: the gland fills up with secretions). Therefore, endoreplication might not be required for the replenishment of gland contents (recall that there is no increase in the secondary cell DNA content as the fly matures after eclosion), and might instead represent an off-target effect that results from excessive stimulation of the EcR/BMP signalling axis after mating (if this stimulation is required for a 'normal' function, like, say, the increased production of proteins). The hyperfunction hypothesis is less spectacular, but equally (if not more) plausible, and in the absence of data to either support or disprove this interpretation it must be entertained

alongside the hypothesis that the nuclear growth is functional/adaptive as discussed elsewhere in this Dissertation.

To differentiate between the hypotheses of the mating-dependent nuclear growth being an *adaptation* (mating-dependent nuclear growth promoted the male's reproductive fitness) or a *side-effect* (mating-dependent nuclear growth has no impact on the male's reproductive fitness), further work needs to be performed. For example, such experiments could take the form of comparing the reproductive fitness of males expressing *cyclin E* RNAi after multiple matings to see if his ability to suppress the female post-mating response is deficient compared to males whose secondary cells are still able to undergo endoreplication in response to mating. To use a downstream component like *cyclin E* for these experiments is preferable, since overexpression of *Dad* or RNAi of *EcR* have (or are likely to have) more pleiotropic effects that will confound the results. Concerning the expected effect size of the male reproductive fitness after multiple matings when lacking *cyclin E*, I would believe it to be small (because of the inherent variability in this type of experiment). Therefore, any experiment testing the male's reproductive fitness (*e.g.* his ability to induce female rejection of other males and to increase her ovulation rates) would need to be performed at a large enough scale for these potential differences to be discerned.

The *D. melanogaster* male accessory gland as a model of prostate cancer

The *D. melanogaster* male accessory gland is analogous to the mammalian prostate, and the two organs have evolved from different progenitor tissues to nevertheless perform similar functions in male reproductive biology. Therefore, although our understanding of secondary cell biology is not directly transferable to increase our

understanding of prostate cell biology, it can still be interesting to compare the cell biology of the two organs to understand when they differ, and how, and what they share in common.

Commonalities between cell types increase our understanding of cell biology more generally, allowing us to better understand how (and why) cells function the way they do, and what might be the limitations that govern their functions. For example, Aaron Leiblich (through his *D. Phil.* work) identified aspects of how steroid receptor availability is regulated in secondary cells: with both BMP signalling and E2F1 activity increasing levels of EcR protein (LEIBLICH, 2015). Similar mechanisms of regulation can be identified in the prostate (as discussed in LEIBLICH, 2015), with both BMP-6 treatment and increased E2F1 activation leading to increased levels of AR protein (LEE *et al.,* 2013; SHARMA *et al.,* 2010). Identification of such similarities suggests that that the secondary cells may be used to model the molecular dynamics of steroid receptor expression and regulation. It might be worth noting, however, that therapeutic hormone independence of the AR in the human prostate seems to be primarily driven by *AR* gene amplification; gain-of-function mutations permitting AR activation via non-androgenic ligands; or deregulation of AR co-activators (KNUDSEN *et al.,* 2008). This suggests that the potential hormone independence of the EcR in the secondary cells of mated males in the *ecd*^{1} background (*p.* 453) might not be a good model for the behaviour of the AR in human prostate cancer.

Building on Aaron's work on the role for BMP/EcR signalling in the regulation of secondary cell nuclear growth, I identified in CHAPTER 4 a requirement for Hedgehog signalling in this process. It might be interesting to pursue further work using the secondary cell as a system for exploring the exact interaction between

Hedgehog signalling and BMP signalling on EcR protein levels and/or receptor activity to inform future work in more appropriate human models. There are reports in the literature suggesting that ectopic Hedgehog signalling in the adult mouse prostate might promote the formation of androgen-independent prostate cancer-like cells (*e.g.* CHANG & CHEN *et al.,* 2011). Indeed, other workers have found that one of the mammalian Hedgehog-dependent transcription factors, GLI1, can directly interact with the AR and act as a co-repressor to block AR-mediated transactivation and promote the AR-independent growth of prostate cancer cells (CHEN, GOTO *et al.,* 2011). This is consistent with the data discussed in CHAPTER 5 (*pp.* 308–9) implying a possible role for Hedgehog signalling in the negative regulation of BMP signalling in the secondary cells.

Conversely, differences in the behaviour between different cell types allow us to understand the mechanisms underlying the functional diversity of cells; allowing us to understand the different ways in which different mechanisms might evolve. Overall, comparative cell biology therefore informs us of the diversity in cellular form and function—and of the limitations thereof. For example, in CHAPTER 3, I have shown that the caspase, DRONC, might act non-apoptotically to regulate intracellular trafficking dynamics in secondary cells, by comparing the phenotypes I have observed through my work with those observed through work in other secretory cell types. This comparison suggests that non-apoptotic caspase function might represent a more general mechanism governing the mobilisation of intracellular compartments from the restrictive meshwork formed by the actin cytoskeleton. It would be interesting to expand this work and see if a similar mechanism might regulate the mobilisation of secretory compartments in, for example, the mammalian prostate.

Yet, it is important to remember that "when you take an intellectual ride on a metaphor, it is important that you know where to get off" (HANNERZ, 2002: *p.* 6). The 'metaphor' in this context would be using *D. melanogaster* as a model for any aspect of human biology, since the fruit fly is not human. All results obtained using the fruit fly therefore tell us more about fruit fly biology than about human biology, although the results can be used to infer and approximate what is going on in the human context. The emphasis on work on *D. melanogaster* (or any model, for that matter) should always be that they tell us about the model first and about what is modelled second. Indeed, fundamental differences in endocycling have been found when comparing the processes in *D. melanogaster* and the mouse—including faster genome replication times and forced replication of heterochromatic regions due to the differences in chromatin organisation between the two species (SHER & VON STETINA *et al.,* 2013). Such differences reduce the degree to which we can extrapolate from one model to another and increase the vigilance which we must exercise when making such extrapolations.

Yet, as statistician E. P. Box said (and as quoted at the beginning of this Chapter), although "all models are wrong; some models are useful" (BOX & HUNTER, 2005: *p.* 440). Indeed, the fruit fly has been integral to identifying and characterising the function of both oncogenes and tumour suppressors important in human cancers (BRUMBY & RICHARDSON, 2005). Therefore, work in the fruit fly can inform our understanding of human biology and pathology—although this primarily takes the form of inspiring novel hypotheses and further work in more appropriate models, like, for example, human tissue and cancer cell lines. Fortunately, the Wilson laboratory has such collaborations in place via the laboratory of Deborah Goberdhan, which will allow the findings presented in this Dissertation

to inform future experiments. For example, Kristie McCormick (a *D. Phil.* student in the Goberdhan laboratory) is working to test some of the predictions Aaron Leiblich made in his Dissertation on the regulatory role of BMP signalling on steroid receptor activity) with interesting results. My hope is that the work presented in this Dissertation will become similarly relevant for future work on this topic, for example by exploring the role of BMP/steroid receptor signalling in the biosynthesis of male-specific secretion products, and exploring the potential role for Hedgehog signalling in this process. Overall, I feel that the work presented in this Dissertation has primarily helped inform our understanding of secondary cell biology—and fruit fly biology by extension. The hope of any such work is that by better understanding our models, and how their biology differs from that of humans, that we will become ever-better at asking and formulating the right questions to infer knowledge and to better understand more general concepts.

Concluding remarks

The work presented in this Dissertation improves our understanding of secondary cell biology and function; allowing us to understand one aspect of male reproductive biology in *D. melanogaster* a bit better. For example, I have provided additional evidence to show that the BMP/EcR signalling axis is an important regulator of secondary cell biology, to strengthen previous work on this topic. It is likely that this signalling helps promote male reproductive function; allowing gland contents to replenish after mating so the male is prepared to benefit from possible future mating opportunities.

The work presented in this Dissertation also shows a role for Hedgehog signalling in the regulation of nuclear growth, and it is possible that Hedgehog

signalling helps co-ordinate the genetic interaction between the EcR and the BMP signalling pathways. This Dissertation has also shown that EcR/BMP signalling can regulate the expression of the caspase, DRONC; which acts non-apoptotically to regulate secondary cell intracellular trafficking and possibly help mobilise secretory compartments from the cell. Overall, the findings presented in this Dissertation have helped explain how secondary cell nuclei grow in response to mating, leading to the formulation of a model that attempts to explain how EcR/BMP signalling might regulate several aspects of secondary cell biology.

To conclude, the work presented here has shown that sex, signalling, and steroids, are all important inputs in the regulation of secondary cell biology. It will be very exciting to see future work elucidate the relationship between these in more detail, and to see if a similar logic or inputs help regulate the secretory output of other cells in other glandular tissues—in both flies and in other organisms. However, to be true to the spirit in which this Dissertation has been written, the real value in the work presented herein lies not in speculating how these results might provide any clinical (human) benefit, but in increasing our understanding of one small aspect of biology to serve as the incremental foundation of further work on this topic. It will be very exciting to see how this work develops in the future.

ACKNOWLEDGEMENTS



PREVIOUS PAGE:
One of the muses atop the old home of Oxford University Press, on Broad Street in central Oxford.

ACKNOWLEDGEMENTS

Every scientist with a PhD knows that it takes years of study to be an expert—you not only have to ingest hundreds of books and papers, you have to digest this information, sifting it and evaluating it, and converting it into knowledge and judgment.

– Arlin Stoltzfus
(STOLTZFUS, 2013)

Life proceeds by a twisted route—where each turn allowing new knowledge to be acquired and new insights to be made, and with new opportunities arising as a result. My academic path has wound from the leafy green suburbs of Stockholm through the grit of London, to the wisteria-lined streets of Cambridge, and on to the dreaming spires of Oxford. A number of people have helped me navigate the options available to me during this time, and without their guidance and advice my journey would have been made much harder.

In Stockholm, I am indebted to my teachers, particularly at Viktor Rydberg Gymnasium, for having provided me with the resources and support to work at my own pace, so to not lose the joy and wonder of learning. I also owe an ambiguous debt of gratitude to the nursing college where I realised that I had taken a wrong turn

and the thought of studying abroad occurred to me; proving that there is a silver lining on every cloud.

In London, my undergraduate degree at University College London was very much defined by the world-class education that I received at the Department of Genetics, Evolution and Environment, where I was taught by some of the most inspiring people I have ever met, and where I was rewarded with the friendship of some truly outstanding people. In particular, I would like to thank Daniel Jeffares, who was my first-ever laboratory supervisor, and who allowed me to try my fledgling scientist's wings for the very first time. Dan, there are so many things that you taught me that have served me throughout my subsequent scientific career; ranging from the mundanely pragmatic (mistakes happen and that's all right) to the value of dogged commitment (to keep pursuing even the most elusive of discoveries). At UCL, I would also like to thank Joseph Cain who took the time to talk me through the pros and cons of doing a doctorate degree in the life sciences—at a point in my life when I was unsure if a *Ph.D.* was the right path for me; without your advice, Joe, it is possible that this Dissertation and the discoveries within it would never have happened. Similarly, I owe a lot to David Gems, who supervised my undergraduate research project and who was never shy to offer me sage life advice. Without his influence, I would have been a very different person indeed, and although I didn't follow his advice to the letter, this Dissertation would never have happened without him—since he encouraged me to spread my wings beyond UCL; setting the wheels in motion that eventually made me end up in Oxford.

A twist in the road to Oxford led me through Cambridge, where I was fortunate to spend a year under the supervision of George Salmond and Miguel Matilla. Both imbued me with a refreshed passion for science, and I'm forever indebted to them

both for helping me navigate the challenges and the excitement that comes with a research student's first full-scale research project; one which culminated with my first first-author manuscript (HELLBERG *et al.,* 2014). It would have been much the poorer if not for their co-authorship. It was also while I was in Cambridge that I met my friend Calum Nicholson, who, as a *Ph.D.* student himself, helped me get through all the times when I had decided that doing a doctorate was not for me. Thank you for helping me to get through this degree, Calum—and thank you for encouraging me to pursue a career outside of academia. In Oxford, my friend Hugh Dickinson was equally important in keeping me on track with my *D.Phil.* studies and thinking of diverse career paths: thank you, Hugh, for always being available for a chat over coffee to brighten even the rainiest of days, and for sticking by me as I've struggled with my science—and with what to do next.

In the Wilson laboratory, I owe much support to all the friends who I made during my stay. I owe great thanks to Aaron Leiblich, whose paper in *PNAS* was my first introduction to the laboratory, and which sparked an interest in the regulation of nuclear size in secondary cells that has lasted me throughout my degree. Thank you, Aaron, for paving the way and for allowing me to work alongside you on a very exciting project and manuscript—as well as for having engaged me in enjoyable discussions on an endless list of arcane topics. I am also particularly grateful to Carina Gandy who cheered me on daily, and proved a valuable and infatigable friend. Thank you, Carina, for all the rants you so patiently have listened to, and for all the practical life advice you have shared. Similarly, my *D.Phil.* would have been much different without Siamak Redhai, who, in his position as the senior *D.Phil.* student during most of my time in the laboratory, helped me to navigate the intricacies of the laboratory, and I am grateful for our discussions on the deepest of philosophical

issues; keeping us amused while pushing flies. To my long-time friend, Benjamin Kroeger, I would like to say: thank you, Ben, for your enthusiasm; for helping me out with the milestones of my *D.Phil.*; and for always having been willing to offer a helping hand. Finally, my knowledge of fruit flies would have been much poorer if not for Mark Wainwright, who is eternally patient in answering even the silliest of questions; thank you, Mark, for sharing all your knowledge with me and for humouring all the observations I made over the years—ranging from the insignificant to the not-so-very profound.

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[1] Stocks obtained from the Bloomington Drosophila Stock Center (NIH P40OD018537) were used in this study. Transgenic fly stocks were also obtained from the Vienna Drosophila Resource Center.

the laboratory, invigorated, and ready to tackle a final year. In particular, I am grateful to Laura Belligan for her kind words of support and encouragement regarding my work on her team, and of career options outside of academia.

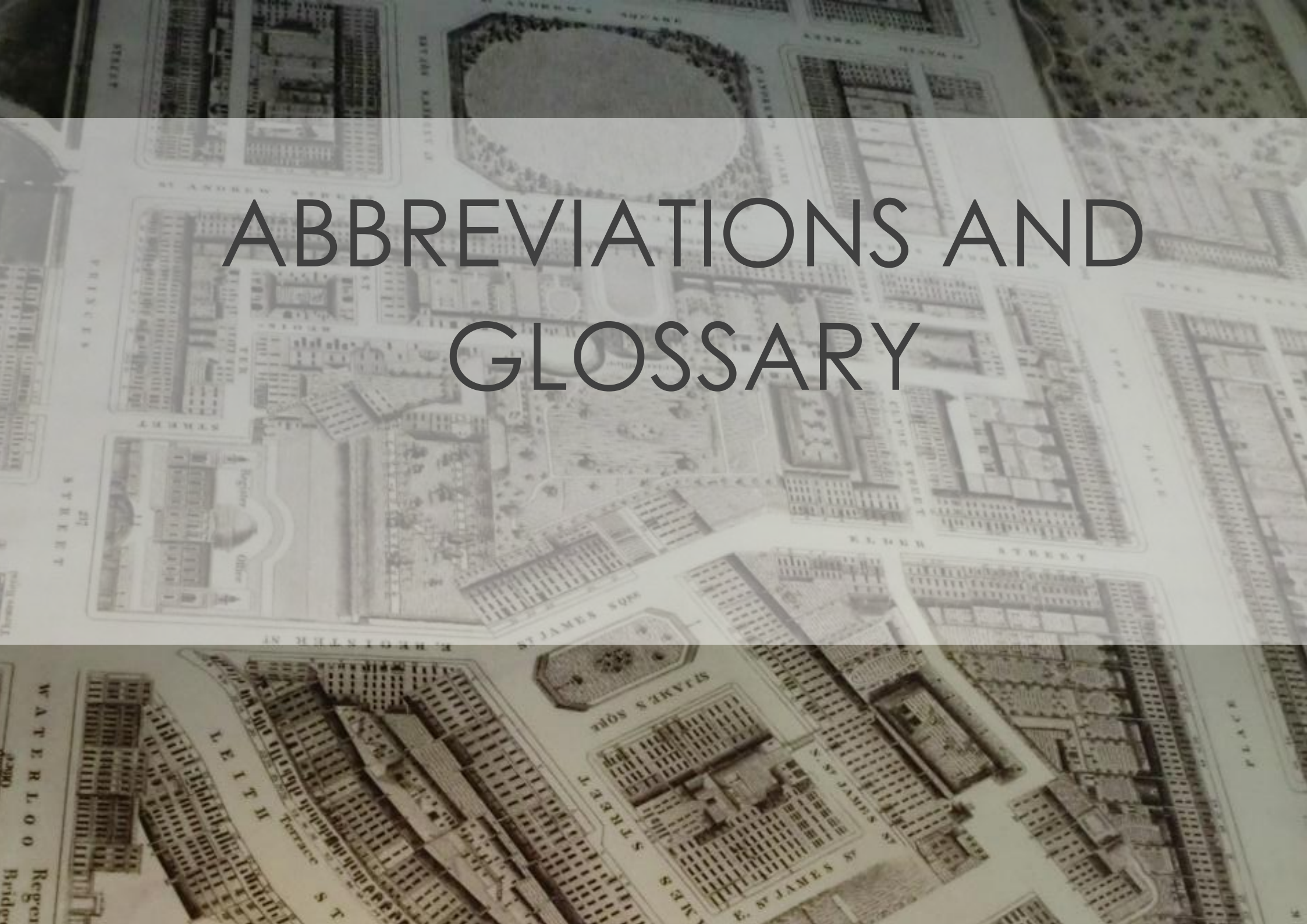
Finally, I owe multitudes to my parents, who, from the very beginning, allowed me to pursue my scientific whims: Mamma and Pappa, I am so grateful to you for having put up with me (and all the tadpoles and caterpillars and grasshoppers and guppy fish) over the years, and thank you for your financial support—without which my nascent academic career would not have been possible. I am also very grateful for the never-ending emotional support of my sisters: Henrietta, Ebba, Siri, and Iris; thank you for having listened to and comforted all my worries over the years, and for having come to visit to make England a little bit less lonely. In particular, I would like to thank Henrietta, who is the most incredible and wonderful person I know, and whose unrelenting support I don't know what I would have done without. I would also like to thank my cat, Leora, who made writing this Dissertation a much more enjoyable process through her constant interruptions; thank you, sweetest little darling thing, for all of your love and purrs and attention.

As I continue on the path that led me here in the first place, it is with some pensiveness that I realize my time in Oxford has come to an end. With a new career as an investment analyst at the investment management firm, Baillie Gifford, I however am confident that everything I have learnt during my time in science will serve me well.



Josephine E.E.U. Hellberg

Edinburgh, Hilary 2018



ABBREVIATIONS AND GLOSSARY


Pantheon
KING STREET
SOUTH STAMFORD STREET
CHEESESIDE
STREET
CATHERINE
STREET
NOTTINGHAM PLACE
HIGH CALTON
STREET
STREET
UNION
PLACE
BROUGHTON

PREVIOUS PAGE:
A map of Edinburgh's New Town.

LIST OF ABBREVIATIONS AND GLOSSARY

While I have done my best to avoid using abbreviations in the text of this Dissertation, provided here is a list of those that nevertheless do occur.

	Term	Description
#	**3D-SIM**	**3D-Structural illumination microscopy**
	20-HE	**20-hydroxyecdysone** The main *Drosophila* steroid hormone.
A	**A**	**A**lanine, the amino acid
	Å	**Å**ngström
	Abd-B	**Abd**ominal-**B**
	Acp	**Ac**cessory gland **p**rotein A protein that is synthesised by the male accessory gland.
	Acp26Aa	**Ovulin** A main cell protein product that is thought to be taken up by and modified by secondary cells in the male accessory gland. When transferred to the female upon mating, the protein goes into her haemolymph, where it has several physiological effects, including regulating her ovulation rates.

	Term	Description
	act	**Actin**
	AF	**Activation function**
	AG	**Accessory gland** A tissue in the male reproductive system that is responsible for the synthesis and secretion of many of the proteins that go into seminal fluid. It is the insect functional analogue of the mammal prostate gland.
	ANCE	**Angiotensin-converting enzyme** A protease that is found in the protein granules in the secondary cell secretory compartments and is secreted into the seminal fluid.
	ANOVA	**Analysis of variance** A collection of statistical models used to analyse differences between group means.
	APAF-1	**Apoptosis protease-activating factor 1** The mammal orthologue of dARK and an apoptosome component responsible for the autocatalytic activation of APAF-3 (a DRONC orthologue).
	AR	**Androgen receptor** A nuclear receptor in mammals that is regulated by androgens. It is orthologous to the EcR in insects.
B	**BDRC**	**Bloomington *Drosophila* Resource Center**
	BFI	**Background fluorescence intensity**
	BMP	**Bone morphogenetic protein** The ligand of the eponymous BMP signalling pathway. There are two BMP proteins in Drosophila: Dpp and Gbb. The BMP ligands bind BMP signalling receptors to activate the pathway.
	Boi	**Brother of Ihog**
C	**C**	Carboxy (-**COOH**)
	C	**Cysteine**
	CA	**Constitutively active**
	CARD	**Caspase-recruitment domain**
	Cas9	**CRISPR-associated protein 9**
	CD	**Cluster of differentiation**
	cDNA	**Coding DNA**

	Term	Description
	CDK	**Cyclin-dependent kinase** A type of kinase involved in the regulation of the cell cycle, and whose activity is dependent on interaction with an activating subunit called a 'cyclin'.
	CDKI	**CDK inhibitor**
	CG	**Computed gene**
	Ci	**Cubitus interruptus** A zinc-finger transcription factor that regulates the transcription of Hedgehog-responsive genes. Depending on the availability of Hedgehog, it can act as either a transcriptional activator or as a transcriptional repressor.
	CiA	**Ci (activator form)**
	CiFL	**Ci (full-length)**
	CiR	**Ci (repressor form)**
	CkIα	**Casein kinase Iα**
	Cos2	**Costal2**
	CRISPR	**Clustered regularly interspaced short palindromic repeats**
	CTNF	**Corrected total nuclear fluorescence**
	C-value	**Constant value** A measure of the number of haploid genome copies contained within a cell or nucleus.
	cycD	**Cyclin D**
	cycE	**Cyclin E**
D	**D**	The aspartic acid amino acid.
	D	**Dorsal**
	d	**Day**
	Da	**Dalton**
	Dad	**Daughters against Dpp** A negative regulator of the BMP signalling pathway. Since Dad is also regulated by BMP signalling, Dad completes the BMP signalling pathway as the final link in a negative feedback mechanism.
	Dally	**Division abnormally delayed**
	DAPI	**4',6-diamidino-2-phenylindole** A fluorescent stain that binds strongly to A=T-rich regions in DNA.

Term	Description
dARK	***Drosophil*a APAF-1–related killer** A protein that is part of the *D. melanogaster* apoptosome, with a role in the autocatalytic activation of DRONC.
DCG	**Dense core granule** A secretory granule that appears dense (strongly stained) when imaged using electron microscopy.
DCP-1	**Death caspase 1**
DED	**Death-effector domain**
DIC	**Differential interference contrast**
Disp	**Dispatched** A multipass membrane protein with similarity to RND-type transmembrane transporters, and can be involved in Hedgehog protein secretion.
Dlp	**Dally-like protein**
DMSO	**Dimethyl sulphoxide** An organosulphur, polar, aprotic solvent that is used in many molecular biology applications.
DN	**Dominant-negative**
Dp	**Dimerisation partner**
D. Phil.	***Doctor Philosophiae***
dpp	**Decapentaplegic** One of the two BMP-signalling ligands in *D. melanogaster*.
DREDD	**Death related ced-3/Nedd2-like caspase**
drICE	***Drosophila* interleukin-1 β-converting enzyme** A *D. melanogaster* effector caspase that is downstream of DRONC.
DRONC	***Drosophila* Nedd2-like caspase** An initiator caspase that initiates the apoptotic cascade in response to an apoptotic signal.
DSHB	**Developmental Studies Hybridoma Bank**
dsRNA	**Double-stranded RNA**
Dsx	**Doublesex** A transcription factor that is part of the sex-determination hierarchy in *D. melanogaster*.

	Term	Description
E	**E2F1**	**E2-factor 1** A transcription factor with a role regulating genes important for the cell cycle.
	ecd	**ecdysoneless** A gene involved in the biosynthesis of the *D. melanogaster* steroid hormone, ecdysone.
	EcR	**Ecdysone receptor** A *D. melanogaster* nuclear receptor regulated by the steroid hormone, ecdysone.
	EdU	**5-ethynyl-2'-deoxyuridine** A thymidine analogue that is incorporated into newly-synthesised DNA and can be used to assay DNA synthesis.
	ELISA	**Enzyme-linked immunosorbent assay** A plate-based assay technique designed for detecting and quantifying substances such as peptides, proteins, antibodies and hormones.
	esg	**Escargot** A Snail-family transcription factor.
F	**F/O**	**Flip-out**
	FAS-III	**Fascilin-III** A cell-adhesion molecule that is common in the nervous system.
	FLP	**Flippase** A *S. cerevisiase* recombinase that mediates recombination between DNA sequences flanked by FRT sites.
	FRT	**FLP recognition target** A nucleotide sequence recognised by the FLP recombinase.
	fru	**Fruitless**
	Fu	**Fused**
G	**GAL4**	**Galactose-responsive transcription factor 4** A transcription factor in *S. cerevisiae* that regulates the expression of genes involved in galactose metabolism.
	GAL80	**Galactose-responsive transcriptional regulator 80** A transcriptional regulator in *S. cerevisiae* that inhibits the activity of GAL4.
	Gbb	**Glass-bottom boat** One of the two BMP-signalling ligands in *D. melanogaster*.

	Term	Description
	GCE	**Germ cells-expressed** A juvenile hormone receptor that is mutually redundant with Met.
	GDP	**Guanine diphosphate**
	GFP	**Green fluorescent protein**
	Gpc	**GPI-linked glypican**
	GPCR	**G-protein–coupled receptor**
	GPCRK	**GPCR kinase**
	G-phase	**Growth phase**
	GPI	**Glycosyl phosphatidylinositol** A glycolipid that can be attached to the C-terminus of a protein during posttranslational modification.
	Gsk3β	**Glycogen synthase kinase 3β**
	GTP	**Guanine triphosphate**
H	**HA**	**Hemagglutinin**
	HDAC1	**Histone deacetylase 1**
	Hh	**Hedgehog** The ligand of the epynomous Hedgehog signalling pathway and potent tissue-patterning morphogen. It is post-translationally modified with cholesterol and palmitic acid, which reduces its aqueous spread.
	HSC	**Hedgehog signalling complex** A multi-protein complex that relays the Hedgehog signal downstream of Smoothened.
	HSPG	**Heparan sulphate proteoglycan**
I	**Ihog**	**Interference hedgehog**
	ID	**Integrated density**
	iPSC	**Induced pluripotent stem cell**
	IR	**Inverted repeat**
J	**JH**	**Juvenile hormone**
K	**Kyoto DGRC**	**Kyoto *Drosophila* Genetic Resource Centre**

	Term	Description
M	**Mad**	**Mothers against Dpp** An intracellular component of the BMP signalling pathway that acts as a BMP-dependent transcription factor together with Medea.
	MC	**Main cell** A secretory cell that forms the main part of the secretory epithelium of the *Drosophila* male accessory gland and is responsible for the synthesis of many accessory gland proteins.
	Med	**Medea** An intracellular component of the BMP signalling pathway that acts as a BMP-dependent transcription factor together with pMad.
	Met	**Methoprene-tolerant** A juvenile hormone receptor that is mutually redundant with GCE.
	mm	**Multiply mated**
	M-phase	**Mitotic phase**
	M-strain	**Maternal strain**
	MVBL	**Multivesicular body/lysosome**
N	**N**	Amino (**-NH_3**)
	N	**Notch** A single-pass transmemebrane receptor protein with a role in cell-cell communication.
	Nedd2	**NPC-expressed, developmentally down-regulated [gene] 2**
	NPC1a	**Niemann-pick type 1a** A multipass membrane protein with similarity to RND-type transmembrane transporters.
	n.s.	**Not significant**
	NTD	**N-terminal domain** The amino (-NH_3)-terminal part of a protein.
O	**O/N**	**Overnight**
P	***p***	**Probability**
	P13	**Pupal stage 13**
	PBS	**Phospate-buffered saline**

	Term	Description
	PBST	**PBS·Triton X**
	P-element	**Paternal element**
	PFA	**Paraformaldehyde**
	PKA	**Protein kinase A**
	pMad	**Phosphorylated Mad protein**
	P-strain	**Paternal strain**
	Ptc	**Patched** The Hedgehog receptor, and an inhibitor of the Hedgehog signalling pathway activator, Smoothened. It has similarity to multidrug efflux pump (RND-type) membrane proteins with a role in lipid transport and metabolism.
	PTTH	**Prothoracicotropic hormone**
Q	**Q**	The amino acid, glutamine.
	qRT-PCR	**Quantiative RT-PCR** A molecular biology technique for the quantification of RNA.
R	**RAB**	**RAS in the brain** A group of membrane-associated GTPase that localise to distinct subcellular compartments.
	RBF	**Retinoblastoma-family protein** The *D. melanogaster* orthologue of the retinoblastoma protein, and a negative regulator of the cell cycle.
	RFP	**Red fluorescent protein**
	RHO1	**RAS-homologous protein 1** A GTPase signaling protein that acts as a molecular switch and functions as key regulator of the actin cytoskeleton.
	RI	**Refractive index**
	RNAi	**RNA interference**
	RND	**Resistance-nodulation-division** A family of multipass transmembrane transporters that includes the proteins Patched, Dispatched and NPC1a.
	ROI	**Region of interest**
	RT-PCR	**Reverse transcriptase polymerase chain reaction** A molecular biology technique for the detection of RNA.
S	**S2 cells**	**Schneider 2 cells** One of the most common *D. melanogaster* cell lines.

	Term	Description
	SC	**Secondary cell** A large, secretory cell that is located at the distal end of the *D. melanogaster* accessory gland and that has large internal secretory compartments.
	SD	**Standard deviation**
	Sgp	**Salivary gland protein**
	SHH	**Sonic hedgehog** One of the mammal orthologues of the Hedgehog protein.
	Ski	**Skinny hedgehog**
	Smo	**Smoothened** The activator/signal transducer of the Hedgehog signalling pathway.
	SP	**Sex peptide** A small, main cell-derived accessory gland protein that is responsible for many of the physiological and behavioural changes that occur in female *D. melanogaster* after mating.
	S-phase	**Synthetic phase**
	spi	**Spitz** A *D. melanogaster* EGF-like growth factor.
	SSD	**Sterol-sensing domain**
	STRICA	**Ser/Thr-rich caspase**
	SuFu	**Suppressor of Fused**
T	**Tkv**	**Thickveins** A type I BMP receptor in *D. melanogaster*.
	TOR	**Target of rapamycin** A nutrient-sensitive kinase that regulates several aspects of cell growth, like protein translation and autophagy.
	TRR	**Trithorax-related** An EcR co-activator in *D. melanogaster.*
	ts	**Temperature-sensitive**
U	**UAS**	**Upstream activating sequence** A regulatory site within the promoters of galactose-responsive genes in *S. cerevisiae*.
	Usp	**Ultraspiracle**
V	**V**	**Ventral**
	VDRC	**Vienna *Drosophila* Resource Centre**

	Term	Description
	VP16	**Viral protein 16**
W	**w**	**White**
	Wt	**Wild-type**
Y	**YCA**	**Yeast-cornmeal agar**
	YFP	**Yellow fluorescent protein**
	yw	**Yellow white**

REFERENCES



PREVIOUS PAGE:
A summer view of the Magdalen College Cloisters.

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Itaque non invidebis […] nostris antecessoribus
suam his laudem.

[Do not […] begrudge our predecessors their proper credit.]

— Johannes Kepler, writing to Galileo Galilei
(in KEPLER, 1611: *pp*. 41-2)

– A –

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APPENDICES



PREVIOUS PAGE:
A view of one of the ornate vaulted ceilings in the Bodleian Library. (Note the many spandrels.)

APPENDIX 1

Numerical results tables for CHAPTER 3

There are many ways in which data can be presented. For the purposes of this Dissertation, I have made the decision to present data visually, in the form of graphs. The choice of graph is, in itself, important, and I have followed the advice of (WEISSGERBER *et al.,* 2015) in choosing the scatter dot-plot graphs—because this type of graph allows data points to be individually examined, as well as examined in the context of the larger data set itself. However, as with all representations, some aspects of the data are lost, regardless of what type of graph is chosen to represent it. To amend this loss of data to some extent, while also trying to avoid filling this Appendix with spreadsheets containing the raw data itself (however much I would like to do just that), I have prepared brief tables containing the numerical information presented in the graphs, to supplement the visual aspects of the data. For example, the number of samples that have gone into each data set can easily be lost in a scatter dot-plot, where many individual data points overlap in a graph with restricted space and give the appearance that 'one' and 'two' and 'three' (*etc.*) number of data points yield 'many' rather than the actual number (*n*) that was used.

Therefore, over the following pages, I have included supplementary tables relevant for the data otherwise presented in CHAPTER 3, and these have been referred to in the text for ease of cross-reference. The decision to not include these tables in the chapters themselves was made to maximize the clarity of the text and the impact of the figures, by not including any element that did not directly improve on these.

SUPPLEMENTARY TABLE 3. 1. | *dronc*^{ts} > RFP^{NLS} REPORTER ACTIVITY APPEARS TO NOT CHANGE OVER TIME

Age	Proportion *dronc*^{ts} > RFP^{NLS}-positive secondary cells ± SD (%)	n_{gland}
day 1	13.89 ± 5.9	13
day 3	21.36 ± 9.1	6
day 6	16.8 ± 8.2	16

SUPPLEMENTARY TABLE 3. 2 | THE EcR REGULATES THE PROPORTION OF SECONDARY CELLS POSITIVE FOR *dronc*^{ts} > RFP^{NLS}

Genotype	Proportion *dronc*^{ts} > RFP^{NLS}+ secondary cells ± SD (%)	n_{gland}
Control	16.8 ± 8.2	16
EcR^{RNAi}	1.5 ± 3.5	12
EcR-C	0 ± 0	15

SUPPLEMENTARY TABLE 3. 3. | BMP SIGNALLING REGULATES THE PROPORTION OF SECONDARY CELLS POSITIVE FOR *dronc^{ts}* > RFP^{NLS}

Genotype	Proportion *dronc^{ts}* > RFP^{NLS}+ secondary cells ± SD (%)	n_{gland}
Control	16.8 ± 8.2	16
Dad	0.03 ± 0.01	15
tkv^{Q253D}	43.3 ± 26.5	16

SUPPLEMENTARY TABLE 3. 4. | SECONDARY CELLS SHOW EVIDENCE OF LOW-LEVEL BMP SIGNALLING ACTIVATION

Age	Proportion pMad+ secondary cells ± SD (%)	n_{gland}
day 3	9.2 ± 8.5	8
day 6	10.1 ± 5.3	10

SUPPLEMENTARY TABLE 3. 5. | DRONC REGULATES NUMBERS OF NON-ACIDIC COMPARTMENTS IN SECONDARY CELLS

Driver	Age	Genotype	Number CD63::GFP^{+} non-acidic compartments ± SD	n_{gland}
dsx^{ts} > CD63::GFP	day 3	Control	24.16 ± 5.5	9
		dronc^{RNAi}	44.26 ± 24.4	15
	day 6	Control	37 ± 13.7	13
		dronc^{RNAi}	61.2 ± 23.1	16
	day 15	Control	48.1 ± 22.3	13
		dronc^{RNAi}	69.7 ± 41.4	13

Driver	Age	Genotype	Number GPI::GFP^{+} cored compartments ± SD	n_{gland}
spi^{ts} > GPI::GFP	day 6	Control	8.17 ± 2.3	17
		dronc^{RNAi}	10.76 ± 2.2	15

SUPPLEMENTARY TABLE 3. 6. | DRONC REGULATES ACIDIC COMPARTMENT NUMBERS IN SECONDARY CELLS

Age	Genotype	Acidic compartment number ± SD	n_{gland}
day 3	Control	4.56 ± 1.3	9
	dronc^{RNAi}	3.66 ± 3.0	15
day 6	Control	3.8 ± 1.7	13
	dronc^{RNAi}	5.2 ± 5	16
day 15	Control	3.9 ± 2.2	13
	dronc^{RNAi}	5.3 ± 5.3	13

SUPPLEMENTARY TABLE 3. 7. | DRONC REGULATES THE VOLUME OF THE LARGEST SECONDARY CELL ACIDIC COMPARTMENT

Age	Genotype	Acidic compartment volume (μm^3) ± SD	n_{gland}
day 3	Control	356.7 ± 37.9	9
	dronc^{RNAi}	305.2 ± 207	15
day 6	Control	751 ± 227.5	13
	dronc^{RNAi}	521.8 ± 193.5	16
day 15	Control	1187.9 ± 345.3	13
	dronc^{RNAi}	581.5 ± 219.8	13

SUPPLEMENTARY TABLE 3. 8. | THE EFFECT OF CATALYTICALLY INACTIVE DRONC ON SECONDARY CELL COMPARTMENTS

Genotype	Number CD63::GFP^{+} non-acidic compartments ± SD	n_{gland}
Control	49.7 ± 12.4	14
dronc$^{C>A}$	47.7 ± 16.3	11

Genotype	Acidic compartment number ± SD	n_{gland}
Control	3.4 ± 1.2	14
dronc$^{C>A}$	4.4 ± 2.1	11

Genotype	Acidic compartment volume (μm^3) ± SD	n_{gland}
Control	705.7 ± 285.7	14
dronc$^{C>A}$	533.3 ± 225.1	11

SUPPLEMENTARY TABLE 3. 9. | THE EFFECT OF CATALYTICALLY ACTIVE DRONC ON SECONDARY CELL COMPARTMENTS

Genotype	Number CD63::GFP^{+} non-acidic compartments ± SD	n_{gland}
Control	49.7 ± 12.4	14
dronc$^{\Delta N}$	25.1 ± 8.7	11

Genotype	Acidic compartment number ± SD	n_{gland}
Control	3.4 ± 1.2	14
dronc$^{\Delta N}$	5.0 ± 2.2	11

Genotype	Acidic compartment volume (μm^3) ± SD	n_{gland}
Control	705.7 ± 285.7	14
dronc$^{\Delta N}$	341.8 ± 163.1	11

SUPPLEMENTARY TABLE 3. 10. | DRONC SEEMS TO REGULATE SECONDARY CELL NUMBERS

Genotype	Time at 29 °C	Number of secondary cells	n_{gland}
dsx^{ts} CD63::GFP × *dsx*^{ts} CD63::GFP	Since eclosion (3 days)	44.85 ± 4.0	13
dsx^{ts} CD63::GFP × *UAS-dronc*^{RNAi}	Since eclosion (3 days)	53.82 ± 3.8	11
	Overnight (16 hours)	46.38 ± 4.4	13
UAS-dronc^{RNAi} × *UAS-dronc*^{RNAi}	Since eclosion (3 days)	44.63 ± 3.3	12

SUPPLEMENTARY TABLE 3. 11. | THE EFFECT OF KNOCKING DOWN *drICE* ON SECONDARY CELL COMPARTMENTS

Genotype	Number CD63::GFP^{+} non-acidic compartments ± SD	n_{gland}
Control	49.7 ± 12.4	14
drice^{RNAi}	122.9 ± 37.2	11

Genotype	Acidic compartment number ± SD	n_{gland}
Control	3.4 ± 1.2	14
drice^{RNAi}	7.3 ± 3.1	11

Genotype	Acidic compartment volume (μm^3) ± SD	n_{gland}
Control	705.7 ± 285.7	14
drice^{RNAi}	343.5 ± 161.8	11

SUPPLEMENTARY TABLE 3. 12. | THE EFFECT OF KNOCKING DOWN *dARK* ON SECONDARY CELL COMPARTMENTS

Genotype	Number CD63::GFP^{+} non-acidic compartments ± SD	n_{gland}
Control	45.4 ± 9.9	16
dark^{RNAi}	29.3 ± 12.0	14

Genotype	Acidic compartment number ± SD	n_{gland}
Control	3.8 ± 1.2	16
dark^{RNAi}	3.1 ± 1.2	14

Genotype	Acidic compartment volume (μm^3) ± SD	n_{gland}
Control	597.3 ± 205.7	16
dark^{RNAi}	528.1 ± 207.0	14

SUPPLEMENTARY TABLE 3. 13. | THE EFFECT OF OVEREXPRESSING *dIAP1* ON SECONDARY CELL COMPARTMENTS

Genotype	Number CD63::GFP^{+} non-acidic compartments ± SD	n_{gland}
Control	46.0 ± 13.1	8
diap1	24.1 ± 7.2	4

Genotype	Acidic compartment number ± SD	n_{gland}
Control	4.1 ± 1.0	8
diap1	3.8 ± 1.3	4

Genotype	Acidic compartment volume (μm^3) ± SD	n_{gland}
Control	597.8 ± 160.7	8
diap1	645.0 ± 170.6	4

APPENDIX 2

Activated BMP signalling changes the refractive appearance of secondary cells in the male accessory gland under DIC microscopy

Over the course of my investigations I have cursorily examined many accessory glands using DIC, as I have been preparing the sample for examination using confocal microscopy. Through these brief glimpses of the accessory gland tissues, I over time, I noticed a peculiar pattern emerging.

The accessory glands of virgin males normally appear nondescript; with the secretory granules of the secondary cells typically being the only notable structures present. However, after mating, a new structural feature is easy to observe, whose refraction pattern is different from that of the surrounding epithelial tissues (**SUPPLEMENTARY FIGURE 3. 1. A.**). These refractionally-different structures correspond to secondary cells, as confirmed using fluorescent confocal microscopy, and are very similar to the dead (apoptotic) cells that I helped examine in *C. elegans* gonads at the Institute of Healthy Ageing at University College London in the 2012–2013 academic year. The appearance of these atypical-looking cells in accessory glands in the DIC channel after mating is common, although further work will be needed to quantify their numbers properly (for example by capturing DIC and confocal micrographs of the glands to determine the proportion of refractionally-different cells to the total secondary cell population and see if this proportion changes significantly between virgin and mated glands).

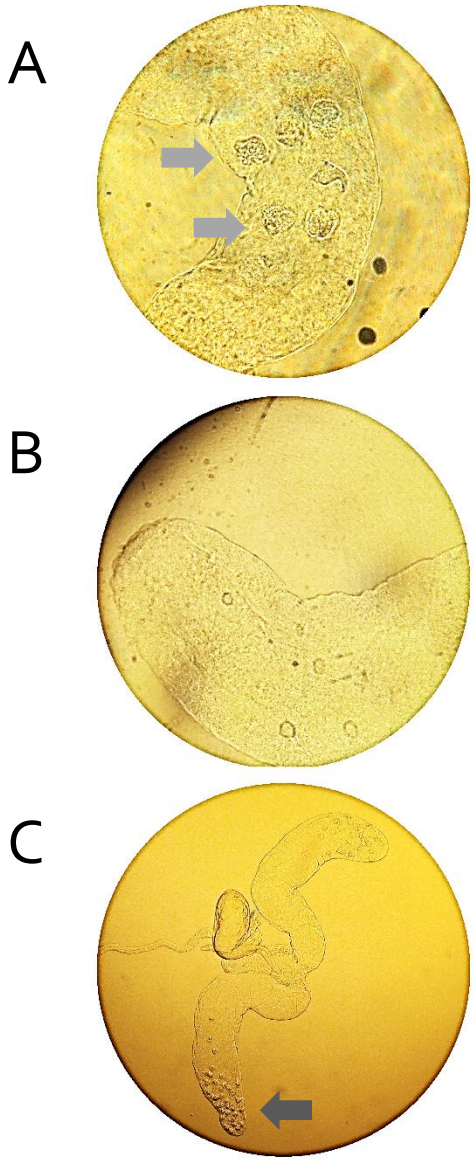
Having established that BMP signalling is activated upon mating, and having also observed an increase in the number of these refractionally-different cells when

UAS-tkv^{Q253D} is expressed in secondary cells (to numbers far exceeding those observed after mating in wild-type glands), I have concluded that BMP signalling is somehow causal of the appearance of these disc-like structures. Intrigued, when I had the samples available, I also examined the appearance of accessory glands where the secondary cells overexpress the BMP signalling inhibitor, *Dad*, and was interested to see that these atypical-looking cells are not seen in this genetic background—even after mating (**SUPPLEMENTARY FIGURE 3. 1. B**). Conversely, when BMP signalling is activated through expression of *tkv*^{Q253D}, the number of refractive cells is dramatically increased in virgin material (**SUPPLEMENTARY FIGURE 3. 1. C**).

My cursory examinations of accessory gland material using DIC microscopy suggest to me that BMP signalling is responsible for the appearance of these structural changes in the accessory gland epithelium. My previous experience of detecting apoptotic cells using this method leads me to believe that activated BMP signalling is detrimental to secondary cells, and that BMP signalling activation (either transgenic or after mating) reduces the viability of the secondary cells. This work would need to be performed more rigorously, and in conjunction with markers that identify stressed and/or dying cells, in order to better understand this phenomenon. For example, antibodies that detect activated caspase-3 (commercially available via Cell Signaling Technology) would be useful in this context, as would the various assays for apoptotic cells that are available commercially.

I believe there is merit to this work, since it suggests that the normal function of the accessory gland (filling/refilling in combination with sexual activity) contributes to the development of (what looks like a) pathology characterized by cell loss. This, in itself, would be an interesting topic of further study; with particular regard to biogerontology—where understanding the normal cellular processes that contribute

to pathology are a crucial component to understanding the ageing process itself (*e.g.* GEMS, 2015).



SUPPLEMENTARY FIGURE 3. 1. | ACTIVATED BMP SIGNALLING ALTERS THE REFRACTIVE APPEARANCE OF THE MALE ACCESSORY GLAND EPITHELIUM.

A: A subset of secondary cells appear refractionally different (arrows) after mating in the male accessory gland.

B: When the secondary cells overexpress the BMP pathway inhibitor, Dad, the secondary cells are not easily distinguished from the rest of the epithelial tissues, and the gland itself looks like the accessory gland of a virgin fly.

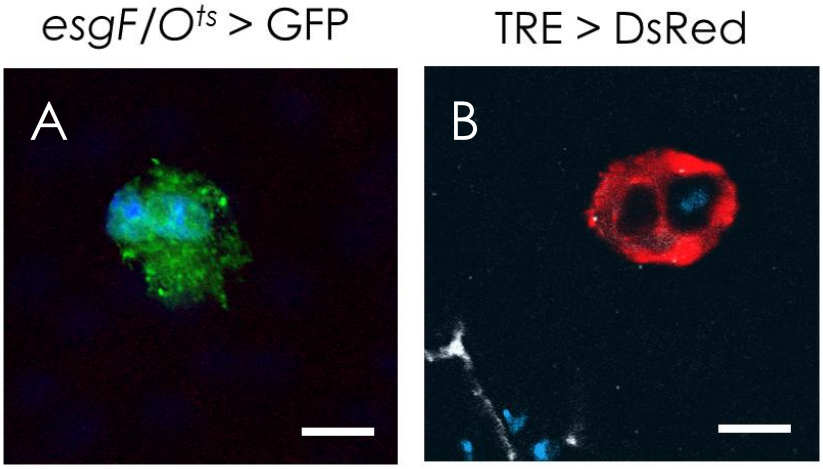
C: When secondary cells express an activated form of the Thickveins receptor, the proportion of secondary cells appearing refractionally different in the DIC channel is vastly increased; being easily detectable at the tips of both accessory glands which take on a granular-, nodular-looking appearance (arrow).

All images were captured down the eye piece of a confocal microscope by holding an iPhone 4 (Apple) up to it.

N.B. The images shown in this figure are not to scale, and no scale bar is provided.

Elevated BMP signalling has previously been associated with an increased frequency of secondary cell delamination from the epithelium (LEIBLICH *et al.,* 2012). Secondary cells delaminate from the epithelium at low levels in wild-type glands, and the number of delaminated cells increases over time (J. HELLBERG & C. WILSON, unpublished observations). This might suggest that the delamination represents a genuine age-related pathology of the male accessory gland—perhaps driven by increased BMP signalling activity over time? This would be interesting to investigate further.

LEIBLICH (2015) found that expression of *EcR-C* reduces the number of secondary cell delamination events. This suggests that the EcR-C isoform might be protective against secondary cell delamination. Intriguingly, as LEIBLICH (2015) has shown, and we have seen previously in CHAPTER 3, the activity of the EcR-C differs from that of the endogenous EcR isoforms. For example, expression of the EcR-C abolishes the expression of *dronc* in secondary cells (as measured by expression of the $drons^{fs}$ > RFP expression reporter) (**FIGURE 3. 5.**: *p.* 82). Although very hypothetical, this nevertheless suggests the *possibility* that the secondary cell delamination might depend of EcR-dependent DRONC activity. If it does, is it possible that the delaminated secondary cells are not actively delaminating after having acquired a more 'migratory' phenotype (as proposed by LEIBLICH *et al.* (2012), but that they are delaminating as the result of being 'unwell' and passively falling out of/being ejected from the epithelium? In support of this possibility, delaminated secondary cells appear generally unwell: containing granular-looking cytoplasm (**SUPPLEMENTARY FIGURE 3. 2. A**) and evidence of expression from a JNK-dependent stress reporter (TRE-DsRed; CHATTERJEE & BOHMANN, 2012) (**SUPPLEMENTARY FIGURE 3. 2. B**).



SUPPLEMENTARY FIGURE 3. 2. | DELAMINATED SECONDARY CELLS CONTAIN GRANULAR-LOOKING CYTOPLASM AND SIGNS OF ACTIVATED STRESS-SIGNALLING.

A: A delaminated secondary cell that has been imaged in the proximal portion of the male accessory gland. Do note how the secondary cell is present above the plane of the surrounding main cells (whose nuclei are out-of-focus in this focal plane). The secondary cell cytoplasm is labeled with *esgF/O*^{ts} > GFP that appears granular, suggesting that the cell is unwell. In this panel, nuclei are stained with DAPI (blue/cyan) and GFP stains the secondary cell cytoplasm (green).

B: A delaminated secondary cell in the proximal part of the male accessory gland. Again, note how the secondary cell is present in a plane apart from the main cells (the gland epithelium is visible to the bottom-left of the image), and appears to be floating free in the gland lumen. A JNK-signalling stress reporter (TRE-DsRed) stains the secondary cell cytoplasm strongly (red), but is absent from the cytoplasm of the proximal main cells. In this panel, TRE-DsRed is shown in red, whereas DNA is stained with DAPI (cyan), and FASIII stains epithelial cell junctions (white).

The scale bar in both panels represents 10 μm.

Altogether, regardless of what gives secondary cells their distinctive, refractory appearance and causes them to delaminate after mating or after ectopic BMP signalling activation, I think it represents an interesting, potential age-related pathology/phenotype that would be interesting to characterise further. Doing so might provide information on how secondary cells do and do not cope with the signalling and physical stress of being part of a dynamic, secretory epithelium in the male *D. melanogaster* reproductive tract.

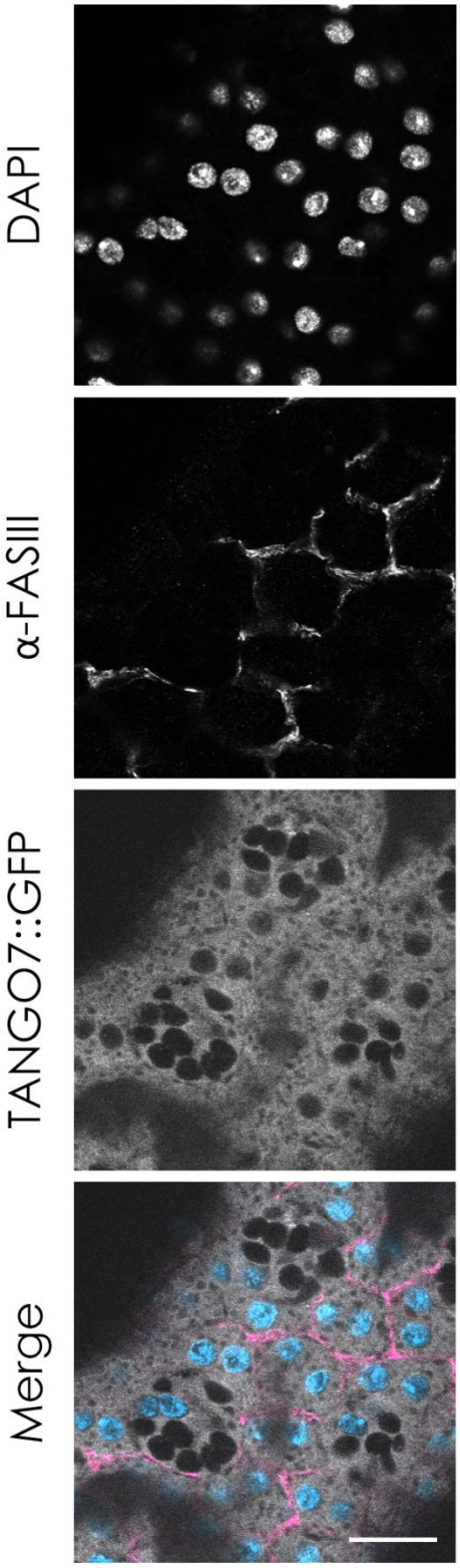
Supplementary reference:

Chatterjee, N. & Bohmann, D., (2012). A versatile ΦC31 based reporter system for measuring AP-1 and Nrf2 signaling in *Drosophila* and in tissue culture. *PLOS One, 7*(4): e34063. DOI: 10.1371/journal.pone.0034063

APPENDIX 3

TANGO7 is present in the male accessory gland

The transport and Golgi organisation protein, TANGO7, has been proposed to function as a regulator of non-apoptotic apoptosome function. A TANGO7 gene trap is available (SAROV *et al.,* 2016), and can be detected in the male accessory gland: it being present in the cytoplasm of both main cells and secondary cells and being excluded from the intracellular compartments in both cell types (**SUPPLEMENTARY FIGURE 3. 3.**).



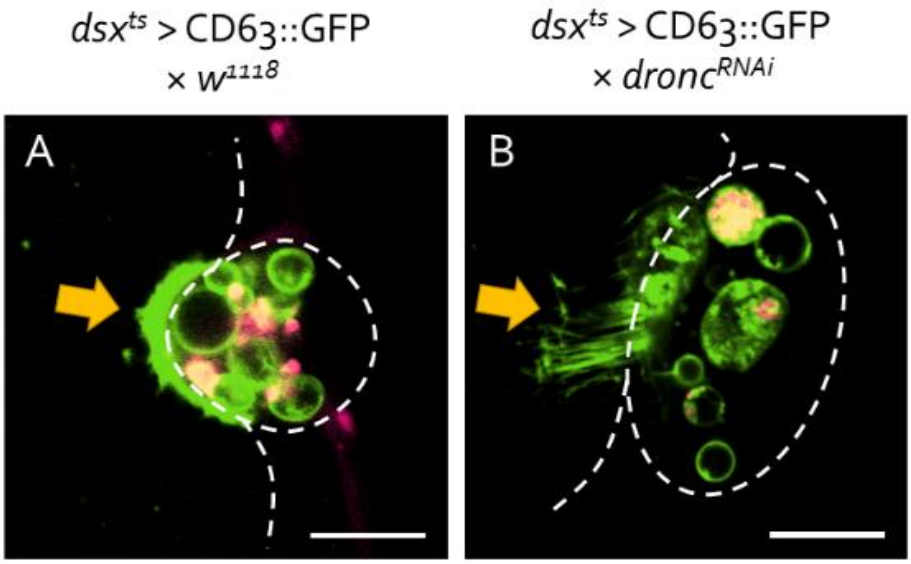
SUUPLEMENTARY FIGURE 3. 3. | TANGO7 IS PRESENT IN THE MALE ACCESSORY GLAND EPITHELIUM.

The Golgi-associated protein, TANGO7, has been shown to interact with the non-apoptotic apoptosome in *D. melanogaster*, and can be detected in the male accessory gland epithelium. The GFP-tagged TANGO7 protein seems to be present in the cytoplasm of both main cells and secondary cells, and seems to be excluded from the intracellular compartments (and nuclei) of these. In the bottom panel TANGO7::GFP is shown in white, whereas FASIII labels cell junctions magenta and DAPI is shown in cyan. The scale bar represents 20 μm.

APPENDIX 4

Secondary cells support apical filaments that extend into the accessory gland lumen

Imaging secondary cells labelled with the membrane marker, CD63::GFP, reveals that the apical face of the secondary cell supports filamentous extensions that reach into the accessory gland lumen (**SUPPLEMENTARY FIGURE 3. 4. A**). Whereas it remains to be determined whether these extensions represent a genuine secondary cell feature, or if they are an artefact resulting from overexpression of the CD63::GFP tetraspanin, I have nevertheless noticed that the length of these filaments is increased upon expression of an inverted repeat that targets the *D. melanogaster* caspase, *dronc* (**SUPPLEMENTARY FIGURE 3. 4. B**). However, this observation should be considered tentative, since the length of the extensions would need to be properly quantified and analysed via confocal micrography.



SUPPLEMENTARY FIGURE 3. 4. | RNAi-MEDIATED KNOCKDOWN OF *dronc* SEEMS TO INCREASE THE LENGTH OF EXTENSIONS PROTRUDING FROM THE SECONDARY CELL APICAL SURFACE.

When secondary cells express the membrane marker, CD63::GFP, the apical surface is easy to visualize (shown in green).

A: In control secondary cells, the CD63::GFP-positive apical surface (yellow arrows) is evident, and has a slight;y 'fuzzy' appearance.

B: In secondary cells expressing an inverted repeat that targets *dronc*, the CD63::GFP-positive apical surface supports much longer extensions (yellow arrow), that reach farther into the gland lumen.

In both panels the secondary cell outline and extent of the surface of the neighbouring main cells has been approximated with the dashed lines. CD63::GFP is shown in green whereas LysoTracker-Red is shown in magenta. The scale bar represents 10 μm.

APPENDIX 5

Numerical results tables for CHAPTER 4

SUPPLEMENTARY TABLE 4. 1. | THE PROPORTION OF *hh* > GFP+ SECONDARY CELLS INCREASES OVER TIME

Age (post-eclosion)	Proportion *hh* > GFP$^+$ secondary cells ± SD (%)	n_{gland}
0 hours	0.0 ± 0.0	8
8 hours	5.9 ± 10.6	10
24 hours	37.0 ± 13.9	9
72 hours	72.3 ± 15	10
144 hours	77.8 ± 14.8	13

SUPPLEMENTARY TABLE 4. 2. | THE PROPORTION OF *ptc* > GFP+ SECONDARY CELLS INCREASES OVER TIME

Age (post-eclosion)	Proportion *ptc* > GFP$^+$ secondary cells ± SD (%)	n_{gland}
0 hours	2.0 ± 4.6	8
8 hours	34.9 ± 13.5	13
24 hours	98.2 ± 3.0	10
72 hours	98.4 ± 2.4	10
144 hours	100.0 ± 0.0	6

SUPPLEMENTARY TABLE 4. 3. | *hh*^{RNAi} REDUCES SECONDARY CELL NUCLEAR SIZE

Genotype	Secondary cell sum nuclear size ± SD (μm^2)	n_{gland}
Control	56.4 ± 11.7	14
hh^{RNAi} I	43.2 ± 10.9	16
hh^{RNAi} II	42.3 ± 10.1	14
hh^{RNAi} III	45.1 ± 11.0	16

SUPPLEMENTARY TABLE 4. 4. | *hh*^{RNAi} DOES NOT REDUCE SECONDARY CELL SIZE

Genotype	Secondary cell maximum area ± SD (μm^2)	n_{gland}
Control	406.0 ± 67.8	14
hh^{RNAi} I	409.4 ± 105.5	16
hh^{RNAi} II	385.0 ± 79.1	14
hh^{RNAi} III	372.1 ± 97.0	16

SUPPLEMENTARY TABLE 4. 5. | OVEREXPRESSION OF *hedgehog* REDUCES SECONDARY CELL NUCLEAR SIZE

Genotype	Secondary cell sum nuclear area ± SD (μm^2)	n_{gland}
Control	52.1 ± 10.0	14
Hh::GFP	39.5 ± 8.1	14
HhN::GFP	38.6 ± 9.8	14
HhC85S::GFP	42.8 ± 9.2	13
HhC85SN::GFP	44.6 ± 8.7	5

SUPPLEMENTARY TABLE 4. 6. | DISPATCHED APPEARS TO REGULATE SECONDARY CELL NUCLEAR GROWTH

Genotype	Secondary cell sum nuclear area ± SD (μm^2)	n_{gland}
Control	52.7 ± 9.3	17
$disp^{RNAi}$ I	47.7 ± 7.3	16
$disp^{RNAi}$ II	41.4 ± 7.6	15

SUPPLEMENTARY TABLE 4. 7. | SMOOTHENED REGULATES SECONDARY CELL NUCLEAR SIZE

Genotype	Secondary cell sum nuclear area ± SD (μm^2)	n_{gland}
Control	49.2 ± 7.1	14
smo^{RNAi}	41.5 ± 6.5	13
smo	28.8 ± 3.8	12

SUPPLEMENTARY TABLE 4. 8. | PATCHED REGULATES SECONDARY CELL NUCLEAR SIZE

Genotype	Secondary cell sum nuclear area ± SD (μm^2)	n_{gland}
Control	50.5 ± 6.8	9
ptc^{RNAi} I	36.2 ± 6.2	16
ptc^{RNAi} II	40.1 ± 9.5	15

SUPPLEMENTARY TABLE 4. 9. | *ci*^{RNAi} REDUCES SECONDARY CELL NUCLEAR SIZE

Genotype	Secondary cell sum nuclear area ± SD (μm^2)	n_{gland}
Control	52.9 ± 9.3	17
ci^{RNAi} I	45.2 ± 7.8	16
ci^{RNAi} II	41.0 ± 10.4	15
ci^{RNAi} III	51.8 ± 12.4	10

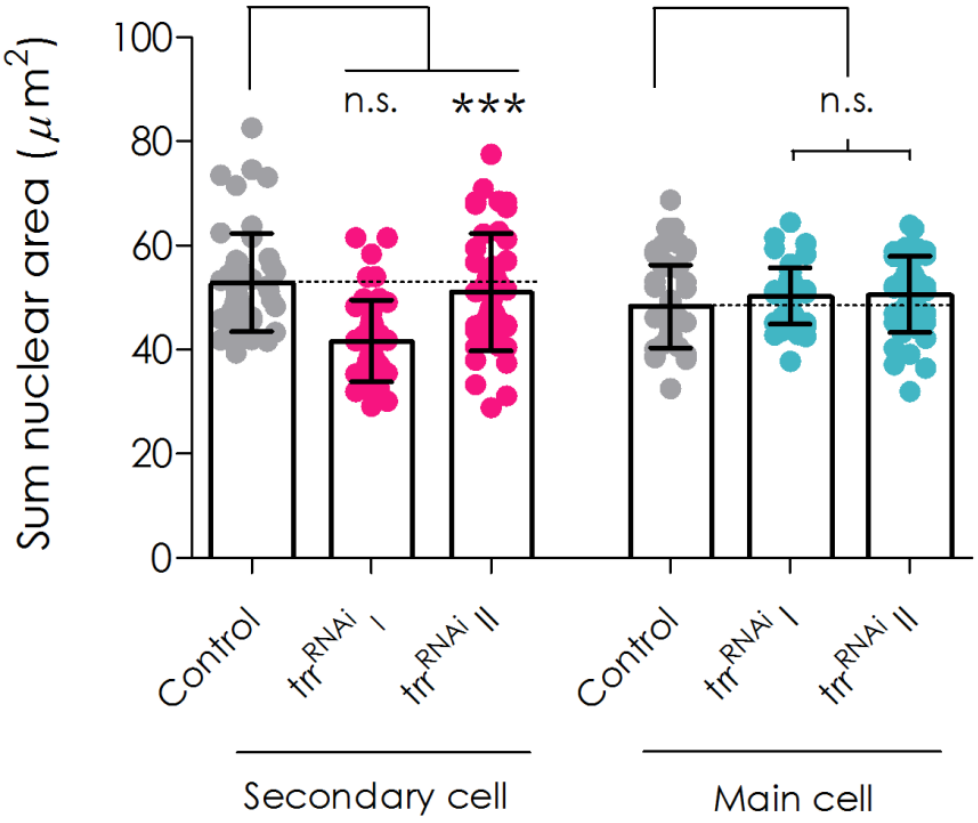
SUPPLEMENTARY TABLE 4. 10. | OVEREXPRESSION OF *ci* CAN DRIVE SECONDARY CELL NUCLEAR GROWTH

Genotype	Secondary cell sum nuclear area ± SD (μm^2)	n_{gland}
Control	49.2 ± 7.1	14
ci	53.4 ± 12.7	13
Ci::VP16	59.0 ± 11.4	12

APPENDIX 6

The EcR co-activator, Trithorax-related (TRR), regulates secondary cell nuclear size

The *hedgehog* gene promoter contains EcR-binding motifs, and EcR-dependent gene expression has been shown to be regulated by the EcR co-activator, TRR (SEDKOV & CHO *et al.,* 2003). Since RNAi of *EcR* (LEIBLICH, 2015) and *hedgehog* reduce secondary cell nuclear size, I was interested to see if RNAi of *TRR* would have an effect. To do so, I used *esg F/O*^{ts} to express two different RNAis targeting *TRR* (*TRR*^{RNAi} I and II; DIETZL *et al.,* 2007) in secondary cells (*esg F/O*^{ts} > *TRR*^{RNAi} I and II). Quantifying secondary cell nuclear size after six days at the non-permissive temperature shows that one of the *TRR* hairpin constructs (*TRR*^{RNAi} I) has a significant effect on secondary cell nuclear size (**SUPPLEMENTARY FIGURE 4. 1.**). Knocking-down *TRR* in secondary cells seems to have no effect on main cell nuclear size. Altogether, this suggests that TRR might have a role regulating secondary cell nuclear size, and it would be interesting to confirm the exact relationship between TRR, EcR, and Hedgehog in these cells.



SUPPLEMENTARY FIGURE 4. 1. | RNAi-MEDIATED KNOCKDOWN OF *TRR* REDUCES SECONDARY CELL NUCLEAR SIZE.

Scatterplot bar chart showing how secondary cell and main cell nuclei respond to the expression of an inverted repeat targeting *TRR* in the secondary cells. Each dot represents the value of an individual cell, and error bars show the standard deviation within the sample.

The number of stars represent the *p*-value obtained by a one-way ANOVA with Dunnett's correction for multiple comparisons (all columns compared to the relevant control): 'n.s.' = $p > 0.1$; *** = $p < 0.0001$.

APPENDIX 7

Numerical results tables for CHAPTER 5

SUPPLEMENTARY TABLE 5. 1. | SECONDARY CELL SUM NUCLEAR AREA CHANGES OVER TIME

Age	Sum nuclear area ± SD (μm^2)	n_{gland}
day 0	37.7 ± 5.9	16
day 1	40. 0 ± 8.6	14
day 3	44.2 ± 7.8	16
day 6	39.9 ± 8.5	14
day 6 (mated)	46.3 ± 9.1 8	15
day 10	32.9 ± 5.6	13

SUPPLEMENTARY TABLE 5. 2. | MAIN CELL SUM NUCLEAR AREA CHANGES OVER TIME

Age	Sum nuclear area ± SD (μm^2)	n_{gland}
day 0	40 ± 6.7	16
day 1	41.6 ± 6.4	14
day 3	43.6 ± 5.9	16
day 6	43.5 ± 8.4	14
day 6 (mated)	44.36 ± 1.0	15
day 10	41.4 ± 6.8	13

SUPPLEMENTARY TABLE 5. 3. | SECONDARY CELL SUM NUCLEAR AREA CHANGES RELATIVE TO THE NUMBER OF FEMALES SUPPLIED

Number of females	Sum nuclear area ± SD (μm^2)	n_{gland}
virgin (0 females)	39.9 ± 8.5	14
3 females	39.1 ± 7.3	12
5 females	43.7 ± 12.8	14
7 females	46.3 ± 9.1 8	15

SUPPLEMENTARY TABLE 5. 4. | SECONDARY CELL VOLUME DOES NOT SEEM TO CHANGE MUCH OVER TIME

Age	Cell volume ± SD (μm^3)	n_{gland}
day 0	21,735.0 ± 4,128.5	16
day 1	28,201.3 ± 6,431.0	14
day 3	24,579.7 ± 8,457.6	16
day 6	21,870.7 ± 6,099.0	14
day 6 (mated)	35,888.7 ± 11,019.7	15
day 10	25,089.6 ± 7,346.3	13

SUPPLEMENTARY TABLE 5. 5. | MATING ACTIVATES BMP SIGNALLING IN SECONDARY CELLS

Genotype	Mating status	Proportion pMad+ secondary cells ± SD (%)	n_{gland}
Control	Virgin	10.1 ± 5.3	10
	Mated	27.7 ± 9.3	7
dpp^{RNAi} I	Virgin	2.5 ± 3.2	11
	Mated	2.5 ± 5.1	10
dpp^{RNAi} II	Virgin	6.4 ± 4.9	13
	Mated	3.4 ± 2.9	12
Dad	Mated	0.0 ± 0.0	11

SUPPLEMENTARY TABLE 5. 6. | MATING-DEPENDENT NUCLEAR GROWTH REQUIRES ACTIVE BMP SIGNALLING

Genotype	Mating status	Sum nuclear area ± SD (μm^2)	n_{gland}
Control	Virgin	49.6 ± 8.9	14
	Mated	61.1 ± 11.9	13
Dad	Virgin	31.3 ± 4.2	15
	Mated	27.4 ± 4.0	15

SUPPLEMENTARY TABLE 5. 7. | MATING FURTHER SUPPLEMENTS BMP-DEPENDENT SECONDARY CELL NUCLEAR GROWTH

Genotype	Mating status	Sum nuclear area ± SD (μm^2)	n_{gland}
Control	Virgin	49.6 ± 8.9	14
	Mated	61.1 ± 11.9	13
tkv^{Q253D}	Virgin	84.8 ± 18.3	15
	Mated	159.16 ± 53.2	12

SUPPLEMENTARY TABLE 5. 8. | MATING CAN FURTHER SUPPLEMENT NUCLEAR GROWTH IN RESPONSE TO BMP/ECR SIGNALLING AXIS ACTIVATION

Genotype	Mating status	Sum nuclear area ± SD (μm^2)	n_{gland}
Control	Virgin	49.6 ± 8.9	14
	Mated	61.1 ± 11.9	13
EcR-C	Virgin	53.1 ± 8.4	16
	Mated	67.4 ± 17.7	14
EcR-C tkv^{Q253D}	Virgin	117.6 ± 37.5	16
	Mated	150.7 ± 35.7	16

SUPPLEMENTARY TABLE 5. 9. | MATING-DEPENDENT NUCLEAR GROWTH SEEMS TO REQUIRE THE EcR

Genotype	Mating status	Sum nuclear area ± SD (μm^2)	n_{gland}
Control	Virgin	49.6 ± 8.9	14
	Mated	61.1 ± 11.9	13
EcR^{RNAi}	Virgin	40.4 ± 8.2	14
	Mated	46.5 ± 8.4	7
EcR^{RNAi} *tkv*^{Q253D}	Virgin	41.7 ± 7.9	5
	Mated	52.6 ± 12.3	4

SUPPLEMENTARY TABLE 5. 10. | HEDGEHOG SIGNALLING AND MATING-DEPENDENT SECONDARY CELL NUCLEAR GROWH

Genotype	Mating status	Sum nuclear area ± SD (μm^2)	n_{gland}
Control	Virgin	52.9 ± 9.3	17
	Mated	69.9 ± 16.3	14
hh^{RNAi}	Virgin	41.1 ± 8.0	15
	Mated	62.8 ± 12.7	11
Ci::VP16	Virgin	58.5 ± 11.6	19
	Mated	67.0 ± 15.3	9

SUPPLEMENTARY TABLE 5. 11. | CYCLIN D MIGHT REGULATE MATING-DEPENDENT SECONDARY CELL NUCLEAR GROWH

Genotype	Mating status	Sum nuclear area ± SD (μm^2)	n_{gland}
Control	Virgin	43.0 ± 9.0	13
	Mated	65.5 ± 12.2	15
cycD^{RNAi}	Virgin	37.8 ± 7.1	14
	Mated	53.5 ± 13.8	14
cycD·CDK4	Virgin	288.2 ± 91.6	14
	Mated	365.6 ± 121.6	15

SUPPLEMENTARY TABLE 5. 12. | CYCLIN E SEEMS TO BE REQUIRED FOR THE MATING-DEPENDENT SECONDARY CELL NUCLEAR GROWH

Genotype	Mating status	Sum nuclear area ± SD (μm^2)	n_{gland}
Control	Virgin	43.0 ± 9.0	13
	Mated	65.5 ± 12.2	15
cycE^{RNAi} I	Virgin	44.6 ± 7.9	14
	Mated	49.6 ± 8.2	15
cycE^{RNAi} II	Virgin	37.9 ± 8.0	14
	Mated	48.6 ± 9.1	16
cycE I	Virgin	106.6 ± 41.5	15
	Mated	110.6 ± 4.3	16
cycE II	Virgin	158.1 ± 47.6	14
	Mated	138.9 ± 41.1	15

SUPPLEMENTARY TABLE 5. 13. | EdU LABELLING REVEALS THAT GENETIC MANIPULATIONS THAT ALTER SECONDARY CELL NUCLEAR SIZE DRIVE THE SYNTHESIS OF NEW DNA

Genotype	Proportion EdU+ secondary cells ± SD (%)	n_{gland}
Control	1.1 ± 2.6	11
tkv^{Q253D}	56.3 ± 33.2	11
EcR-C	99.7 ± 1.0	10
EcR^{RNAi} tkv^{Q253D}	6.0 ± 11.6	7

SUPPLEMENTARY TABLE 5. 14. | MATING PROMOTES BMP AND EcR-DEPENDENT EdU UPTAKE BY SECONDARY CELLS

Genotype	Mating status	Proportion EdU+ secondary cells ± SD (%)	n_{gland}
Wild-type	Virgin	1.0 ± 2.1	11
	Mated	14.7 ± 11.3	6
Control	Virgin	1.1 ± 2.6	11
	Mated	30.2 ± 25.5	9
Dad	Mated	1.3 ± 2.4	12
EcR^{RNAi}	Mated	11.0 ± 15.3	11

SUPPLEMENTARY TABLE 5. 15 | EdU UPTAKE CORRELATES WITH SECONDARY CELL NUCLEAR GROWTH AFTER MATING

Mating status	EdU uptake	Sum nuclear area ± SD (μm^2)	n_{gland}
Virgin	EdU^-	34.3 ± 7.8	7
Mated	EdU^-	47.4 ± 11.1	5
	EdU^+	60.9 ± 17.3	

SUPPLEMENTARY TABLE 5. 16. | MATING INCREASES THE DAPI STAINING INTENSITY OF SECONDARY CELL NUCLEI RELATIVE TO MAIN CELL NUCLEI

Age	DAPI staining intensity ± SD (SC:MC ratio)	n_{gland}
day 0	0.91 ± 0.28	16
day 3	1.02 ± 0.26	16
day 6	1.13 ± 0.35	14
day 6 (mated)	1.41 ± 0.67	15
day 10	1.08 ± 0.31	13

SUPPLEMENTARY TABLE 5. 17. | THE ROLE OF E2F1 AND RBF IN MATING-DEPENDENT NUCLEAR GROWTH

Genotype	Mating status	Sum nuclear area ± SD (μm^2)	n_{gland}
Control	Virgin	39.2 ± 6.6	14
	Mated	58.0 ± 12.7	11
E2F1^{RNAi}	Virgin	37.5 ± 6.1	14
	Mated	54.1 ± 12.6	15
RBF^{CA}	Virgin	30.3 ± 14.2	13
	Mated	65.8 ± 23.6	6

SUPPLEMENTARY TABLE 5. 18. | MAIN CELL NUCLEAR GROWTH CAN BE PROMOTED BY POSITIVE REGULATORS OF THE CELL CYCLE

Genotype	Sum nuclear area ± SD (μm^2)	n_{gland}
Control	51.5 ± 6.7	15
cycD·CDK4	171.5 ± 46.3	16
E2F1·Dp	170.4 ± 55.6	15
RBF^{CA}	67.0 ± 9.4	14

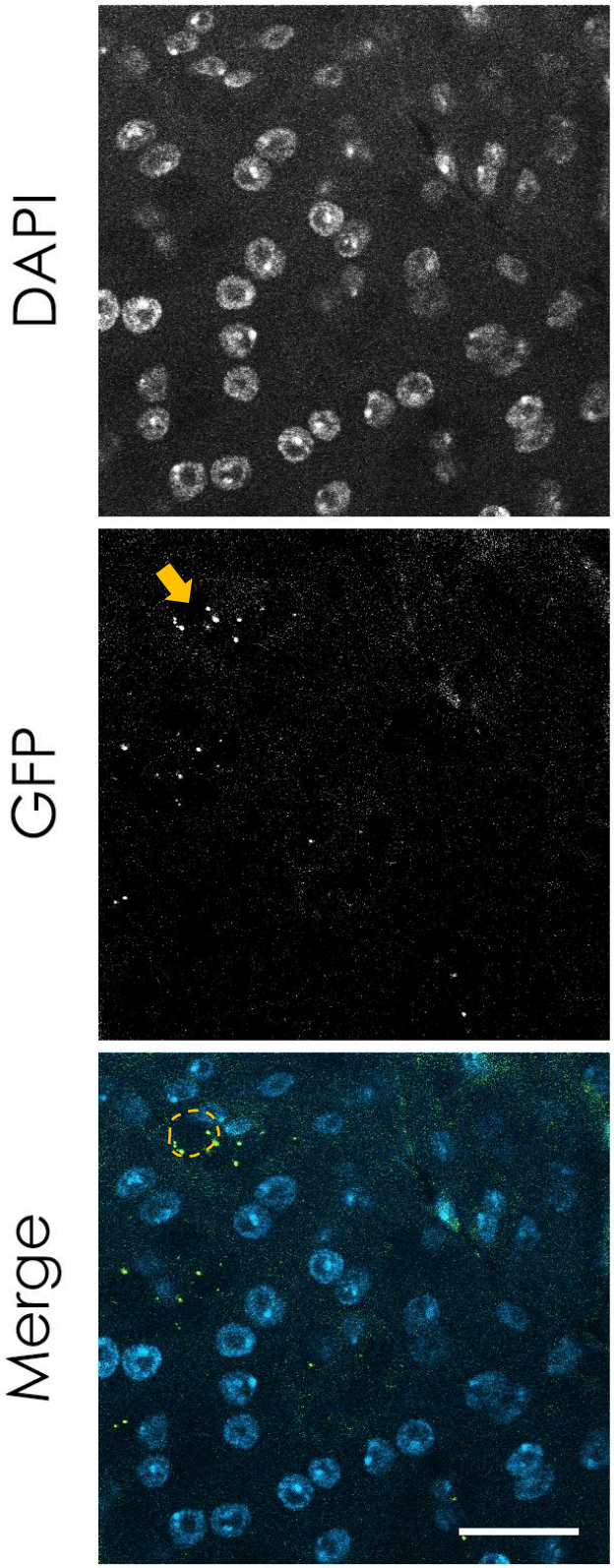
APPENDIX 8

Notch appears to be present in secondary cells

Notch signalling can regulate endoreplication in *D. melanogaster* (e.g. SUN & DENG, 2007). A Notch gene trap is available (N::GFP; COUTURIER *et al.* 2012), and dissecting the accessory glands of six-day old males reveals GFP-positive *puncta* in secondary cells, but not in main cells (**SUPPLEMENTARY FIGURE 5. 1.**). These *puncta* appear to appear in association with the secondary cell intracellular compartments. This is consistent with the internalisation of activated Notch (SCHWEISGUTH, 2004). However, further work will be required to confirm this work in secondary cells. For example, higher-resolution microscopy of accessory gland material from the Notch gene-trap line, co-stained with α-FASIII (to label the epithelial cell junctions) and α-Chaoptin (to label the secondary cell intracellular compartment membranes), might better reveal the exact localisation N::GFP in secondary cells. In addition, although preliminary work has shown that RNAi of Notch (NI *et al.*, 2009) does not affect secondary cell nuclear size when expressed over a six-day period in adult males (J. HELLBERG & C. WILSON, preliminary results), because of the relationship between Notch signalling and endoreplication in other *D. melanogaster* tissues (*e. g.* follicle cells; SUN & DENG, 2007), it would be interesting to determine if Notch participates in this process in secondary cells.

Supplementary reference

Schweisguth, F., (2004). Regulation of Notch signaling activity. *Current Biology*, *14*(3): R129–38. DOI: 10.1016/j.cub.2004.01.023



SUPPLEMENTARY FIGURE 5. 1. | NOTCH APPEARS TO BE PRESENT IN THE MALE ACCESSORY GLAND EPITHELIUM.

A Notch gene trap (N::GFP) appears to be present as punctate structures on the large intracellular compartments of secondary cells in the male accessory gland (yellow arrow in the middle panel, and outlined in the bottom panel). In the bottom panel N::GFP is shown in green, whereas DAPI is shown in cyan. The scale bar represents 20 μm.

APPENDIX 9

Mating stimulates secondary cell nuclear growth in *ecdysoneless* males

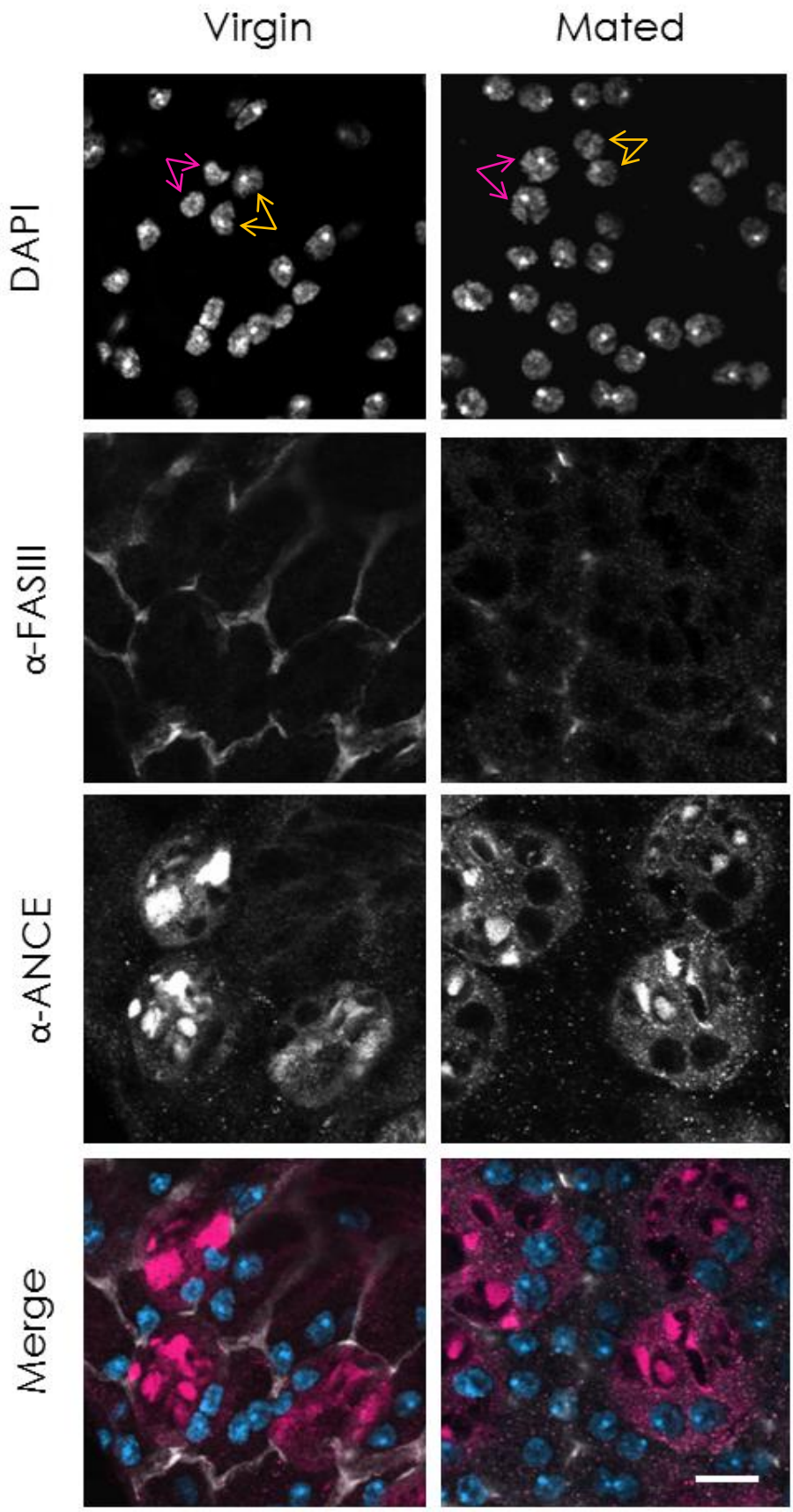
When present in double copy, and flies are shifted to the non-permissive temperature (28.5 °C), the recessive allele *ecd*^{1} results in a decreased fruit fly whole-body titer of ecdysone (GAREN *et al.*, 1977). Since ecdysone is the EcR ligand, one would expect this treatment to reduce EcR activity. When male *ecd*^{1} flies are shifted to the non-permissive temperature at eclosion and maintained there for six days, the nuclei of their secondary cells are smaller than those of similarly treated controls (*w*^{1118} male flies, although Canton-S would make a better control for future experiments) (**SUPPLEMENTARY FIGURE 5. 2.**, **SUPPLEMENTARY FIGURE 5. 3.**) (LEIBLICH, 2015). Having shown in CHAPTER 5 that the EcR is important for the mating-dependent nuclear growth phenotype, I was intrigued to determine how the nuclei would behave when temperature-shifted *ecd*^{1} males are mated multiple times. Based on the behaviour of the nuclei in *ecd*^{1} virgin males, I expected the nuclei to fail to grow after mating in this genotype. Surprisingly, this is not the case: *ecd*^{1} temperature-shifted secondary cell nuclei *do* grow after mating (**SUPPLEMENTARY FIGURE 5. 2.**, **SUPPLEMENTARY FIGURE 5. 3.**). This suggests that although mating-dependent nuclear growth requires the EcR, the EcR can function in secondary cells even in the absence of high titres of ecdysone. This implies that the EcR might function in a potentially ligand-independent manner in secondary cells, potentially due to BMP-dependent stabilisation of the receptor.

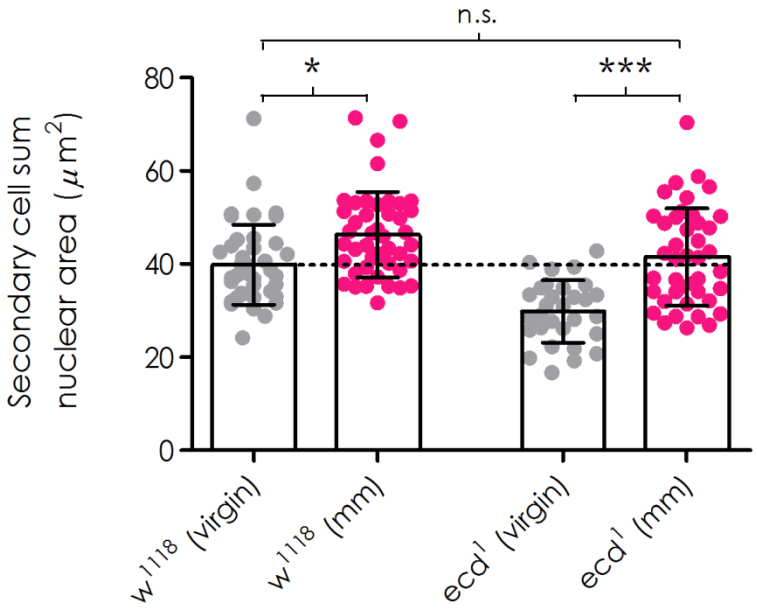
Having determined that secondary cell nuclei grow after mating in temperature-shifted *ecd*^{1} males, I was curious to determine if this growth is supported by new DNA being synthesised. Maintaining *ecd*^{1} male flies on 0.2 mM EdU medium for six days at the non-permissive temperature, together with seven females, I could determine the proportion of secondary cells having synthesised new DNA. Unfortunately, variation within these glands was quite high compared to the other genotypes I have examined, and the average proportion of EdU-positive secondary cells was not different between *w*^{1118} and *ecd*^{1} males (**SUPPLEMENTARY FIGURE 5. 4.**). However, there appears to be a trend towards a potential increase in EdU incorporation in the *ecd*^{1} genotype, meriting further experiments with higher n-numbers (and the appropriate virgin and Canton-S controls) to determine if this is indeed the case.

SUPPLEMENTARY FIGURE 5. 2. | THE SECONDARY CELL NUCLEI OF *ecd*^{1} MALES GROW AFTER MATING.

Secondary cell nuclei (pink arrows) are smaller in size than those of the surrounding main cells (yellow arrows) in the accessory gland of a virgin male. After mating, the secondary cell nuclei have increased in size, compared to the surrounding main cell nuclei.

The scale bar corresponds to 10 μm and α-FASIII labels main cell junctions (white). Secondary cells are labelled with α-ANCE (magenta), and DAPI stains DNA (cyan).

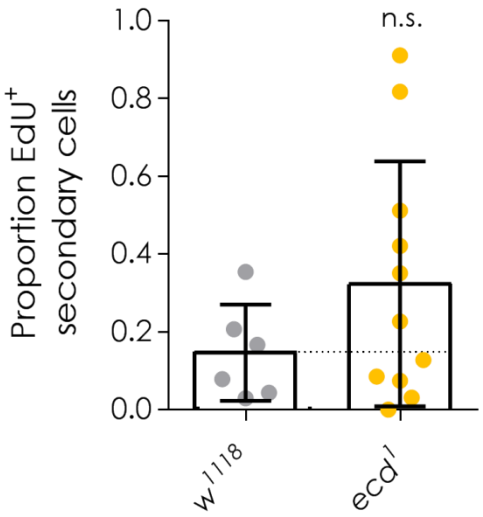

Virgin
Mated
DAPI
α-FASIII
α-ANCE
Merge



SUPPLEMENTARY FIGURE 5. 3. | SECONDARY CELL NUCLEI APPEAR TO GROW AFTER MATING IN *ecd¹* MALES.

Quantifying the nuclear size of secondary cells before (virgin) and after mating (mated) shows that the nuclei increase in size after mating in both *w¹¹¹⁸* and *ecd¹* males. Although the *ecd¹* secondary cell nuclei are smaller than those in *w¹¹¹⁸* males in virgin flies, they reach a similar size to the *w¹¹¹⁸* virgin secondary cell nuclei after mating.

Each dot on the plot represents the value of an individual secondary cell, and error bars show the standard deviation within the sample. The number of stars represent the *p*-value obtained by one-way ANOVA with Tukey's correction for multiple comparisons (all columns): 'n.s.' = $p > 0.1$; * = $p < 0.01$; *** = $p < 0.0001$.



SUPPLEMENTARY FIGURE 5. 4. | MATING MIGHT INDUCE ENDOREPLICATION IN *ecd¹* SECONDARY CELLS.

Scatterplot bar chart showing how the proportion of EdU-positive secondary cells after mating compare between *w¹¹¹⁸* and *ecd¹* males.

Each dot represents the value of an individual accessory gland, and error bars show the standard deviation within the sample. The number of stars represent the *p*-value obtained by one-way ANOVA with Tukey's correction for multiple comparisons (all columns): 'n.s.' denotes $p > 0.1$.

PLOS | GENETICS

RESEARCH ARTICLE

Regulation of Dense-Core Granule Replenishment by Autocrine BMP Signalling in *Drosophila* Secondary Cells

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Abstract

Regulated secretion by glands and neurons involves release of signalling molecules and enzymes selectively concentrated in dense-core granules (DCGs). Although we understand how many secretagogues stimulate DCG release, how DCG biogenesis is then accelerated to replenish the DCG pool remains poorly characterised. Here we demonstrate that each prostate-like secondary cell (SC) in the paired adult *Drosophila melanogaster* male accessory glands contains approximately ten large DCGs, which are loaded with the Bone Morphogenetic Protein (BMP) ligand Decapentaplegic (Dpp). These DCGs can be marked in living tissue by a glycophosphatidylinositol (GPI) lipid-anchored form of GFP. In virgin males, BMP signalling is sporadically activated by constitutive DCG secretion. Upon mating, approximately four DCGs are typically released immediately, increasing BMP signalling, primarily via an autocrine mechanism. Using inducible knockdown specifically in adult SCs, we show that secretion requires the Soluble NSF Attachment Protein, SNAP24. Furthermore, mating-dependent BMP signalling not only promotes cell growth, but is also necessary to accelerate biogenesis of new DCGs, restoring DCG number within 24 h. Our analysis therefore reveals an autocrine BMP-mediated feedback mechanism for matching DCG release to replenishment as secretion rates fluctuate, and might explain why in other disease-relevant systems, like pancreatic β-cells, BMP signalling is also implicated in the control of secretion.

Author Summary

Glands and neurons concentrate and store the signals that they secrete in dense-core granules (DCGs). They are released in response to the appropriate stimulus in a regulated fashion. Since release rate varies with the level of stimulation, it must be matched to DCG biogenesis to replenish the DCG pool rapidly after secretion. However, the mechanisms by

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Competing Interests: The authors have declared that no competing interests exist.

which DCG release is sensed and then co-ordinated with biogenesis are not understood. Here we genetically dissect this process in the prostate-like secondary cells of the adult male *Drosophila* accessory gland. These cells form large DCGs that are secreted into seminal fluid. We find that these DCGs are loaded with a specific signalling molecule, the Bone Morphogenetic Protein (BMP), Decapentaplegic (Dpp). When males mate, a number of DCGs are rapidly secreted, stimulating BMP signalling primarily via an autocrine mechanism. This BMP signal accelerates DCG biogenesis, so that DCG number is fully replenished within 24 h. Our work therefore suggests that at least in secondary cells, BMP signalling plays a simple autocrine role in matching DCG release to new biogenesis. Interestingly, BMP signalling affects secretion in other cell types, such as pancreatic β-cells, suggesting possible evolutionary conservation of this homeostatic mechanism.

Introduction

Regulated secretion of neurotransmitters, hormones and enzymes from neuronal, endocrine and exocrine cells typically involves fusion of specialised secretory compartments with the plasma membrane. Prior to secretion, the bioactive molecules are packaged into dense-core granules (DCGs), which form via a series of membrane trafficking and maturation events [1,2]. Cargos are first clustered together at the trans-Golgi network, and bud off in membrane-bound compartments. They are then sorted from non-DCG proteins by specific interactions with adaptor molecules [2] and condense together in a maturation process that requires intraluminal Ca^{2+} ions and acidification [3,4].

By studying DCG biogenesis in cultured mammalian secretory cells, some key regulators have been identified. For example, intraluminal proteins called granins complex with cargos and drive formation and budding off of immature DCGs from the Golgi [5,6]. Cargo clustering at the trans-Golgi network also involves cholesterol and lipid raft-like structures [7], and requires the activity of specific enzymes associated with lipid metabolism [8,1].

When stimulated by secretagogues, mature DCGs fuse to the plasma membrane in a Ca^{2+}-dependent fashion via a process involving synaptotagmins, SNAP (Soluble NSF Attachment Protein) Receptors (SNAREs) and vesicle-associated membrane proteins (VAMPs) [9,10]. Secretory cells must increase DCG biogenesis to rapidly replenish released DCGs. There is good evidence for elevated transcription and translation of mRNAs encoding DCG proteins after secretion [11]. But the mechanisms by which either these processes or membrane trafficking itself are modulated in response to secretion are unknown, despite their likely importance in maintaining secretion rates in several disease-relevant systems, particularly pancreatic β-cells [1]. A number of signalling molecules have been implicated in regulating secretion. For example, insulin release from β-cells is affected by Hedgehog proteins [12], Wnts [13] and BMPs [14]. However, models to genetically dissect out DCG biogenesis control *in vivo* are very limited, and none of these signals has yet been implicated in triggering elevated DCG biogenesis in response to exocytosis.

Secondary cells (SCs) of the paired exocrine *Drosophila* male accessory glands (AGs) form multiple large intracellular secretory compartments [15,16] (Fig 1A). A minority of these compartments are Rab7-positive, acidic, mature late endosomal multivesicular bodies or lysosomes (MVBLs) [17]. The remaining compartments have been referred to as secretory vacuoles (SVs) [16]. Many, but not all, of these contain DCGs [16,17]. To date, the only characterised molecule identified within these DCGs is the homologue of angiotensin-I converting enzyme, ANCE [16].

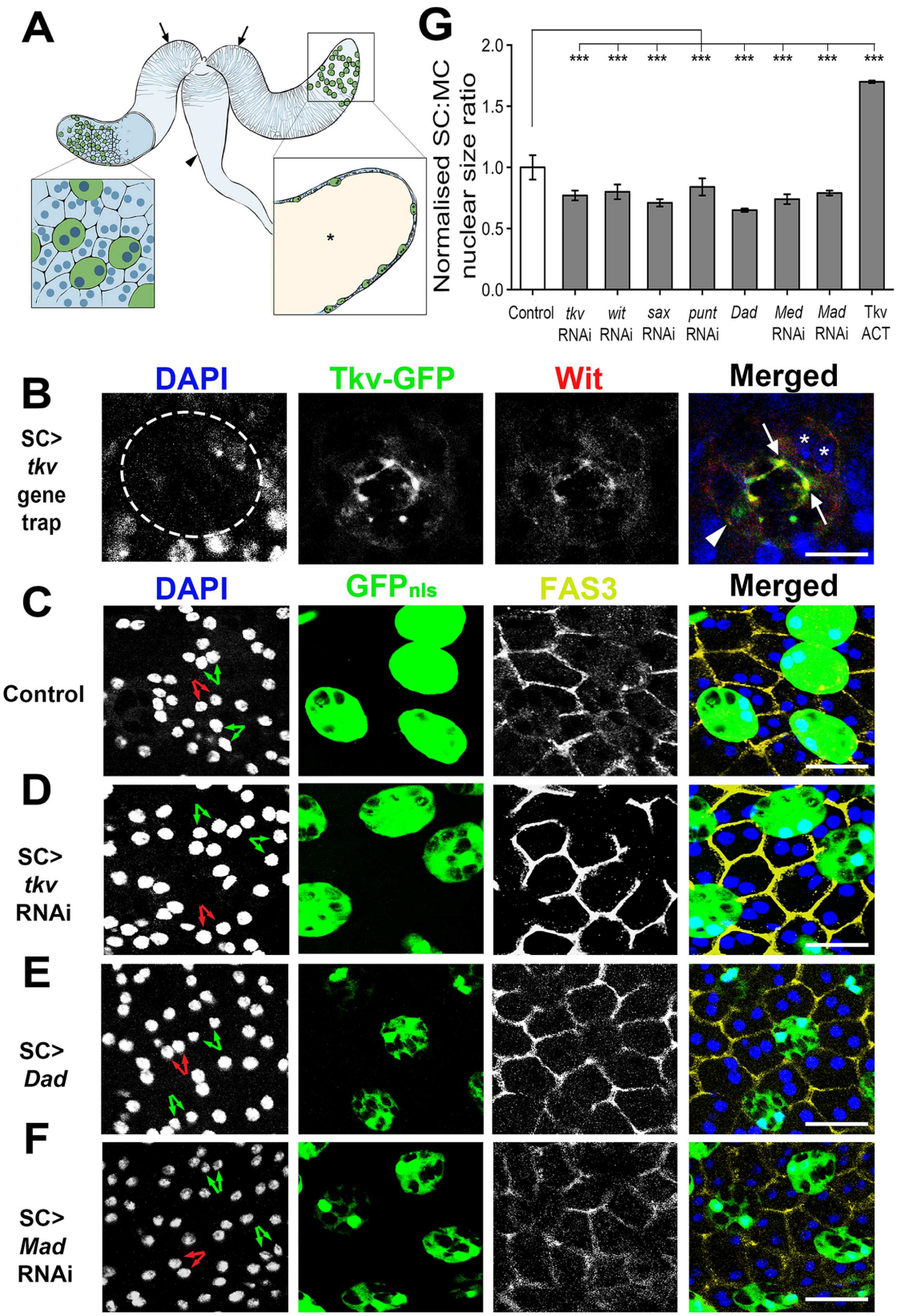

A
G
Normalised SC:MC nuclear size ratio
2.0
1.5
1.0
0.5
0.0
***
Control
tkv RNAi
wit RNAi
sax RNAi
punt RNAi
Dad
Med RNAi
Mad RNAi
Tkv ACT
B
SC> tkv gene trap
DAPI
Tkv-GFP
Wit
Merged
C
Control
DAPI
GFPnls
FAS3
Merged
D
SC> tkv RNAi
E
SC> Dad
F
SC> Mad RNAi

Fig 1. Multiple BMP signalling components regulate nuclear growth in SCs. A. Schematic of the paired male accessory glands (arrows), which pump their contents into the ejaculatory duct (arrowhead) during mating. Left inset shows the epithelial secretory monolayer containing secondary cells (SCs; green) and main cells (MCs), all of which are binucleate. Right inset is a section through the gland revealing the large lumen (asterisk). **B.** SC (circled) expressing a gene trap for the BMP type I receptor *tkv*, and stained with an antibody against the BMP type II receptor Wit. These receptors are present both on the plasma membrane (arrowhead) and co-localise in intracellular compartments (arrow). DAPI marks nuclei blue in SCs (asterisks) and surrounding non-expressing MCs. **C-F.** Accessory glands (AGs) from 6-day-old controls (C) and males expressing RNAis targeting *tkv* (D) or *Mad* (F) or expressing the BMP signalling antagonist *Dad* (E) in adult SCs under the control of esgF/O^{ts} after temperature shift at eclosion. AGs were dissected, fixed and imaged by confocal microscopy. Glands were stained with DAPI (blue) to mark nuclei and an anti-Fas3 antibody (yellow) to mark cell boundaries. SCs express nuclear GFP, which is also present in the cytosol. Pairs of nuclei from binucleate SCs and MCs are indicated by green and red arrows respectively. **G.** Bar chart showing SC nuclear size relative to that of MC neighbours for glands expressing different transgenes in SCs under esgF/O^{ts} control, normalized to the ratio for controls. Note that all manipulations that decrease BMP signalling significantly reduce SC nuclear size. Genotypes for images are: *w; PBac[544.SVS-1]tkv^{CPTI002487}* (B); *w; esg-GAL4 tub-GAL80^{ts} UAS-FLP; UAS-GFP$_{nls}$ actin>FRT>CD2>FRT>GAL4* combined with no other transgene (C); *P[TRiP.JF01485]attP2* (III) (D); *P[w^{+} UAS-Dad]* (II) (E); *P[TRiP.JF01263]attP2* (III) (F). ***P<0.001, Kruskal-Wallis test, n = 10. Scale bar for (B) is 10 μm, for all other images it is 20 μm.

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Like their mammalian counterparts, the prostate and seminal vesicles, the AGs secrete a complex mixture of proteases, protease inhibitors and other glycoproteins into seminal fluid, which are transferred to females during mating [18]. The glands' secretome modulates female behaviour, for example by increasing egg laying and feeding, and reducing the female's receptivity to subsequent mating [19]. Many of these functions are attributed to sex peptide [20], which is secreted by the second epithelial cell type in the AG, the main cells (MCs).

More recent data also implicate SCs in modifying female behaviours. Abnormal development of SCs in males is associated with reduced ability to induce female egg laying and inhibit female receptivity [21,22]. MVBLs in SCs generate large numbers of intraluminal vesicles (ILVs) that are labelled by a GFP-tagged form of the human tetraspanin CD63, which is typically associated with secreted vesicles called exosomes in mammalian systems [17]. Indeed, CD63-GFP-positive vesicles are released into the AG lumen. Blocking exosome formation or trafficking regulators in adult SCs specifically affects the male's ability to inhibit female remating [17].

Interestingly, post-mitotic adult SCs grow as males age, a process enhanced by mating [23]. Bone Morphogenetic Protein (BMP) signalling is essential for this growth, for formation and maturation of MVBLs and for exosome secretion [17]. It is also required for reprogramming of female remating behaviour, although it is unclear whether this is linked to its role in exosome secretion [23].

Here we show that Decapentaplegic (Dpp), the homologue of mammalian BMP ligands, BMP2 and BMP4, is packaged into DCGs of SCs and is the primary driver of BMP-dependent processes in SCs. We demonstrate that regulated DCG release involves the SNARE protein SNAP24 and is stimulated by mating. Secretion drives a rapid Dpp-dependent autocrine feedback mechanism and some paracrine signalling between SCs, which accelerates the replenishment of new DCG compartments in preparation for subsequent matings. Previously reported roles for BMPs in DCG secretion suggest that this autocrine mechanism may be shared by other higher eukaryotic cells, potentially offering a new route for modulating defective secretion in disease.

Results

Multiple BMP signalling components control SC growth

We previously showed that expression of a constitutively activated form of the Type I BMP receptor Thick veins, Tkv (Tkv^{ACT}, containing a Q199D substitution) [24], drives SC growth

and secretion, while knockdown of the co-Smad *Medea* (*Med)* [25], a BMP-dependent transcriptional regulator, or overexpression of the downstream BMP antagonist *Daughters against Decapentaplegic*, *Dad* [26], blocks adult SC growth and endolysosomal trafficking [23,17]. To determine which BMP receptors are expressed in SCs, we first analysed a yellow fluorescent protein- (YFP-) tagged *tkv* gene trap line, $tkv^{CPTI002487}$ [27]. It was expressed in all SCs, primarily associating with the plasma membrane and small intracellular compartments distinct from the large SC compartments (Fig 1B). In addition, an antibody against the Type II BMP receptor Wishful Thinking (Wit) [28], which forms a heterodimer with Type I receptors, labelled a subset of the Tkv-YFP-positive intracellular compartments and the cell surface (Fig 1B). These data suggest that Tkv and Wit are normally expressed in SCs.

To test which BMP signalling genes normally control SC growth, they were knocked down with specific RNAis in adult SCs using the esgF/O^{ts} driver line [23]. In this line, a ubiquitously expressed, temperature-sensitive form of the GAL4 inhibitor GAL80 blocks *esg*-GAL4-dependent expression until the temperature is shifted to 28.5°C in newly eclosed virgin males, driving SC-specific RNAi and nuclear GFP expression in the AG.

We knocked down the Type I BMP receptors, *tkv* and *saxophone* (*sax*), and the Type II receptors, *punt* (*put*) and *wit*, in adult SCs, using two independent RNAis per gene, several of which have been used successfully in previous analyses of BMP signalling in other cell types (see Materials and Methods). SC growth was assessed in 6-day-old adults by measuring nuclear size relative to neighbouring MC nuclei. We have found previously that this provides a simple assay to gauge overall SC growth, because nuclear size typically correlates with SC size [23,17].

RNAi-induced silencing of each receptor, but not a control RNAi (targeting MC-specific *Sex Peptide*) significantly decreased relative SC:MC and absolute SC nuclear size compared to controls, demonstrating that they play non-redundant roles in regulating growth (Figs 1C, 1D and 1G, S1A–S1D and S2A–S2C). It was not feasible to gauge the level of knockdown in these experiments by RT-PCR of AG mRNA, because SCs represent about 2% of all the cells in the AG, and it is unclear whether the different BMP signalling components are exclusively expressed in SCs. However, Wit expression levels, as judged by immunostaining, were greatly reduced following SC-specific *wit* knockdown, indicating strong knockdown in this experiment (S2G and S2H Fig). Consistent with previous findings [23], downregulation of BMP signalling by knockdown of *Med* or *Mad*, the gene encoding Med's binding partner, or by overexpressing Dad also significantly inhibited SC growth, while up-regulating BMP signalling through overexpression of Tkv^{ACT} increased growth (Figs 1E–1G, S1E, S1F and S2A–S2C). None of the UAS-linked constructs affected growth in the absence of a GAL4 driver (S2D Fig). We conclude that SCs express the signalling machinery required to transduce BMP signalling via more than one non-redundant receptor complex.

BMP signalling regulates SV numbers, endolysosomal maturation and exosome secretion

We previously reported that blocking BMP signalling in SCs by overexpressing Dad decreases the total number of large non-acidic SVs, prevents MVBL maturation and inhibits exosome secretion [17]. We tested whether all BMP signalling components are similarly involved in these processes. A GFP-tagged form of the human exosome marker CD63 [29] labels all or most large (>1 μm diameter) internal SC compartments and exosomes released by SCs (Fig 2A and 2F) [17]. Since incorporating new GFP-tagged transgenes into the esgF/O^{ts} line was technically challenging, we employed the *dsx*-GAL4 driver, which is expressed only in SCs within the AG epithelium and has been used previously with UAS-CD63-GFP to mark SC exosomes [17]. Expressing Dad and Tkv^{ACT} with *dsx*-GAL4 produces the same effects as the esgF/O^{ts} driver

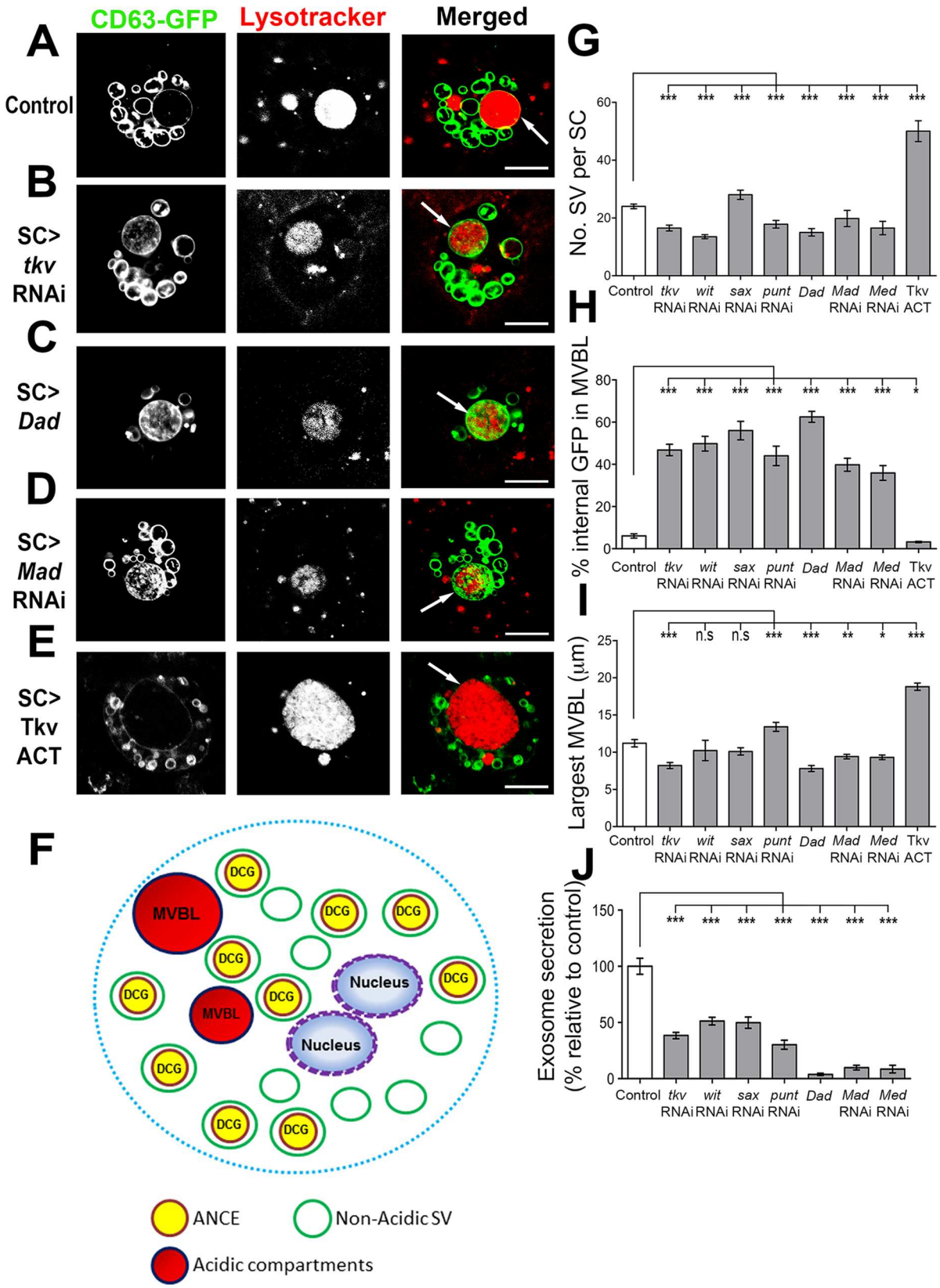

CD63-GFP
Lysotracker
Merged
A
Control
B
SC> tkv RNAi
C
SC> Dad
D
SC> Mad RNAi
E
SC> Tkv ACT
F
MVBL
DCG
Nucleus
ANCE
Non-Acidic SV
Acidic compartments
G
No. SV per SC
H
% internal GFP in MVBL
I
Largest MVBL (μm)
J
Exosome secretion (% relative to control)
Control
tkv RNAi
wit RNAi
sax RNAi
punt RNAi
Dad
Mad RNAi
Med RNAi
Tkv ACT
n.s

Fig 2. Multiple BMP signalling components in SCs regulate numbers of SVs, endolysosomal maturation, largest MVBL size and exosome secretion. A-E. SCs from 6-day-old males expressing CD63-GFP and either no additional transgene (A), or RNAis against *tkv* (B) or *Mad* (D), or overexpressing *Dad* (C) or an activated form of the *Tkv* receptor (Tkv^{ACT}; E) throughout adulthood. They were stained with the acid-sensitive dye Lysotracker Red (red), and imaged under live conditions using confocal microscopy. Images represent a single z-plane, so do not show all SVs. **F**. Schematic representation of different intracellular SC compartments, showing large non-acidic compartments (SVs), most of which contain DCGs. **G**. The number of large CD63-GFP-positive SVs (>1μm diameter) per SC was counted. Inhibiting BMP signalling (except when using *sax*-RNAi) results in fewer SVs, while activating the pathway produces more. **H**. In control glands (A), GFP fluorescence is largely undetectable in the lumen of the largest, most mature MVBL (arrow). Inhibition of BMP signalling, however, results in significant accumulation of GFP fluorescent signal in the MVBL lumen. **I**. Reducing BMP signalling often reduces the diameter of the largest MVBL, while activating the pathway (by overexpressing Tkv^{ACT}) increases the diameter. **J**. Inhibiting BMP signalling in SCs reduces the number of CD63-GFP-positive exosomes inside the AG lumen. Genotypes for images are: *w; UAS-CD63-GFP tub-GAL80^{ts}; dsx-GAL4* combined with no other transgene (A); *P[TRiP.JF01485]attP2* (III) (B); *P[w$^+$ UAS-Dad]* (II) (C); *P[TRiP.JF01263]attP2* (III) (D); *P[w$^+$ UAS-Tkv^{ACT}]* (E). The *w; UAS-CD63-GFP tub-GAL80^{ts}; dsx-GAL4* line was employed to produce data in G-J. *P<0.05, **P<0.01, ***P<0.001, Kruskal-Wallis test, n = 10. Scale bar for all images is 10 μm.

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on SC nuclear size (S2E Fig), suggesting similar levels of GAL4 expression with these two drivers. Furthermore, SC nuclear size was unaffected in 6-day-old males by expression of CD63-GFP under *dsx*-GAL4 control from eclosion onwards (S2F Fig), indicating that SC growth was not affected by expression of this marker.

RNAi knockdown of *tkv*, *put*, *wit*, *Mad*, or *Med* using *dsx*-GAL4 resulted in an overall decrease in SV number, while Tkv^{ACT} produced an increase (Figs 2B–2E, 2G and S3B–S3G). Surprisingly, both *sax*-RNAi lines appeared to increase SV numbers (Figs 2G, S3D and S3G), suggesting that this receptor might have a different effect to other signalling components on SC membrane trafficking.

Fluorescence of GFP fusion proteins, including CD63-GFP, is quenched by the acidic microenvironment of MVBLs [17,30]. Analysis of confocal z-sections through each SC's largest MVBL in control 6-day-old flies showed that less than 10% of the total area was marked by ILV-associated fluorescent GFP in live glands (Fig 2A and 2H), even though anti-GFP antibody staining revealed that this compartment was full of cross-reacting GFP (S3A Fig). Silencing any BMP signalling component resulted in significantly increased accumulation of fluorescent CD63-GFP inside the largest MVBL (Figs 2B–2D, 2H, S3B–S3F and S3H). Conversely, Tkv^{ACT} expression significantly reduced the GFP signal (Fig 2E and 2H), supporting the idea that BMP signalling through multiple receptors promotes MVBL maturation.

In agreement with our previous findings [17], the size of the largest MVBL was also often reduced when BMP signalling was inhibited (Fig 2I and S3I Fig), consistent with a role in driving trafficking into this compartment. However, we found that knockdown of some BMP signalling components did not produce this phenotype (eg. both *punt* RNAis), perhaps because degradation and export of MVBL contents are reduced by these treatments as well as endolysosomal trafficking. Knockdown of all BMP receptors, *Mad* or *Med* in adult SCs resulted in a significant reduction in CD63-GFP-positive puncta in the AG lumen, which we have previously shown represent exosomes [17], as did overexpression of Dad (Fig 2J and S3J Fig). In conclusion, all *Drosophila* BMP receptors and major downstream transcriptional regulators are required to retain normal SV number, promote endolysosomal maturation and drive exosome release from SCs, demonstrating that this signalling pathway plays a key role in secretory trafficking events through multiple receptor combinations.

BMP-dependent SC growth and membrane trafficking are primarily regulated via autocrine Dpp signalling

Drosophila melanogaster synthesises three BMP ligands, two of which are closely related to mammalian BMPs: Decapentaplegic (Dpp) is homologous to BMP2 and BMP4, and Glass

bottom boat (Gbb) is more closely related to BMP5-8. The third ligand Screw (Scw) is a more distant BMP relative, which does not appear to be expressed in adults [31] and was therefore not analysed.

Knockdown of either *dpp* or *gbb* in adult MCs using the MC-specific driver, *Acp26Aa*-GAL4 [32], suggested that neither gene controls SC growth in these cells (eg. S4F Fig). However, SC-specific silencing of *dpp*, but not *gbb*, resulted in a significant reduction in SC nuclear growth (Figs 3A and 3B, S4D, S4E, S4G and S4H), indicating that SC-expressed Dpp normally controls SC growth. Moreover, overexpression of GFP-tagged Dpp, Dpp-GFP [33], but not Gbb-GFP [34], in SCs increased SC growth (Figs 3B, S4A, S4D and S4E), even though both fusion proteins can activate BMP signalling in other tissues. Interestingly, overexpression of Dpp-GFP in MCs had no effect on growth of these cells, but did increase SC growth (S4C and S4F Fig).

To further investigate whether *dpp* is normally synthesised in SCs, we analysed the expression of a previously characterised *dpp*-GAL4 transgene, dpp^{blk}-GAL4, which recapitulates endogenous *dpp* expression in several developmental scenarios [35,36]. In the adult AG, dpp^{blk}-GAL4 drives UAS-GFP expression exclusively in SCs (Fig 3C). Although we cannot be certain that this reflects normal *dpp* expression, combined with the *dpp* knockdown data, we conclude that SCs are the primary source of SC-regulatory BMP activity in the AG.

To determine whether Dpp signals cell-autonomously or non-autonomously to control SC growth, *dpp*-RNAi-expressing SC mosaics were produced using the esgF/O^{ts} system [23]. Growth of those SCs expressing *dpp*-RNAi, which are GFP-positive, was most severely reduced (Fig 3D and 3E). However, SCs not expressing the RNAi were also affected, albeit to a lesser extent. Conversely, mosaic overexpression of UAS-*dpp-GFP* increased nuclear growth only in SCs expressing the transgene (Fig 3E and S4B Fig). We conclude that SC-expressed Dpp primarily regulates growth via an autocrine mechanism, but can also exert paracrine effects on other SCs.

To test whether Dpp, like other BMP signalling components, affects membrane trafficking events within SCs, it was knocked down in SCs expressing CD63-GFP during the first 6 days of adulthood. The number of SVs was reduced and endolysosomal maturation inhibited (Fig 3F–3I), mirroring effects seen with knockdown of other BMP signalling components. The size of the biggest acidic compartment, which is more variably affected by changes in BMP signalling (Fig 2I), was slightly enlarged with one *dpp*-RNAi treatment and unaffected by the other (Fig 3J). Dpp was required for the secretion of CD63-GFP-positive exosomes into the AG lumen (Fig 3K). Our results demonstrate that Dpp signals mainly in an autocrine way in SCs and is the primary regulator of BMP-dependent growth, SV number, endolysosomal maturation and exosome secretion in these cells.

Dpp is stored in DCGs of SCs prior to secretion

To determine how SC-expressed Dpp is trafficked and secreted, Dpp-GFP was overexpressed specifically in these cells. Live confocal imaging revealed that Dpp-GFP is concentrated in spherical structures resembling DCGs, but is typically absent from MVBLs (Fig 4A). Co-staining Dpp-GFP-expressing SCs with the DCG marker ANCE [16] confirmed that Dpp-GFP is mostly trafficked to DCGs (Fig 4B). Consistent with this, an RFP-tagged form of Dpp [37] primarily trafficked into CD63-GFP-positive SVs (S5A Fig). DCG localisation does not appear to be the result of overexpression of tagged Dpp, because even a short one hour pulse of Dpp-GFP expression is chased into DCG compartments over a subsequent12 h period (S5B Fig).

Previously, we reported that a subset of non-acidic SVs is marked by the slow recycling endosome marker Rab11 [17,38]. Co-staining SCs from flies expressing Rab11-YFP from a

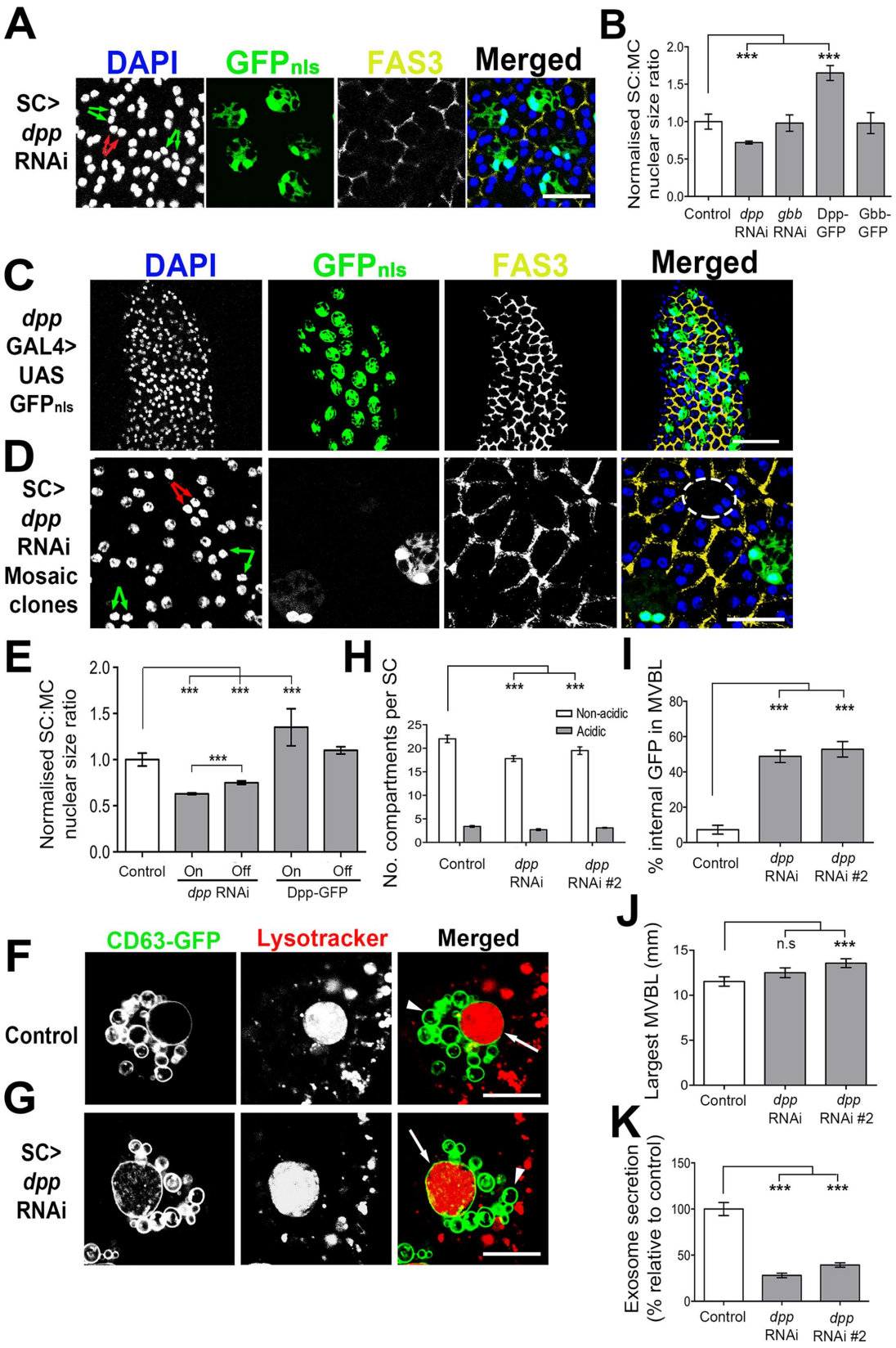

A
DAPI
GFPnls
FAS3
Merged
SC>
dpp
RNAi
B
Normalised SC:MC nuclear size ratio
Control
dpp RNAi
gbb RNAi
Dpp-GFP
Gbb-GFP
C
dpp
GAL4>
UAS
GFPnls
D
SC>
dpp
RNAi
Mosaic
clones
E
Normalised SC:MC nuclear size ratio
On
Off
dpp RNAi
Dpp-GFP
H
No. compartments per SC
Non-acidic
Acidic
dpp RNAi #2
I
% internal GFP in MVBL
J
Largest MVBL (mm)
n.s
K
Exosome secretion (% relative to control)
F
CD63-GFP
Lysotracker
Merged
Control
G
SC>
dpp
RNAi

Fig 3. Autocrine Dpp regulates SC growth, SV number, endolysosomal trafficking and exosome secretion. A. Expression of *dpp*-RNAi during the first six days of adulthood using the esgF/O^{ts} driver reduces the size of SCs and their nuclei (green arrows) relative to MCs (red arrows). **B**. Relative SC:MC nuclear size for SCs expressing RNAis targeting *dpp* and *gbb*, or GFP-tagged Dpp and Gbb, revealing specific effects of Dpp on growth. **C**. *dpp^{blk}*-GAL4 drives expression of a UAS-coupled nuclear GFP exclusively in SCs of the AG. **D, E.** Mosaic expression of *dpp*-RNAi or Dpp-GFP in a subset of SCs has a stronger effect on nuclear growth in expressing cells (on–green arrows) than in non-expressing (off–red arrows; white dashed circle) SCs, although *dpp* knockdown also reduces growth in the latter. **F-K.** Co-expression of *dpp*-RNAi with CD63-GFP using the *dsx*-GAL4 driver (G) reduces non-acidic SV number (eg., marked by arrowhead) and increases GFP fluorescence in largest MVBL (arrow; stained with Lysotracker Red) compared to controls (F); the statistical analysis of these changes for two independent RNAis is shown in H and I respectively. Knockdown of *dpp* either results in a small increase in the size of the largest MVBL or no significant size change (J), and reduces exosome secretion (K). Confocal images are from fixed glands (A, C, D) stained with DAPI (blue) and for Fas3 (yellow) or from living glands (F, G). Genotypes for images are: *w; esg-GAL4 tub-GAL80^{ts} UAS-FLP; UAS-GFP$_{nls}$ actin>FRT>CD2>FRT>GAL4/P[TRiP.HMS00011]attP2* (A and mosaic in D; the esgF/O^{ts} driver was also used to generate data in E); *w; P[w^{+} UAS-GFP$_{nls}$]; P[w^{+} dpp^{blk}-GAL4]* (C); *w; UAS-CD63-GFP tub-GAL80^{ts}; dsx-GAL4* combined with no other transgene (F) or *P[TRiP.HMS00011]attP2* (III) (G). ***P<0.001, Kruskal-Wallis test, n = 10. Scale bar for A, D is 20 μm, F, G, 10 μm, and for C, 50 μm.

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tubulin promoter [38] with an anti-ANCE antibody revealed that all ANCE-containing DCG compartments were lined with Rab11 (Fig 4C), indicating that DCGs are located in Rab11-positive compartments. Although we have previously reported that the tub-Rab11-YFP transgene suppresses formation of large MVBLs in SCs [17], the number of Rab11-YFP-positive compartments was identical to the number of ANCE-positive compartments in wild type cells from 3-day-old males (~10; S4I Fig). Therefore, the subset of SVs that contains DCGs appears to be unaffected by expression of this Rab fusion protein. Like ANCE, Dpp-GFP was secreted from SCs into the AG lumen (Fig 4E), from where it potentially exerts its paracrine effects on other SCs.

DCGs can be marked by a glycophosphatidylinositol-anchored form of GFP

Since overexpressing Dpp-GFP in SCs over extended periods has a significant effect on membrane trafficking and cell growth (Fig 3E), we investigated alternative markers to label DCG compartments in living cells. We found that when expressed in SCs, UAS-regulated GFP-GPI [39], a form of GFP that binds to the outer leaflet of lipid bilayers via a glycophosphatidylinositol (GPI) anchor, also traffics to the limiting membrane of DCG compartments and surprisingly concentrates in ANCE-positive DCGs, even though they do not appear to contain membranes (Fig 4D).

Since we found it difficult to make a line containing both UAS-*GFP-GPI* and *dsx*-GAL4, we combined the GFP-GPI transgene with a ubiquitously expressed GAL80^{ts} construct and *spitz*-GAL4 (*spi*-GAL4), which drives GAL4 expression strongly and selectively in SCs of the AG (Fig 4D). *spi*-GAL4 induces similar nuclear growth phenotypes to *dsx*-GAL4 and esgF/O^{ts} when combined with UAS-*Dad* and UAS-*Tkv^{ACT}* constructs (S2E Fig). It also has a similar effect to *dsx*-GAL4 on largest MVBL size when combined with these regulators of BMP signalling (S3K Fig). These data indicate that *spi*-GAL4 promotes UAS-dependent gene expression at roughly the same level as the other two drivers. In virgin 3-day-old males, GFP-GPI expressed under *spi*-GAL4 control from eclosion onwards marked the same number of DCGs as ANCE and Rab11-YFP (S4I Fig). Furthermore, GFP-GPI overexpression with *spi*-GAL4 did not significantly affect SC nuclear growth (S2F Fig) or MVBL numbers (S6A Fig) over the first six days of adulthood compared to other genotypes. However, it did increase the size of the largest MVBL when compared to SCs from *w^{1118}* control males, albeit not as much as CD63-GFP overexpression (S6B Fig). In fact, we also found that largest MVBL size was increased in SCs from esgF/O^{ts} males compared to *w^{1118}* control males (S6B Fig), suggesting that this specific

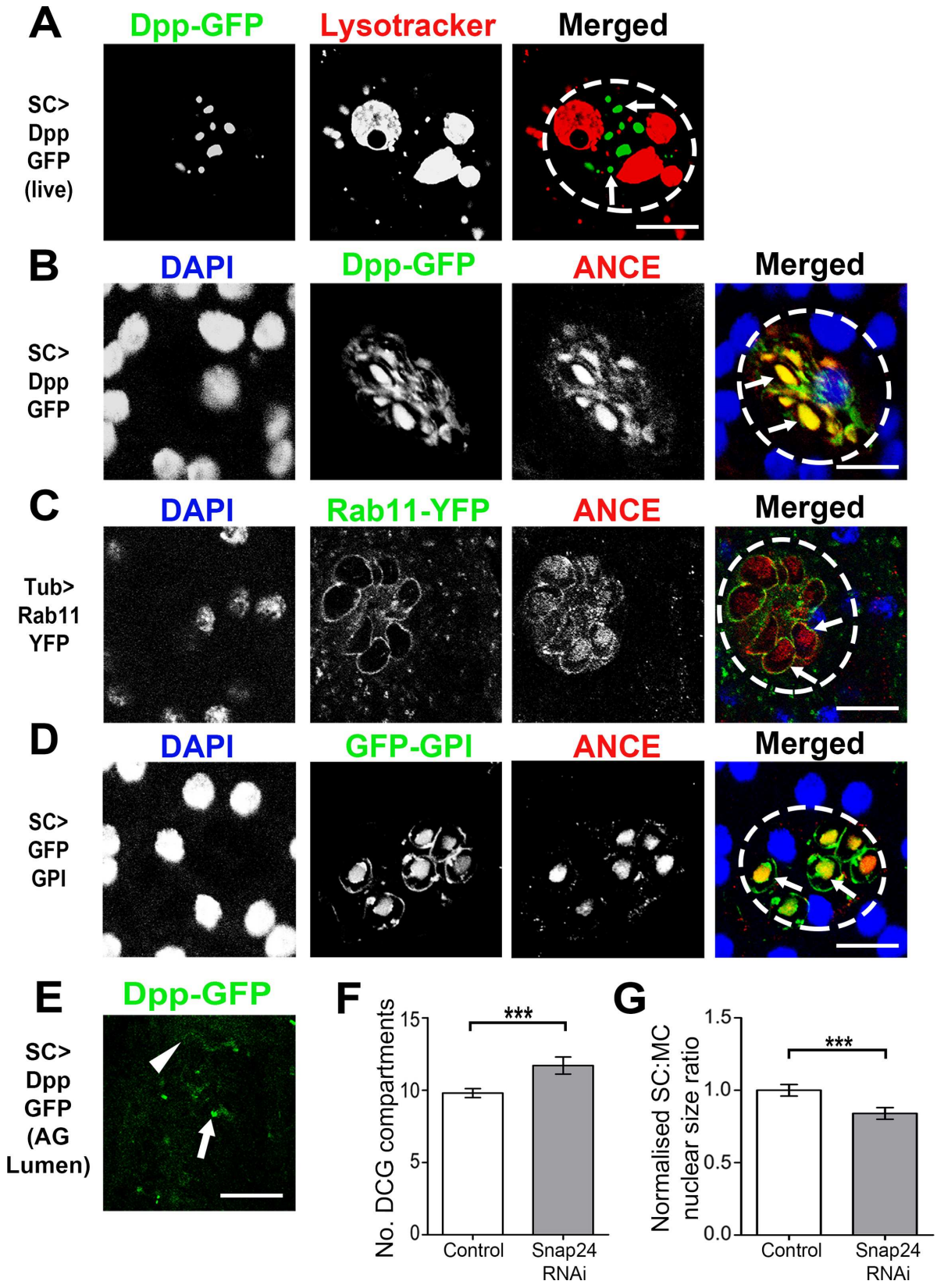

A
Dpp-GFP
Lysotracker
Merged
SC>
Dpp
GFP
(live)
B
DAPI
Dpp-GFP
ANCE
Merged
SC>
Dpp
GFP
C
DAPI
Rab11-YFP
ANCE
Merged
Tub>
Rab11
YFP
D
DAPI
GFP-GPI
ANCE
Merged
SC>
GFP
GPI
E
Dpp-GFP
SC>
Dpp
GFP
(AG
Lumen)
F
No. DCG compartments
15
10
5
0
***
Control
Snap24
RNAi
G
Normalised SC:MC
nuclear size ratio
1.5
1.0
0.5
0.0
***
Control
Snap24
RNAi

Fig 4. Dpp is stored in SC dense-core granules. A. Live image of Dpp-GFP-expressing SC stained with Lysotracker Red to identify acidic compartments. Note that Dpp-GFP localises to spherical structures (arrows) that are distinct from acidic compartments. **B**. Image of fixed SC, stained with anti-ANCE antibody (red) and DAPI (blue) after 24 h pulse of Dpp-GFP expression, reveals co-localisation of GFP fluorescence with ANCE-positive DCGs (arrows). **C**. Rab11-YFP-positive compartments in SCs from flies ubiquitously expressing this fusion protein under tubulin promoter control contain DCGs that stain positive for ANCE (red). **D**. Fixed SC expressing GFP-GPI and stained with anti-ANCE (red) and DAPI (blue), showing co-localisation of ANCE and GFP in DCGs (arrows). **E**. GFP-positive puncta (arrow) and filaments (arrowhead) are detected in the AG lumen when Dpp-GFP is expressed in SCs. **F, G.** SC-specific expression of *Snap24* RNAi in adults significantly increases number of GFP-GPI-labelled DCGs in 6-day-old virgin males (F; using *spi*-GAL4 driver), and reduces nuclear size (G; using esgF/O^{ts} driver). All images are from 3-day-old virgin males and individual SCs are outlined by dashed circles. Genotypes for images are: *w; tub-GAL80^{ts}; dsx-GAL4/UAS-Dpp-GFP* (A, B, E); *w; tub-rab11-YFP* (C); *w; spi-GAL4 tub-GAL80^{ts} UAS-GFP-GPI/CyO* (D). ***P<0.001, Mann-Whitney *U* test, n = 10. Scale bar for A-D is 10 μm and E is 20 μm.

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cellular property may be modulated by overexpression of any GFP-marked transgene. However, aside from this change in MVBL size, we conclude that the *spi*-GAL4 UAS-*GFP-GPI* reporter line has no obvious effect on normal SC biology.

In undertaking this analysis, we noticed that the total number of SVs in CD63-GFP-expressing SCs was considerably greater than the number of DCG compartments in GFP-GPI-expressing cells (24 ± 0.8 versus 9.8 ± 0.3), and that the former compartments appeared smaller than the latter. To determine whether CD63-GFP overexpression altered DCG compartments in SCs, we co-expressed this transgene with GFP-GPI. This combination increased the number of GFP-GPI-positive DCGs in SCs from 6-day-old males (S6C–S6E Fig), suggesting that although compartment identity is not obviously defective in CD63-GFP-overexpressing SCs, the number of large non-acidic compartments is increased (and their size reduced). We speculate that this reflects an increased rate of trafficking through the secretory and endolysosomal systems. By contrast, when expressed alone, the GFP-GPI transgene does not appear to affect the size or number of large compartments in SCs.

Since BMP signalling stimulates SC growth even in virgin males, we reasoned that Dpp-containing DCGs must be steadily released from SCs during adulthood. SNARE proteins have been implicated in regulating fusion of DCG compartments and synaptic vesicles to the plasma membrane in several eukaryotic systems [9,40]. In the *Drosophila* salivary gland, the granule-associated SNARE, SNAP24, a homologue of mammalian SNAP25, is involved in glue granule release [41,42]. Expression of an RNAi targeting *Snap24* in adult SCs significantly increased the number of GFP-GPI-positive DCGs, suggesting that SNARE-mediated fusion events are required for normal DCG release in virgin males (Fig 4F). Nuclear growth was also decreased in these cells (Fig 4G), as would be expected when BMP signalling is reduced.

Activation of BMP signalling in SCs is detected by an anti-pMad antibody

Activation of BMP signalling leads to phosphorylation of cytosolic Smad proteins, which then translocate to the nucleus to regulate gene transcription [43]. Nuclear phospho-specific anti-Mad antibody staining is a commonly used readout for BMP pathway activity in flies [44,45]. Although no pMad signal was observed in MCs, nuclear pMad was detected in some SCs from virgin 3- and 6-day-old esgF/O^{ts} males (9% and 20% respectively) (Figs 5A, 5E and S7D). Knockdown of BMP signalling components in SCs strongly reduced the number of pMad-positive nuclei (S8A Fig), suggesting BMP signalling is sporadically activated in these cells, perhaps upon DCG release.

To further validate pMad staining as a marker for BMP signalling in SCs, we overexpressed either Dpp-GFP or Tkv^{ACT} in SCs. This induced detectable nuclear pMad staining in all SCs and also in those SCs expressing Dpp-GFP in clones, while no nuclear pMad staining was observed in SCs when BMP signalling was blocked by overexpressing Dad (S4A, S4B and S7A–

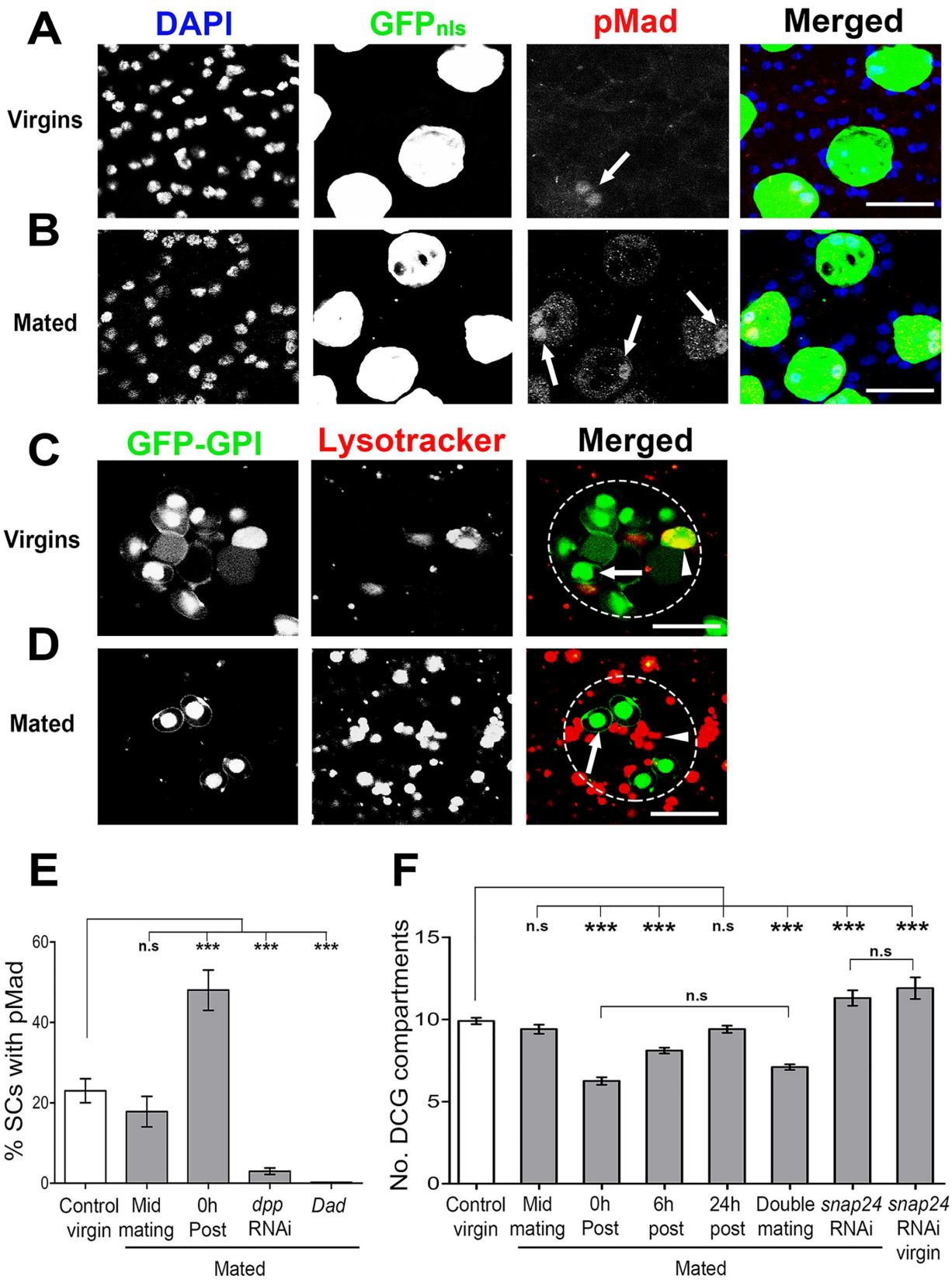

A
DAPI
GFPnls
pMad
Merged
Virgins
B
Mated
C
GFP-GPI
Lysotracker
Merged
Virgins
D
Mated
E
% SCs with pMad
60
40
20
0
n.s
***
***
***
Control virgin
Mid mating
0h Post
dpp RNAi
Dad
Mated
F
No. DCG compartments
15
10
5
0
n.s
***
***
n.s
***
***
***
n.s
n.s
Control virgin
Mid mating
0h Post
6h post
24h post
Double mating
snap24 RNAi
snap24 RNAi virgin
Mated

Fig 5. DCGs are released from SCs during mating, activating BMP signalling. A, B. SCs from 6-day-old esgF/O^{ts} virgin males (A) and from males immediately after mating (B) were fixed and stained with an anti-pMad antibody (red) and DAPI (blue), revealing that the proportion of SCs with detectable nuclear pMad is higher in mated animals. **C, D.** Immediately after mating, living SCs (D) have less GFP-GPI-labelled DCGs than virgins (C). Image shows a single z-plane of gland stained with Lysotracker Red; not all compartments are in the focal plane. Note that the largest MVBL in (C; arrowhead) contains GFP, probably because of fusion between a DCG compartment and the MVBL [17]. **E**. Graph shows proportion of SCs with nuclear pMad in 6-day-old virgin, and mated males (dissected 8 min into mating [Mid] and immediately after mating), and mated males expressing *dpp*-RNAi and Dad in SCs from eclosion onwards using the *w; esg-GAL4 tub-GAL80^{ts} UAS-FLP; UAS-GFP$_{nls}$ actin>FRT>CD2>FRT>GAL4* driver. **F**. Graph shows number of GFP-GPI-positive DCG compartments in 6-day-old virgin and mated males (using the *w; spi-GAL4 tub-GAL80^{ts} UAS-GFP-GPI* driver line; Double is twice mated in 2 h), and at different times after single mating in control SCs. Compartments were also counted in SCs expressing *Snap24* RNAi post-eclosion in virgins and immediately after mating. Labelled compartments were counted using a complete z-series for each cell. Genotypes for images are: *w; esg-GAL4 tub-GAL80^{ts} UAS-FLP/+; UAS-GFP$_{nls}$ actin>FRT>CD2>FRT>GAL4/+* (A, B); *w; spi-GAL4 tub-GAL80^{ts} UAS-GFP-GPI/CyO* (C, D).***P<0.001, Kruskal-Wallis test, n>15. Scale bar for A-B is 20 μm and C-D is 10 μm.

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S7C Figs). MC-specific expression of Dpp-GFP had no effect on MCs themselves, but increased pMad levels in SCs (S4C Fig). Therefore pMad staining marks BMP signalling activity in AGs. Although all SCs can respond to BMPs when Dpp is overexpressed, there is only stochastic activation of signalling in SCs in normal virgin males.

Mating induces rapid DCG compartment release from SCs resulting in activation of BMP signalling

When males mate, part of the luminal content of the AG is transferred to females. Western blot analysis of reproductive tracts from females mated with males expressing GFP-GPI in SCs revealed that this marker is transferred during mating (S7E Fig), consistent with previous findings using the DCG marker ANCE [17]. We hypothesised that DCG secretion from SCs might increase to restore luminal content in preparation for subsequent matings.

Interestingly, when GFP-GPI-expressing SCs of 6-day-old males were imaged immediately after a single mating, approximately four GFP-positive DCG compartments had been lost (Figs 5C, 5D and 5F), indicating that DCGs are secreted upon mating. Approximately two of these compartments were replenished within 6 h and by 24 h, the original number of DCGs was restored (Fig 5F). When flies expressing *Snap24*-RNAi in SCs were mated, no decrease in DCG numbers was observed compared to virgins, indicating that *Snap24* is required for mating-induced DCG release (Fig 5F). AG luminal contents are only transferred to females during the second half of the ~20 min mating process [46,47]. Males separated after only 8 minutes of mating did not show a significant reduction in GFP-GPI-positive DCGs (Fig 5F), suggesting that secretion coincides with transfer of AG luminal content.

Since mating stimulates increased BMP-dependent growth of SCs [23], we reasoned that this might result from release of Dpp-containing DCGs during mating. To test this hypothesis, control 3- and 6-day-old esgF/O^{ts} males were mated and their AGs immediately dissected and stained for pMad. Although we observed no significant change in nuclear pMad staining between virgins and males separated 8 min into mating, there was a significant increase in the number of pMad-positive SCs immediately after mating in both 3- (S7D Fig) and 6-day-old (Fig 5E) males with approximately 50% of all SCs stained compared to 10% and 20% respectively in virgins. This effect was completely suppressed by expressing *dpp* RNAi or Dad in SCs (Fig 5E). These results indicate that Dpp contained within DCGs is released upon mating and stimulates BMP signalling via a mechanism that requires *Snap24*, and that ultimately promotes increased SC growth.

Autocrine Dpp signalling is required to replenish DCGs after mating

SC-specific BMP signalling, which is required to fully inhibit female receptivity after mating [17], is also needed to maintain normal numbers of CD63-GFP-marked SVs, some of which

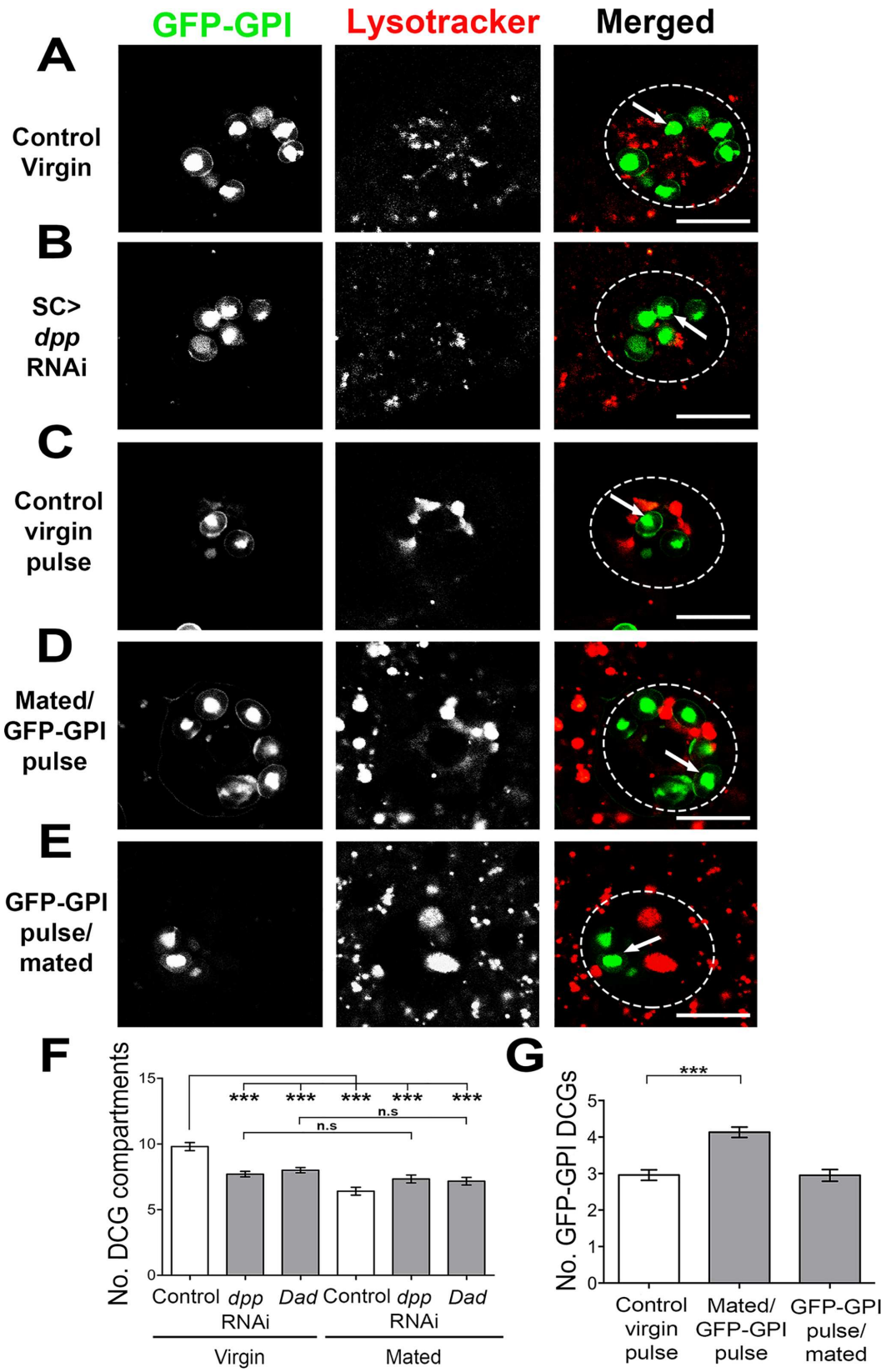

GFP-GPI
Lysotracker
Merged
A
Control Virgin
B
SC> dpp RNAi
C
Control virgin pulse
D
Mated/ GFP-GPI pulse
E
GFP-GPI pulse/ mated
F
No. DCG compartments
15
10
5
0
***
***
***
***
***
n.s
n.s
Control
dpp RNAi
Dad
Control
dpp RNAi
Dad
Virgin
Mated
G
No. GFP-GPI DCGs
5
4
3
2
1
0
***
Control virgin pulse
Mated/ GFP-GPI pulse
GFP-GPI pulse/ mated

Fig 6. Mating rapidly accelerates the rate of new DCG compartment formation. A, B. Single z-plane images of SCs from 6-day-old virgin males labelled with GFP-GPI, either co-expressing (B) or not expressing (A) *dpp*-RNAi. Reducing Dpp signalling decreases the number of labelled DCGs (arrow). **C-E**. 6-day-old males were shifted to 28.5˚C to induce GFP-GPI expression for 16 hrs in virgins (C) or immediately after (D) or before (E) mating. Their AGs were dissected and imaged; each image is from a single z-plane. **F**. Graph showing number of GFP-GPI-positive DCGs in SCs from either 6-day-old virgin or mated control flies or flies in which BMP signalling is inhibited. There is no decrease in total DCG number in SCs expressing either *dpp*-RNAi or *Dad* after mating. **G**. Graph showing GFP-positive DCG number after a 16 h pulse of GFP-GPI in SCs from 6-day-old males, as in C-E above. Genotypes for images are: *w; spi-GAL4 tub-GAL80^{ts} UAS-GFP-GPI* combined with no other transgene (A, and pulse-labelled in C, D, E) or *P[TRiP.HMS00011]attP2* (III) (B). The *w; spi-GAL4 tub-GAL80^{ts} UAS-GFP-GPI* line was used to generate data in F and G. ***P<0.001, Kruskal-Wallis test, n = 15. Scale bar for A-E, 10 μm.

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contain DCGs. We tested more directly whether BMP signalling controls the number of DCGs in virgin flies using the GFP-GPI marker, which unlike CD63-GFP, does not noticeably affect DCG numbers in SCs when overexpressed (S4I and S6E Fig). Inhibiting the BMP pathway by knockdown of *dpp*, BMP receptors, *Mad* or *Med*, or by overexpression of *Dad* in adult SCs reduced the number of DCGs by about 20% (Figs 6A, 6B, 6F and S8B), except in the case of *sax*, which also has a unique effect on SV number in CD63-GFP-overexpressing SCs (Fig 2G and S3G Fig). These data indicate that normal biogenesis of DCGs is dependent on BMP signalling. To test whether mating-dependent DCG secretion also requires BMP signalling, we analysed flies after a single mating. There was no significant decrease in DCGs between mated and virgin flies when BMP signalling was inhibited by *dpp* knockdown or Dad overexpression (Fig 6F), suggesting that DCG release is blocked, perhaps because the compartments have failed to mature normally.

One explanation for the rapid replenishment of DCGs after mating is that BMP signalling upregulates biosynthesis of new DCG compartments. To test this, we conducted a series of experiments using pulses of GFP-GPI expression. A 16 h pulse of GFP-GPI labelled 3 ± 0.1 DCG compartments in virgin males (Fig 6C and 6G). When the 16 h GFP-GPI pulse occurred immediately after mating, there was a significant increase in the number of labelled DCGs (4 ± 0.1; Fig 6D and 6G), supporting a model whereby mating accelerates cargo loading into new DCGs. An alternative explanation is that some DCG compartments formed during the 16 h pulse are secreted in virgins, but not after mating. In mammals, immature DCGs are typically not secreted, but must undergo a stepwise maturation process involving luminal acidification by the v-ATPase and selective removal of non-DCG proteins prior to exocytosis [1,4]. To test whether pulse-labelled DCG compartments could be secreted from SCs upon mating, we induced GFP-GPI for 16 h before mating, then dissected the AGs immediately after mating. We did not observe any decrease in the number of labelled DCG compartments compared to virgin males (Fig 6E and 6G), indicating that GFP-GPI is trafficked over a 16 h period to new immature compartments, which cannot be immediately secreted. Indeed, if males expressing GFP-GPI throughout adulthood were remated within two hours for a second time, no additional DCGs were released (Fig 5F), suggesting that normally only about 4 DCGs are sufficiently mature to be secreted.

To assess whether the acceleration of DCG biosynthesis after mating involved BMP signalling, we co-overexpressed Dad during a 24 h GFP-GPI pulse to test whether this might induce sufficient inhibition of signalling to affect new DCG formation. Mating control flies 8 h after starting a 24 h GFP-GPI pulse increased DCG numbers when compared to virgins expressing GFP-GPI for the same duration (4.4 ± 0.2 DCGs in virgins versus 6.2 ± 0.2 DCGs in mated flies; Fig 7A–7C and 7E). However, when Dad was co-expressed with GFP-GPI in virgin flies for 24 h, the number of labelled DCGs was significantly reduced (Fig 7D and 7E), suggesting that BMP signalling is required for biogenesis of new immature DCGs. When we overexpressed GFP-GPI with Dad for 8 h, then mated male flies once and continued the pulse for a further 16 h (24 h in total) (Fig 7A), we observed a marked decrease in the number of labelled

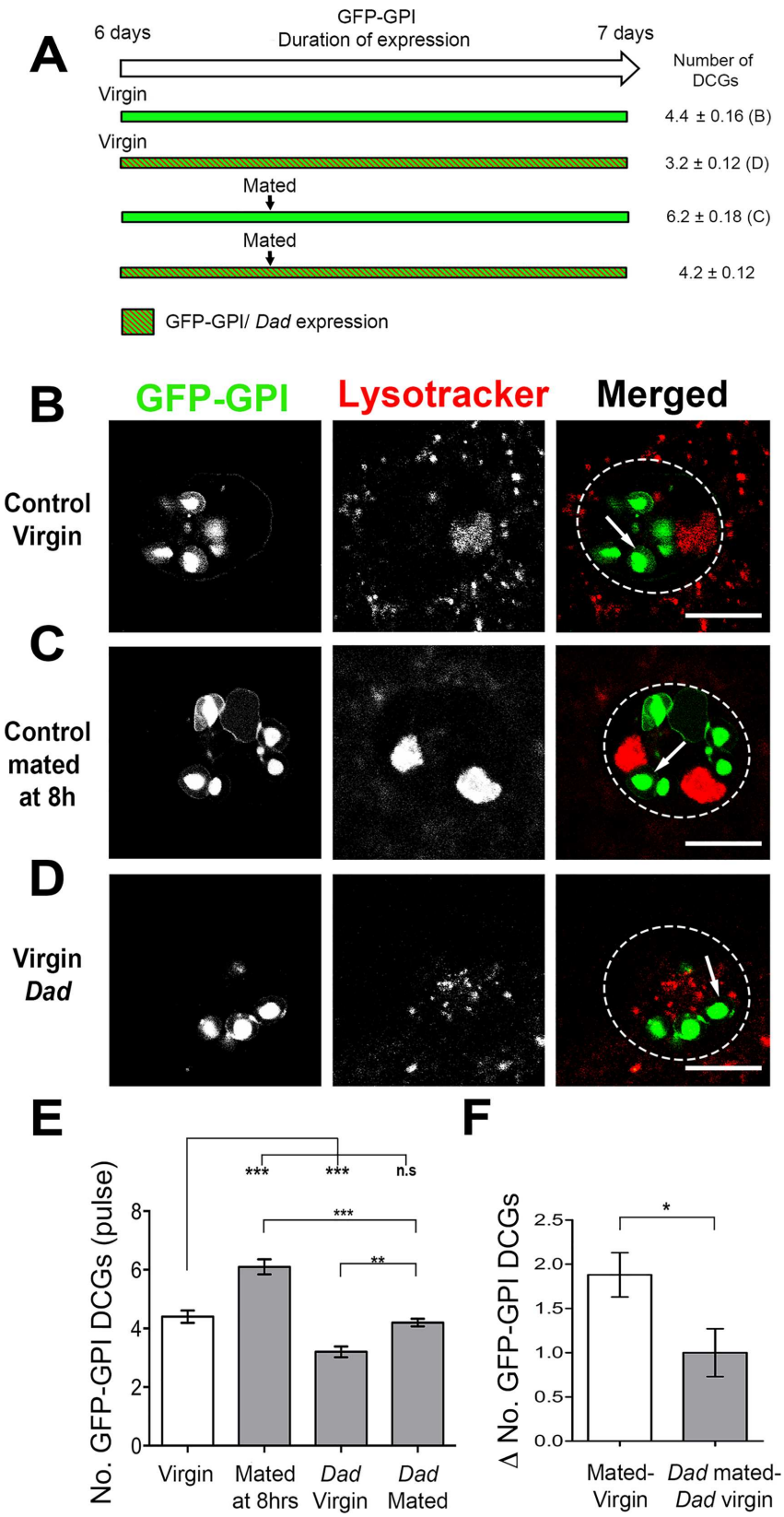

A
6 days
GFP-GPI
Duration of expression
7 days
Number of DCGs
Virgin
4.4 ± 0.16 (B)
Virgin
3.2 ± 0.12 (D)
Mated
6.2 ± 0.18 (C)
Mated
4.2 ± 0.12
GFP-GPI/ Dad expression
B
GFP-GPI
Lysotracker
Merged
Control Virgin
C
Control mated at 8h
D
Virgin Dad
E
No. GFP-GPI DCGs (pulse)
***
***
n.s
***
**
Virgin
Mated at 8hrs
Dad Virgin
Dad Mated
F
Δ No. GFP-GPI DCGs
*
Mated-Virgin
Dad mated-Dad virgin

Fig 7. Rapid replenishment of DCGs after mating is BMP-dependent. A. Schematic representation of pulse-chase experiments shown in B-F, indicating the duration of GFP-GPI and *Dad* overexpression and timings of mating events. **B-D**. 6-day-old flies were shifted to 28.5˚C for 24 h to allow expression of GFP-GPI in virgins (B) or in males mated 8 h after the start of the pulse (C). The number of GFP-GPI-labelled DCGs in SCs was reduced in virgin males co-expressing Dad (D). **E.** Graph shows a significant increase in the number of labelled DCGs if males are mated at 8h during a 24 h GFP-GPI pulse. The number of DCGs labelled in virgin and mated males is reduced if *Dad* is co-expressed. **F**. The increase in labelled compartments after mating is also reduced by Dad co-expression. The *w; spi-GAL4 tub-GAL80^{ts} UAS-GFP-GPI* line was used to generate data in E and F. *P<0.05, ***P<0.001, Kruskal-Wallis test, n = 15. Scale bar in B-D, 10 μm.

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DCGs in comparison to mated control flies (Fig 7E). The number of marked DCGs was slightly higher than in Dad-expressing virgins, presumably because BMP signalling is not fully inhibited by a 24 h pulse of Dad. However, the difference between mated and virgin DCG numbers in Dad-expressing versus control males was significantly reduced (Fig 7F), demonstrating that BMP signalling is required to drive increased biosynthesis of new DCGs after mating.

Discussion

Dense-core granule secretion is a fundamental and evolutionarily conserved process by which glandular epithelia release their bioactive contents in a regulated fashion. Importantly, to be sustained under different physiological conditions, this regulated output must be matched to new biogenesis. Here we show that *Drosophila* SCs with their large and easily counted DCGs provide a powerful new *in vivo* system to study this process. We demonstrate a critical role for BMP signalling in co-ordinating biogenesis and release, primarily via an autocrine mechanism. This signalling upregulates formation of new DCGs after rapid mating-induced secretion (Fig 8), a process that may be evolutionarily conserved.

DCG release from SCs is accelerated during mating

We find that adult SCs from virgin males consistently contain approximately 10 Rab11-positive DCG compartments, as well as a number of other non-acidic compartments that do not contain DCGs and whose Rab signature is yet to be established. These other compartments are most clearly visualised using the CD63-GFP marker, which also labels DCG compartments and MVBLs. However, our study suggests that after expression in adult SCs for 6 days, this marker increases the number of SVs and DCG compartments, while reducing their size and increasing MVBL size. Therefore we have not yet been able to accurately determine the normal total number of SVs in SCs. Although these findings suggest that some aspects of secretion, including exosome secretion, may be modulated by CD63-GFP activity, it is important to emphasise that vesicles are observed inside wild type SC compartments by transmission EM [17], and are therefore not merely induced by CD63-GFP marker expression.

Our data further suggest that DCG compartments are turned over continuously. Expression of GFP-GPI in pulses indicates that one new immature DCG is formed about every 6 h. Furthermore, BMP signalling is sporadically activated in virgin males in a process requiring Dpp expression in SCs (Fig 5). Pancreatic β-cells, parotid and other glandular cells also have a basal constitutive-like secretory pathway [48,49].

Mating lasts about 20 min in flies. We find that approximately four DCG compartments are lost from each SC during the second half of this period (Fig 5). Newly-formed DCGs that are labelled during a 16 h GFP-GPI pulse are not released during mating, suggesting that only mature compartments are secreted in a regulated fashion, mirroring mammalian DCG secretion [1]. Indeed, since additional DCGs are not released after a second mating (Fig 5F), only about four DCGs normally appear to be sufficiently mature to be released. Knockdown

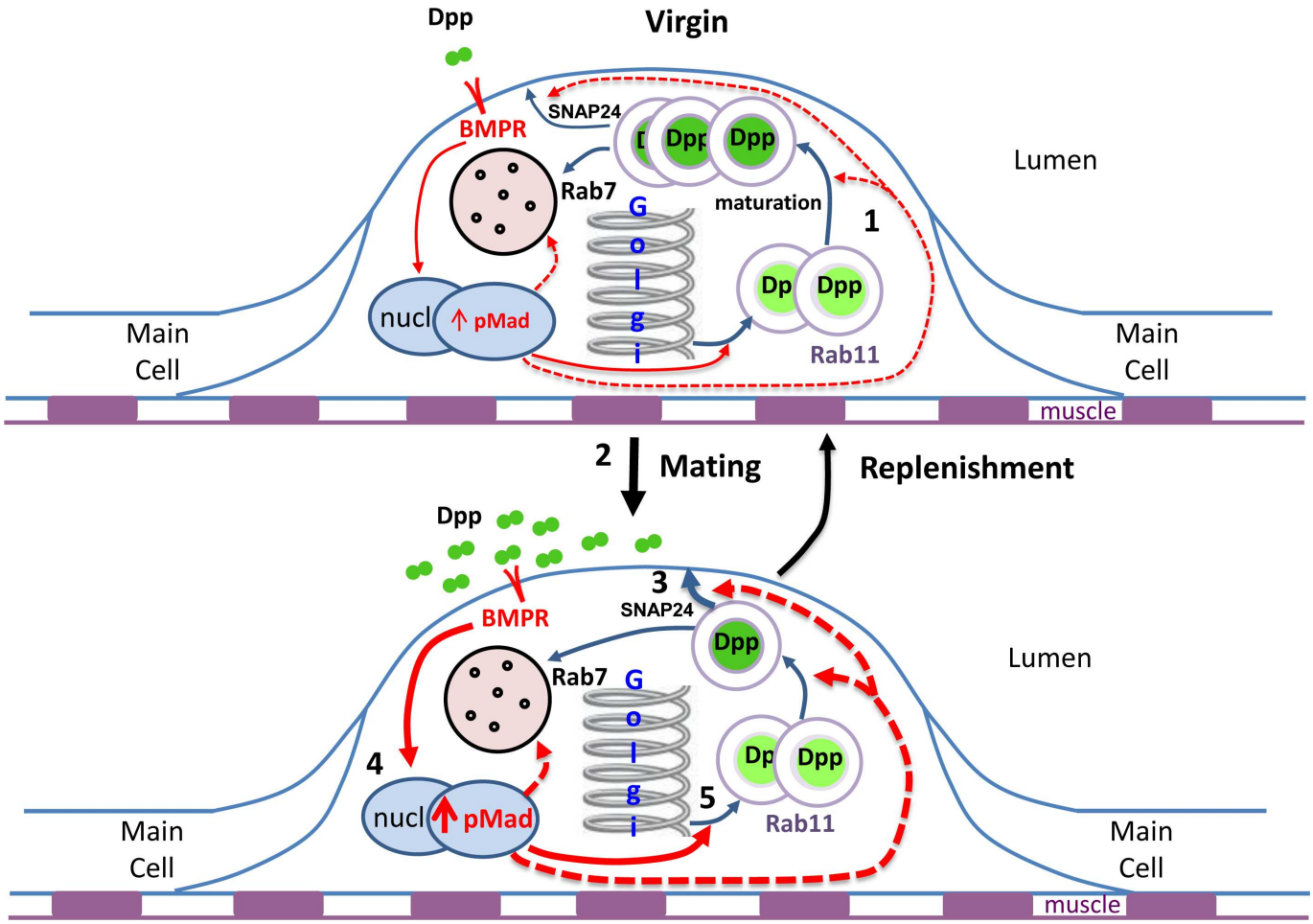


Fig 8. Model to explain autocrine regulation of DCG replenishment by BMP signalling in SCs. Schematics of a single SC immediately before and after mating. (1) In virgin males, Dpp is trafficked to and stored in DCGs. Sporadic release of these DCGs activates BMP signalling, and sustains a basal level of growth, DCG biogenesis and exosome secretion. (2) During mating, about 4 mature DCGs are released (3), resulting in an increase in BMP signalling (4), primarily via an autocrine mechanism and probably in pulses. This stimulates growth, but also increases biosynthesis of new DCG compartments (5; solid arrow), ensuring that the total number of DCGs is fully replenished within 24 h. Dashed arrows highlight other parts of the secretory/endolysosomal system that might be affected by altered BMP signalling. Previous data (Corrigan et al., 2014) and data presented here suggest that long-term elevated BMP signalling enhances endolysosomal trafficking.

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experiments revealed that both constitutive and regulated secretion of DCGs is dependent on the SNARE protein, SNAP24 (Figs 4 and 5F), which has been shown to control regulated secretion from the fly salivary gland [41]. This function appears to be very broadly conserved in evolution, since the mammalian SNAP24 homologue, SNAP-25, is also implicated in insulin secretion from rat pancreatic β-cells [50] and DCG release from other secretory cells [9].

Other observations also suggest further parallels between SCs and other secretory cells. For example, DCGs contain proteases involved in processing bioactive secretory products; in SCs, ANCE has been proposed to play a role in this process [16]. Although multiple Rabs are implicated in DCG formation and secretion, homologues of Rab11, a key signature of DCGs in SCs (Fig 4C), have been highlighted in the secretory control of several mammalian DCGs, including pancreatic acinar cell zymogen [51] and β-cell insulin [52] granules. Finally, cholesterol-rich membranes and lipid raft structures, which associate with GPI-anchored proteins like GFP-GPI, are involved in mammalian DCG formation [7].

Ultimately GFP fluorescence associated with GFP-GPI in SCs traffics into the DCG core, even though there do not appear to be membranes inside these structures [16]. This could be explained in a number of ways. For example, the GPI anchor may be removed in DCG compartments to permit release into the dense core; indeed, mammalian ANCE (or ACE) has been reported to exhibit GPIase activity [53], although this finding has subsequently been challenged [54]. Alternatively, the GPI fusion protein may lose its association with the limiting membrane of the compartment membrane or much of the overexpressed GFP may lack a GPI anchor because it saturates the GPI anchor synthesis enzymes. It will therefore be interesting to test whether other GPI-anchored proteins expressed at more physiological levels also behave in this way in this and other systems. However, overall, the similarities in regulation of SC and mammalian DCGs indicate that SCs provide a powerful new *in vivo* model to genetically dissect fundamental aspects of DCG biogenesis.

BMP signalling is increased during mating

Our findings that dpp^{blk}-GAL4 drives gene expression only in SCs of the AG and that SC-specific knockdown of *dpp* has a strong effect on BMP-mediated SC functions (Fig 3), clearly show that Dpp expression from SCs plays a central role in controlling the biology of these cells. Staining with an anti-pMad antibody reveals the sporadic activation of BMP signalling in SCs, and an increase in cells with activated signalling during mating. Although it seems that all SCs release DCGs during mating and that overexpressing Dpp-GFP in SCs can activate BMP signalling in all expressing cells (S4A Fig), only about 50% of SCs from mated males contain nuclear pMad at any specific time, suggesting that activation after DCG release may be short-lived.

Interestingly, we previously found that a *Dad-GFP* transgene, which has been used as a BMP signalling reporter in other *Drosophila* cell types [55], is active in all SCs and at lower levels in MCs [23]. Its expression was not obviously increased by mating. It is unclear whether this reporter activity in SCs reflects cumulative activation of BMP signalling in SCs and/or non-BMP-dependent expression. However, since normal SC growth is primarily regulated cell-autonomously by BMP signalling [23,17] (Fig 3), we conclude that autocrine BMP signalling is probably independently activated in a pulsatile way in every SC as adults age. Phenotypic analysis of SC-specific *dpp*-RNAi expression in mosaics suggests that Dpp can also have paracrine effects on other SCs, but these cannot compensate for the effects of cell-autonomous loss of BMP signalling. Surprisingly, mosaic expression of Dpp-GFP does not affect growth of non-expressing SCs. One possible explanation is that this fusion protein is not released from SCs or detected by neighbouring SCs in the same way as wild type Dpp, although it does function normally in other cellular contexts [33]. Alternatively, there may be a threshold requirement for paracrine Dpp signalling or negative feedback signalling between SCs, while as more autocrine Dpp is produced, BMP signalling continues to increase.

Given the autocrine and secretion-dependent nature of BMP signalling in SCs, it is critical that Dpp is separated from its receptors prior to release. We found that at least one Type I and one Type II receptor (Tkv and Wit respectively) are trafficked to compartments that are distinct from the Rab11-positive DCGs. Unfortunately, we could not use a previously characterised fluorescence-based reporter for Tkv activation, TIPF [37], to investigate receptor activation further, because its expression under either UAS or ubiquitin promoter control activated BMP signalling constitutively and increased SC growth. Interestingly, work in *C. elegans* has shown that Type I and Type II receptors are separated after endocytosis [56], potentially terminating signalling. Our analysis suggests that Tkv and Wit may be in the same intracellular compartments in SCs, but their separation from Dpp presumably prevents continued activation.

One key question that now needs to be addressed is how DCG secretion is stimulated upon mating. One possibility is that during mating, the physical forces induced by peristaltic movement of the muscular sheath surrounding the AG could stimulate the fusion of DCG compartments docked close to the SC plasma membrane. Alternatively, a neuropeptide circuit has recently been identified in males that is critical for sperm and seminal fluid transfer and innervates cells in the AG [57]. It will be important to test whether this circuit makes connections with SCs or can stimulate DCG secretion through diffusion of the neurotransmitter(s) it releases.

BMP signalling and the regulation of DCG biology in SCs

Overall our data support a model in which DCG-loaded Dpp acts primarily as an autocrine signal that matches regulated release of DCG compartments to new biogenesis (Fig 8). New DCG formation occurs continuously, even in virgin males, to replenish the constitutive release of these compartments. Upon mating, the rate of biogenesis is increased in a BMP-dependent fashion, so that two of the four DCGs lost at mating are replaced within 6 h. Blocking BMP signalling in SCs reduces biogenesis of new DCG compartments in both virgin and mated males, but also inhibits release of DCGs, which may explain why the number of DCGs in knockdown cells are only reduced by about 20% (Fig 6F and S8B Fig). One possible interpretation is that BMP signalling also controls DCG compartment fusion to the plasma membrane. However, currently we favour a model in which BMP signalling regulates maturation of DCGs, so they can be released upon stimulation. Indeed, it appears that only some DCGs are sufficiently mature to be secreted in normal SCs, because rapidly mating males a second time does not lead to further DCG release (Fig 5F). Identification of markers for DCG maturation status will help to test this specific aspect of our model further.

How could BMP signalling be driving elevated rates of DCG biogenesis? Downstream Smad signalling primarily regulates gene transcription. Previous studies in pancreatic beta-cells have revealed that BMP signalling induces insulin secretion and increases levels of many transcripts associated with the secretory process, such as the *Snap24* homologue, *SNAP25* [14]. Pheochromocytoma cells also express higher levels of the DCG assembly protein Chromogranin A in response to secretion [11]. Since SCs constitute only a small proportion of cells in the AG, we have not been able to use qRT-PCR methods to determine whether secretory regulators like *Snap24* are induced by mating or BMP signalling activation in SCs. We will need to identify SC-specific BMP target genes to study this in the future.

BMP signalling co-ordinately controls compartment biogenesis, endolysosomal trafficking, exosome secretion and growth in SCs

Interestingly, we have found that BMP signalling has a role in several biosynthetic processes in SCs. We have employed three different GAL4 drivers to analyse BMP functions in SCs, esgF/O^{ts}, *dsx*-GAL4 and *spi*-GAL4, which produce similar BMP-mediated effects on SC nuclear growth, and secretory and endolysosomal trafficking. Although expressed selectively in SCs within the AG, none of the drivers we use are only expressed in SCs within adult flies. Importantly, the consistent phenotypes induced with different drivers (eg. S2E Fig) therefore supports our conclusion that effects are induced by cell-autonomous changes in BMP signalling and not by altered signalling in other tissues.

Signalling appears to involve multiple BMP receptors working in a non-redundant way ([17] and this study) and acting through Mad activation, since knockdown of each receptor reduces numbers of pMad-positive SCs by about 75% (S8A Fig). Both Type I [58] and Type II [59] receptors have previously been shown to act non-redundantly in other *Drosophila* cell

types. In SCs, knockdown of different BMP receptors can produce different effects; most notably *sax* RNAi did not reduce SV or DCG number in the same way as inhibiting other BMP signalling components (Figs 2G, S3G and S8B). Sax has previously been reported to have both positive and negative effects on BMP signalling through its different responses to Dpp and Gbb ligands [58]. To date, our knockdown analyses have not revealed a function for Gbb in SCs, although it appears to be expressed strongly in the AG [31]. Additional tests are required to determine whether Gbb might play a role in specific aspects of SC membrane trafficking.

In addition to controlling secretory and endolysosomal trafficking, BMP signalling also regulates SC nuclear size. Although in some *Drosophila* post-mitotic cells, like larval salivary gland cells and ovarian follicle cells, nuclear growth is achieved through endoreplication of the genome, nuclei can grow in other organs, such as the eye, without replicating the genome [60]. We previously were unable to detect new DNA synthesis in adult SCs using bromodeoxyuridine (BrdU) labelling [23], although we cannot exclude the possibility that this merely reflects the relatively low sensitivity of this method.

Another important consideration is whether the different effects of BMP signalling on SCs are mechanistically linked. For example, could the changes in MVBL maturation and exosome secretion observed when BMP signalling is inhibited result from BMP-dependent modulation of DCG membrane trafficking, or vice versa? The compartment phenotypes induced by blocking BMP signalling are consistent with a general reduction in membrane flux through the secretory and endosomal systems in SCs. However, a better understanding of the DCG, MVBL and exosome biogenesis pathways in these cells will be required to determine whether these processes are interdependent in other ways. For example, it will be important to determine how vesicles formed inside endosomal compartments are released into the AG lumen as exosomes, and whether the acidification of maturing DCG compartments reported in other systems [1,4] takes place in SCs and involves cross-talk with more acidic late endosomal compartments. Interestingly, cells within glands and neurons involved in regulated secretion are also reported to communicate via exosomes [61,62], and so our discovery that there is overlap in the control of these processes in SCs may also be relevant to these other systems.

It also remains to be investigated whether defective maturation of late endosomes and lysosomes, major sites for activation of the growth regulatory kinase complex, mechanistic Target of Rapamycin Complex 1 (mTORC1) [63,64], could underlie the inhibition of SC growth and membrane biogenesis observed after BMP signalling blockade. The potentially complex interplay between growth, trafficking and secretion makes it difficult to identify what changes are responsible for the defect in reprogramming female receptivity that accompanies BMP signalling inhibition in SCs [23]. Although we have previously provided evidence that this may be partly explained by altered exosome secretion [17], our findings here suggest that other secretory processes and a general reduction in macromolecular biosynthesis in SCs may also be involved.

Overall, we conclude that SCs match secretion of DCG compartments with their replenishment by regulating DCG biogenesis rates via BMP signalling induced by DCG-packaged Dpp. Evidence from other systems suggests that autocrine BMP signalling may provide a general mechanism for control of DCG biogenesis rates in response to regulated secretion. For example, neuronal Gbb synthesis is required for normal synaptic secretion at the neuromuscular junction [65]. Furthermore, pancreatic β-cell signalling by the Dpp orthologue, BMP4, has been implicated in regulating glucose-stimulated insulin secretion via the BMP receptor BMPR1A [14]. It will now be important to test whether this signalling is truly autocrine and whether it could also match DCG biogenesis to secretion, particularly given the significance of secretory mis-regulation in some forms of diabetes.

Materials and Methods

Fly stocks

The following fly strains were used: esgF/O^{ts} (*w; esg-GAL4 tub-GAL80^{ts} UAS-FLP/CyO; UAS-GFP$_{nls}$ actin>FRT>CD2>FRT>GAL4/TM6* [66];*UAS-CD63-GFP* [29]; *Acp26Aa-GAL4* [31]; *dsx-GAL4* [67]: *spi-GAL4*; *spi^{NP0261}* [68]; *UAS-GFP-GPI* [39]; *tub-rab11-YFP* [38]; *UAS--Dad* [25]; *UAS-tkv^{QD}* [23]; *UAS-gbb-GFP* [34]: *tkv^{CPTI002487}* [27]: *dpp^{blk}*-GAL4 [35]; *UAS-Dpp-GFP* [33]; *UAS-tRFP-Dpp* [37]. Multiple lines were obtained from the Bloomington, Vienna and Kyoto Stock Centres including the following UAS-RNAi lines [69,70], some of which have been previously employed in other studies: *dpp*, JF01090 (RNAi#1; [71]), HMS00011 (RNAi# 2): *gbb*, HMS01243 [72]: *tkv*, JF01485 [73], JF01486: *wit*, HMS02298, JF01969: *sax*, v46358 [72], JF03431, HMS00758: *punt*, JF02664 [74], GL00069, HMS01944: *Mad*, JF01263 [75], JF01264: *Med*, JF02218 [72, 73], GL01313: *Snap24*, JF03146, v48034.

Genetics

To achieve adult SC-specific expression of transgenes and RNAi, flies carrying UAS-coupled transgenes were crossed to flies containing *tub-GAL80^{ts}*, a *UAS-GFP* transgene, and a GAL4 driver (*esg*, *spi*, *dsx*) that is only expressed in SCs of the AG [17, 23]. Typically, the progeny of this cross were shifted to 28.5°C on eclosion to inhibit the temperature-sensitive GAL4 repressor, GAL80^{ts}, thus driving transgene expression specifically in adults. Mosaic SC expression was achieved by a temperature shift of esgF/O^{ts} progeny to 28.5°C in 5-day-old adults, when some SCs have stopped expressing *esg*-GAL4, as previously described [23]. For pulse experiments, flies were incubated from eclosion at 25°C then shifted to 28.5°C for the expression pulse.

Mating experiments

To analyse the effects of mating on BMP signalling activity in SCs, control male flies were mated to a single female at either 3 days or 6 days post-eclosion. Immediately after mating, the male flies were separated and the AGs were dissected in fix and stained as described below. In experiments where a GFP-GPI expression pulse was employed, matings were set up at different times, as described in the text.

Immunostaining and imaging

Immunostaining were performed as previously reported in [17,23]. Samples were incubated overnight with primary antibodies (diluted in PBST [PBS with 0.3% Triton X-100]) at 4°C before washing in PBST for 6 × 10 min. Incubation with fluorescently conjugated donkey secondary antibodies was for 2 h at 22°C followed by a further six10-min washes in PBST. Stained glands were mounted in Vectashield with DAPI. The following primary antibodies were used: ANCE (1:1000) [16], Wit (1:10; Developmental Biology Hybridoma Bank [DSHB]), Phospho-Smad1/5 (1:100; Cell Signalling; 41D10), Fasciclin 3 (1:10; DSHB), GFP (1:1000; Abcam; #6556). Secondary antibodies were conjugated to Alexa Fluor 649 and Cy3 (both diluted 1:400; Jackson ImmunoResearch Laboratories, Inc.) and incubated for 2 hours with AGs in the dark. All samples were imaged on a Zeiss Axioplan 2 (LSM 510 Meta) scanning confocal microscope, using LSM 510 Meta software. Low magnification images were obtained using the x10 objective (0.45NA, Plan-APOCHROMAT), whilst high magnification images used the x63 objective (1.4NA, Oil DIC, Plan-APOCHROMAT).

Live cell imaging

AGs were dissected in ice-cold PBS and transferred to ice-cold PBS containing Lysotracker Red DN-99 (1:1000; Invitrogen) for 5 min. They were then washed in ice-cold PBS for 5 minutes, mounted in PBS on a coverslip bridge and imaged at approximately 16°C using an LSM 510 Axioplan 2 scanning confocal microscope (Zeiss) as described above.

Counting compartment numbers

SVs were classified as CD63-positive, non-acidic compartments with a diameter of >1 μm. DCGs were defined as non-acidic compartments with a compact dense core marked by GFP-GPI or ANCE-cross-reactivity, and >1 μm in diameter. MVBLs were CD63-GFP-positive (on limiting membrane), LysoTracker Red-positive and > 2 μm in diameter, as in Corrigan et al. (2014). The compartments within each SC were counted by scanning through the entire cell. Three SCs were analysed per gland and the mean compartment number calculated.

Assaying exosome release by SCs

For exosome lumen counts, 3-day-old UAS–CD63-GFP; *dsx*-GAL4 males expressing an RNAi against a target gene together with paired controls were mated with at least two w^{1118} virgin females at 29°C overnight to expel as much of the luminal AG content as possible. These males were isolated overnight at 29°C to allow replenishment of AG content and then dissected and fixed as previously outlined. Exosome secretion was measured using the approach described in [17], in which luminal CD63-GFP fluorescent puncta (exosomes or exosome aggregates) within the central third of each gland were counted in three different z-planes (spaced by 5 μm and imaged at identical settings using 1.7x zoom with the 63x oil objective) using ImageJ.

Nuclear size measurement

Measurements were performed as described in [23]. The two nuclei of an individual SC and 3 surrounding MCs were imaged and their areas measured using Axiovision software (Zeiss). Three SC/MC clusters were imaged per gland. The relative SC:MC nuclear size was calculated and the ratios averaged across a minimum of 6 glands.

Measurement of largest MVBL in SCs

The largest MVBL was identified by scanning through SCs and then imaged in the plane where the diameter was at its greatest. All images were taken using the same confocal settings. The diameter of the MVBL compartment was calculated using ImageJ.

Measurement of fluorescent GFP inside MVBL

All images were taken using the same confocal settings, ensuring that the signal associated with MVBLs was not saturated. The z-plane in which the largest MVBL was at its greatest diameter was selected and a binary calculation was used to measure the proportion of the area covered by fluorescent GFP using ImageJ.

Western blot analysis

Male AGs expressing SC-specific GFP-GPI from eclosion onwards and w^{1118} controls were dissected in ice-cold PBS. To generate female samples, each male expressing GFP-GPI was mated to a single w^{1118} female. The whole reproductive tract with ovaries removed was subsequently dissected from these females and from controls in ice-cold PBS.

The AG and female reproductive tract lysates were then prepared as previously described in [17]. Briefly, dissected samples were pooled and homogenised using a motorised pellet pestle (Sigma-Aldrich) in 100μl of lysis buffer (Radioimmunoprecipitation assay buffer; Sigma-Aldrich) supplemented with anti-protease and anti-phosphatase cocktails (Sigma-Aldrich). The lysate was then centrifuged (13000 g; 5 min) and protein content in the supernatant quantified using a bicinchoninic acid assay kit (Thermo Fisher Scientific).

Blots were prepared as previously described [16,17]. Samples containing 2.5 μg homogenized protein (made up to 15 μl) were added to 5 μl of 4× sampling buffer (0.2 M Tris-HCl, pH 6.8, 12% SDS, 40% glycerol, 20% β-mercaptoethanol, and 0.008% bromophenol blue) and heated to 95°C for 5 min. Proteins were separated on 10% mini-Protean TGX precast gels (Bio-Rad Laboratories) for 30 min along with a prestained ladder and then transferred to a polyvinylidene fluoride membrane (Immobilon-P; EMD Millipore) at 100 V for 1 h. The membrane was blocked for 1 h in 5% skimmed milk in TBS with Tween 20 (TBST), then incubated with anti-GFP antibody at 1:500 (rabbit; ab6556; Abcam) in 5% milk in TBST overnight at 4°C. It was washed in TBST for 3 × 5 min, incubated at room temperature for 1 h with a HRP-conjugated anti–rabbit secondary antibody (W4018; Promega) at 1:20,000 in 5% milk in TBST, and then washed in TBST for 3 × 5 min. Antibody binding was detected using Clarity Chemiluminescent Substrate (Bio-Rad) and a ChemiDoc Touch Imaging System (Bio-Rad). Band intensities were measured using the Image Lab software (Bio-Rad).

Statistical analyses

For all compartment counts, measurements of intracellular compartments and nuclear size quantifications, three SCs per gland were analysed from a minimum of 10 animals (6 for nuclear size), typically involving 2–3 independent experiments. For analysis of pMad antibody staining patterns, all SCs in each AG were counted. All data were analysed using GraphPad Prism. The Shapiro-Wilk test was used to test for normality. Since not all data in each experiment were normally distributed, a Mann-Whitney *U* test or Kruskal-Wallis test was typically employed. Error bars represent ±1 SEM.

Supporting Information

S1 Fig. BMP signalling controls SC growth. A-F. Images of SCs from 6-day-old control male flies (A) or flies expressing RNAis targeting either, *wit* (B), *sax* (C), *punt* (D), or *Med* (E) or expressing an activated form of the Tkv receptor (Tkv^{ACT}) (F) under the control of esgF/O^{ts} after temperature shift at eclosion. Green and red arrows indicate SC and MC nuclei respectively. Glands were stained with DAPI (blue) to mark nuclei and an anti-Fas3 antibody (yellow) to mark cell boundaries. Genotypes for images are: *w; esg-GAL4 tub-GAL80^{ts} UAS-FLP; UAS-GFP$_{nls}$ actin>FRT>CD2>FRT>GAL4* combined with no other transgene (A); *P[TRiP.HMS02298]attP2* (III) (B); *P[GD2546]v46358* (II) (C); *P[TRiP.JF02664]attP2* (III) (D); *P[TRiP.JF02218]attP2* (III) (E); *P[w$^+$ UAS-Tkv^{ACT}]* (III) (F). Scale bar is 20 μm.
(TIF)

S2 Fig. BMP signalling controls SC growth. A. Graph showing the effects on SC growth when BMP signalling is reduced using a second independent RNAi or increased after expression of an activated form of Tkv (expression driven by esgF/O^{ts} in A-F). **B**. The absolute size of SC nuclei is reduced when BMP signalling is inhibited. **C**. In contrast, MC nuclei are not affected when SCs express different genes targeting the BMP pathway. **D.** Flies carrying UAS-transgenes used in this study do not affect the SC:MC nuclear size ratio in the absence of an SC-specific driver. **E.** Driving expression of Dad and Tkv^{ACT} in adult SCs for 6 days with esgF/

O^{ts}, *dsx*-GAL4 and *spi*-GAL4 produces similar effects on SC nuclear growth. **F.** The esgF/O^{ts}, *dsx*-GAL4 UAS-*CD63-GFP* and *spi*-GAL4 UAS-*GFP-GPI* lines all produce SCs with equivalent nuclear size relative to MCs when expression is induced for 6 days immediately after eclosion. **G**, **H**. SC-specific knockdown of *wit* using the esgF/O^{ts} driver line eliminates anti-Wit antibody staining in SCs (H) compared to control (arrow; G). Glands were stained with DAPI (blue) to mark nuclei. Gain settings on confocal are increased in (H) to demonstrate absence of specific signal. Genotypes for images are: *w; esg-GAL4 tub-GAL80^{ts} UAS-FLP; UAS-GFP$_{nls}$ actin>FRT>CD2>FRT>GAL4* combined with no other transgene (G) or *P[TRiP.HMS02298] attP2* (III) (H). ***P<0.001, Kruskal-Wallis test, n = 10. Scale bar for G, H is 10 μm.
(TIF)

S3 Fig. BMP signalling in SCs controls numbers of non-acidic compartments, endolysosomal maturation, largest MVBL size and exosome secretion. A. SC from dissected AG of 6-day-old male expressing CD63-GFP throughout adulthood and stained with an anti-GFP antibody and DAPI. Note absence in one large compartment of fluorescent GFP signal, which is still detected with the antibody (arrow). **B**-**F**. SCs expressing no RNAi (B) or RNAis targeted against transcripts encoding *wit* (C), *sax* (D), *punt* (E) and *Med* (F), and stained with Lysotracker Red (red). **G**. The number of SVs is reduced by inhibiting BMP signalling, using a second independent RNAi, except after *sax* knockdown, but increased by an activated form of Tkv. **H**. Inhibition of BMP signalling induces a significant accumulation of fluorescent GFP inside the largest MVBL (arrows in **B**-**F**), indicating a disruption in endolysosomal maturation. **I.** In most cases, inhibiting BMP signalling reduces the size of the largest MVBL. **J**. The number of CD63-GFP-positive exosomes released from SCs is reduced when BMP signalling is decreased. **K.** Expressing Dad and Tkv^{ACT} in SCs using either the *dsx*-GAL4 or *spi*-GAL4 driver produces a similar effect on the size of the largest MVBL. Genotypes for images are: *w; UAS-CD63-GFP tub-GAL80^{ts}; dsx-GAL4* combined with no other transgene (A, B); *P[TRiP.HMS02298]attP2* (III) (C); *P[GD2546]v46358* (II) (D); *P[TRiP.JF02664]attP2* (III) (E); *P[TRiP.JF02218]attP2* (III) (F). The *w; UAS-CD63-GFP tub-GAL80^{ts}; dsx-GAL4* line was employed to produce data in G-J. * P<0.05, ** P<0.01 ***P<0.001, Kruskal-Wallis test, n = 10. Scale bar for A-F 10 μm.
(TIF)

S4 Fig. Dpp controls adult SC growth. A. Image of SCs from 6-day-old male expressing Dpp-GFP under the control of esgF/O^{ts} after temperature shift at eclosion and stained for pMad (red), which is present in all SC nuclei (arrows). **B**. Mosaic clones of SCs using esgF/O^{ts} system to express Dpp-GFP in some SCs, but not others. Note the presence of high levels of nuclear pMad only in SC expressing Dpp-GFP (white arrow) and not in adjacent non-expressing cell, which has smaller nuclei (white dashed circle). **C**. Expression of Dpp-GFP in MCs leads to nuclear pMad accumulation only in SCs (arrows). Glands in A-C were stained with DAPI (blue) to mark nuclei. **D**. Absolute SC nuclear size changes significantly when *dpp* is either silenced or Dpp-GFP is overexpressed in SCs, but no obvious change is seen for *gbb* (expression driven by esgF/O^{ts} in D-H). **E**. The same treatments produce no significant change in absolute MC nuclear size. **F**. Expression of Dpp-GFP in MCs induces SC-specific nuclear growth, but there is no clear change if *dpp*-RNAi or *gbb*-RNAi is expressed in MCs using the MC-specific *Acp26Aa*-GAL4 driver. **G**. SCs expressing a second independent RNAi targeting *dpp* transcripts show reduced relative nuclear growth. **H.** The UAS-*dpp*-RNAi construct has no effect on SC nuclear size in the absence of a GAL4 driver. **I**. Bar chart shows number of large compartments per cell labelled by anti-ANCE, GFP-GPI *w; spi-GAL4 tub-GAL80^{ts} UAS-GFP-GPI* line) or Rab11-YFP (*tub-Rab11-YFP* line). Counts were made in independent experiments using 3-day-old virgin males and do not differ significantly. Genotypes for images are: *w; esg-GAL4 tub-GAL80^{ts} UAS-FLP; UAS-GFP$_{nls}$ actin>FRT>CD2>FRT>GAL4/*

UAS-Dpp-GFP (A and mosaic in B); *w Acp26Aa*-GAL4; *UAS-Dpp-GFP (III)* (C). ***P<0.001, Kruskal-Wallis test, except for G and H, where a Mann-Whitney *U* test was used. Scale bar is 20 μm.
(TIF)

S5 Fig. Fluorescently tagged Dpp traffics to DCGs. A. Males aged for 3 days were dissected and fixed after a 24 h pulse of CD63-GFP and Dpp-RFP, and SCs imaged. Dpp-positive cores (white arrows) are observed inside CD63-GFP-positive compartments. Note the increase in SC nuclear size (green arrows) even after one day of Dpp-RFP expression. **B.** Dpp-GFP expressed in a one hour pulse in adult males previously aged for 3days at 25°C after eclosion is chased into a number of spherical structures (arrows) that are located in ANCE-positive (red; eg. arrow in merged image) DCGs. Genotypes for images are: *w UAS-Dpp-tagRFP; tub-GAL80^{ts}; dsx-GAL4* (A); *w; tub-GAL80^{ts}; dsx-GAL4/UAS-Dpp-GFP* (B, C) genotypes; Scale bar is 10μm.
(TIF)

S6 Fig. Expression of CD63-GFP in adult SCs increases DCG and SV compartment number. A. SCs in esgF/O^{ts}, *dsx*-GAL4 *UAS-CD63-GFP* and *spi*-GAL4 *UAS-GFP-GPI* 6-day-old males that express their respective transgenes from eclosion onwards (after *tub-GAL80^{ts}* inactivation) all have the same number of MVBLs. **B**. The diameter of the largest MVBL in SCs from 6-day-old males is increased compared to wild type cells in esgF/O^{ts}, *esg-GAL4 tub-GAL80^{ts} UAS-GFP-GPI*, *spi*-GAL4 *tub-GAL80^{ts} UAS-GFP-GPI*, *esg-GAL4 tub-GAL80^{ts} UAS-CD63-GFP*, *dsx*-GAL4 *tub-GAL80^{ts} UAS-CD63-GFP*, and *spi*-GAL4 *tub-GAL80^{ts} UAS-CD63-GFP* males. **C, D.** SC from 6-day-old *spi*-GAL4 *UAS-GFP-GPI* (C) and *spi*-GAL4 *UAS-GFP-GPI UAS-CD63-GFP* (D) males stained with Lysotracker Red to mark acidic compartments. **E.** Such co-expression of CD63-GFP with GFP-GPI for 6 days following eclosion produces SCs with increased numbers of GFP-GPI-positive DCG compartments compared to controls. Genotypes for images are: *w; spi-GAL4 tub-GAL80^{ts} UAS-GFP-GPI* combined with no other transgene (C) or with *UAS-CD63-GFP* (D). * P<0.05, ** P<0.01 ***P<0.001, Kruskal-Wallis test for A and B, for E a Mann-Whitney *U* test was used. Scale bar for C, D is 10 μm.
(TIF)

S7 Fig. BMP signalling and mating control number of SCs with nuclear pMad staining. A-C. SCs from 6-day-old virgin males expressing either Dad (B), activated Tkv (C) or no BMP signalling regulator (A) stained with pMad antibody, revealing different BMP signalling activities. pMad-positive nuclei are marked with arrows. **D**. Graph showing the proportion of SCs that contain pMad-positive nuclei in 3-day-old esgF/O^{ts} male virgins and males dissected immediately after mating. **E**. Western blot probed with anti-GFP antibody. AG extracts from either *w^{1118}* males, or virgin and mated *w; spi-GAL4 tub-GAL80^{ts} UAS-GFP-GPI* males were analysed. Females were either virgins or mated to *w^{1118}* males or SC>GFP-GPI-expressing males.). Genotypes for images are: *w; esg-GAL4 tub-GAL80^{ts} UAS-FLP; UAS-GFP$_{nls}$ actin>FRT>CD2>FRT>GAL4* combined with no other transgene (A); *P[w^{+} UAS-Dad]* (II) (B); *P[w^{+} UAS- Tkv^{ACT}]* (III) (C).***P<0.001, Mann-Whitney *U* test, n>15. Scale bar is 20 μm.
(TIF)

S8 Fig. BMP signalling components in SCs regulate BMP pathway activation and DCG number. A. Graph showing the proportion of SCs that contain pMad-positive nuclei in 6-day-old esgF/O^{ts} virgin males expressing RNAis against different BMP signalling components after eclosion. **B.** Graph showing that the number of GFP-GPI-labelled DCGs in SCs from 6-day-old *w; spi-GAL4 tub-GAL80^{ts} UAS-GFP-GPI* virgin males is reduced when BMP signalling components other than *sax* are knocked down post-eclosion.
(TIF)

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Otack är världens lön.