Cell biology and genetic regulation of secondary cells in the *Drosophila* accessory gland

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i. Acknowledgments

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ii. Abstract: Cell biology and genetic regulation of secondary cells in the *Drosophila* accessory gland

The *Drosophila* adult male accessory gland (AG), which secretes many components of seminal fluid and modifies many behaviours of mated females, is a surprisingly understudied organ compared to many other fly tissues. This has been partly because of a lack of genetic tools allowing manipulation of the cell types that make up the AG epithelium. I have defined a set of transcriptional GAL4 drivers that display specific expression in a subset of AG epithelial cells known as secondary cells (SCs), and used these to investigate SC cell biology and whether there are biological parallels between SCs and the secretory cells of the human prostate. I show that these cells grow in an age- and mating-dependent manner relative to the rest of the epithelium. Furthermore, I have observed that these cells display the remarkable ability to delaminate apically from the epithelium after mating, migrate proximally within the gland in a directional manner, and can even be transferred intact to females during mating.

A small-scale genetic screen was performed which established that BMP signalling plays a crucial role in these interesting features of SC biology. Specifically, BMP signalling activity is required for normal SC growth and migratory activity. Hyper-activation of the BMP signalling pathway drives SC overgrowth and promotes spontaneous delamination and migration at a high frequency. Intriguingly, I have also shown that ecdysone receptor (EcR) signalling, a steroid hormone signalling pathway, is an important positive regulator of SC growth. This is of particular interest given an analogous role that the androgen receptor (AR), also a steroid hormone receptor, plays as a potent driver of cell growth in the prostate epithelium. I also show that BMP signalling activity increases EcR expression levels in a cell type-specific fashion, via a potential protein stabilisation mechanism. A surprising discovery has been the observation that the cell cycle regulators, Rbf and E2F1, also genetically interact with EcR and promote SC growth. E2F1 activation drives normal SC growth and positively regulates EcR expression and activity. Many of these findings are reminiscent of biology observed in the human prostate and prostate cancer, where for example, AR expression and activity is driven by E2F1 activity. This mechanism is postulated to be of importance in the progression of prostate cancer to a disease that no longer responds to androgen deprivation therapy.

In summary, my research in the *Drosophila* AG highlights several novel features of adult SC biology, including growth and migration, which are regulated by BMP and steroid hormone signalling activity. Much of this biology appears to closely parallel equivalent cell physiological phenomena in the prostate epithelium and in prostate cancer. In addition, as a consequence of this research, the *Drosophila* AG is emerging as an important model to study a number of conserved aspects of cell biology, particularly membrane trafficking, relevant to human diseases, including cancer.

iii. List of abbreviations

Abbreviation	Description
20-HE	**20-H**ydroxy-**ec**dysone
Acp	**A**ccessory gland **p**roteins
AG	**A**ccessory **g**land
ALiX	**A**poptosis **li**nked factor **X** or **AL**G-2 **i**nteracting protein **X**
ANCE	**An**giotensin-**c**onverting **e**nzyme
AR	**A**ndrogen **r**eceptor
BMP	**B**one **m**orphogenic **p**rotein
Bnl	**B**ra**n**ch**l**ess (FGF)
Btl	**B**rea**th**l**e**ss (FGFR)
Dad	**D**aughters **a**gainst **D**pp
CA	**C**onstitutively **a**ctive
Dib	**Disembodied**
DN	**D**ominant **n**egative
Dp	**Dp transcription factor**
Dpp	**D**eca**p**enta**p**legic
Dsx	**D**ouble**sex**
E2F1	**E2F** transcription factor **1**
Ecd	**Ecdysoneless**
EcR	**Ec**dysone **r**eceptor
ED	**E**jaculatory **d**uct
EGFR	**E**pidermal **g**rowth **f**actor **r**eceptor
Esg	**Es**car**g**ot
ESCRT	**E**ndosomal **s**orting **c**omplexes **r**equired for **t**ransport
EV	**E**xtracellular **v**esicles (exosomes and microvesicles)
FGFR	**F**ibroblast **g**rowth **f**actor **r**eceptor
Gbb	**G**lass **b**ottom **b**oat
l(2)gl	**L**arval **(2) g**iant **l**arvae
Mad	**M**others **a**gainst **d**pp
MAPK	**M**itogen-**a**ctivated **p**rotein **k**inase
Med	**Med**ea
MC	**M**ain **c**ell
Put	**Pu**n**t**
mTOR(C1)	**M**echanistic **t**arget **o**f **r**apamycin (complex 1)
Ovi	**Ovi**duct
PI3K	**P**hospho**I**nositide 3-**K**inase
PMR	**P**ost-**m**ating **r**esponse
PtdIns()P	**P**hospho**tidylinos**itol-()**p**hosphate
Pten	**P**hosphatase and **ten**sin homologue
RTK	**R**eceptor **t**yrosine **k**inase
Rbf	**R**etino**b**lastoma **f**amily protein

Abbreviation	Description
RNAi	**RNA i**nterference
Sad	**S**h**ad**ow
Sax	**Sax**ophone
SC	**S**econdary **c**ell
Shd	**Sh**a**d**e
SP	**S**ex **P**eptide
spi	**S**pitz
Spt	**Sp**erma**t**hecae
SR	**S**eminal **r**eceptacle
SV	**S**ecretory **v**acuole
(m)TORC	**M**echanistic **t**arget **o**f **r**apamycin **c**omplex
Tkv	**T**hic**kv**eins
TGF-β	**T**ransforming **g**rowth **f**actor-**beta**
UAS	**U**pstream-**a**ctivating **s**equence
VDRC	**V**ienna ***D****rosophila* **R**NAi **C**entre
Wit	**Wishful thinking**
Wt	**w**ild **t**ype

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Chapter One – Introduction

1 – Chapter One - Introduction

1.1 Drosophila as a model for genetic research

There is a rich and vibrant history stretching back for more than a century of scientists using the fruit fly, *Drosophila melanogaster,* as a model system to interrogate fundamental questions in biology (Rubin and Lewis 2000, Bellen, Tong et al. 2010, Green 2010). More so than ever, scientists today using *Drosophila* are working at the vanguard of genetic research and continue to innovate and shed new light on biological mysteries. The history of *Drosophila* as a stand-out performer in genetic research can be traced back to the great American evolutionary biologist and geneticist, Thomas Hunt Morgan, and his seminal experiments of the 1910s conducted in his famous Fly Room, housed at Columbia University (Muller 1946, Kyle and Shampo 1981). These studies mark a milestone in our understanding of the mechanisms that underlie the heredity of genetic material; specifically illustrating the role chromosomes play in this process (Morgan 1910, Morgan 1911a, Morgan 1911b, Morgan 1915). The importance of Thomas Hunt Morgan's work within the scientific community was affirmed when he was awarded the Nobel Prize for Physiology or Medicine in 1933, a prize that not only acknowledged his eminence as a geneticist, but also drew attention to the fruit fly as an extraordinarily powerful research tool (Muller 1946, Raju 1999).

Tellingly, this most prestigious of academic awards has been bestowed on no fewer than four additional *Drosophila* biologists in the last twenty years. In 1995, the award was shared between three *Drosophila* researchers: Edward Lewis, Christiane Nüsslein-Volhard and Eric Wieschaus. In a landmark *Nature* paper, Lewis characterised and named the *bithorax*

complex, demonstrating the crucial role of this cluster of genes in determining segment identity in *Drosophila* (Lewis 1978). This work essentially marked the beginning of developmental genetics as distinct field of study.

Armed with this greater understanding of the importance of the genome in defining developmental programmes, Eric Wieschaus and Christiane Nüsslein-Volhard worked collaboratively to identify genes responsible for patterning the early embryo and beyond. Much of their work entailed designing and performing large scale genetic screens and examining the effects of random mutations on fly development. By characterising mutations affecting normal embryogenesis, they were able to identify a vast number of genes implicated in developmental patterning (Nusslein-Volhard and Wieschaus 1980, Anderson and Nusslein-Volhard 1984, Wieschaus, Nusslein-Volhard et al. 1984, Anderson, Bokla et al. 1985, Gergen and Wieschaus 1986, Lehmann and Nusslein-Volhard 1986, Nusslein-Volhard, Frohnhofer et al. 1987, Wieschaus and Riggleman 1987, Zusman and Wieschaus 1987, Driever and Nusslein-Volhard 1988, Klingler, Erdelyi et al. 1988).

Indeed, the 1980s marked something of a high point for *Drosophila* biologists studying developmental genetics, reaching its apotheosis in work done by Walter Gehring and his group, who cloned and characterised a number of *Hox* genes. This work led to the discovery that these genes are conserved and play a similarly central role in directing developmental programmes in all metazoans (Carrasco, McGinnis et al. 1984, Hafen, Levine et al. 1984, McGinnis, Garber et al. 1984, McGinnis, Levine et al. 1984, Shepherd, McGinnis et al. 1984). Of the *Drosophila*-based Nobel laureates, the most recently recognised was Jules Hoffmann, who received the award, along with Bruce Beutler and Ralph Steinman, in 2011 for pioneering work into the mechanisms underpinning the activation of innate immunity via

the Toll developmental signalling pathway (Paul 2011). Specifically, Hoffmann and his lab demonstrated that Toll signalling, via activation of Toll-like receptors (TLRs), initiates transcriptional programmes responsible for the innate immune response to fungal infection in *Drosophila*, providing the first description in any system of TLRs as regulators of innate immunity (Lemaitre, Nicolas et al. 1996). Subsequent work by Bruce Beutler and his colleagues showed that this mechanism is highly conserved in vertebrates, and that a large family of TLRs exist in humans, many of which are required for the activation of different aspects of innate immunity (Poltorak, He et al. 1998).

A common theme that emerges from all of the above examples of *Drosophila*-centred research is that the significance of these scientific discoveries extends well beyond the fly, with direct relevance to human biology. This illustrates the point that fundamental cell biological processes in *Drosophila*, and the genetic regulation of that biology, are often highly conserved across the animal kingdom (Rubin 1988, Anderson and Ingham 2003, Bier 2005).

1.1.1 Genetic manipulation in *Drosophila*

The enormous number of easily accessible and highly sophisticated genetic tools available to fly biologists lies at the heart of the popularity of *Drosophila* amongst scientists dedicated to exploring questions aimed at answering how genetic mechanisms underpin fundamental cell biology. In the early 1990s a *Drosophila*-based GAL4/UAS expression system was developed, permitting cell type-specific genetic manipulation (Brand and Perrimon 1993, Duffy 2002). The power and simplicity of this approach was soon evident, and widespread adoption of this technique by the fly community followed quickly after. Over the subsequent two decades, numerous GAL4 ‘driver’ lines have been developed that allow

selective genetic control over almost all *Drosophila* cell types. Concurrently, a huge quantity of compatible lines carrying UAS-regulated transgenes have been created, many of which are available from publicly accessible stock centres, notably the Bloomington *Drosophila* Stock Centre (Rasmuson-Lestander 1995, Cook, Parks et al. 2010) and the vast RNAi library of the Vienna *Drosophila* Resource Centre (Dietzl, Chen et al. 2007).

1.1.2 The GAL4/UAS expression system

As alluded to above, the development of the GAL4/UAS expression system has marked a watershed advance in the ability of *Drosophila* geneticists to manipulate gene expression in a tissue-specific manner (Brand and Perrimon 1993), and is used extensively in the work presented in this thesis. In the early 1980s, *GAL4* was characterised in the yeast *Sacchromyces cerevisiae* as a galactose-inducible gene, encoding a protein of 881 amino acids that acts as a transcription factor regulating the expression of genes such as *GAL1* and *GAL10* (Laughon, Driscoll et al. 1984, Laughon and Gesteland 1984). Subsequently, target DNA binding sequences and transcriptional activation functions of GAL4 were meticulously characterised (Giniger and Ptashne 1988, Ptashne 1988). It was shown that GAL4 activates the expression of the divergently transcribed GAL1 and GAL10 genes by directly binding to four related 17 base pair (bp) sites located between these two loci (Giniger, Varnum et al. 1985). These sequences define what are now referred to as Upstream Activating Sequences (Oemar, Masaoka et al. 1982), which are functionally akin to eukaryotic enhancer elements and are essential for activation of GAL4-regulated genes.

Shortly after these data emerged, it was shown that GAL4 expression was able to induce transcription of a reporter gene under UAS control in *Drosophila* (Fischer, Giniger et al. 1988) and also in mammalian cells (Kakidani and Ptashne 1988). Crucially, no deleterious

phenotypic effects were observed in flies expressing GAL4, paving the way for the development of a comprehensive GAL4/UAS expression system (Brand and Perrimon 1993). In this system, GAL4 expression is used to drive the transcription of transgenes containing UAS sequences. Many GAL4 lines employ a method known as 'enhancer trapping' to drive GAL4 expression. An enhancer trap consists of a transposable element (most commonly a P-element) and a reporter controlled by a basal promoter, usually P[lacZ] or P[GAL4], such that the expression of the reporter reflects the activity of the 'trapped' enhancer (O'Kane and Gehring 1987, Bellen, O'Kane et al. 1989, Wilson, Pearson et al. 1989).

Most GAL4 drivers are created by random P-element insertion of GAL4 into the enhancer region of a gene, such that the activity of an affected enhancer is reflected by expression of GAL4. Trapped enhancers that show tissue- or cell type-specific activity can provide specific driver activity. Performing genetic crosses to place both a GAL4 driver and a UAS-regulated transgene into the same fly results in GAL4-driven expression of the transgene only in cell types where GAL4 is expressed. The illustrative example overleaf depicts a genetic cross using a line where P[GAL4] is located in the enhancer region of *engrailed* (Johnson, Grenier et al. 1995), a gene that displays selective expression in the posterior wing (Brower 1984, Kassis 1990), crossed to a line containing a GFP reporter transgene under UAS control (Yeh, Gustafson et al. 1995). Only progeny containing both en-GAL4 and UAS-GFP display expression of GFP, which is observed in the posterior wing (Figure 1.1).

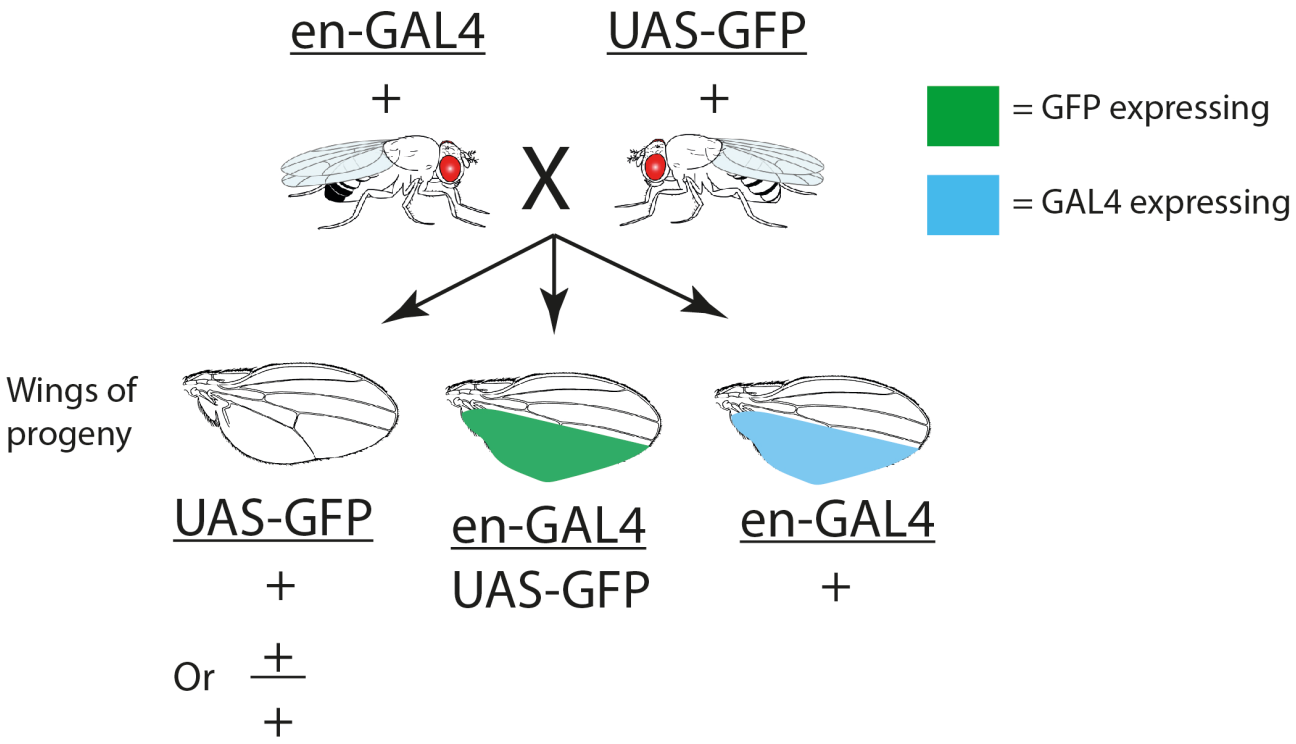


Figure 1.1: Spatial control of UAS-regulated transgenes in *Drosophila*. A GFP transgene under the control of UAS sequences is expressed only in progeny also containing the en-GAL4 insert. In wings, GFP is observed only in the posterior territory where en-GAL4 is expressed.

A drawback associated with the GAL4/UAS system is the lack of temporal control over transgene expression. The effect of transgene expression being active during development limits the usefulness of the system as a tool for examining gene function specifically in adult tissues. Often, phenotypic changes or lethality occur as a consequence of transgene expression during development, and render the interpretation of adult phenotypes an impossible exercise (Duffy 2002).

An early adaptation developed to circumvent this problem entailed the use of the GAL4 repressor, GAL80 (Lee and Luo 1999). In combination with FLP/FRT mediated mitotic recombination (Golic and Lindquist 1989, Xu and Rubin 1993), the GAL4/GAL80/UAS system was used to create mosaic animals in a technique subsequently named 'Mosaic Analysis

with a Repressible Cell Marker' (MARCM). In mosaic animals created using MARCM technology, small populations of genetically defined cells lacking GAL80 and expressing transgenes are induced, whilst neighbouring 'un-flipped' cells and cells homozygous for the GAL80 construct do not express the transgenes (Lee and Luo 1999, Lee and Luo 2001).

An important advance in the ability to control transgene expression with temporal as well spatial control has emerged using a temperature-sensitive form of GAL80, GAL80^{ts} (McGuire, Le et al. 2003, McGuire, Mao et al. 2004). GAL80^{ts} was cloned and placed under the control of the tubulin promoter, creating a tubGAL80^{ts} construct that drives ubiquitous expression of GAL80^{ts}. This is combined with the GAL4/UAS system so that, at restrictive temperatures, GAL80^{ts} represses the transcriptional activity of GAL4, whilst shifting flies to permissive temperatures (28.5°C and above) results in the degradation of GAL80^{ts}, thus permitting GAL4-regulated transcription (McGuire, Le et al. 2003, McGuire, Mao et al. 2004) (Figure 1.2).

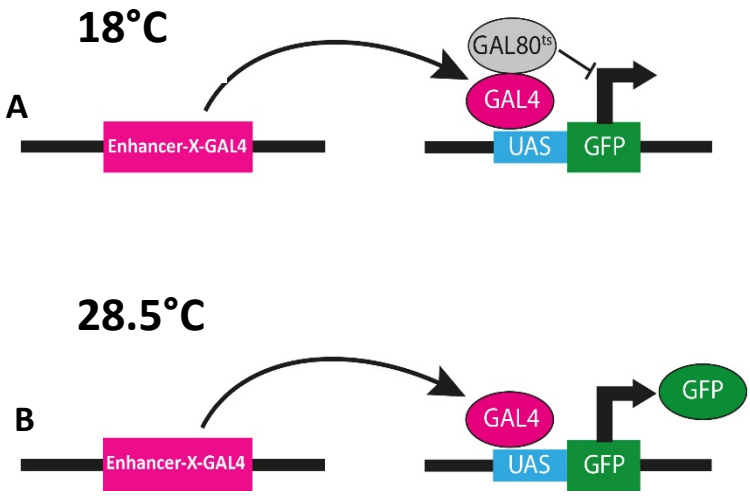


Figure 1.2: The GAL4/GAL80^{ts}/UAS system. GAL80^{ts} is ubiquitously expressed under the control of the tubulin promoter. At restrictive temperatures, GAL80^{ts} inhibits the transcription factor GAL4, preventing expression of UAS-regulated transgenes (A). At permissive temperatures of 28.5°C and above, GAL80^{ts} protein is degraded permitting GAL4-driven expression of UAS-transgenes (B). The ability to control transgene expression according to temperature provides temporal control over the GAL4/UAS expression system.

This system, GAL4/GAL80^{ts}/UAS, has been used extensively to generate many of the data presented in this thesis, as much of the focus of my work has entailed genetic analyses specifically of adult tissues.

1.1.3 *Drosophila* human disease models

Given the tractability of *Drosophila* as a laboratory organism and the associated track record of success in genetic research, interest has emerged in developing fly-based, disease-focused models. An often-quoted piece of evidence cited to support this approach is the remarkable statistic that 75% of all human disease genes have a direct homologue in the fly genome (Reiter, Potocki et al. 2001). Models established in *Drosophila* encompass the range of human pathologies and include studies into conditions as diverse as diabetes (He, Ludwig et al. 2014, Park, Ludwig et al. 2014), dementia (Prussing, Voigt et al. 2013, Mhatre, Michelson et al. 2014), cancer (Read, Goodfellow et al. 2005, Vidal and Cagan 2006, Gonzalez 2013), alcoholism (Devineni and Heberlein 2013), heart disease (Kaushik, Zambon et al. 2012, Na, Musselman et al. 2013, Viswanathan, Kaushik et al. 2014) and even kidney stones (Chen, Liu et al. 2011, Miller, Chi et al. 2013).

1.1.4 *Drosophila* cancer models

Historically, *Drosophila* research has played a significant and, I think, rather under-valued role in cancer studies. It is a little-known fact that the first ever *in vivo* description of a tumour suppressor gene was provided by *Drosophila* research nearly eighty years ago, with work done by the Swiss biologist, Ernst Hadorn, who observed lethality due to over-growth in animals mutant for the *lethal (2) giant larvae* (*l(2)gl*) gene (Hadorn 1937). Subsequent

work studying *l(2)gl* mutants showed that this excessive growth occurred as a consequence of uncontrolled proliferation of imaginal disc cells and larval brain neuroblasts, confirming the tumour suppressor role of this gene (Bryant and Schubiger 1971). This gene was later identified as coding for an adhesion protein that plays an important role in defining cell polarity (Manfruelli, Arquier et al. 1996). By now, the reader should not be surprised to discover that there is a close homologue of *l(2)gl* in the human genome, LLGL1, with practically identical functions to the fly gene (Strand, Unger et al. 1995). It is implicated in the progression of a variety of human malignancies (Schimanski, Schmitz et al. 2005, Kuphal, Wallner et al. 2006, Tsuruga, Nakagawa et al. 2007, Lu, Feng et al. 2009).

Indeed, *l(2)gl* is just one of many genes first identified in flies that have subsequently been implicated in human oncogenesis and progression. Furthermore, fly work has contributed to the discovery and characterisation of a number of highly conserved signalling pathways that are now known to play important roles in driving the development and progression of malignant disease. These insights include research that led to the discovery of the Hippo (Tapon, Harvey et al. 2002, Udan, Kango-Singh et al. 2003, Wu, Huang et al. 2003), Wnt (Nusslein-Volhard and Wieschaus 1980, Rijsewijk, Schuermann et al. 1987), Hedgehog (Nusslein-Volhard and Wieschaus 1980, Forbes, Nakano et al. 1993, Tabata and Kornberg 1994, Collins and Cohen 2005) and Notch signalling pathways (Kidd, Kelley et al. 1986, Artavanis-Tsakonas, Delidakis et al. 1991).

Aside from the discovery of cancer-related genes and signalling pathways, a small number of fly models have been developed in recent years in order to study specific malignancies, including colorectal cancer (Wang, Zhao et al. 2013), endocrine neoplasia (Read, Goodfellow et al. 2005) and glioma (Read, Cavenee et al. 2009, Witte, Jeibmann et al. 2009). A notable

example of a fly model of human malignancy is the system developed by Ross Cagan and his lab to study the cancer syndrome, Multiple Endocrine Neoplasia Type II (MEN2) (Read, Goodfellow et al. 2005, Vidal, Wells et al. 2005, Vidal and Cagan 2006), an autosomal dominant disease typified by a heritable predisposition for developing medullary thyroid cancer and neoplastic transformation of other endocrine tissues, including the adrenal and parathyroid glands (Moline and Eng 2011). Genetic studies of MEN2-affected families have shown that the vast majority of cases are due to gain-of-function mutations in the RET oncogene (Hofstra, Landsvater et al. 1994, Mulligan, Eng et al. 1994), a gene that codes for the Receptor Tyrosine Kinase (RTK), Ret (Takahashi and Cooper 1987, Takahashi 1988, Takahashi, Buma et al. 1988). Inevitably, there is a close homologue of this gene in flies, *dRet*, which encodes an analogous RTK (Sugaya, Ishimaru et al. 1994).

The *Drosophila* MEN2 model was developed by cloning a version of *dRet* containing a gain-of-function mutation, $dRet^{MEN2}$, mimicking the constitutively activating mutations seen in human MEN2, and placing it under the control of a UAS-enhancer. This transgene was then expressed in the adult *Drosophila* eye by using the GMR-GAL4 driver, resulting in proliferative over-growth. Using this system, a screen was performed to search for inhibiting or enhancing *dRet*-interacting genes, and a number of promising candidates were identified (Read, Goodfellow et al. 2005).

Impressively, this system has been used to show that an RTK inhibitor, ZD6474 (Vandetanib), can rescue the tumorigenic phenotypes associated with $dRet^{MEN2}$expression with few toxic effects (Vidal, Wells et al. 2005). Remarkably, this same compound has subsequently been shown to inhibit proliferation of human medullary thyroid cancer cells, the principal malignancy associated with MEN2 (Vitagliano, De Falco et al. 2011), and

efficacy in patients has been demonstrated in clinical trials (Wells, Gosnell et al. 2010, Wells, Robinson et al. 2012). Significantly, Vandetanib has now been approved in the U.S. for therapeutic use in advanced medullary thyroid cancer, and treatment is associated with significantly increased progression-free survival (Thornton, Kim et al. 2012, Sim and Cohen 2014). This success story serves as an exemplar; illustrating how *Drosophila* cancer models can be used to investigate disease biology, identify candidate genes and screen for therapeutic compounds. Indeed, *Drosophila*-based compound screens are emerging as an attractive adjunct to drug discovery efforts, proving to be both high-throughput and cost-effective (Tickoo and Russell 2002, Gladstone and Su 2011, Pandey and Nichols 2011).

These valuable contributions of fly research have provided important insights into cancer biology that would be difficult to recognise in any other system and have been a source of personal inspiration in the approach I have taken to my nascent scientific career to date. A key reason that I chose to pursue doctoral research in a fly lab was to explore the feasibility of developing a system in *Drosophila* that could be studied to provide novel insights into the biology of the human prostate and prostate cancer.

1.2 The human prostate

The prostate is a fibromuscular and glandular organ of the male reproductive tract located in the pelvis directly beneath the urinary bladder and anterior to the rectum (Figure 1.3). The normal prostate is ovoid in shape, weighs roughly 20 g and is traversed by a 2.5 cm portion of the urethra, known as the prostatic urethra. The prostatic urethra contains several openings, including the sinuses where the ejaculatory ducts drain, carrying sperm from the testes and fluid from the seminal vesicles. The ejaculatory ducts are formed from

the union of the vas deferens and the duct of the seminal vesicle, and penetrate the substance of the prostate to reach the urethra (Figure 1.4).

Prostate-derived secretions drain directly from the prostatic ducts into this portion of the urethra to mix with sperm and seminal fluid, forming the complex cocktail of the ejaculate. Anatomically, the prostate is divided into four distinct zones: the transition zone, surrounding the prostate, the central zone, surrounding the ejaculatory ducts, the peripheral zone, where most prostate cancers arise, and the anterior fibromuscular zone. The prostate is surrounded by a dense fibrous capsule composed of elastin, collagen and smooth muscle. Approximately 70% of the prostate's mass is composed of glandular elements, whilst the rest is stroma.

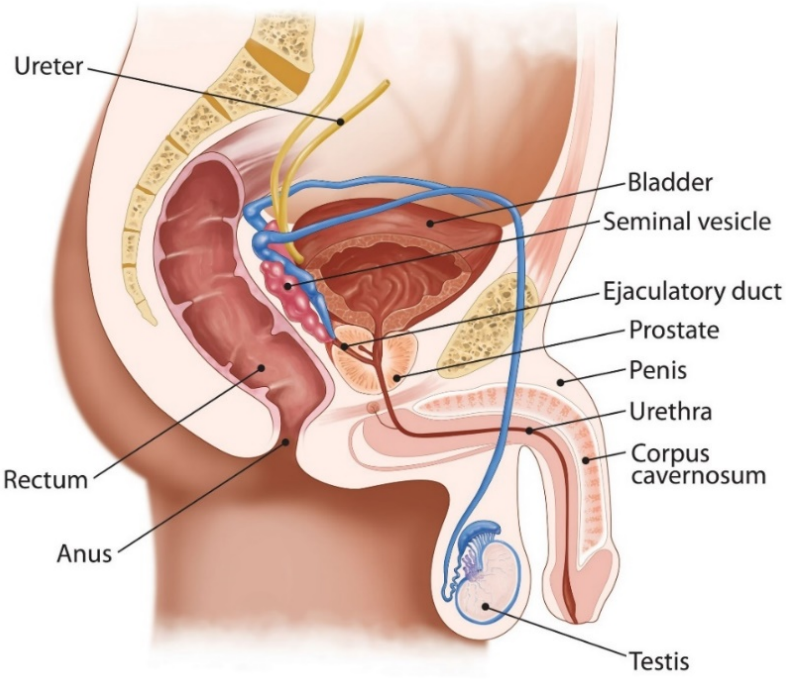


Figure 1.3: Sagittal section through the human male pelvis. The human prostate, an organ of the male reproductive tract is a walnut-sized structure located in the pelvis directly beneath the bladder and anterior to the rectum. It is traversed by both a short section of urethra and the ejaculatory ducts. Image downloaded from http://healthfavo.com

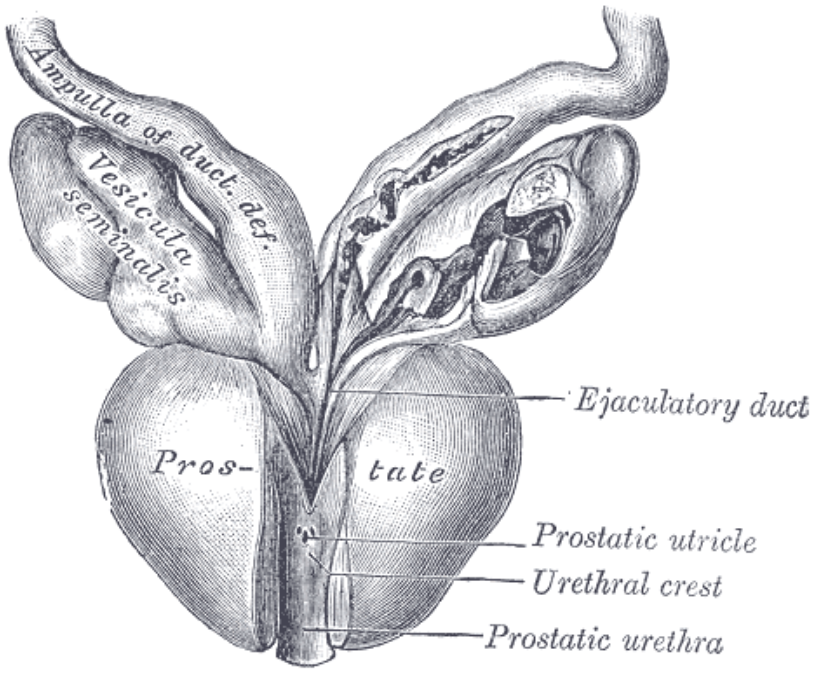


Figure 1.4: Anatomic relationship between the prostate, prostatic urethra and ejaculatory ducts. Prostatic ducts drain directly into the prostatic portion of the urethra where secreted products mix with seminal fluid products derived from the seminal vesicles, via the ejaculatory ducts. Image from Gray's Anatomy (Standring and Gray 2008).

1.2.1 The prostate epithelium

There are three major cell types that make up the prostate epithelium; luminal cells, basal cells and neuroendocrine cells (Figure 1.5). Luminal cells are the most populous cell type of the prostate epithelium and express a number of proteins characteristic of prostate differentiation, including prostate-specific antigen (PSA), androgen receptor (AR), acid phosphatase and 15-lipoxygenase-2 (Shappell, Boeglin et al. 1999, Bhatia, Maldonado et al. 2003). Additionally, luminal cells are rich in keratin filaments (van Leenders and Schalken 2003). The apical aspect of these tall, columnar cells projects into the lumen of the prostatic acini, whilst the basal aspect of these cells is tethered to the underlying basement membrane by baso-lateral integrins (Knox, Cress et al. 1994). The cytoplasm of luminal cells is abundant in Golgi apparatus and the upper portion of the cell is rich in secretory granules.

The basal cells are smaller than luminal cells and are fewer in number. These cells rest on the basement membrane, wedged between the bases of adjacent luminal cells. Unlike luminal cells, basal cells do not express PSA or AR and express distinct keratin sub-types (5 and 14, compared to 8 and 18, which are expressed by luminal cells). Neuro-endocrine cells are found sparsely scattered throughout the prostate epithelium and are thought to be able to influence differentiation, growth and secretion of the rest of the epithelium via paracrine mechanisms (Abrahamsson 1999, Vashchenko and Abrahamsson 2005).

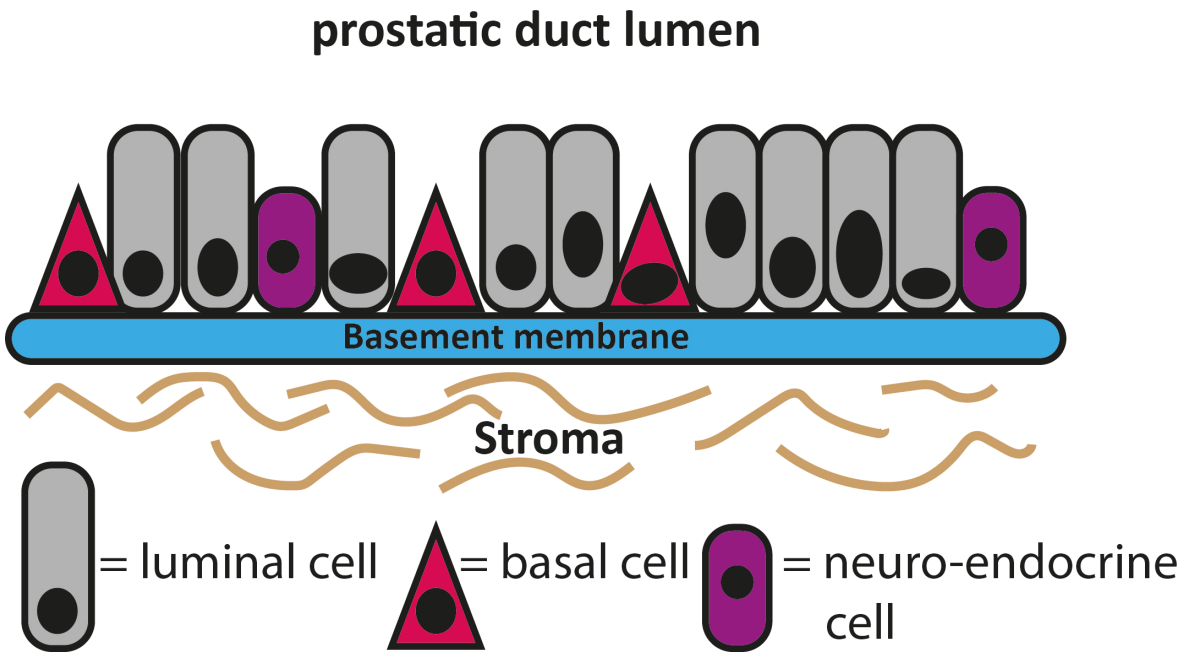


Figure 1.5: schematic of the prostate epithelium: Three distinct cell types make up the prostate epithelium. Luminal cells (also referred to as secretory cells), the most populous cell type, are tall columnar cells and rich in apical secretory vacuoles. These cells express markers consistent with prostatic differentiation, including androgen receptor (AR) and prostate-specific antigen (PSA). Basal cells are found interposed between the bases of luminal cells and lack AR and PSA expression. Occasional neuroendocrine cells are found scattered throughout the glandular epithelium. The function of these cells is poorly understood but are thought to regulate aspects of luminal and basal cell biology via paracrine mechanisms.

1.2.2 Prostatic secretions

Seminal vesicle-derived secretions contribute roughly two thirds of the volume of the human ejaculate, whilst prostatic secretions make up the remaining third. A key product secreted by the seminal vesicles is the sugar fructose, which functions to provide an anaerobic and aerobic energy source for spermatozoa (Campbell, Wein et al. 2007). The prostate secretes a cocktail of proteins that contribute to the ejaculate, including a number of serine proteases, such as PSA and human kallikrein 2 (HK2), protease inhibitors, including protein C inhibitor (PCI) and acid phosphatases, like prostatic acid phosphatase (PAP) (Mann 1964). It is postulated that the role of PSA is to lyse the clot in the ejaculate (Bilhartz, Tindall et al. 1991). Similarly, the secreted kallikreins are all thought to play a role in regulating seminal fluid coagulation, whilst anti-proteases, like PCI, counteract these functions to maintain a balance of coagulation and liquefaction in human semen (Michael, Pampalakis et al. 2006, Emami, Deperthes et al. 2008).

1.2.3 Hormonal influences on prostate epithelial biology

The Leydig cells of the testes are the major site of testosterone synthesis and secretion in adult human males. Leydig cell number is regulated by the action of the anterior pituitary hormones, follicle stimulating hormone (FSH) and luteinising hormone (LH); LH also promotes testosterone synthesis (Hall and Guyton 2011). In the plasma, testosterone circulates either in a complex with soluble proteins, such as sex-hormone binding globulin (SBG) and albumin, or as free, un-bound testosterone. Free testosterone is transported into prostate epithelial cells by passive diffusion, where it is converted by the enzyme 5α-reductase into dihydrotestosterone (DHT) (Hall and Guyton 2011). DHT then binds to and

activates cytoplasmic AR, which forms a homo-dimer that translocates to the nucleus and binds to cognate DNA sequences, known as androgen response elements (Januschke, Reina et al.), to regulate expression of AR target genes (Shaffer, Jivan et al. 2004) (Figure 1.6).

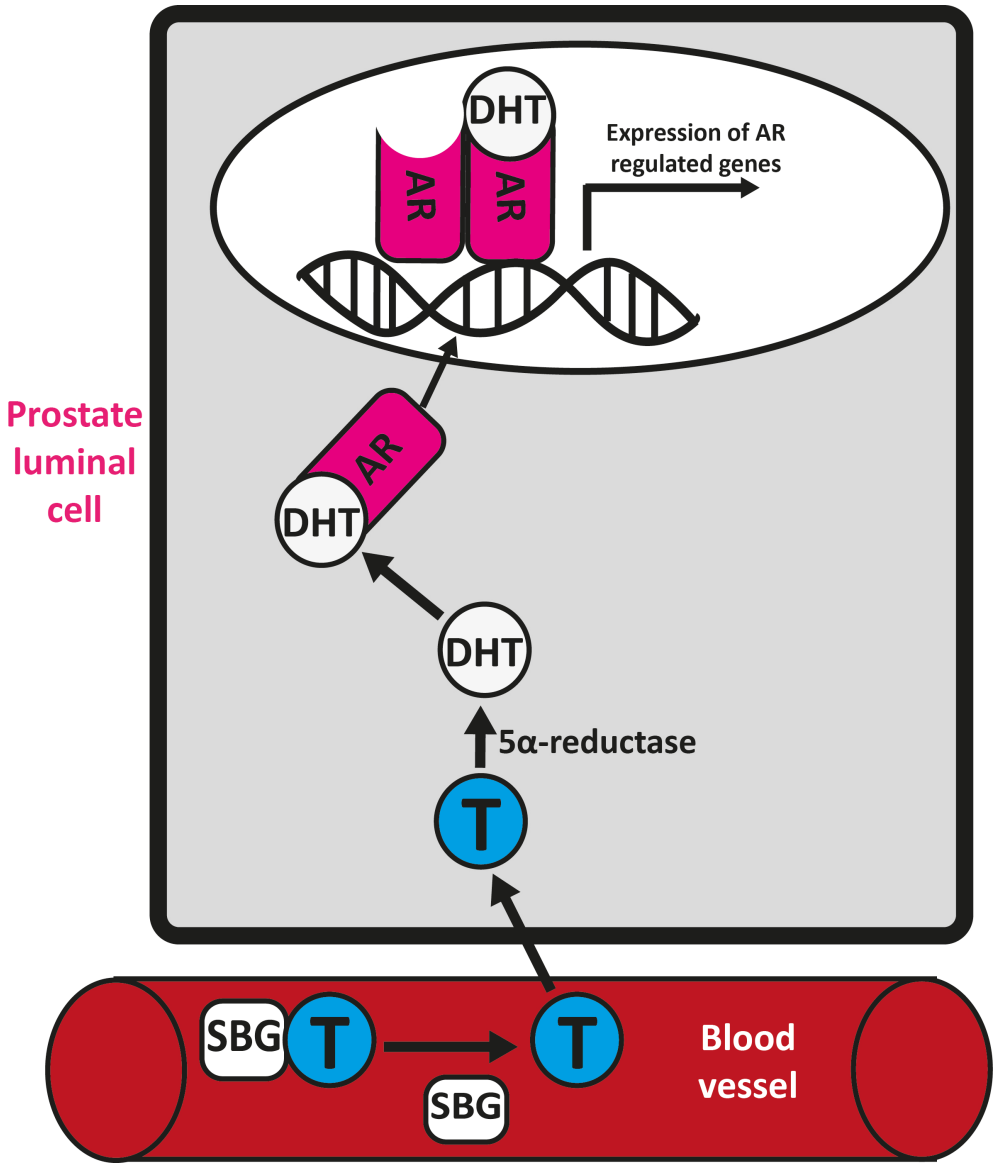


Figure 1.6: Schematic depicting prostate epithelial AR signalling. Testosterone (T) circulates bound to soluble proteins, notably sex-binding globulin (SBG). Free T crosses the plasma membrane of prostate epithelial luminal cells. T is converted to the active metabolite dihydrotestosterone (DHT) in the cytoplasm by the action of the enzyme, 5α-reductase. DHT interacts with the androgen receptor (AR) via the ligand-binding domain of AR. Upon ligand binding, AR forms homo-dimers and translocates to the nucleus where the complex binds to DNA containing cognate ARE sequences via the DNA-binding domain.

The role of androgens in regulating the physiology of the prostate was first illustrated by the American Nobel Prize-winning urological surgeon, Charles Huggins, whose experiments in dogs demonstrated the growth- and secretion-promoting effects of testosterone on the prostate (Huggins, Masina et al. 1939, Huggins and Clark 1940, Huggins and Sommer 1953). Subsequently, a number of prostatic secretory proteins, including PSA, have been shown to be transcriptionally regulated by AR (Montgomery, Young et al. 1992, Wolf, Schulz et al. 1992, Young, Andrews et al. 1992, Shan, Porvari et al. 1997). In the adult human, androgens maintain cells in the normal prostate, and androgen withdrawal is associated with prostatic atrophy (Wu and Gu 1987, Isaacs, Furuya et al. 1994).

Androgens do not appear to have any pro-mitotic effects in adult prostate epithelium, and seem to contribute to growth regulation through anti-apoptotic mechanisms (Coffey, Watson et al. 2002). As outlined below, androgens play crucial roles in promoting proliferative growth in the developing prostate epithelium, and these mechanisms may be recapitulated in malignant disease (Schaeffer, Marchionni et al. 2008).

1.2.4 Prostate development

The prostate arises from an embryonic urethral epithelium, known as the urogenital sinus epithelium, initially as simple tubular outgrowths, in a process that begins at the end of the 11th week of gestation (Thomson, Cunha et al. 2008). Throughout the 2nd trimester these ducts undergo a process of extensive arborisation, forming a complex ductal system. Mid-way through the 2nd trimester, this developing epithelium comes into contact with mesenchymal cells, which organise around the developing tubules and ultimately

differentiate into components that make up the mature fibro-muscular stroma (Thomson and Marker 2006, Thomson, Cunha et al. 2008).

There is a complex interaction between epithelial and mesenchymal elements of the developing prostate that is regulated by foetal androgens. During this stage of development only the mesenchymal cells express the androgen receptor (AR), and the action of androgens on the prostatic mesenchyme induces epithelial bud formation, regulates growth and branching, and promotes the differentiation of the secretory epithelium (Thomson 2001, Marker, Donjacour et al. 2003, Prins and Putz 2008). Members of the fibroblast growth factor family, FGF7 and FGF10, have been shown to be expressed by the prostatic mesenchyme and play an essential role in organising development of epithelial components, although these mechanisms are thought to be independent of the androgen-related regulation (Thomson and Cunha 1999, Donjacour, Thomson et al. 2003, Thomson 2008). These data intimate that FGFs act as mesenchymal paracrine regulators of prostate epithelial development (Thomson 2008).

1.3 Prostate cancer

1.3.1 Incidence and mortality

Prostate cancer is the most commonly diagnosed cancer in European and North American men, and is second only to lung cancer as the leading cause of male cancer-related deaths in the UK (Hussain, Gunnell et al. 2008, Jemal, Bray et al. 2011, Ferlay, Steliarova-Foucher et al. 2013, Siegel, Ma et al. 2014). In 2008, prostate cancer accounted for 14% of all newly diagnosed cancer cases in men worldwide, and 6% of cancer deaths (Jemal, Bray et al. 2011). Incidence varies greatly worldwide, with the rates higher in the developed regions of Europe, North America and Oceania than developing countries (Jemal, Bray et al. 2011). This

is largely because of the widespread utilisation of PSA testing that detects clinically significant cancer as well as slow-growing tumours that may well have otherwise remained undetected (Etzioni, Penson et al. 2002). Indeed, detection of prostate cancer in the U.S. rose sharply after PSA-testing became widespread in the late-1980s, reaching a peak incidence in 1992 (Stephenson and Stanford 1997).

Throughout the 1970s and 80s, prostate cancer mortality rose steadily in the U.S. and began to decline slowly from 1990 onwards (Etzioni, Legler et al. 1999, Wachtel, Nelius et al. 2013). It is thought that this decline is unlikely to be explained by PSA testing alone. Based on current understanding of lead-time, it is thought that the fall in mortality would have occurred too soon after the introduction of PSA testing (Etzioni, Legler et al. 1999, Etzioni, Gulati et al. 2012). Rather, it is felt that this trend in mortality reflects a more aggressive approach that emerged in the late 1980s to treating early disease (Walsh 2000, Etzioni, Gulati et al. 2012).

1.3.2 Natural history and prognosis in prostate cancer

The natural history of prostate cancer is poorly understood, meaning that disease progression is highly unpredictable (Johansson, Andren et al. 2004, Popiolek, Rider et al. 2013). Men diagnosed with cancer that is entirely confined to the prostate can consider their diagnosis with an optimistic outlook, as the 5 year survival rate of organ-confined disease exceeds 90% (Sammon, Abdollah et al. 2015). Conversely, individuals who present at initial diagnosis with skeletal metastases have a much bleaker prognosis, with a 5 year survival of less than 30% (Sammon, Abdollah et al. 2015). Clearly, the development of metastatic disease represents a watershed in survivability from prostate cancer, however

the propensity for the disease to progress is highly unpredictable (Popiolek, Rider et al. 2013).

The vast majority of those diagnosed with organ-confined prostate cancer do no die of this disease, but from unrelated pathologies that occur in the general population at a high frequency, such as ischaemic heart disease (Popiolek, Rider et al. 2013). An important factor that contributes to this failure of being able to differentiate which cancers pursue an indolent course, and which will turn out to be aggressive and life-limiting, can be attributed to the relative lack of research into the fundamental biology of the prostate and prostate cancer. The ability to predict the natural history of prostate cancer is particularly cryptic given the deficiencies in understanding of the cell biology and genetic regulation of the prostate epithelium.

1.3.3 Model systems for studying prostate cancer

1.3.3.1 Cell lines

Efforts to characterise cell biology in prostate cancer have involved the establishment of a number of prostate cancer cell lines, including the PC3 (Kaighn, Narayan et al. 1979), DU145 (Stone, Mickey et al. 1978) and LNCaP lines (Horoszewicz, Leong et al. 1983). However, the general view is that none of these lines are particularly representative of prostate cancer. For instance, the most commonly used line in prostate cancer research, PC3, does not express PSA, which can be detected in serum and is routinely used as a clinical biomarker to identify and track the progress of prostatic malignancy (Sobel and Sadar 2005). Furthermore, the PC3 line does not express the androgen receptor (AR) and cell growth is therefore insensitive to androgens. This is in contrast to the vast majority of all early prostate cancers, which do express high levels of PSA and grow in an androgen-dependent

manner (Sobel and Sadar 2005). Indeed, it has been speculated that PC3 cells more closely resemble a rare form of prostate cancer known as prostatic small cell neuroendocrine carcinoma, rather than prostate adenocarcinoma (Tai, Sun et al. 2011). Supporting this, PC3 cells (as well as DU145 cells) have been shown to express the neuroendocrine markers Chromogranin A and PGP 9.5 (Leiblich, Cross et al. 2007).

Similarly, the DU145 cell line lacks important characteristics of classical prostatic adenocarcinoma in that, like PC3 cells, this line does not express PSA and is insensitive to androgens (Sobel and Sadar 2005). Furthermore, this line was isolated from a brain metastatic lesion, a highly unusual site for metastasis in prostate cancer. The LNCaP cell line in many ways shares more parallels with the natural history of prostate cancer than the other cell lines discussed. Unlike PC3 and DU145 cells, LNCaP cells express PSA and Prostate Specific Membrane Antigen (PSMA), and growth of these cells is androgen-dependent (Sobel and Sadar 2005). Additionally, nude mice that have LNCaP cells injected into their prostates develop skeletal metastases that, as in prostate cancer, form osteoblastic, rather than osteolytic, lesions (Wu, Sikes et al. 1998). Conversely, both PC3 and DU145 cells have a propensity to form osteolytic bone metastases in similar experiments (Nemeth, Harb et al. 1999).

1.3.3.2 Mouse models of human prostate cancer

Over the past two decades a number of prostate cancer mouse models have been developed and studied. These models can be characterised as either xenografts or genetically engineered mouse models (GEMMS) (Valkenburg and Williams 2011). Xenografts entail the implantation of human prostate cancer cells or tissue into a recipient immunodeficient mouse. These mice are selected for their inability to mount an immune

response against foreign material, therefore permitting growth of grafted tissue (Morton and Houghton 2007). Typically, prostate cancer xenografts utilise one of the established cell lines discussed above, PC3, DU145 or LNCaP cells (Billstrom, Lecander et al. 1995, Shamsuddin and Yang 1995, Wang, Corr et al. 1995). Prior to implantation, the donor cells may be manipulated genetically, for example using virally transfected RNAi techniques, to probe gene function in growing tumours. Typically, prostate cancer cells are grafted subcutaneously (Wang, Corr et al. 1995), into bone (Wu, Sikes et al. 1998) or directly into the prostate (known as orthotopic grafts) (Pettaway, Pathak et al. 1996). The mouse prostate is relatively small and anatomically dissimilar to the human prostate (Castrillon and DePinho 2001), with a much more elaborate arrangement of the lobes (Figure 1.7). These anatomic features make orthotopic grafts particularly difficult to establish, hence the popularity of extra-prostatic grafts. Although xenograft systems provide an opportunity to study how prostate cancer cells grow *in vivo*, they bear the inherent limitations associated with the cell lines, as detailed above, and transplantation into a heterologous system.

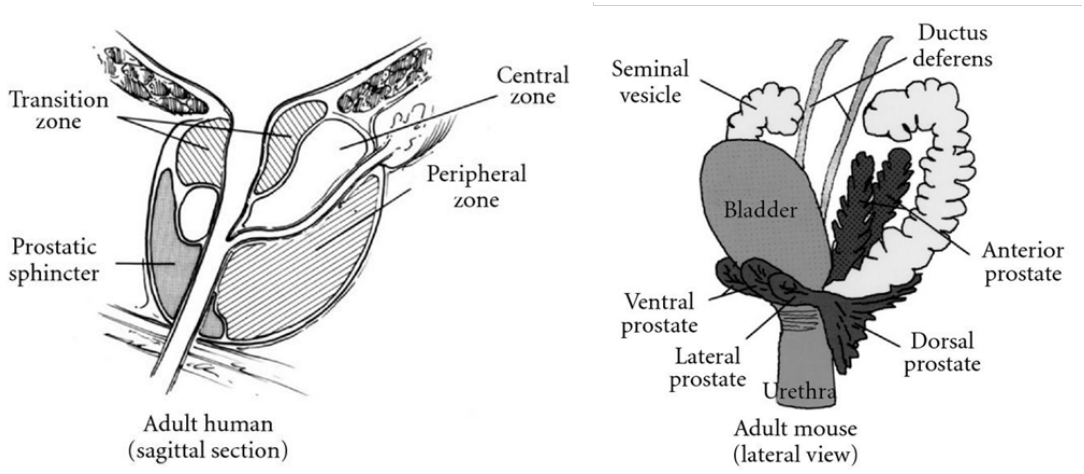


Figure 1.7: Comparison of human and murine prostates. Sagittal section through the human prostate (A) depicts the relationship of the three zones; transitional, central and peripheral zones are closely related and all confined within the prostate capsule. The mouse prostate (B) is a great deal smaller and more elaborate than the human prostate, with four distinct lobes; the anterior prostate, the dorsal prostate and two ventral lobes, that do not show the same close anatomic relationship as the zones of the human prostate.

The development of GEMM models of prostate cancer goes some way to circumvent some of the limitations associated with cell line and xenograft models. The most widely used GEMM prostate cancer system is the TRAMP (transgenic adenocarcinoma of the mouse prostate) model, which emerged in the mid-to-late 1990s (Greenberg, DeMayo et al. 1995, Gingrich, Barrios et al. 1996, Gingrich, Barrios et al. 1997). The TRAMP model uses the prostate-specific rat-Probasin promoter to drive gene expression in the mouse prostate. In particular, this promoter is used to drive the expression of simian virus 40 (SV40) Large T antigen (Tag), a protein that has been selected for its ability to induce malignant transformation. By 8 weeks of age, these mice develop epithelial hyperplasia, histological evidence of prostatic epithelial neoplasia (PIN) exists by 18 weeks and at 28 weeks virtually all mice have developed metastatic disease, although skeletal metastases are very rare. This system has established the first *in vivo* model of prostate cancer, and undoubtedly has, and will continue to provide mechanistic insights into metastasis. However, the translational value of TRAMP is somewhat diluted by the observation that the tumours that develop in these animals are most frequently neuroendocrine in origin, whereas neuroendocrine neoplastic disease of the human prostate is relatively uncommon.

The drawbacks associated with cell line and murine models of prostate cancer have served as an important stimulating factor in my own search for alternative platforms in which to study the basic biology relevant to prostate cancer and have driven my interest in exploring the feasibility of using *Drosophila*-based genetic techniques to understand prostate biology.

1.4 The *Drosophila* accessory gland

The male *Drosophila* accessory glands (AGs) are paired, blind-ended tubular structures that, together with the testes, seminal vesicles and ejaculatory duct, form the male reproductive tract (Figure 1.8A). The AGs are composed of a monolayer epithelium that is encircled by muscle, which has recently been identified as striated rather than smooth muscle (Susic-Jung, Hornbruch-Freitag et al. 2012). Epithelial cells are arranged on a basement membrane and secrete apically into the AG lumen (Bairati 1968) (Figure 1.8C). During mating, the proteinaceous contents of the lumen are expelled from the glands as a consequence of muscle contraction, facilitating the transfer of AG luminal contents to the female.

Two morphologically distinct epithelial cell types make up the AG epithelium; main cells (MCs) and secondary cells (SCs) (Bairati 1968). Main cells are the most populous cell type, numbering in the region of 1000 cells per gland (Figure 1.8D). They are found throughout the organ and are flat and polygonal in shape (Bairati 1968). Secondary cells, however, number only 40 or so cells per gland, and their location is restricted to the distal tip of each gland (Figure 1.8B&C). These cells are larger than MCs, are more rounded in shape, with an apical surface that bulges into the AG lumen (Bairati 1968). Unusually, both cell types are binucleate, although the underlying function of binucleation has yet to be fully characterised. One group has argued that binucleation helps maintain the integrity of the epithelium when changes in cell shape occur upon mating as a consequence of the gland expelling its contents (Taniguchi, Kokuryo et al. 2012).

Differences between SCs and MCs are not limited to the distinct morphological characteristics described. Indeed, a number of genes are differentially expressed between these two cell types, indicating that MCs and SCs contribute unique products to the ejaculate (Bertram, Akerkar et al. 1992)

Figure 1.8 The *Drosophila* male reproductive tract and AG epithelium

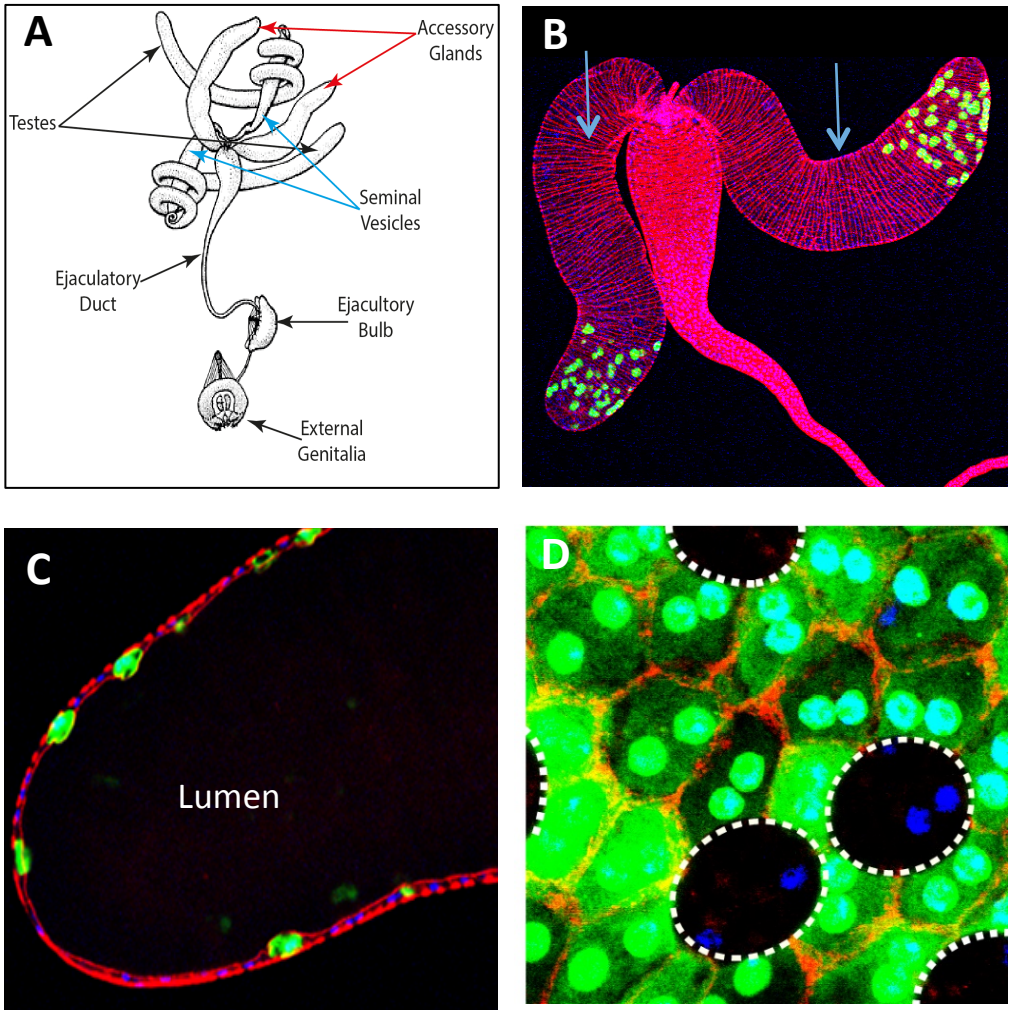


Figure 1.8: The *Drosophila* male reproductive tract: The male AGs (A, red arrows, B, blue arrows) are paired, ductal organs composed of a monolayer epithelium, encircled by striated muscle. The muscle layer is indicated in pink in B using a TRITC-conjugated phalloidin stain. The cells of the AG epithelium secrete products directly into the duct lumen, shown in cross-section in C. Two bi-nucleate cell types make up the AG epithelium; main cells, marked with GFP in D, are the most populous cell type and are flat and polygonal in shape. Secondary cells, expressing GFP in B and circled in D (where they are non-GFP-expressing), are larger and much more rounded than main cells, and their location is restricted to the distal tip of the gland. (Figure A courtesy of www.flybase.org)

1.4.1 Accessory gland function

Interestingly, mutant males lacking mature AGs are sterile, suggesting that these organs play an important role in normal reproductive physiology and fertility (Xue and Noll 2002). As alluded to earlier, a number of peptides secreted by the AG epithelium have been characterised and are required for the induction of stereotyped physiological and behavioural responses in females after mating (Wolfner 1997, Wolfner 2007). These responses are known collectively as the post-mating response (PMR), and will be covered in more detail below.

The secreted proteins derived from the accessory glad are commonly referred to as Acps, many of which are proteases and anti-proteases (Laflamme and Wolfner 2013). This cocktail of proteases and anti-proteases contributes to the formation and integrity of the mating plug, but also serves to process a number of pre-pro-peptides, promoting their activation and function in the female, a process that is highly reminiscent of the function of mammalian prostate-derived secretions, such as PSA (Laflamme and Wolfner 2013). The secretory activity of the AG epithelium appears to be modulated by a range of exogenous and endogenous cues. For instance, both RNA and protein synthesis within the gland are promoted by mating activity (Schmidt, Chen et al. 1985, DiBenedetto, Harada et al. 1990), whilst the synthesis of at least two Acps has been shown to be induced by the steroid hormone, 20-hydroxyecdysone (20-HE) (Wolfner, Partridge et al. 1997).

1.4.2 The female *Drosophila* post-mating response

The female PMR is largely evoked by peptide signals derived from the male AGs (Wolfner 2002, Wolfner 2007). It includes increased egg-laying (Aigaki, Fleischmann et al. 1991,

Chapman, Herndon et al. 2001, Chapman, Bangham et al. 2003), increased food consumption (Carvalho, Kapahi et al. 2006, Kubli 2010) and decreased receptivity to further mating (Chen, Stumm-Zollinger et al. 1988, Aigaki, Fleischmann et al. 1991, Chapman, Liddle et al. 1995, Wolfner 1997). The PMR is divided into short- and long-term response effects, with the short-term effects appearing in the first 24 hours after mating (Kalb, DiBenedetto et al. 1993), whilst long-term responses may last up to 10 days (Manning 1962, Ram and Wolfner 2009).

The most well-studied of the AG-derived PMR-inducing signals include the Acps, Sex Peptide (SP or Acp70A) (Chen, Stumm-Zollinger et al. 1988) and Ovulin (Acp26A) (Monsma and Wolfner 1988), both of which are expressed by main cells (DiBenedetto, Harada et al. 1990, Kalb, DiBenedetto et al. 1993). SP plays complex roles in females that include changes in behaviour. Normally, a female rejects the courtship advances of further males once she has mated and will not normally re-mate for a period of up 8 days. Transfer of SP from males to females is an absolute requirement in order to prevent females from re-mating. It can therefore be surmised that an important function of the AG, and main cells in particular, is to induce physiological and behavioural changes in females after mating.

1.4.3 Parallels between the *Drosophila* AG and human prostate

This project developed from an idea that research using *Drosophila* might yield useful insights into cell biology relevant to prostate function and prostate cancer. To this end, I chose to study the epithelium of the male fly accessory gland, an organ that seemed to share similarities with the mammalian prostate. Both are accessory organs of the male reproductive tract that secrete a complex cocktail of glycoproteins and kallikrein-like molecules into seminal fluid that are required for normal fertility. As I will discuss, my

studies identified several other parallels that are beginning to inform experimental work in human prostate biology and cancer, while subsequent analysis in the lab (Corrigan, Redhai et al. 2014) revealed that secondary cells secrete exosomes, small, membrane-bound nanoparticles that are implicated in cell-cell signalling and target cell reprogramming (Raposo and Stoorvogel 2013). Interestingly, cells of the human prostate also secrete highly specialised exosomes that, like fly AG-derived exosomes, are secreted into the seminal fluid and fuse with sperm (Arienti, Carlini et al. 1999, Palmerini, Carlini et al. 1999). It is speculated that prostate-derived exosomes play a role in modulating sperm function and motility (Arienti, Carlini et al. 2004, Park, Kim et al. 2011).

1.5 Aims

When my DPhil work began, I set myself the following aims:

- To establish drivers and tools to facilitate study of the AG epithelium
- To develop high resolution fluorescence imaging of these epithelial cells
- To screen for prostate-like activities and to dissect out their genetic regulation
- To start to use the accessory gland system to develop new models to explain prostate biology and disease

The resulting work has been the foundation for a major expansion of research in the lab to a programme that focuses on several broad cell biological processes, such as extracellular vesicle secretion and function, intracellular membrane trafficking and its control by cell-cell signalling, regulation of cell growth, migration and ageing, and the regulation of secretion, all of which can be studied in the living gland in remarkable detail. In discussing my data, I

will overview how my studies have informed this research and how they now integrate into an integrated research programme with wide-reaching applications.

2 – Chapter two – Materials and Methods

2.1 Fly stocks

The *Drosophila* strains used in this work were largely sourced from the major stock centres, including; the Bloomington Stock Centre, Indiana USA http://flystocks.bio.indiana.edu/, the Vienna *Drosophila* Resource Centre (VDRC), Austria http://stockcenter.vdrc.at/control/main and the Kyoto *Drosophila* Genetic Resource Centre (DGRC), Japan https://kyotofly.kit.jp/cgi-bin/stocks/index.cgi. A small number of stocks were kindly donated from various labs around the world, and the sources are acknowledged in this chapter with appropriate references. A number of genetic crosses were made during the course of this work, largely to create combination genotypes to test genetic interactions or to create new genetic tools. Details of how these genetic crosses were created will be described in this chapter.

2.1.1 BMP signalling related fly strains

Genotype	**Source/Ref**	**Description**
w; P{w[+mC]=UAS-tkv.Q253D.Nb}/TM3	Bloomington stock 36536 (Nellen, Burke et al. 1996)	Constitutively active form of Tkv receptor, under UAS control
yw; P{w[+mC]=UAS-Dad} on II	Donation – Dr D.Bennett, Univ. Liverpool, UK. (Tsuneizumi, Nakayama et al. 1997)	BMP pathway repressor, Dad, under UAS control
w; P{w[+mC]=UAS-tkv-GFP}/TM3	Donation – Prof T. Kornberg, UCSF, USA. (Roy, Hsiung et al. 2011)	GFP-tagged Tkv receptor, under UAS control

Genotype	Source/Ref	Description
w; P{w[+mC]=UAS-tkv.Q253D.Nb}/TM3	Bloomington stock 36536 (Nellen, Burke et al. 1996)	Constitutively active form of Tkv receptor, under UAS control
yw; P{w[+mC]=UAS-Dad} on II	Donation – Dr D.Bennett, Univ. Liverpool, UK. (Tsuneizumi, Nakayama et al. 1997)	BMP pathway repressor, Dad, under UAS control
w; P{w[+mC]=UAS-tkv-GFP}/TM3	Donation – Prof T. Kornberg, UCSF, USA. (Roy, Hsiung et al. 2011)	GFP-tagged Tkv receptor, under UAS control
yv ;P{y[+t7.7]v[+t1.8]=TRiP.JF02218}attP2 -MedRNAi	Bloomington stock 31928 (Beckwith, Gorostiza et al. 2013)	RNAi directed against Med, under UAS control
yv; P{y[+t7.7]v[+t1.8]=TRiP.JF01263}attP2 -MadRNAi	Bloomington 31315 (Guo, Driver et al. 2013)	RNAi directed against Mad, under UAS control
yv; P{y[+t7.7]v[+t1.8]=TRiP.JF01264}attP2 -MadRNAi	Bloomington 31316 (Ni, Markstein et al. 2008)	RNAi directed against Mad, under UAS control
yv; P{y[+t7.7]v[+t1.8]=TRiP.JF01485} attP2/TM3, Ser- tkvRNAi	Bloomington 31040 (Deshpande, Willis et al. 2014)	RNAi directed against Tkv, under UAS control
yv; P{y[+t7.7]v[+t1.8]=TRiP.JF01486} attP2 -tkvRNAi	Bloomington 31041 (Ni, Markstein et al. 2008)	RNAi directed against Tkv, under UAS control

yv; P{y[+t7.7]v[+t1.8]=TRiP.JF01371}attP2 - Dpp RNAi	Bloomington 25782 (Guo, Driver et al. 2013)	RNAi directed against Dpp, under UAS control
yv; P{y[+t7.7]v[+t1.8]=TRiP.HMS00011}attP2 - Dpp RNAi	Bloomington 33618 (Guo, Driver et al. 2013)	RNAi directed against Dpp, under UAS control
yv; P{y[+t7.7] v[+t1.8]=TRiP.HMS01243}attP2 – gbb RNAi	Bloomington 34898 (Ballard, Jarolimova et al. 2010)	RNAi directed against Gbb, under UAS control
w; P{w[+mC]=UAS-dpp.S}42B.4	Bloomington 1486 (Tracey, Ning et al. 2000)	Dpp expression construct, under UAS control
w; P{w[+mC]=UAS-dpp.GFP}/TM3	Bloomington 53716 (Teleman and Cohen 2000)	Dpp expression construct, under UAS control, GFP-tagged
yw; P{w[+mC]=dpp-GAL4.PS}l(3)8B[8B] P{w[+mC]=dpp-GAL4.PS}6A/TM3	Bloomington 7006 (Takaesu, Johnson et al. 2002)	Dpp-Gal4 line
yw; P{w[+mC]=dpp-GAL4.PS}6A/TM3,	Bloomington7007(Takaesu, Johnson et al. 2002)	Dpp-Gal4 line
w; P{w[+mC]=dad-eGFP-lacZ}.1 on II	Donation – Prof E. Martin-Blanco, Molecular Biology Institution of Barcelona, Spain. (Ninov, Menezes-Cabral et al. 2010)	BMP pathway activity reporter construct

2.1.2 EcR-related fly strains

Genotype	Source/Ref	Description
w; P{w[+mC]=UAS-EcR.A} on III	Bloomington 6470 (Lee, Marticke et al. 2000)	EcR-A expression construct, under UAS control
w; P{UAS-EcR.B1} on III	Bloomington 6469 (Lee, Marticke et al. 2000)	EcR-B1 expression construct, under UAS control
w; P{UAS-EcR.B2}on III	Bloomington 6468 (Lee, Marticke et al. 2000)	EcR-B2 expression construct, under UAS control
w; P{UAS-EcR.C} on III	Bloomington 6868 (Cherbas, Hu et al. 2003)	An N-terminally truncated EcR form, EcR-C, expression construct, under UAS control
w; P{w[+mC]=UAS-EcR-RNAi}	Bloomington 9326 (Colombani, Bianchini et al. 2005)	RNAi directed against all EcR isoforms, under UAS control
w; P{w[+mC]=UAS-EcR-RNAi}	Bloomington 9327 (Bernardi, Romani et al. 2009)	RNAi directed against all EcR isoforms, under UAS control
w; P{w[+mC]=UAS-EcR.A.dsRNA/TM3	Bloomington 9328 (Bernardi, Romani et al. 2009)	RNAi directed against EcR-A isoform, under UAS control
w; P{w[+mC]=UAS-EcR.B1.dsRNA}/TM3	Bloomington 9329 (Bernardi, Romani et al. 2009)	RNAi directed against EcR-B1 isoform, under UAS control
yv; P{y[+t7.7]v[+t1.8]=TRiP.JF02546}attP2 - uspRNAi	Bloomington 27258 (Costantino, Bricker et al. 2008)	RNAi directed against Usp, under UAS control
w; PBac{y[+mDint2] w[+mC]=usp-GFP.FLAG}VK00033	Bloomington 38672	Usp gene trap

2.1.3 Other driver lines

Genotype	**Source/Ref**	**Description**
w; esgGAL4, UAS-GFPnls/CyO	Donation – Prof. B Edgar University of Heidelberg, Germany (Jiang, Patel et al. 2009)	SC driver line (Esg-GAL4), expresses GFPnls in SCs
w; P{w[+mC]=esg-GAL4 tub-GAL80ts UAS-GFPnls/CyO; UAS-flp act>CD2>GAL4/TM6	Donation – Prof. B Edgar University of Heidelberg, Germany (Jiang, Patel et al. 2009)	Temperature-sensitive, flip-out SC driver line, expresses GFPnls in SCs
w; P{w[+mC]=spi-GAL4}/CyO	Kyoto DGRC 112-114 (Jiang and Edgar 2009)	SC driver line (*spi*-GAL4)
w; spi-GAL4, UAS-GFPnls/CyO	Combination line generated by C Wilson	SC driver line, expresses GFPnls in SCs
w; spi-GAL4; UAS-GFPnls tubGAL80ts/SM5.TM6	Combination line generated by L Corrigan (Corrigan, Redhai et al. 2014)	Temperature sensitive SC driver line, expresses GFPnls in SCs
w; P{w[+mC]= dsx-GAL4}/TM6B	Donation - Prof S. Goodwin University of Oxford, UK (Rideout, Dornan et al. 2010)	SC driver line (*dsx*-GAL4)
w; UAS-GFPnls tub-GAL80ts; dsx-GAL4/SM5.TM6	Combination line generated by L Corrigan (Corrigan, Redhai et al. 2014)	Temperature sensitive SC driver line, expresses GFPnls in SCs
yw; P{w[+mW.hs]=GawB}1btl[NP6593]/TM6	Kyoto DGRC 105-276 (Ahmad and Baker 2002)	SC driver line (*btl*-GAL4)
w[*]; P{w[+mW.hs]=GawB}NP2211 / TM3	Kyoto DGRC 112-825 (Ahmad and Baker 2002)	SC driver line (*bnl*-GAL4)

w,P{w[+mC]=Acp26Aa-GAL4} on X	Donation - Prof S. Goodwin University of Oxford, UK(Chapman, Bangham et al. 2003)	MC driver line (Acp26Aa-GAL4)
w,acp26Aa-GAL4; UAS-GFP^{nls},tubGAL80^{ts}(Tamai, #325)	Combination line generated by L Corrigan (Corrigan, Redhai et al. 2014)	Temperature sensitive MC driver line (Acp26-GAL4), expresses GFPnls in MCs

2.1.4 Rbf/E2F1 stocks

Genotype	**Source/Ref**	**Description**
w; P{w[+mC]=UAS-E2f1.N}3B P{w[+mC]=UAS-Dp.D}1-4b/TM6B, Tb[1]	Bloomington 4770 (Neufeld, de la Cruz et al. 1998)	E2F1 & Dp expression construct, under UAS control, on 3rd chromosome
w; P{w[+mC]=UAS-E2f1.N}5A P{w[+mC]=UAS-Dp.D}8C/CyO	Bloomington 4774 (Neufeld, de la Cruz et al. 1998)	E2F1 & Dp expression construct, under UAS control, on 2nd chromosome
yv; P{y[+t7.7]v[+t1.8]=TRiP.JF02718}attP2	Bloomington 27564 (Ji, Miles et al. 2012)	RNAi directed against E2F1, under UAS control
yv; P{y[+t7.7] v[+t1.8]=TRiP.JF01741}attP2	Bloomington 31214 (Ji, Miles et al. 2012)	RNAi directed against E2F1, under UAS control
w; P{w[+mC]=UAS-Rbf.280}3/TM3, Sb[1]	Bloomington 50748 (Xin, Weng et al. 2002)	Rbf^{CA} constitutively active construct, under UAS control.
w; P{w[+mC]=UAS-Rbf.D}II	Bloomington 50746 (Neufeld, de la Cruz et al. 1998)	Rbf expression construct, under UAS control, on 2nd chromosome
w; P{w[+mC]=UAS-Rbf.D}III	Bloomington 50747 (Neufeld, de la Cruz et al. 1998)	Rbf expression construct, under UAS control, on 3rd chromosome

yv; P{y[+t7.7]v[+t1.8]=TRiP.HMS03004}attP2/TM3	Bloomington 36744 (Bayarmagnai, Nicolay et al. 2012)	RNAi directed against Rbf, under UAS control
yv; P{y[+t7.7] v[+t1.8]=TRiP.GL01293}attP40	Bloomington 41863(Bayarmagnai, Nicolay et al. 2012)	RNAi directed against Rbf, under UAS control

2.1.5 Miscellaneous stocks

Genotype	**Source/Ref**	**Description**
w; Pten^{1}/CyO Roi	Dr. D. Goberdhan, University of Oxford (Goberdhan, Paricio et al. 1999)	Used as white-eyed 2nd chromosome balancer stock
w;Df(2L)170BFRT40A/SM5^TM6	Dr. D. Goberdhan, University of Oxford (Goberdhan, Paricio et al. 1999)	Used as white-eyed 2nd and 3rd linked-chromosome balancer stock
yw;{Mae-UAS Rheb^{AV4}}/TM3	Donation from the late Prof. J. Lengyel, UCLA,USA. (Patel, Thapar et al. 2003)	Used as yellow mutant, white-eyed 3rd linked-chromosome balancer stock
w;FRT2A Nedd4T119FS/TM6	Donation from Prof. S. Hayashi, RIKEN centre for Developmental Biology, Japan. (Sakata, Sakaguchi et al. 2004)	Used as white-eyed 3rd linked-chromosome balancer stock
w;UAS-EcR-B1,UAS-Tkv^{QD}/ TM6	C. Wilson and A. Leiblich	Combination line
w;UAS-EcR-B2, UAS-Tkv^{QD}/TM6	C. Wilson and A. Leiblich	Combination line
w; UAS-EcRB-A, UAS-Tkv^{QD}/TM6	C. Wilson and A. Leiblich	Combination line

w; UAS-EcR-C, UAS-Tkv^{QD}/TM6	C. Wilson and A. Leiblich	Combination line
w; UAS-EcR-RNAi (Bl 9327); UAS-TkvQD/SM5.TM6	C. Wilson and A. Leiblich	Combination line
W; UAS-EcR-RNAi; UAS-E2F1, UAS-Dp/SM5.TM6	C. Wilson and A. Leiblich	Combination line
y,w,P{w[+mC]=UAS-Pi3K92E.CAAX}1, y[1] w[1118]	Bloomington 8294 (Leevers, Weinkove et al. 1996)	PI3K^{Act} constitutively active construct, under UAS control.
yv; P{y[+t7.7]v[+t1.8]=TRiP.JF01366} attP2	Bloomington 31387 (Biteau and Jasper 2011)	RNAi directed against Rolled, under UAS control
yv; P{y[+t7.7]v[+t1.8]=TRiP.JF01080} attP2	Bloomington 31524 (Biteau and Jasper 2011)	RNAi directed against Rolled, under UAS control

2.2 Reagents

2.2.1 Buffers and solutions

The table below details the buffers and solution used during the immunostaining protocol.

Buffer/solution	**Recipe**
Phosphate buffered Saline (PBS)	One tablet dissolved in 200ml of ddH20, yielding: 0.01M Phosphate buffer 0.0027M KCl 0.137 M NaCl pH 7.4
PBST	PBS with 0.3% (v/v) Triton X-100
10% PBSTG	PBST with 10% (v/v) Goat serum
4% PFA	4% Paraformaldehyde in PBS

- PBS tablets - Sigma-Aldrich #P4417
- Triton X-100 - Sigma-Aldrich #X100
- Goat serum – Sigma-Aldrich #G9023
- Paraformaldehyde – Sigma-Aldrich #P6148

2.2.2 Primary antibodies

Antigen	**Host species**	**Source**	**Reference**	**Dilution**	**Protocol**
Fasciclin 3 (Fas3)	Mouse	Developmental Studies Hybridoma Bank (DSHB), Iowa, USA. http://dshb.biology.uiowa.edu/	(Patel, Snow et al. 1987)	1/10	**IF**
Ecdysone receptor (EcR)	Mouse	Developmental Studies Hybridoma Bank (DSHB), Iowa, USA. http://dshb.biology.uiowa.edu/	(Talbot, Swyryd et al. 1993)	1/10	**IF**
Glass bottom boat (Gbb)	Mouse	Developmental Studies Hybridoma Bank (DSHB), Iowa, USA. http://dshb.biology.uiowa.edu/	(Akiyama, Marques et al. 2012)	1/10	**IF**
Angiotensin converting enzyme (Ance)	Rabbit	Donation from Prof. E. Isaac, Univeristy of Leeds, UK	(Rylett, Walker et al. 2007)	1/10	**IF**
Bromodeoxy-uridine (BrdU)	Mouse	Developmental Studies Hybridoma Bank (DSHB), Iowa, USA. http://dshb.biology.uiowa.edu/	(George-Weinstein, Foster et al. 1993)	1/10	**IF**

2.2.3 Secondary antibodies

Reactivity	Host species	Conjugate	Source	Reference	Dilution	Protocol
Mouse	Donkey	Alexa Fluor 647	Life Technologies Cat #A-31571	(James, Levine et al. 2005)	1/400	IF
Mouse	Donkey	Alexa Fluor 555	Life Technologies Cat #A-31570	(Andersen, Schmidt et al. 2006)	1/400	IF
Rabbit	Donkey	Alexa Fluor 647	Life Technologies Cat # A-21245	(Szymborska, de Marco et al. 2013)	1/400	IF
Rabbit	Donkey	Alexa Fluor 555	Life Technologies Cat #A-31572	(Kosman, Mizutani et al. 2004)	1/400	IF

2.3 Fly food

A glucose-rich formula for a cornmeal/yeast-based fly food recipe was used for all fly culturing purposes during the course of this work, and was cooked in 25 litre batches using the recipe below. Nipagin, a potent anti-fungal, was added to prevent the growth of mould. After cooking, food was dispensed into individual vials to a depth of between 3 and 6 cm, and allowed to cool overnight before plugging with cotton wool. Food-containing vials were stored at 4˚C for up to 4 weeks to prevent drying.

To make 25 L fly food, the following recipe was used:

Reagent	**Quantity**	**Source/catalogue number**
Agar	241g	Sigma-Aldrich - #G0350500
Glucose	2325g	Sigma-Aldrich - #A5306
Yeast	787g	Sigma-Aldrich - #51475
Cornmeal	1875g	Sigma-Aldrich - #C6304
Calcium	17.g	Sigma-Aldrich - #215147
100% Ethanol	300ml	Sigma-Aldrich - #E7023
Potassium sodium tartrate	215g	Sigma-Aldrich - #1551140
Ethyl-4-hydroxybenzoate (Nipagin)	62.5g	Sigma-Aldrich - #111988

2.4 Experimental procedures

2.4.1 Fly husbandry

During development, flies were reared on cornmeal/yeast-based food at constant temperature of 18˚C. At this temperature $Gal80^{ts}$ is active and inhibits Gal4-mediated expression, thereby preventing transgenic expression interfering with normal development. Virgin males were isolated within 12 h post-eclosion and shifted to 28.5˚C, a permissive-temperature at which $Gal80^{ts}$ is degraded, thus permitting Gal4-mediated transgene expression. Genetic crosses (e.g. to make new combination lines) were maintained at 25˚C.

2.6 Generation of combinatorial *Drosophila* stock lines

2.6.1 Combination lines generated by recombination of constructs carried on the 3rd chromosome

Several quirks of genetic inheritance in *Drosophila* make the fruit fly an organism that is particularly amenable to genetic manipulation, allowing the generation of lines bearing multiple different transgenic inserts and/or mutations. An important feature of *Drosophila* genetics is the total absence of recombination in males (Orr-Weaver 1995), meaning that recombinant lines can by generated with relative ease through selection based on sex. In addition to this, balancer chromosomes, usually bearing phenotypic markers, have been developed over the years, and are used extensively as a tool to allow identification of invisible genotypes and also to prevent unwanted recombination in stable stock lines (Greenspan 2004). The figure below demonstrates a mating scheme used here to create a recombinant line bearing both UAS-Tkv^{Act} and UAS-EcR-B1 constructs on the 3rd chromosome (Figure 2.1).

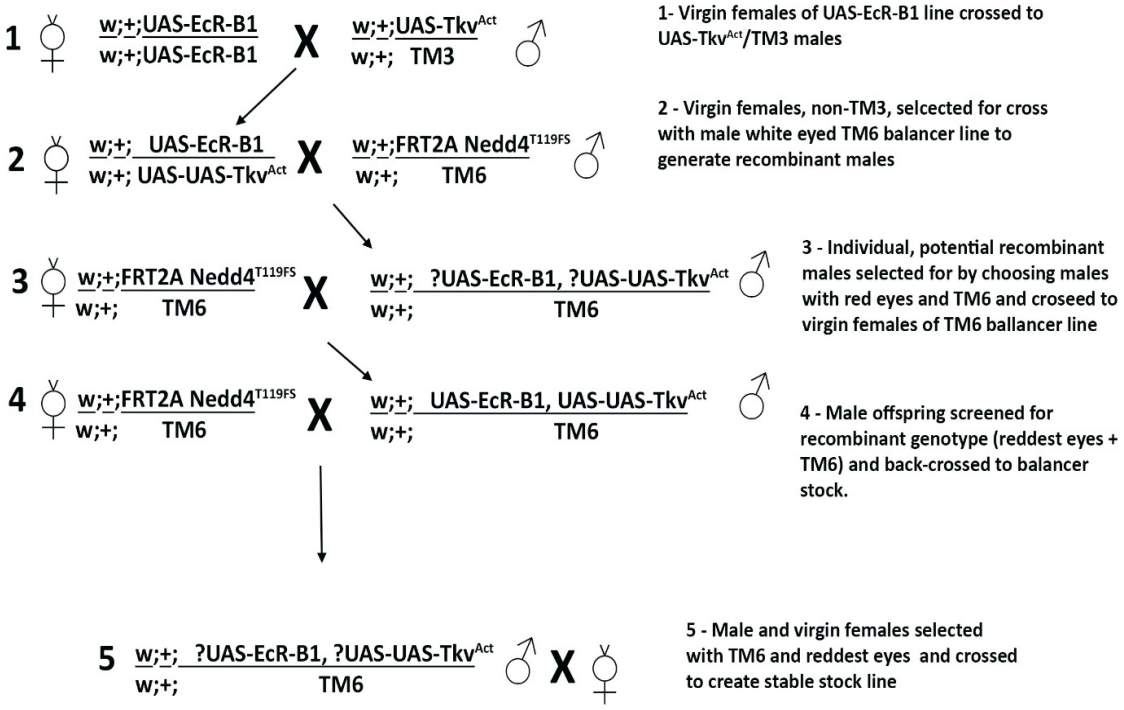


Figure 2.1: Recombinant mating scheme used to generate a UAS-EcR-B1 and UAS-*Tkv*^{Act} combination line. This same mating scheme was used to create UAS-*Tkv*^{Act} combinatorial lines with EcR-B2, EcR-A, EcR-C and EcR-RNAi.

2.4.2 Dissection of male *Drosophila* accessory glands

Adult virgin males, cultured at 28.5˚C, were collected at a given number of days post-eclosion, usually between 3-12 days. Flies were anaesthetised with CO_2 and, using fine-tipped forceps, decapitated and their abdomens separated from the thorax. The abdomens were then placed and dissected in fixative solution, 4% paraformaldehyde in PBS (4% PFA). Dissection was performed by direct visualisation under a light microscope. Fly abdomens were dissected in small pools of 4% PFA sitting on Sylgard gel plated in petri dishes. The accessory glands were removed from the abdomen and separated from adjacent tissues by gentle dissection using fine-forceps, utilising a 'no-touch' technique, whereby direct contact with the glands is avoided and isolation is achieved by manipulation and separation of non-reproductive tissues. Once the reproductive organs were isolated the testes were removed, leaving the glands and ejaculatory duct. Typically, glands remained in fix solution for 20

minutes in total, including time spent during dissection, before being washed in phosphate buffered saline (PBS).

2.4.3 Immunostaining protocol

Once glands had been dissected and fixed in 4% PFA, they were washed 3 times in PBS with 5 minute incubation periods on an orbital shaker for each wash. The glands were then incubated in blocking solution, composed of PBS containing 10% goat serum (10% PBSTG), for a 30 minute period. After blocking, the glands were incubated overnight at 4˚C in primary antibody solution, which had been diluted in 10% PBSTG. After this incubation, glands were washed in PBST 6 times with 5 minute incubations. Once this washing step was complete, the glands were incubated in a secondary antibody solution, again using PBSTG as the diluent. Glands were incubated with the appropriate fluorescent-conjugated donkey secondary antibody for a minimum of 2 hours at room temperature on an orbital shaker, before undergoing 6 further washes in PBST, each wash lasting 5 minutes. The glands were then finally washed once with PBS, prior to mounting on microscopy slides.

2.4.4 Mounting and DAPI staining of tissues

Once the staining protocol was complete, glands were mounted on slides using a glass pipette, and visualising the process using a light microscope. Excess fluid was removed from the slides by gentle wiping using lint free tissue, avoiding contact with the glands. To prevent the tissues drying out, the glands were then immediately immersed in a single drop of mounting solution. We routinely used a mounting solution available from Vectashield containing 4',6-diamidino-2-phenylindole (DAPI), a fluorescent stain that binds avidly to A-T rich regions of DNA, thus acting as a useful marker of nuclei. After treating with mounting

solution, a glass coverslip was placed over the slide and sealed using transparent nail varnish.

2.4.5 Confocal microscopy

Fixed tissues were imaged using a Zeiss AxioPlan (LSM 510Meta) laser scanning confocal microscope. Low magnification images were obtained by using a Zeiss Plan-Apochromat 10x/0.45 objective, whilst image capture at higher magnifications was achieved using a Zeiss Plan-Apochromat 63x/1.4Oil DIC objective.

2.4.6 Image processing and analysis

All images were processed using freely available software, including Zeiss LSM Image Browser (http://www.zeiss.co.uk/microscopy/en_gb/website/downloads/lsm-image-browser.html), Zeiss AxioVision LE (http://www.zeiss.co.uk/microscopy/en_gb/downloads/axiovision.html) Zeiss 2012 ZenLite (http://www.zeiss.co.uk/microscopy/en_gb/products/microscope-software/zen-lite.html) and ImageJ (http://imagej.nih.gov/ij/). Nuclear size measurements for all genotypes assessed were calculated using the Zeiss LSM Image Browser software.

2.4.7 Nuclear size ratio measurement and calculation

Measurement of cell size was not a straightforward method for measuring growth, because large variations were observed within a given genotype in both apical/basal cell shape and cell size. To this end, nuclear area was measured as a proxy for cell growth, as the links between cell growth and nuclear growth are well established, with nuclear size:cell size ratio remaining constant with cell growth (Jorgensen, Edgington et al. 2007, Neumann and

Nurse 2007, Webster, Witkin et al. 2009, Cohen-Fix 2010). The nuclear area of SCs (two nuclei per cell) was measured utilising the area measurement tool in the LSM Image Browser software to outline individual SC nuclei, and expressing the area as pixel number. To estimate SC growth relative to MCs, the nuclear area of 3 MC nuclear pairs adjacent to a particular SC were measured, and an average MC was calculated. This MC nuclear area average was then used to calculate the SC:MC ratio. For each genotype, the SC:MC nuclear ratio was measured for 3 different SCs within a single AG to produce a gland average, and for each genotype this was done for 5 different animals (Figure 2.2).

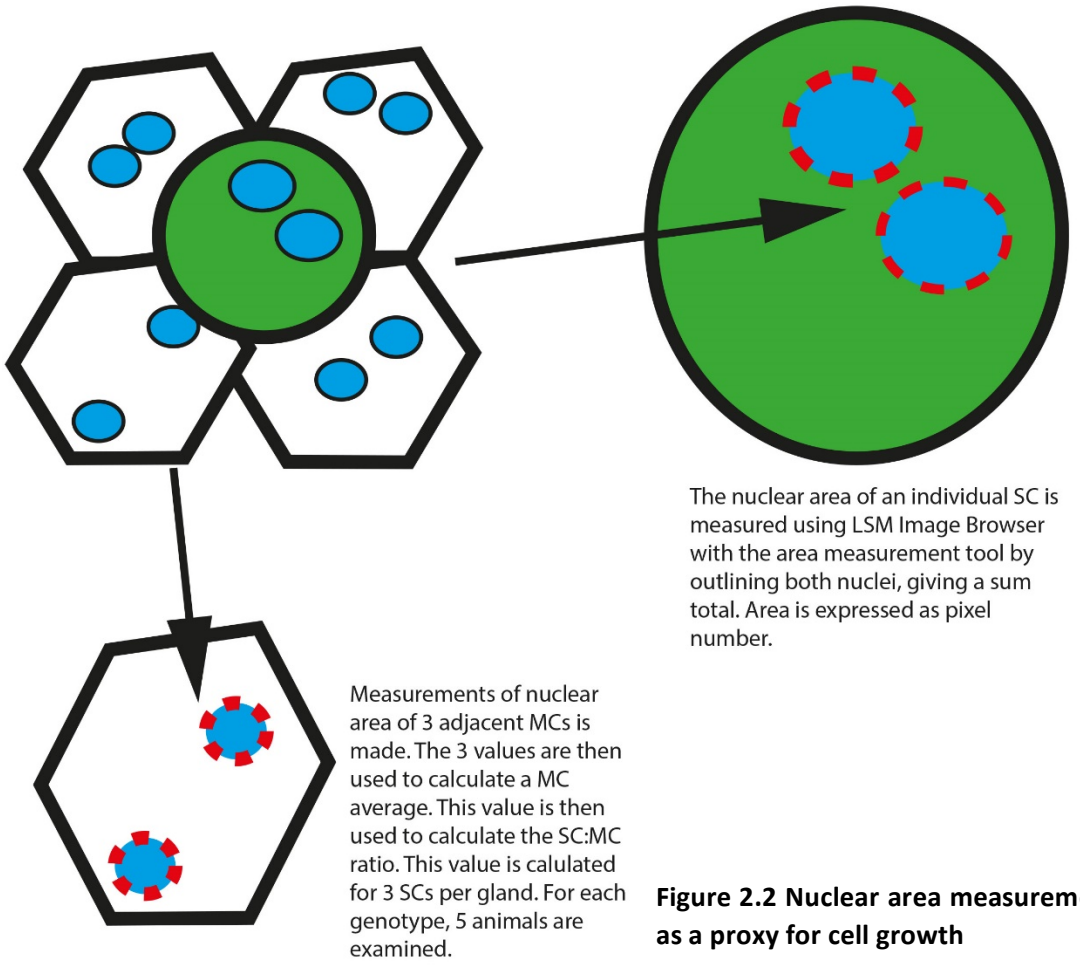


Figure 2.2 Nuclear area measurement as a proxy for cell growth

2.4.8 Statistical analysis of nuclear size

All analyses were performed using the statistical software package, SPSS (Witte, Jeibmann et al.). To determine the appropriate statistical tools that should be applied, an initial test for normality was performed using the Shapiro-Wilk test (Shapiro and Wilk 1965). In all cases, the nuclear size data were shown to be normally distributed, and therefore suitable for analysis using parametric testing tools. In this study, all data were analysed by one-way ANOVA testing (Vajda 1947), and subsequently applying the Bonferroni correction to counteract error associated with multiple comparisons (Dunn 1961).

2.5 Mating protocol

For single-pair matings, individual males were added to a normal 1- to 5-d-old virgin w^{1118} female (for a specific group of control and test flies, females were of identical age), and mating was observed. The flies were separated after mating and females cultured at 25 °C for 4 d, transferring them to new vials each day, so eggs and progeny could be counted. Each female was then introduced to a dominantly marked white-eyed male and left for 3 h to test if it remated. Any progeny from this second mating could be easily distinguished, because all or most of the progeny from the first cross were red-eyed.

Multiply mated males for staining experiments were generated by placing a newly eclosed male with at least six virgin females and leaving the cross for 3 or 6 d at 28.5 °C. For multiple-mating experiments to test effects on female receptivity, individual 3-d-old virgin males either expressing GFP alone or GFP and *Dad* under esg^{ts} F/O control were mated to a group of five virgin females overnight. These males were then separated and introduced to a further three virgin females for 24 h, observing mating during the first 3 h. Females from

the first cross were individually separated into new vials to check for mating. To assay receptivity to remating, the three females from the second round of mating were separated into individual vials for 3 d (to check they had mated and count progeny), before transferring to a new vial and adding two dominantly marked white-eyed males to test if the females remated over a 3-hour period. Scoring transfer of males SCs to females was performed by dissecting out the female reproductive tract within 30 minutes of completion of mating, fixing the organs in PFA and observing for the presence of SCs in the reproductive tract by confocal microscopy. Intact, GFP marked cells with DAPI stained nuclei were scored as male SCs and the number of positive cells per female reproductive tract were counted.

3 - Chapter three – New genetic tools to study *Drosophila* secondary cells reveal unusual growth and migratory behaviours

3.1 Introduction

In this chapter I will describe my efforts to identify a set of genetic tools that allow the study of the genetic regulation of SC cell biological behaviours. In particular, I will describe novel cell-growth and –migratory phenomena exhibited by these cells. The development of tools permitting genetic manipulation of SCs marks an important milestone in my efforts to study these intriguing cells and to characterise the interesting behaviours described in this chapter

3.1.1 A lack of genetic tools has hampered efforts to characterise the biology of AG epithelium

To date, studies of the adult male accessory gland have largely focused on the characterisation of seminal fluid peptides secreted by this organ and their effects on female reproductive physiology (DiBenedetto, Harada et al. 1990, Monsma, Harada et al. 1990, Chapman, Liddle et al. 1995, Herndon and Wolfner 1995, Bertram, Neubaum et al. 1996, Lung and Wolfner 1999, Heifetz, Lung et al. 2000, Lung, Kuo et al. 2001, Lung, Tram et al. 2002, Chapman, Bangham et al. 2003, Liu and Kubli 2003, Peng, Zipperlen et al. 2005, Wolfner 2007, McGraw, Clark et al. 2008, Avila and Wolfner 2009). Indeed, when I started my thesis work, genetic studies detailing the cell biology and regulation of the accessory gland epithelium were conspicuously absent from the literature.

3.1.2 Few GAL4 drivers specifically expressing in AG epithelial cells are currently described

An important reason for the lack of progress in attempts to characterise the cell biology of the epithelium stemmed from an apparent paucity of characterised cell type-specific drivers selectively expressed in this tissue. Previously, expression of the transcriptional driver, prd-GAL4, had been observed in the epithelium of newly eclosed adult accessory glands in both MCs and SCs, although expression is absent by day 10 post-eclosion (Bertuccioli, Fasano et

al. 1996, Xue and Noll 2002). Additionally, a further transcriptional driver, *Acp26*-GAL4, had been shown to express in the adult accessory gland, showing selective expression in main cells and not secondary cells (Chapman, Bangham et al. 2003).

Aside from these two GAL4 lines, no other reports existed describing expression of GAL4 drivers in the adult AG epithelium, although several other enhancer traps were available (Bertram, Akerkar et al. 1992). Indeed, prior to my own research efforts, no SC-specific GAL4 drivers had been identified that allow selective analysis of this cell type. Therefore, an early objective of my research was to screen through a number of GAL4 lines to identify candidate drivers for AG epithelial cells, with a particular emphasis on identifying drivers selectively expressed in SCs. Indeed, the Identification of drivers that allow selective expression of transgenes in SCs has proved an important milestone in facilitating my and other members of the lab's efforts to characterise SC cell biology and is the foundation for the study in Leiblich et al., 2012.

3.1.3 Aims

In this results section, I will describe the results of screen of *Drosophila* GAL4 lines, aimed at providing a characterisation of a set of novel SC-selective drivers. In addition to this, I will describe and define a set of interesting cell biological behaviours exhibited by SCs, including age- and mating-dependent growth and migratory behaviour.

3.2 Results

3.2.1 Screening for secondary cell-specific GAL4 transcriptional drivers

In order to identify transcriptional drivers selectively expressed by secondary cells, I performed a screen of a number of GAL4 lines and examined their expression patterns in the adult male reproductive tract (Fischer, Giniger et al. 1988, Brand and Perrimon 1993). These driver lines were crossed to animals carrying a reporter transgene encoding a nuclear localised GFP (FP^{nls}) (Robertson, Mergliano et al. 2003, Jiang, Patel et al. 2009) under the transcriptional control of upstream activation sequences (Oemar, Masaoka et al.), which are enhancer targets of GAL4 (Webster, Jin et al. 1988). The progeny were then selected for genotypes containing both the GAL4 driver and the UAS-GFP^{nls} reporter construct (Figure 3.1). The reproductive tracts, including the accessory glands, of these adult males were dissected and fixed in the first 1-3 days post-eclosion to examine for GFP expression by confocal microscopy.

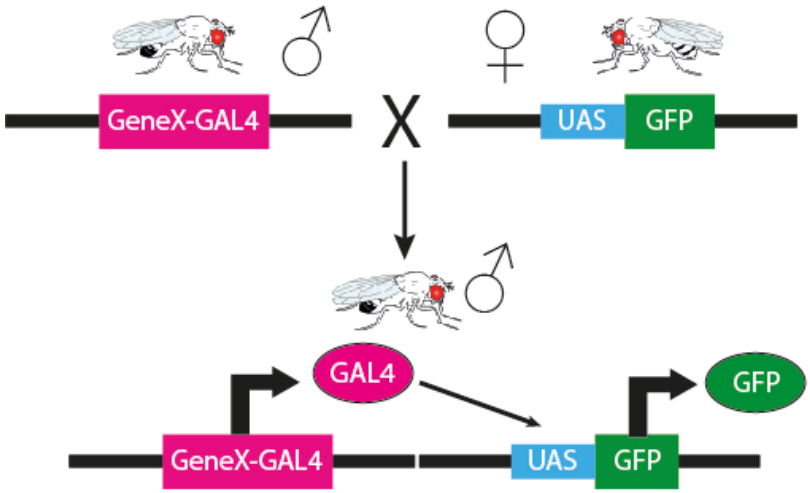


Figure 3.1: Identification of GAL4 transcriptional drivers expressed in the *Drosophila* male accessory gland. A parental line harbouring a GAL4 insertion under the control of a native promoter is crossed to a parental line containing a GFP construct under the control of UAS sequences. Male progeny are selected for genotypes bearing both the GAL4 insertions and UAS-GFP construct. Activity of the GAL4 drivers in the accessory gland is analysed by examining the fixed, dissected glands of adult males for the presence of GFP.

This screen revealed for the first time a number of GAL4 drivers displaying selective expression in secondary cells, including *spi*-GAL4 (Buchon, Broderick et al. 2010), indicating that secondary cells express *spitz (spi)*, a ligand for the EGF receptor (EGFR) (Figure 3.2C). Additionally, *dsx*-GAL4 drives expression of GFP^{nls} in secondary cells and in no other accessory gland cell type (Figure 3.2D), confirming initial observations made in Stephen Goodwin's lab (Rideout, Dornan et al. 2010).

The doublesex (*dsx*) gene plays important roles in somatic sex determination and encodes a transcription factor that is alternatively spliced to give rise to two isoforms, one expressed only in females and the other only expressed in males (Hildreth 1965, Burtis and Baker 1989). Notably, I also observed *dsx*-GAL4 expression in all ejaculatory duct epithelial cells (Figure 3.2E). Furthermore, expression of both the FGF receptor breathless (*btl*) and FGF ligand branchless (*bnl*) was observed in secondary cells and no other AG cell types, perhaps hinting at an autocrine function for FGF signalling in adult secondary cells (Figure 3.2G and 3.2H).

An early aim of my work on AG biology was aimed at determining whether SCs possess any stem cell-like properties. To this end, I screened the AG for expression of the snail transcription factor Esgargot (*esg*), a reliable marker of stem cells in the adult *Drosophila* mid-gut (Jiang and Edgar 2009). Although no evidence has ever emerged to suggest SC possess any stem-like properties (see section 3.2.2), *esg* expression in these cells was demonstrated (Figure 3.1A) by the observation that UAS-GFP^{nls} expression is driven in these cells by *esg*-GAL4.

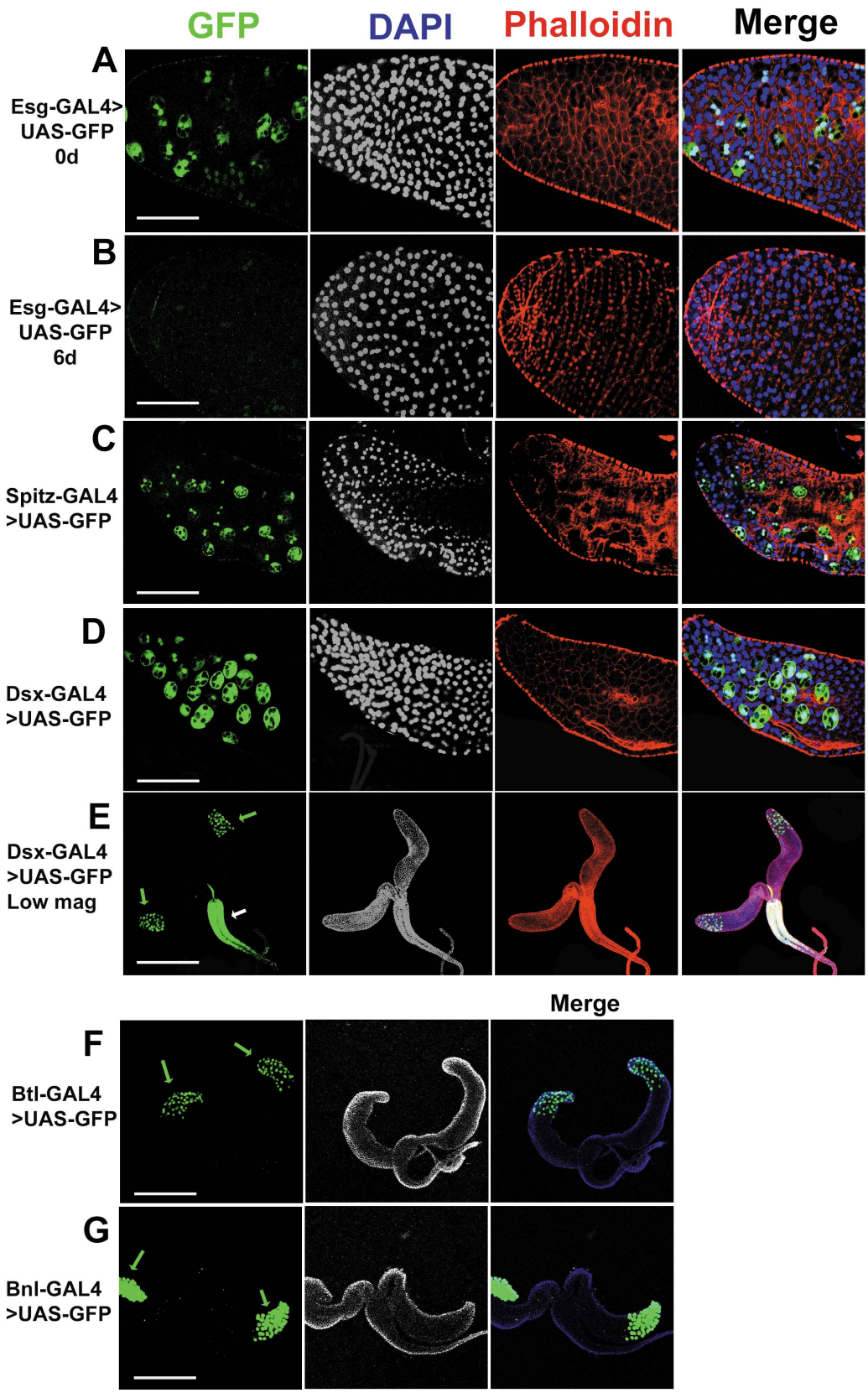


Figure 3.2: Several GAL4 drivers display selective expression in adult SCs. GFP^{nls} is observed in SCs, but not in MCs, driven by the GAL4 transcriptional activator in different fly lines (all 6d unless otherwise stated). These data indicate such expression for *esg* (A), *spi* (C), *dsx* (D,E), *btl* (F) and *bnl* (G). Notably, in addition to displaying expression in SCs (green arrows, E), *dsx* is expressed in all epithelial cells of the ejaculatory duct (white arrow, E). *esg* expression is observed in all SCs at eclosion (A), but is essentially absent in the gland by 6 days post-eclosion. Scale bars = 50µm (A-D), = 250µm (E-G).

Notably, I observed that the expression of *esg*-GAL4 in SCs diminished with time, such that only a handful of cells, if any, expressed GFP by day 6 post-eclosion (Figure 3.2B). Therefore, ordinarily this driver would not be well suited to driving transgene expression in adult secondary cells beyond the first few days of adult life. However, a *FLP-Out* system utilising the *esg*-GAL4 driver, known as *esg*^{ts} F/O, has previously been described (Jiang, Patel et al. 2009) where the *esg*-GAL4 driver is combined with a ubiquitously expressed, temperature-sensitive form of the GAL4 inhibitor, GAL80 (tub-GAL80^{ts}) (McGuire, Le et al. 2003, Suster, Seugnet et al. 2004), and an interrupted copy of an actin-GAL4 construct, which is activated by UAS-FLP-induced recombination (Pignoni and Zipursky 1997). Using this system, GAL4-regulated, FLP-induced events occur in all SCs if adult flies are shifted to 28.5°C from 18°C within 1 day of eclosion (Figure 3.3). These FLP-induced recombination events then activate SC-specific actin-GAL4 expression, placing UAS-containing transgenes under the control of actin-GAL4, ensuring that expression is maintained throughout adulthood provided the flies remain at 28.5°C (see control in Figure 3.4B). This allowed me to reliably express numerous UAS-controlled transgenes in adult secondary cells and facilitated time course experiments, where the effect of these transgenes on secondary cell biology were analysed over several days. A complete table of GAL4 lines tested in the male accessory gland is provided on the next page (table 3.1) and a description of the expression of these lines in the two cell types, main cells (MCs) and secondary cells (SCs), is provided.

GAL4 line	Expressed in SCs?	Expressed in MCs?
Abd-B	Yes	No
bnl	Yes	No
br	No	No
brk	No	No
btl	Yes	No
dally	No	No
Dl	No	No
Dpp	Yes (sporadic)	No
Dr	No	No
dsx	No	Yes
dve	Yes	No
EcR	No	No
Eip78C	No	No
Elav	No	No
en	No	No
ERR	No	No
esg	Yes	No
eve	No	No
ey	No	No
Eya	No	No
GMR	No	No
Hh	No	No
Hr39	No	No
InR	No	No
Mef2	Yes	No
pnt	No	No
shn	No	No
shot	No	No
slbo	No	No
stg	No	No
Tk	No	No
Ubx	No	No
wg	No	No

Table 3.1: List of GAL$ lines tested in the male accessory gland and their expression patterns in main cells (MCs) and secondary cells (SCs).

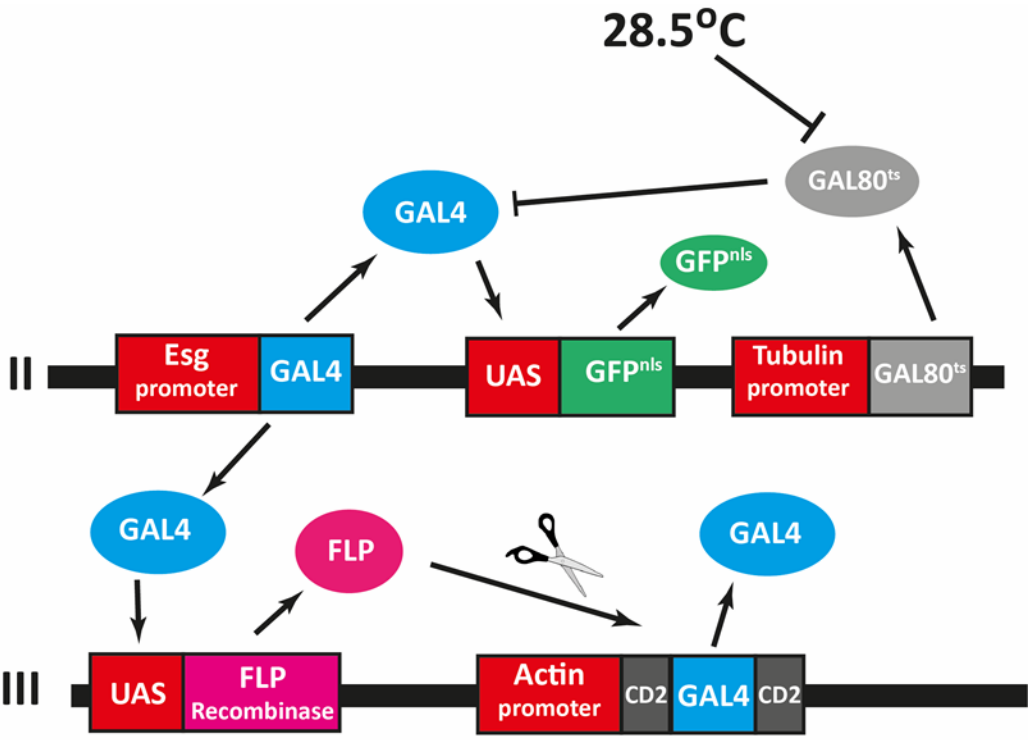
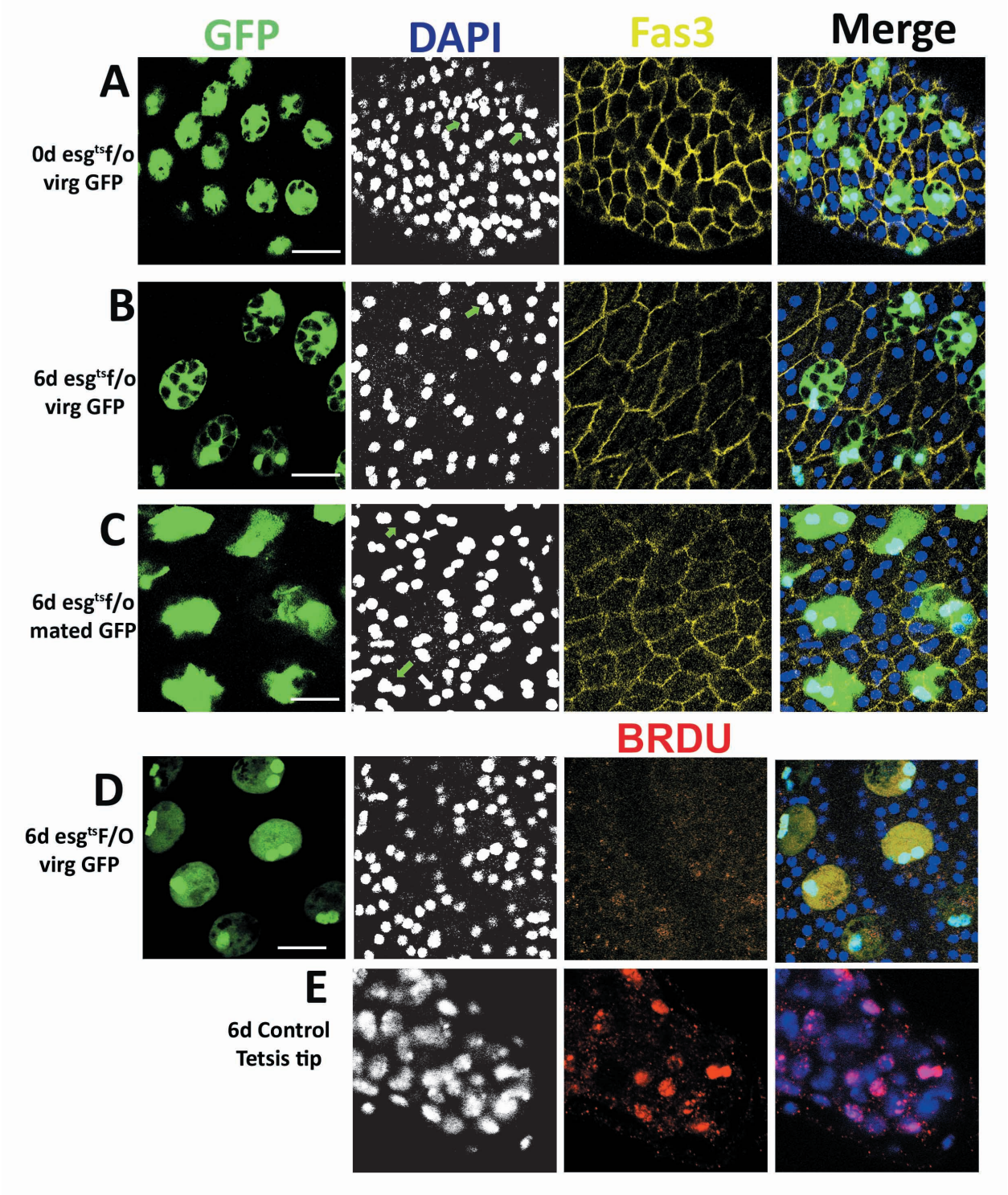


Figure 3.3. The esg^{ts} F/O system: A temperature-sensitive form of GAL80, GAL80^{ts}, is constitutively expressed under the tubulin promoter and acts to inhibit GAL4. Shifting flies to 28.5^{0}C at eclosion results in degradation of GAL80^{ts}, permitting binding of GAL4 to UAS target sequences, and directing transcription of transgenes under UAS control, including transgenic FLP recombinase. A FLP-induced recombination event then places GAL4 under the control of a constitutively active actin promoter, thereby ensuring expression of GAL4 is maintained in SCs at 28.5^{0}C throughout adulthood.

3.2.2 Secondary cells grow in an age- and mating- dependent manner

Using the *esg*^{ts} F/O to distinguish secondary cells by labelling them with GFP and marking apico-lateral cell outlines with an antibody directed against the septate junction protein, Fasciclin 3 (Fas3; one of multiple junctional protein antibodies I tested in these cells) (Snow, Bieber et al. 1989), I observed that the size of secondary cells and their nuclei relative to main cells increased over time in virgin males (Figure 3.4A, B and F). Because the nuclei of secondary cells and surrounding main cells remain roughly spherical in all genotypes studied, I compared nuclear area for these two cell types in confocal sections in the plane of the epithelial monolayer.

There was a significant increase in both the absolute size and the relative size of secondary cell nuclei with age ($P<0.001$, n = 5 glands, one-way ANOVA), and this was accompanied by a visible change in cell size, which was not measured routinely, because the change in cell shape with age and genotype made this a laborious process. For nuclear size, the secondary cell/main cell nuclear ratio of virgin male glands on day 0 of adult life was 0.82+/- 0.04 and rose to 1.26+/- 0.08 by day 6. Main cell nuclear size, however, remained unaltered (Figure 3.4G). Flies fed with the nucleotide analogue bromodeoxyuridine (BrdU) did not incorporate this molecule in secondary cell nuclei (Figure 3.4D, versus control staining in testis in Figure 3.4E), indicating that the SC nuclear size increase was not associated with global genomic DNA replication. Increase in nuclear size without DNA replication is described in other cell types in *Drosophila* and often associated with cell growth (Gao and Pan 2001). Interestingly, these age-related growth effects were accelerated in mated flies versus virgins, with mated flies exhibiting significantly larger nuclei than age-matched virgins (compare Figure 3.4B with 3.4C, and chart in Figure 3.4F).


GFP
DAPI
Fas3
Merge
A
0d esg^ts f/o virg GFP
B
6d esg^ts f/o virg GFP
C
6d esg^ts f/o mated GFP
BRDU
D
6d esg^ts F/O virg GFP
E
6d Control Tetsis tip

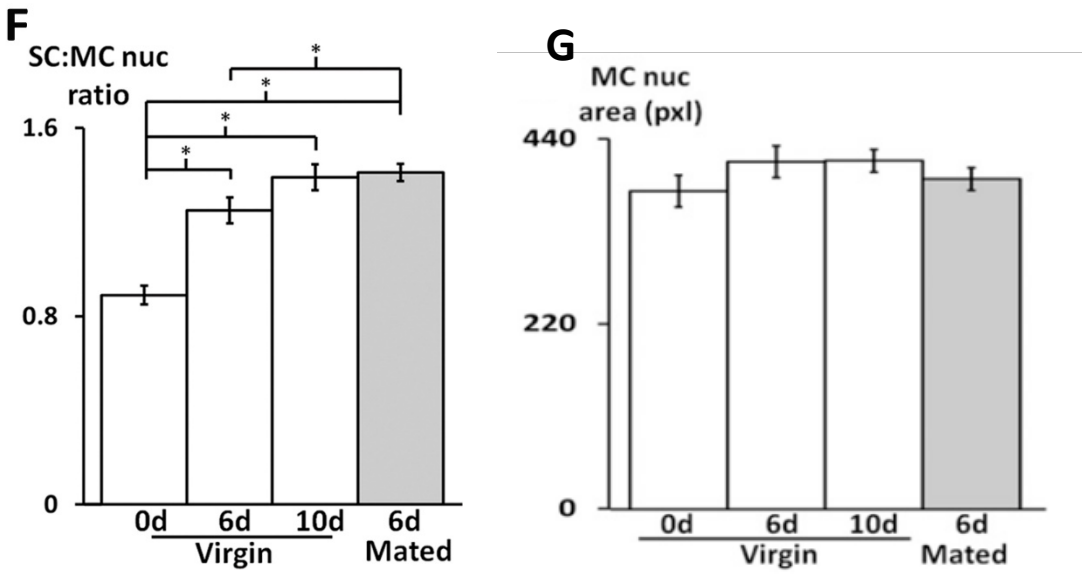


Figure 3.4 Adult SCs grow in an age- and mating-dependent manner. Distal accessory gland epithelium from esg^{ts} F/O>UAS-GFP^{nls} males stained with antibody against the septate junction protein Fasciclin 3 (yellow) and DAPI (blue). SCs marked by esg-GAL4, UAS-GFP^{nls} (green). Comparing the epithelium of newly eclosed virgin males (A) to the epithelium of a 6d old virgin male (B) reveals growth of SC nuclei (green arrows) relative to MC nuclei (white arrows) (also shown in F). SC growth is greater in 6d glands from mated males (C) compared to age-matched glands from virgins (B, see also F). Importantly, there is no statistical significant growth of main cells with age or mating (G). No incorporation of BrdU is observed in the nuclei of growing SCs; only low level non-specific staining is found (D, compare with strong signal in testis control from same animal, E). Scale bars = 10µm. Data analysed by one-way ANOVA; *p<0.001, n=5.

3.2.3 Some secondary cells from multiply mated males migrate proximally and can be transferred to females during mating

Some secondary cells, but not main cells, reduced contact with the basement membrane and started to delaminate apically upon multiple matings. Specific proteins involved in apico-lateral cell-cell adhesion, like Fas3, continued to be localised at cell-cell junctions in delaminating secondary cells, suggesting that the epithelium was not compromised during delamination (Figure 3.5B, C, F and G). Actin-GAL4-induced secondary cell-specific GFP

expression was also consistently elevated in mated males, indicating that the transcription of selected cytoskeletal proteins is also up-regulated by mating. Proximally located secondary cells were observed in a small proportion of flies aged between 3 and 10 days that had mated to multiple females (8 of 200 males), but not in virgin males (Figure 3.5A).

To test whether these cells are transferred to females upon mating, I used males of different ages to set up repeated single pair matings, separated by 3 or 4 days to allow secondary cells to delaminate between matings. The reproductive organs of females were dissected within 1 hour of completion of the final mating. Secondary cells were never transferred from males after a single mating (n>10 for each age, matings at 1, 4, 7 and 11 days). However 4- and 7-day old males, which were mated three separate times over a period of one week, transferred one or more GFP-positive secondary cells to females during the final mating in a significant proportion of cases (4 of 35 females) (Figure 3.5D, E and H). Even more females from a third mating with males mated at 11, 14 and 18 days contained secondary cells (7 of 15 females) (Figure 3.5H).

These data suggest that secondary cells have delaminated in previously mated 18 day-old males. Indeed, all glands (10 of 10) of mated males of this age contained one or more proximally located secondary cells in their accessory glands, when they were dissected rather than mated for a third time. Presumably, the low frequency of migration observed in 6-day-old multiply mated males (8 of 200) is explained by the fact that these cells are transferred upon each mating and it then takes several days for new secondary cells to delaminate and migrate proximally.

No secondary cells or their remnants were observed within the two types of female storage organ, the spermathecae and the seminal receptacle, which begin to accumulate sperm within 5 minutes of the start of mating. Transferred secondary cells are therefore likely to be expelled by the females when she ejects the seminal fluid upon egg laying about 90 minutes after completion of mating.

Overall, these data show that all secondary cells grow as adult males age, a process accelerated by mating, and that a subset of these cells delaminates, migrates proximally along the glandular epithelium and can be transferred to females after multiple matings, particularly in aging males.

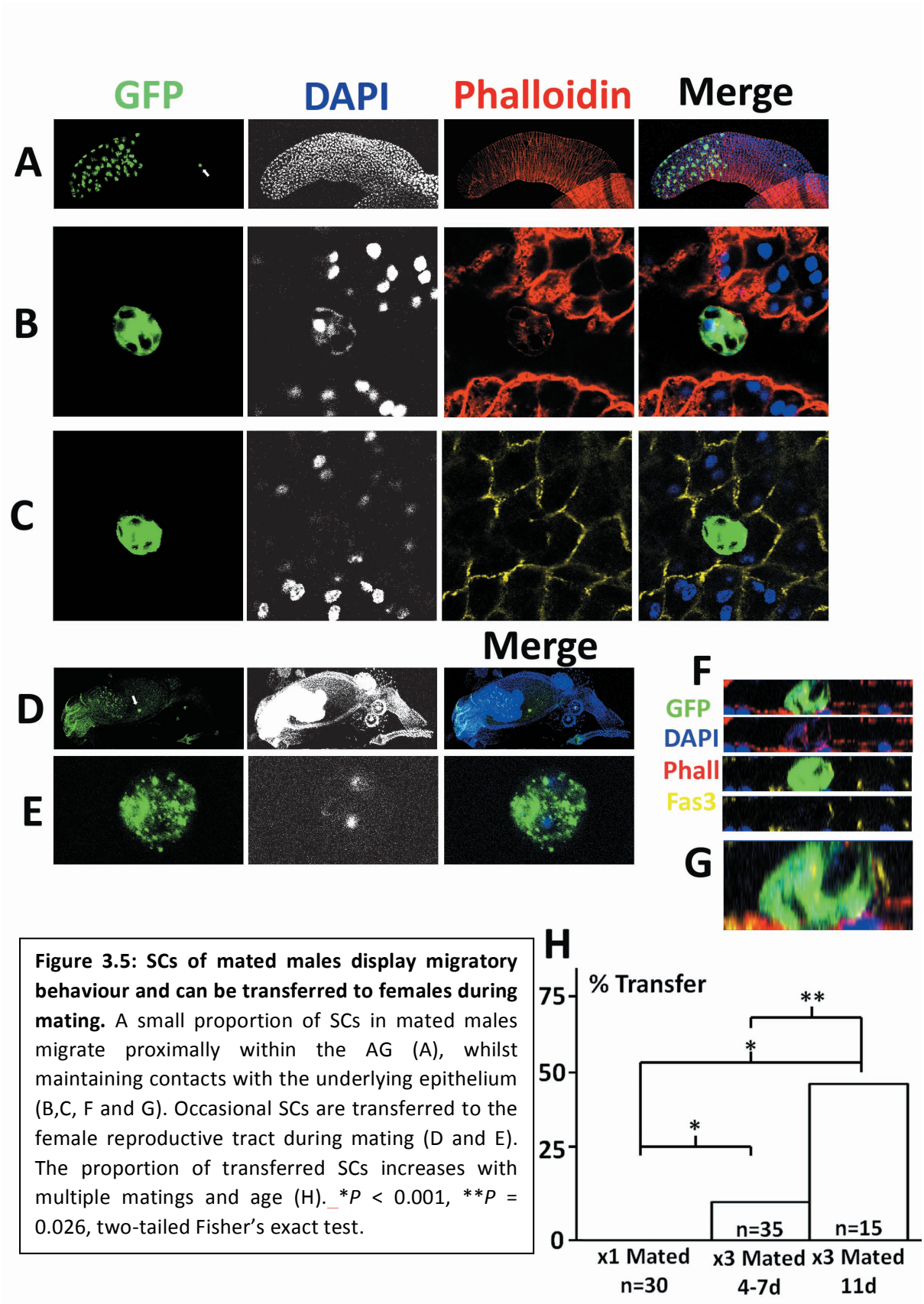


Figure 3.5: SCs of mated males display migratory behaviour and can be transferred to females during mating. A small proportion of SCs in mated males migrate proximally within the AG (A), whilst maintaining contacts with the underlying epithelium (B,C, F and G). Occasional SCs are transferred to the female reproductive tract during mating (D and E). The proportion of transferred SCs increases with multiple matings and age (H). $*P < 0.001$, $**P = 0.026$, two-tailed Fisher's exact test.

3.3 Discussion

I present here the first description of a series of GAL4 drivers displaying selective expression in adult *Drosophila* SCs that can be used to drive transgene expression. These efforts mark an important advance in the endeavour of our lab to characterise the biology and genetic regulation of this cell type. Indeed, the development of an SC-selective expression system has had implications beyond my own work, with other groups beginning to adopt these tools as interest grows in the biology of this cell type (Ito, Ueda et al. 2014).

Importantly, the work here provides the first description of a handful of highly curious and potentially important features of SC biology (now published in Leiblich et al., 2012). Specifically, I have shown that SCs display age-dependent differential growth compared to adjacent main cells and that this growth is altered by reproductive activity. Importantly, I also describe the migratory nature of SCs, which is of particular personal interest to me as I view this phenomenon as a potential tool to probe some of the biological events that underpin the process of metastasis in cancer.

3.5.1 Several genes and GAL4 drivers are specifically expressed in SCs of the accessory gland.

The identification of a set of GAL4 transcriptional drivers selectively expressed in adult secondary cells permits their use as tools for the genetic manipulation of this cell type through the use of the GAL4/UAS expression system (Brand and Perrimon 1993). Additionally, the selective expression of these GAL4 lines in SCs offers an intriguing insight into signalling activity within these cells. Specifically, the observation that both *btl*-GAL4 and

bnl-GAL4 are both expressed in all SCs indicates that this cell type expresses both the FGF ligand branchless (*bnl*) and the FGF receptor breathless (*btl*).

One interpretation of this observation is that FGF signalling activity in SCs forms an autocrine loop, a mechanism of FGF-dependent regulation that, although rare, is not without precedent in *Drosophila*. Indeed, autocrine FGF signalling activity is required for initiating glial cell proliferation in the developing *Drosophila* eye, although in this context the receptor expressed by glial precursors is heartless (htl) rather than *btl* and the ligand these cells express is pyramus (pyr), not *bnl* (Franzdottir, Engelen et al. 2009).

Alternatively, FGF signalling may occur between secondary cells in a paracrine fashion, a mechanism of FGF pathway activation that is well described in *Drosophila*. For instance, during tracheal development, ectodermal cells secrete *bnl*, activating FGF signalling in neighbouring *btl*-expressing tracheal cells, a mechanism that is required for branching morphogenesis (Lee, Hacohen et al. 1996, Sutherland, Samakovlis et al. 1996). In fact, paracrine FGF signalling involving both *btl* and *bnl* is required for male reproductive tract development, including the accessory gland (Ahmad and Baker 2002). A subset of cells within the larval genital disc expresses the FGF ligand, *bnl*, that signal to mesodermal cells, initiating a sequence of developmental steps that involve the migration of these mesodermal cells towards the genital disc and their transition into epithelial cells (Ahmad and Baker 2002). Ultimately, much of the male reproductive tract is believed to derive from these migratory cells, including the seminal vesicles, ejaculatory duct and accessory glands (Ahmad and Baker 2002). Given that my data indicate activity of the FGF pathway in adult SCs, a tantalising theory is that SCs have retained this signalling activity from development,

with the possibility that SCs, or their precursors, play a role in mesodermal recruitment and epithelial transition.

3.5.2 Secondary cells selectively grow in adults

An additional novel observation made in this chapter is that adult SCs exhibit some interesting behaviours with respect to cell-growth. Specifically, the data presented here show that, relative to main cells, secondary cells grow with age, and that this growth is accelerated by mating. This is particularly interesting when drawing parallels between the *Drosophila* accessory gland and the human prostate, an organ also characterised by structural changes with age. Interestingly, prostatic epithelial cells also exhibit cellular growth as a consequence of aging, a process, along with cell proliferation, that plays an important pathophysiological role in the development of Benign Prostatic Enlargement (BPE) (Lepor 2005). Benign enlargement of the prostate with age is universal, but is only considered pathological and termed BPE when symptoms exist (Lepor 2004). Prostatic tissue collected from men typically exhibits epithelial over-growth, with, as seen in secondary cells, age-related increases in cell size and, unlike in the accessory gland, evidence of cellular hyperplasia (Roehrborn 2005). Although no studies exist examining the relationship between sexual activity and size of the human prostate, extensive observations in rats do show that sexual activity promotes prostate growth (Drori and Folman 1964, Thomas and Neiman 1968, Hunt 1969, Aumuller, Braun et al. 1985), again hinting at conserved biology between the mammalian prostate and the *Drosophila* accessory gland, in addition to that previously described (Rylett, Walker et al. 2007).

3.5.3 Secondary cells delaminate apically and migrate proximally in ageing mated males

In this chapter I have also shown that, in mated males, SCs are able to delaminate apically from the AG epithelium and migrate proximally within the gland. Although this appears to occur at a low frequency (~5%) in young (5-10 d) multiply-mated males, the majority of older males that had been multiply mated exhibited at least one SC that had migrated proximally. Additionally, a proportion of these migratory SCs are transferred to females during copulation. Although the function of this phenomenon is unknown, a plausible explanation is that SC transfer to females is an active process aimed at ensuring that SC-derived secreted elements can be released in the female reproductive tract, perhaps via holocrine or other forms of secretion. I speculate that this becomes important as the secretory machinery of SCs in aged, multiply mated males becomes less efficient, when cell transfer provides a sporadic mechanism by which SC secretory products can be increased in some females.

Interestingly, these data again point to cell biological parallels between the *Drosophila* accessory gland and the human prostate. Of note, prostate epithelial secretory cells have been observed in human seminal fluid, and are presumably transferred to the female reproductive tract during mating (Barren, Holmes et al. 1998). Although conventional wisdom has assumed that these cells appear in semen having been sloughed off of the epithelium, our data suggest that this may an evolutionarily conserved active process, requiring regulated cell delamination and migration. That prostate epithelial cells may possess some inherent ability to delaminate and migrate is a particularly intriguing proposition, given that malignant disease of the prostate is often associated with aggressive

metastatic cell behaviours. Subsequent to publishing these data (Leiblich, Marsden et al. 2012), other researchers have studied the migratory behaviours of secondary cells in an effort to identify novel genes implicated in prostate cancer metastasis, with some success (Ito, Ueda et al. 2014), suggesting that the fly model, once properly characterised, may provide insights into prostate cancer biology.

Given the interesting cell behaviours displayed by SCs and their potential relevance to the prostate, I elected to explore and define the cellular mechanisms underpinning these phenomena. Characterising a set of GAL4 drivers selectively expressed in SCs has proved to be an important step forward, which will permit experiments designed to define the genetic regulation of many aspects of SC biology. Indeed, this approach and the subsequent analysis form much of the work that I present in subsequent results chapters.

4 - Chapter four – BMP signalling regulates SC growth, delamination and migration and plays a role in female reproductive reprogramming

Introduction 4.1

My work in the previous chapter identifies a set of novel secondary cell behaviours that appear to be of great interest to researchers concerned with questions addressing the regulatory mechanisms controlling important aspects of cell biology, including cell growth and migration. With this in mind, in this chapter I will describe how I sought to establish which signals regulate these behaviours in secondary cells through a genetic screen, using the GAL4/UAS system to manipulate the SC activity of a set of pathways implicated in cell growth, including PI3K, RAS/MAPK/Erk and BMP signalling pathways. Ultimately, this screen identifies the BMP signalling pathway as crucial regulator, controlling much of this biology in SCs. Furthermore, I establish that BMP activity in SCs acts to modulate some aspects of the normal female post-mating response (PMR) described in chapter 1.

4.1.1 Outline of the BMP signalling pathway in *Drosophila*

In this chapter I outline the role that Bone Morphogenetic Protein (BMP) signalling plays as a key regulator of the cell growth and migratory phenomena observed in SCs, which are described in the previous chapter. Over the past thirty years, *Drosophila* research has been at the heart of efforts aimed at characterising the BMP signalling pathway (summarised in Figure 4.1).

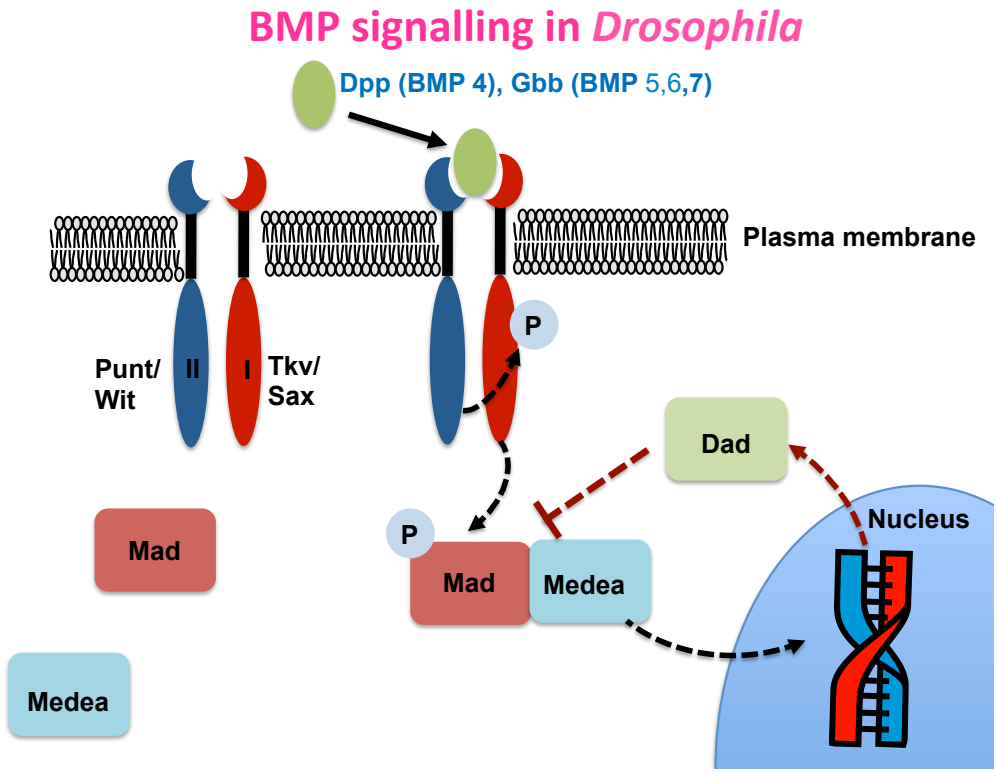


Figure 4.1: Schematic depicting BMP signalling mechanisms in *Drosophila*. Two major fly BMP ligands have been described, Dpp and Gbb. These ligands bind to a BMP receptor complex composed of type I (Tkv or Sax) and type II (Punt or Wit) receptors. Ligand binding induces phosphorylation of the type I receptor. Once activated, this BMP receptor complex phosphorylates Mad, which then interacts with the co-smad, Medea. The activated Mad-Medea complex then translocates to the nucleus to direct transcription of BMP signalling target genes. An example of a target gene is *dad*, which acts to repress BMP signalling activity.

Decapentaplegic (*dpp*), perhaps the most wildly researched BMP ligand across all species, was first identified from a mutagenesis screen aimed at discovering genes required for normal *Drosophila* morphogenesis (Spencer, Hoffmann et al. 1982). Mutations in *decapentaplegic (dpp)* were shown to cause morphological defects in 15 of the 19 imaginal discs, demonstrating a BMP requirement for normal metamorphosis (Spencer, Hoffmann et al. 1982). Subsequently, *dpp* expression has been shown to be vital for even earlier stages of

development, being necessary for normal dorsal-ventral patterning of the embryo (Irish and Gelbart 1987, Ray, Arora et al. 1991). The relevance of dpp-signalling mechanisms in *Drosophila* to human biology became apparent with the discovery of the close homology between dpp and mammalian BMP ligands (Padgett, St Johnston et al. 1987), with BMP-2 and -4 being close vertebrate orthologues that can rescue *dpp* mutant phenotypes (Padgett, Wozney et al. 1993).

A few years after the initial characterisation of *dpp*, mutations of a gene known as *thickveins (tkv)* were noted to cause dorsal-ventral polarity defects in embryos reminiscent of *dpp* mutant phenotypes, implying a role for *tkv* in *Drosophila* BMP-signalling (Nusslein-Volhard and Wieschaus 1980, Terracol and Lengyel 1994). Soon after this observation, a number of reports emerged identifying the *tkv* protein product as a type I BMP serine/threonine kinase receptor and target of dpp ligand, needed for transducing dpp signals (Affolter, Nellen et al. 1994, Brummel, Twombly et al. 1994, Nellen, Affolter et al. 1994, Okano, Yoshikawa et al. 1994, Penton, Chen et al. 1994). Additional *Drosophila* BMP receptors have since been identified, including the type I receptor, *saxophone (sax)* (Penton, Chen et al. 1994, Xie, Finelli et al. 1994) and the type II receptors, *punt (put)* (Letsou, Arora et al. 1995, Ruberte, Marty et al. 1995) and *wishful thinking (wit)* (Aberle, Haghighi et al. 2002, Marques, Bao et al. 2002, Lee-Hoeflich, Zhao et al. 2005). Both dpp and another BMP ligand, known as Glass bottom boat (gbb*)* (Wharton, Thomsen et al. 1991, Haerry, Khalsa et al. 1998), can activate a BMP receptor complex, composed of a type I receptor, either Tkv or Sax, and a type II receptor, either WIT or PUT (Raftery and Sutherland 1999) (Figure 4.1).

Furthermore, *Drosophila* research provided the first description of the mechanisms underlying downstream transduction of BMP signals, with the discovery of the gene, *Mothers against dpp (Mad)* (Sekelsky, Newfeld et al. 1995). Mad was identified as a crucial downstream effector of BMP signalling that is phosphorylated by an activated type-I/type-II BMP receptor complex (Newfeld, Chartoff et al. 1996, Newfeld, Mehra et al. 1997). Once phosphorylated, Mad forms a complex with the co-smad, Medea (MED) (Greenberg, DeMayo et al.) (Raftery, Twombly et al. 1995, Das, Maduzia et al. 1998, Hudson, Podos et al. 1998). The activated Mad/Med complex translocates to the nucleus to direct transcription of BMP signalling targets (Kim, Johnson et al. 1997, Maduzia and Padgett 1997). Shortly after the identification of *Mad* and *Med* in *Drosophila*, researchers searched for and found a family of orthologues in mammals (Derynck, Gelbart et al. 1996, Hoodless, Haerry et al. 1996, Liu, Hata et al. 1996, Zhang, Feng et al. 1996). In recognition of the role of fly research in the discovery of these downstream components, these genes are referred to as smads and co-smads. Interestingly, data obtained from the open access web resource, FlyAtlas (www.flyatlas.org), indicate that *dpp* transcript levels are higher in the AG than almost every other adult tissue (only the crop has higher levels), with a transcript abundance signal of 126+/-9 versus a signal of 32+/-1 for the whole animal.

4.1.2 BMP signals regulate diverse functions, including cell migration and remodelling, in *Drosophila*

A number of cell behaviours, related to those I have described in SCs in the previous chapter, are by BMP signals. For instance, the migratory activity of *Drosophila* tracheal cells during embryogenesis are controlled by BMP signalling in a dpp-dependent manner

(Vincent, Ruberte et al. 1997). In the *Drosophila* wing disc, a larval tissue that ultimately gives rise to adult wing, cell growth and proliferation is positively regulated by BMP pathway activity, through dpp mediate activation of the BMP receptor, Tkv (Burke and Basler 1996, Martin-Castellanos and Edgar 2002). In the developing *Drosophila* neuromuscular junction (NMJ), BMP signals derived from adjacent myocytes activate signalling in motoneurons to promote neuron growth by positively regulating RAC1, a Rho family GTPase involved in cytoskeletal remodelling (Ball, Warren-Paquin et al. 2010).

4.1.3 Male AG secretions regulate multiple aspects of the female post-mating response

Male seminal fluid components transferred during mating induce a number of stereotyped changes in behaviour and physiology in female *Drosophila* (Ram and Wolfner 2007). Two of the best characterised of these behavioural changes include a reduction in readiness to mate or 'receptivity' (Chen, Stumm-Zollinger et al. 1988, Chapman, Bangham et al. 2003) and an increase in the rate of egg laying (Herndon and Wolfner 1995). These post-mating responses are known to persist for up to 1 week. Interestingly, virgin females ectopically expressing sex peptide (SP), a secreted protein derived from male accessory gland main cells, display reduced receptivity, indicating a role for this peptide in inducing this specific response (Aigaki, Fleischmann et al. 1991). Indeed, females mating to males with a loss of function mutation affecting SP do not display a reduction in receptivity, confirming the requirement of SP for inducing the changes in receptivity seen after mating (Liu and Kubli 2003, Peng, Chen et al. 2005).

Interestingly, although virgin females injected with SP phenocopy the reduction in receptivity seen after mating, these effects only last 1-2 days as opposed to the 6-7 days

seen normally after mating. Further work using sperm-less *tudor* males has shown that there is a requirement for sperm to be transferred to achieve sustained reduction in receptivity. Furthermore, the tails of sperm stored in the female reproductive tract bind SP. This peptide is then gradually released from sperm tails by cleavage at a specific trypsin cleavage site. The increase in egg-laying observed after mating is also known to require a main cell-derived seminal fluid peptide, called Ovulin, an AG secreted product that induces egg laying in females and promotes oviposition (Herndon and Wolfner 1995, Heifetz, Lung et al. 2000).

4.1.4 Aims

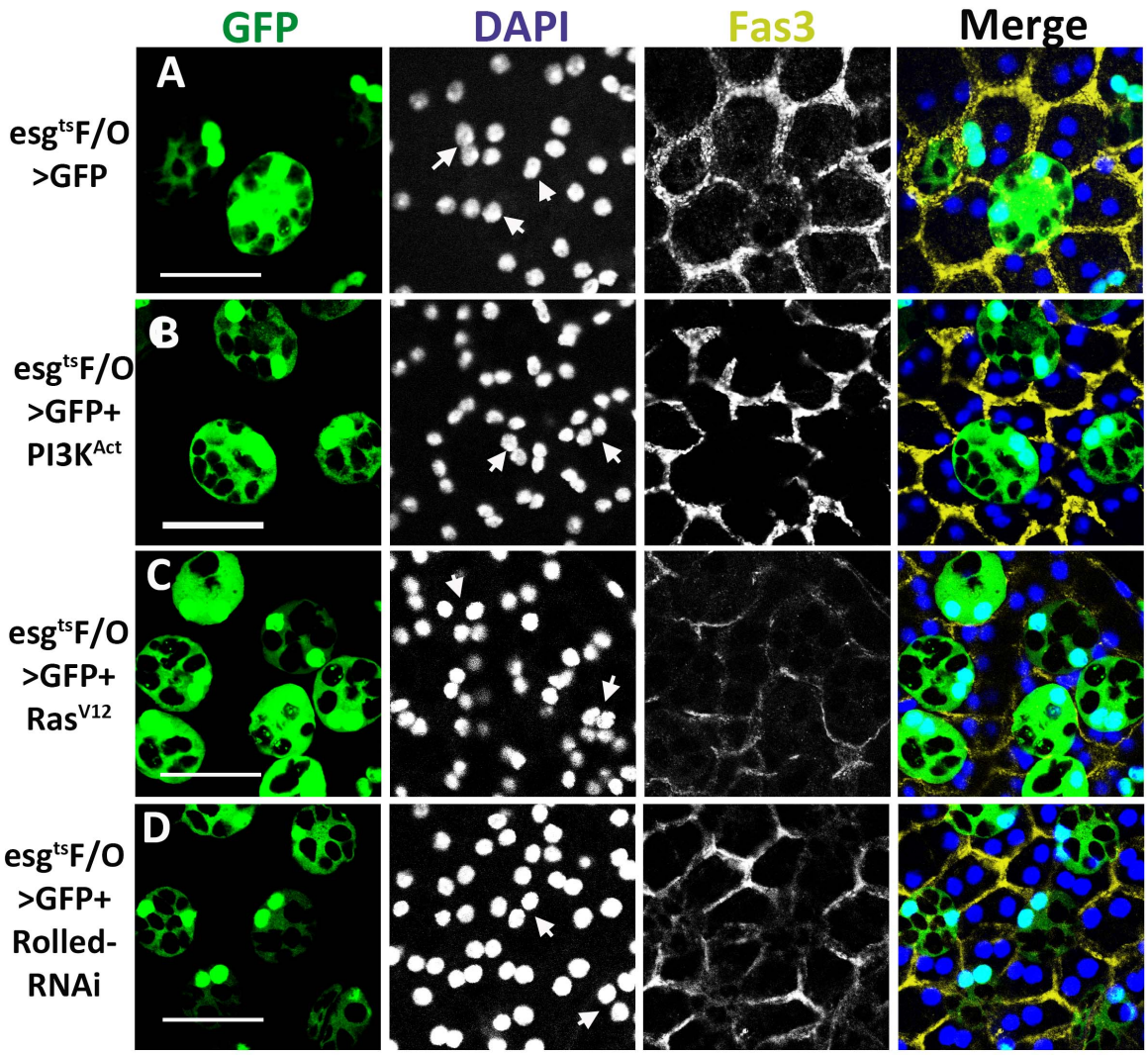
Leading on from the findings in the previous chapter that SCs exhibit growth and migratory phenotypes that can be modulated by mating, I sought to elucidate the mechanisms that underpin these behaviours. To this end, I performed a targeted screen of several signalling pathways previously implicated in cell growth and/or migration. Here, I will present the results of this screen, which identifies the BMP pathway as a key regulator of SC biology. Additionally, I aim to characterise the exact role that BMP signalling plays in modulating the cell biology of SCs. Finally, I will describe a reproductive physiological function for this signalling pathway in SCs as a regulator of specific aspects of the female PMR.

4.2 Results

4.2.1 The PI3K and Ras/MAPK/ERK signalling pathways do not appear to be major regulators of secondary cell nuclear growth

In order to establish which signals might regulate the cell growth and migratory phenomena exhibited by secondary cells, activating and inactivating components of different signalling cascades implicated in growth, including PI3K/Akt (MacDougall, Domin et al. 1995, Leevers,

Weinkove et al. 1996, Goberdhan, Paricio et al. 1999), RAS/MAPK/ERK (Neuman-Silberberg, Schejter et al. 1984, Prober and Edgar 2000) and BMP pathways were over-expressed in secondary cells in virgin males from eclosion onwards using the *esg*^{ts} F/O system. Nuclear size was used as a proxy for measuring cell growth, whereby the area of both SC nuclei where measured and compared to the nuclear area of adjacent MCs to produce a SC:MC nuclear size ratio. I induced hyper-activation of the PI3K/Akt pathway in SCs by expressing a constitutively active PI3K-92E with a farnesylation signal appended to the C-terminus under UAS control (Leevers, Weinkove et al. 1996, Lee, Budanov et al. 2010, Wong, Li et al. 2013). Up-regulating PI3K/Akt in this way had no effect on SC nuclear growth (Figure 4.2B). Up-regulation of RAS/MAPK/ERK pathway signalling in SCs was induced by expressing a constitutively active form of Ras85D, Ras85D^{V12} (Lee, Feig et al. 1996, Prober and Edgar 2000). However, as with PI3K pathway modulation, no growth effects in SCs were observed (Figure 4.2C). Similarly, down regulation of this pathway, by RNAi knockdown of *rolled (rl)* (Nir, Grossman et al. 2012), a gene that codes for the *Drosophila* Mitogen Activated Protein Kinase (MAPK) (Biggs, Zavitz et al. 1994), did not affect SC growth (Figure 4.2D). Taken together, these data indicate that neither the PI3K nor the Ras/MAPK/ERK pathways play a major role in regulating SC nuclear growth.



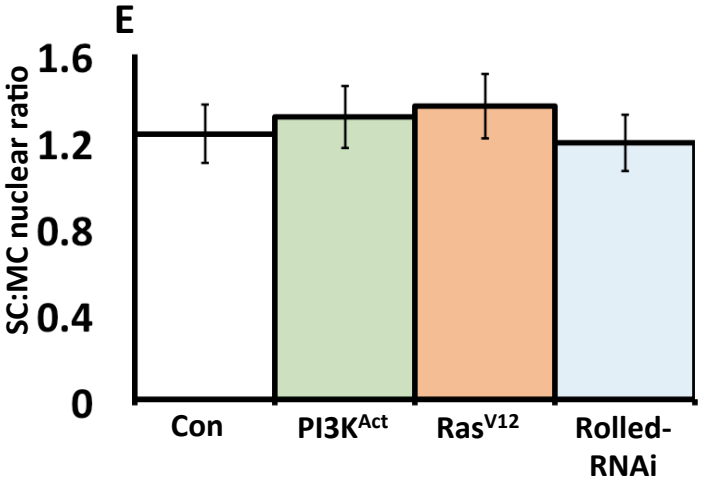
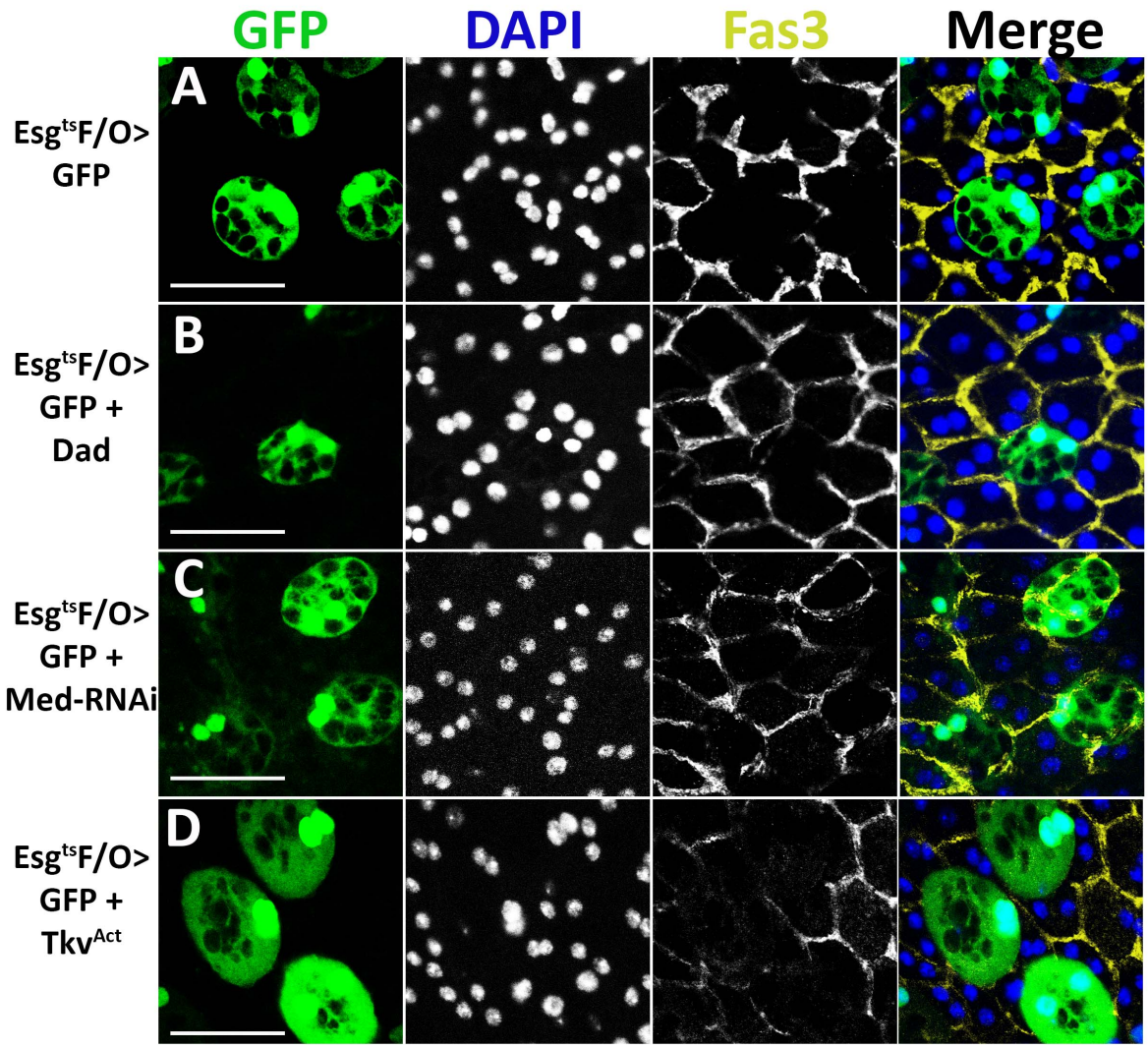


Figure 4.2: Modulating the PI3K and Ras/MAPK/ERK pathways has no effect on SC nuclear growth. Hyper-activating PI3K activity by expressing a constitutively active form of PI3K under UAS control (B) has no effect on growth as observed by measuring nuclear size compared to control (A) (see E for data). Neither up-regulating Ras/MAPK/ERK activity, by SC-specific expression of the constitutively active Ras85D^{V12} construct (C), nor down-regulation by inducing *Rolled* (*Drosophila* ERK MAPK) knockdown using RNAi (D), affected nuclear growth in SCs (see E). White arrows denote SC nuclei, scale bar = 5µm. One-way ANOVA, P=0.001, n=15.

4.2.2 BMP signalling activity regulates secondary cell growth

Although neither PI3K nor Ras/MAPK/ERK pathways appear to be involved in regulating SC nuclear growth, my screen revealed an important role for BMP signalling activity in modulating both SC growth and migration. Down-regulation of BMP signalling activity in secondary cells was achieved either by over-expression of the BMP pathway repressor *Dad* (Tsuneizumi, Nakayama et al. 1997), or by expressing RNAi constructs targeted against the BMP pathway intermediary Medea (Beckwith, Gorostiza et al. 2013) under the control of esg^{ts} F/O. Crucially, by 6 days *Dad* and *Med*-RNAi-expressing secondary cells were smaller than main cells, and their nuclei showed reductions in size of 40% and 38% respectively, compared to controls (for both genotypes, $P<0.001$, $n = 5$, one-way ANOVA) (Figure 4.3C and D, compared to 4.3A; Figure 4.3E).

Up-regulation of BMP signalling activity was achieved by SC-specific expression of a UAS-coupled mutant form of *Tkv* containing a single amino acid substitution (Q253D) in the GS domain (a glycine-serine rich region that is highly conserved amongst all type I BMP receptors) of Tkv, thus conferring ligand-independent, constitutive activity (Nellen, Burke et al. 1996). Expression of this Tkv^{Act} construct strongly stimulates cell and nuclear growth, with the SC:MC nuclear size ratio being nearly 70% larger in SCs expressing Tkv^{Act} than in controls ($P<0.001$, $n = 5$, one-way ANOVA) (Figure 4.3D and E).



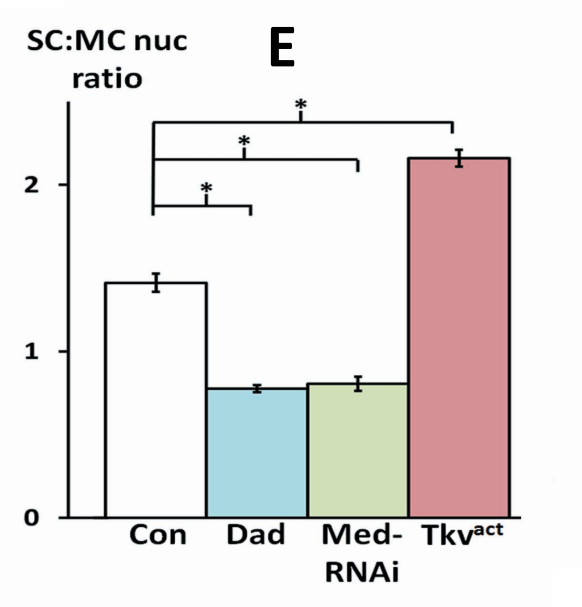


Figure 4.3 BMP signalling modulates SC growth. SC down-regulation of BMP activity by ectopic overexpression of the repressor *Dad* (B) or by expressing Med-RNAi (C) significantly reduces SC nuclear size compared to control (A; see E). Hyper-activation of SC BMP activity by expressing the constitutively active Tkv^{Act} under UAS-control (D) significantly increases nuclear size compared to control (A and E). Changes in basal cell area are also clearly visible in B and D. White arrows denote SC nuclei, scale bar = 5µm. One-way ANOVA, $P<0.001$, $n=15$.

4.2.3 BMP pathway activity modulates the migratory behaviour of secondary cells

In addition to the growth effects observed in SCs expressing *Tkv*^{Act}, expression of this construct induced delamination of a subset of SCs in virgin males (Figure 4.5C, D and E), phenocopying changes normally seen only in mated flies (see previous chapter). If males initiating expression of activated *Tkv* in secondary cells immediately after eclosion were aged for 11-13 days, 9.2 +/- 2.6 of the cells per animal (n=6) fully delaminated and moved more proximally in the gland, a phenotype never observed in normal virgin males (n=6) ($P<0.01$, ANOVA) (Figure 4.4). Additionally, the SC mating-dependent delamination and migration events observed in normal AGs (see section 3.2.4 and Figure 3.5), is prevented by down-regulating SC BMP signalling activity. No delamination events observed in 11-13d old AGs of males mated at least 3 times when the BMP repressor *Dad* is expressed in the SCs. (n=6). Furthermore, cross-sectional imaging indicates that delaminated cells maintain contact with the epithelium (Figure 4.5F), and most cells migrate to the proximal third of the gland (Figure 4.5C and E), suggesting that movement is directional. Additionally, these cells display morphological changes, typified by accumulation of F-actin at the leading edge (Figure 4.4D&E).

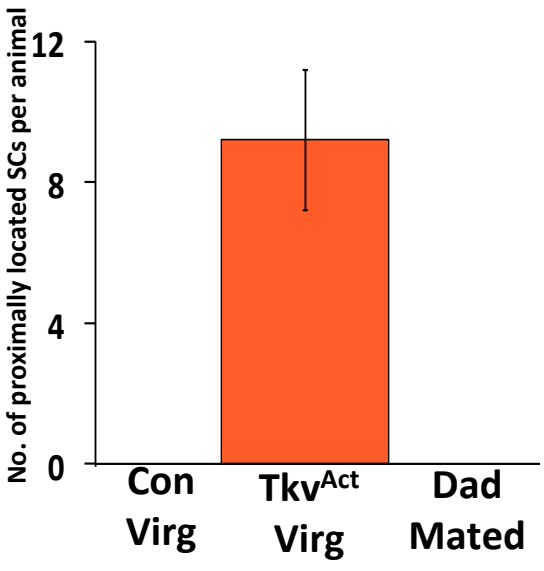
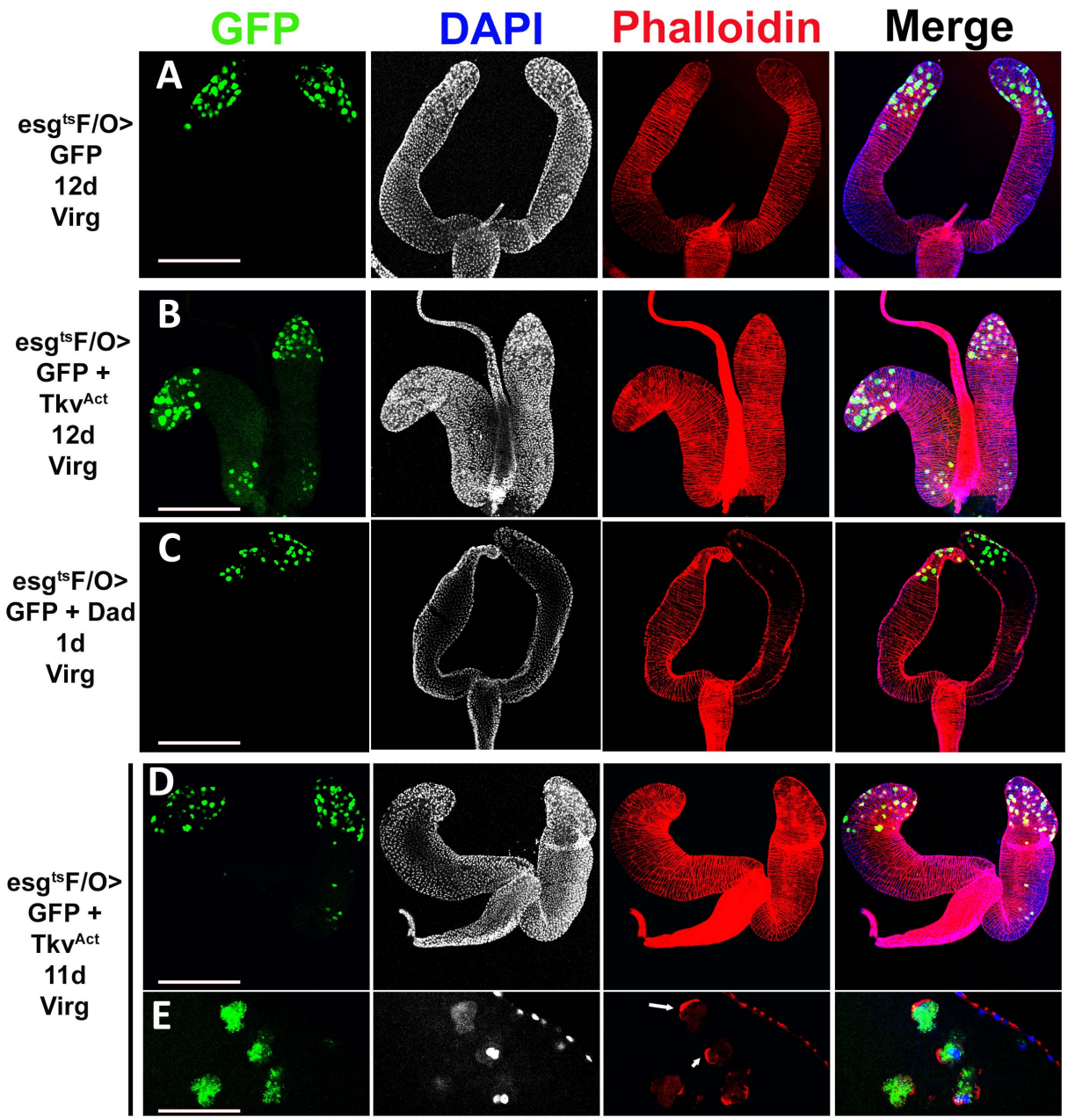


Figure 4.4 BMP signalling activity regulates mating-dependent SC migration. No proximal SCs are observed in the AGs of virgin control or multiply mated (>3) *Dad* males aged 11-13d. Age-matched TkvAct virgin males display a high frequency of SC delamination, with 9.2 +/- 2.6 proxmially situated SCs observed per animal. (n=6, ANOVA<0.01)



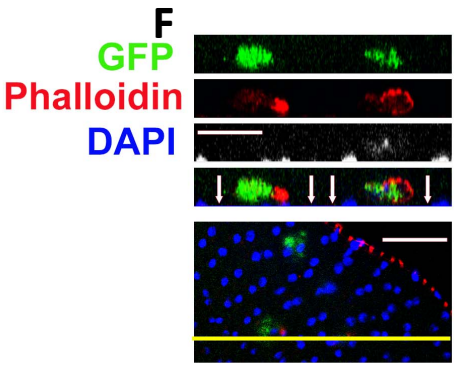


Figure 4.5 BMP signalling activity promotes SC delamination and migration. 12d old virgin AGs containing SCs expressing Tkv^{Act} delaminate and migrate at high frequency (~9 cells/animal, B) compared to age-matched virgin controls (0 cells/animal, A) and Dad expressing mated males (0 cells/animal, C). SCs migrate proximally (B and C) and appear to accumulate F-actin asymmetrically (arrows, E). Cross-sectional Z-stack of proximal migrating SC (in position of yellow line) indicates that these cells retain contact with the underlying epithelium (F, position of underlying epithelium indicated with arrows). Scale bars A-C =30µm, D&E =10ɯm.

4.2.4 Evidence for expression of the BMP ligands, Dpp and Gbb, in the *Drosophila* accessory gland

In an effort to establish the origin of BMP signals that regulate signalling activity in secondary cells, a GAL4 enhancer trap inserted in *dpp*, *dpp*-GAL4.PS (Hazelett, Bourouis et al. 1998) was used to drive the expression of a UAS-GFP construct. Glands of virgin males were dissected at 3 days. Expression of GFP was observed in a small subset of SCs (Figure 4.6A), implying that at least some adult SCs express *dpp*. One interpretation of these data is that this small number of secondary cells express and secrete dpp to regulate BMP activity in all accessory gland SCs, implying a paracrine effect on non-dpp-expressing SCs. Alternatively, it may be the case that SC expression is sporadic and that all SCs are able to express *dpp* in response to as yet unknown cues, in which case dpp-mediated signalling may be either autocrine or paracrine. Indeed, a dynamic and sporadic pattern of *dpp* expression has been observed by others in terminally differentiated adult intestinal epithelial cells (Li, Qi et al. 2013).

Additionally, data obtained from FlyAtlas (www.flyatlas.org), indicate that higher levels of *gbb* transcript are present in accessory glands than any other adult tissue, with a transcript abundance signal of 924 +/-26 versus a whole fly signal of 99 +/-4. In order to determine the localisation of GBB protein, antibody staining was performed using a monoclonal antibody directed against C-terminal sequences of the Gbb protein (Akiyama, Marques et al. 2012). This revealed enrichment of GBB protein in SCs, most notably within compartments adjacent to the large cytoplasmic vacuoles (Figure 4.6B). Clearly, these data indicate that GBB is localised within SCs, although we cannot be certain that these cells express GBB, as it may be secreted by another cell type and endocytosed by SCs.

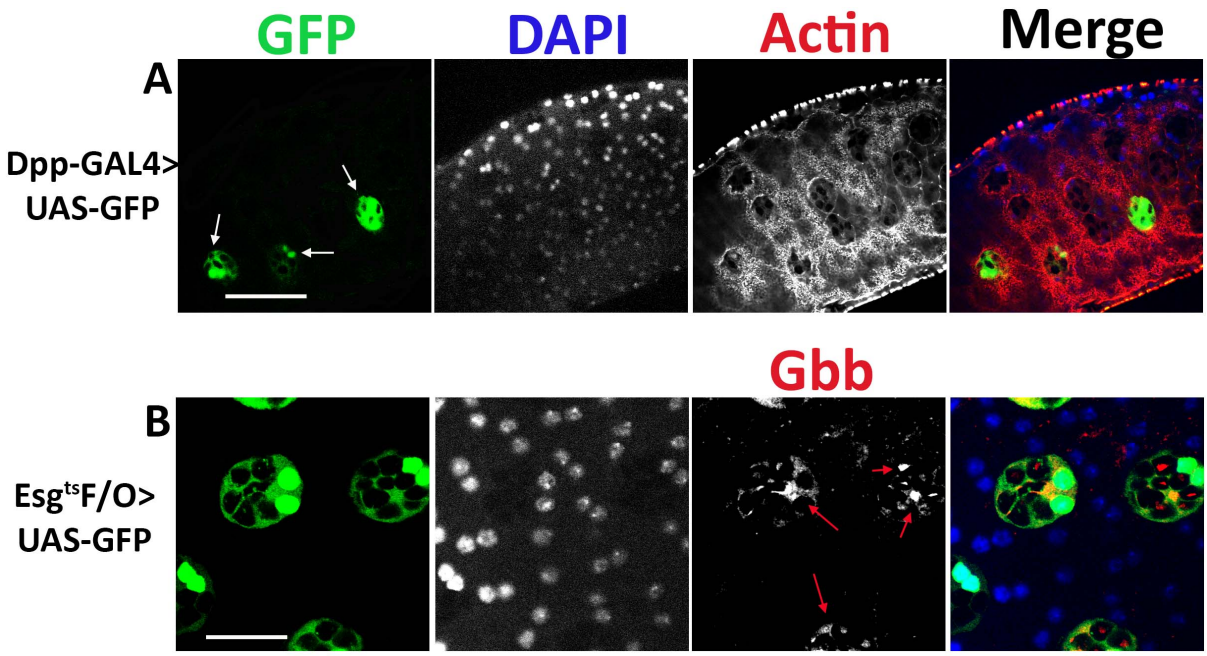


Figure 4.6 BMP ligands, dpp and Gbb, exhibit expression and localisation in adult SCs. (A) Expression of a GFP reporter driven by a *dpp*-GAL4 enhancer trap indicates transcription of *dpp* in a proportion of adult SCs at 3 days (white arrows). (B) Furthermore, antibody staining of 3-day-old glands where SCs are expressing nuclear GFP as a cell marker, reveals the presence of Gbb protein inside SCs (red arrows). Interestingly, Gbb appears to be particularly enriched insidecompartments adjacent to large cytoplasmic vacuoles. Scale bars = 25µm (A), 10µm (B).

4.2.5 BMP signalling in secondary cells is necessary for inducing a normal post-mating response in females

Having shown that BMP signalling plays a crucial role in regulating cell biological behaviours of secondary cells, I sought to establish whether this pathway also plays a role in the reproductive functions of the accessory gland. As described in sections 1.4.2 and 4.1.3, AG derived products are responsible for inducing a range of stereo-typed changes in female physiology and behaviour, known as the post-mating response (PMR), which include reducing the receptivity of females to further mating and increasing the rate of egg laying (Chen, Stumm-Zollinger et al. 1988, Chapman, Bangham et al. 2003) .

To test whether BMP signalling in secondary cells plays a role in modulating the PMR and/or aspects of fertility and fecundity, a series of mating experiments were designed to test the performance of wild-type males with males containing secondary cells expressing *Dad* only in adults.

Overall, there were no significant differences in the progeny number per day or the number of eggs per day laid between females that were mated to control males and females mated to *esg*^{ts} F/O>UAS-*Dad* males (Figure 4.7A, B). Additionally, the total progeny number was no different between the control and test groups (Figure 4.7C)

However, females mated to males containing *Dad*-expressing secondary cells did not show the same decrease in receptivity as females mated to control males. Significantly more *Dad*-expressing males compared with controls (8 of 37 versus 1 of 39; $P<0.01$; two-tailed Fisher's exact test) were unable to fully prevent females from re-mating 4 days later during a 3-hour period (Figure 4.7D). Additionally, while almost all 4-day-old multiply mated control males were able in subsequent matings to block the receptivity of a further three females to re-mating (98%; 45 of 46 males), significantly fewer *Dad*-expressing males (39%; 16 of 44 males; $P<0.001$) were able to do this (Figure 4.6E). Importantly, there were no differences in length of time that the males mated or in the number of males that re-mated within 40 minutes for *Dad*-expressing versus control males, implying that the effect on receptivity observed was not a consequence of changes in male mating behaviour (Figure 4.6F, and G). These data indicate a specific role for secondary cells in modulating receptivity in females after mating and that BMP signalling in these cells is required for this function.

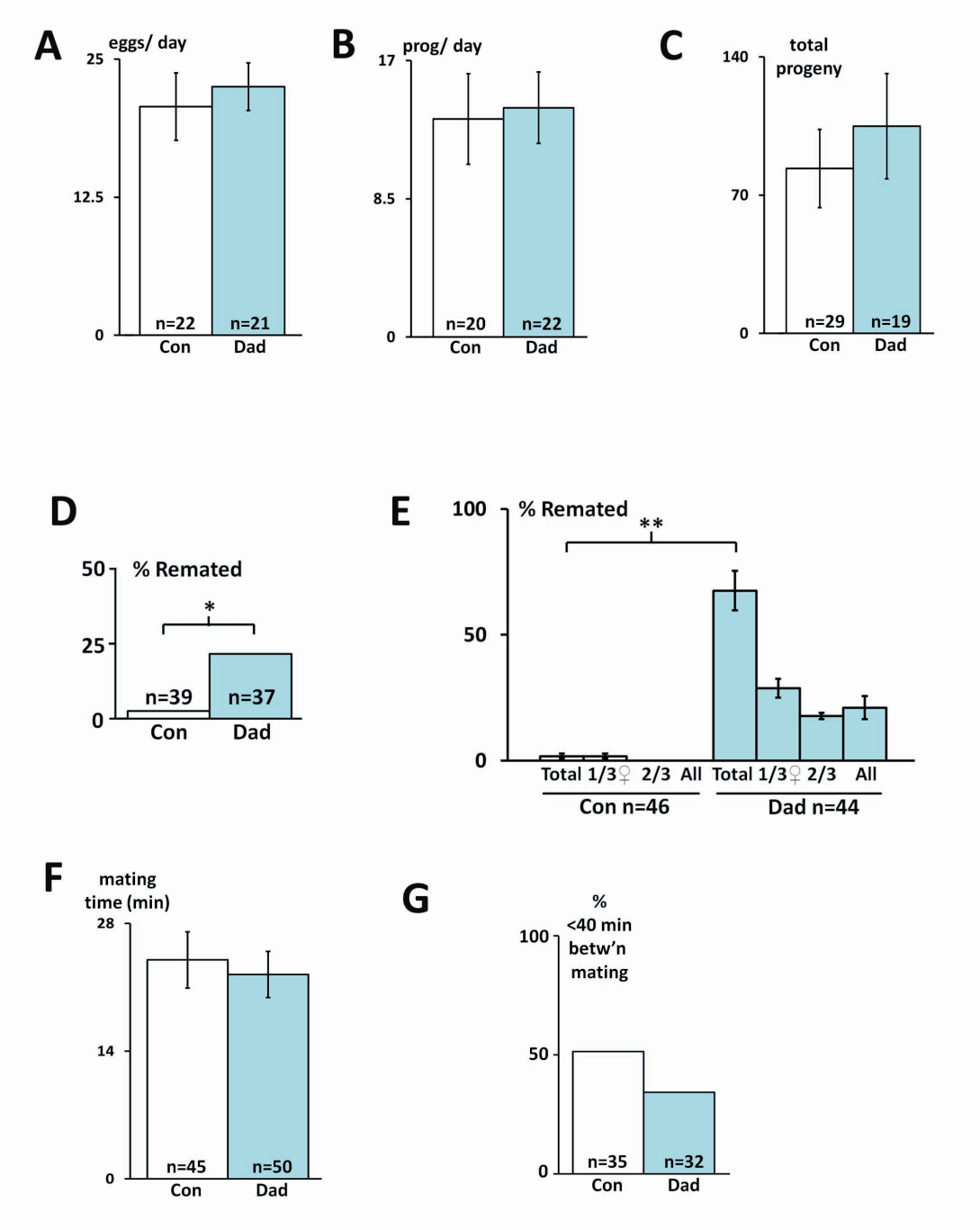


Figure 4.7: SC BMP signalling activity influences female reproductive physiology. Inhibition of BMP activity in SCs by over-expressing Dad has no significant effect on the rate of egg laying (A), progeny/day (B) or total progeny number (C) compared to controls. However, females mated to Dad-expressing males exhibit a higher frequency of re-mating within 4 days of initially mating (D, 8 of 37 versus 1 of 39; $P<0.01$; two-tailed Fisher's exact test). Nearly all 4-day-old multiply mated control males were able to block the receptivity of a further three females to re-mating (98%; 45 of 46 males), significantly fewer Dad-expressing males (39%; 16 of 44 males; $P<0.001$) were able to do this (E). This phenomenon is not a consequence of differences in length of time spent mating (F) nor of differences in the number of males that re-mated within 40 minutes for Dad-expressing versus control males (G).

4.3 Discussion

In this chapter I have described how, through a targeted genetic screen, I have identified the BMP signalling pathway as a crucial regulator of several aspects of SC biology. Specifically, I show that BMP signalling activity is necessary for normal SC growth and abnormal overgrowth is observed if this pathway is hyper-activated. Additionally, the BMP pathway is identified as a positive regulator of SC delamination and migration. I also a that normal levels of BMP signalling activity in SCs are required for a full female PMR, with down-regulation of this pathway associated with specific defects in the PMR. A great deal of the data presented here and in the preceding results chapter are entirely novel, and have contributed greatly to the understanding of the biology of the AG epithelium (Leiblich, Marsden et al. 2012).

4.3.1 SC growth regulation through BMP signalling activity

In this chapter I have presented data showing that the age-dependent growth phenomenon observed in adult SCs (as described in the previous chapter) requires normal levels of BMP signalling activity, and up-regulation of this pathway is sufficient to drive abnormal cell overgrowth. There are other examples in *Drosophila* where BMP activity positively regulates cell growth, but usually in the context of proliferation (Affolter and Basler 2007).

The most prominent example of this role for BMP signalling exists in the developing imaginal wing disc, a developmental epithelial tissue that ultimately gives adult wing structures. In these cells BMP signalling, through Tkv, is required for normal growth and proliferation, and increased cell size and accelerated proliferation are induced in cells expressing Tkv^{Act} (Martin-Castellanos and Edgar 2002). Although this does not mirror exactly the observations

I have made in SCs, as this cell type displays no replicative activity even in the context of high BMP signalling activity, these data do point to important parallels in the two systems that imply that BMP signals act as general regulators of cell growth. Indeed, the studies in the wing disc show that cell growth and proliferation are independently regulated by BMP signals. Indeed, co-expressing Tkv^{Act} with *Rbf*, the *Drosophila retinoblastoma* homolog and a key cell cycle regulator, blocks proliferation of wing disc cells, but they remain larger than wild-type wing discs cells (Martin-Castellanos and Edgar 2002). Therefore, it is plausible that in adult SCs, BMP signalling activity regulates cell growth by the same mechanism as is observed in the wing disc, but no proliferative effects are seen because other signals regulate the activity and expression of cell cycle regulators, such as Rbf, that inhibit this aspect of BMP pathway activity.

4.3.2 The pro-migratory effects of BMP signalling in SCs echoes features of cell migration in *Drosophila* development

In this chapter, I have described how BMP signalling activity also regulates SC delamination and migration. Specifically, up-regulation of BMP activity is associated with high frequency of SC migration, even in virgin animals, whilst delamination and migration are absent in mated males with SCs expressing *Dad*. This role for the BMP signalling is supported by observations made by others that demonstrate an important role for this pathway as a regulator of cell motility during *Drosophila* development. Studying dorsal closure and thorax closure in the developing fly has emerged as powerful model permitting the genetic analysis of the mechanisms that control epithelial cell movements (Zeitlinger and Bohmann 1999, Martin-Blanco, Pastor-Pareja et al. 2000, Ishimaru, Ueda et al. 2004). In this system, thorax closure is disrupted in flies bearing mutations in BMP-pathway genes, including *dpp*, *tkv* and

Med, whilst changes in the actin cytoskeleton are observed, with disruption of actin accumulation at the leading edge (Martin-Blanco, Pastor-Pareja et al. 2000).

This is in agreement with my own data that indicates SC movements are positively regulated by BMP signalling activity and likely involve regulation of cytoskeletal dynamics. An interesting further feature is that mutations in the BMP ligand, dpp, have the same phenotypic effects on cell movement as mutations in the receptor, Tkv, and downstream effector, Med. A possible explanation for this is that the migrating epithelial cells of the developing thorax signal through dpp in an autocrine fashion. Alternatively, these cells are receiving dpp signals from adjacent cells at the dorsal edge in a paracrine fashion. Again, this in keeping the data I have presented that indicate that SCs not only display BMP signalling activity, but also express *dpp* in a subset of cells at any given time point.

Interestingly, the cells of the developing trachea display a very similar migratory phenotype to adult SCs, and, as in SCs, this behaviour is controlled by BMP signalling through the Tkv receptor (Vincent, Ruberte et al. 1997). In a remarkable parallel with my data, tracheal cells lose their migratory capacity when there is diminution of local BMP signals, and up-regulation BMP activity, induced by expressing Tkv^{Act}, is associated with exaggerated cell migration (Vincent, Ruberte et al. 1997). In the light of this evidence, it is appealing to speculate that the migratory behaviour observed in a subset of SCs in aged, mated glands reflects a recapitulation of a developmental process, whereby BMP signals drive and direct the movement of a diverse range of embryonic and larval cell types.

4.3.3 BMP effects on female physiology may occur through regulation of exosome secretion

A surprising aspect of this work was the revelation that BMP signalling activity in SCs is a potent regulator of the physiology and behaviour of the mated female, specifically playing a role in reducing the receptivity of mated females to further mating. Independent research supports the observation that SCs are required for normal post-mating effects in females (Minami, Wakabayashi et al. 2012). The authors of this work noted that adult SCs display high levels of expression of the homedomain repressor, *defective proventriculus* (*dve*). Adult males with mutant *dve* lack secondary cells and fail to protect mated females from subsequent re-mating and have reduced fecundity (Minami, Wakabayashi et al. 2012).

In a similar vein, research conducted in the Karch lab shows that adult SCs express the homeotic gene, *Abdominal-B* (*Abd-B*). Interestingly, males with reduced SC expression of ABD-B are unable to induce the full PMR and, like our *Dad* expressing males, do not block female receptivity to further mating (Gligorov, Sitnik et al. 2013). These studies in SCs, taken together with my own work, clearly demonstrate an important function of SCs as a modulator of the female PMR; although the exact mechanisms by which SCs achieve this are not clear form these studies.

However, Interesting developments have arisen from collaborative experiments within our lab that have gone some way to determine the mechanisms by which BMP signalling activity regulates these behaviours. Work done by a member of our lab has revealed that SC-derived secreted membrane-bound nanoparticles, known as exosomes, are responsible for carrying signals to the female reproductive tract that prevent females from remating (Corrigan, Redhai et al. 2014). Exosomes are formed in multi vesicular bodies (MVBs), and at least one

pathway for their synthesis requires the *endosomal sorting complexes required for transport* (ESCRT) machinery, which includes a set of sorting complexes that regulate many aspects of the membrane dynamics of endosomes, MVBs and lysosomes (Schmidt and Teis 2012, Colombo, Moita et al. 2013). Indeed, blocking ESCRT-dependent exosome synthesis by knockdown of key interactors, such as *ALiX* and *Hrs*, (Tamai, Tanaka et al. 2010, Baietti, Zhang et al. 2012, Colombo, Moita et al. 2013) results in reduced exosome secretion, and ALiX-RNAi knockdown in SCs leads to deficits in female post-mating physiology that closely phenocopy those seen in females mated to males with reduced BMP signalling (Corrigan et al., 2014).

This observation fuelled collaborative studies between Corrigan, myself and other lab members, ultimately leading to the discovery that, in SCs, exosome secretion is regulated by BMP activity. Notably, SCs over-expressing the BMP repressor, *Dad*, showed significantly reduced levels of exosome secretion (Corrigan, Redhai et al. 2014). Taken together, these data indicate that BMP signalling activity in SCs likely influences post-mating female behaviours through effects on exosome secretion, although, as yet, the exact signals carried in exosomes that reprogramme females are unknown.

4.3.4 BMP-mediated effects on endosomal trafficking may link this pathway to mTor signalling, thereby regulating SC growth

In addition to highlighting BMP regulatory effects on exosome secretion, Corrigan et al. (2014) described profound changes in the number, size and arrangement of endosomal compartments in SCs with altered BMP activity, and these effects likely underpin aspects of exosome biology, including biogenesis and synthesis. SCs expressing Tkv^{Act} are shown to contain numerous secretory compartments and extremely large, usually solitary, acidic

compartments, termed MVBLs (multi-vesicular bodies and lysosomes), perhaps indicating that high level BMP signalling activity promotes endolysosomal membrane trafficking. Conversely, *Dad*-expressing SCs have very few non-acidic secretory compartments and possess a single, very small MVB/L; consistent with the explanation that BMP signalling drives endolysosomal trafficking (Corrigan, Redhai et al. 2014).

These findings may be relevant to understanding how BMP signalling modulates SC growth as this provides a possible mechanistic link between BMP and mTORC1 activity. Many aspects of cell metabolism are positively regulated by mTORC1 signalling (Wullschleger, Loewith et al. 2006, Laplante and Sabatini 2012, Tumaneng, Russell et al. 2012), including accumulation of cell mass, which occurs through coordination of protein anabolism (Averous and Proud 2006, Ma and Blenis 2009), nucleotide biogenesis (Ben-Sahra, Howell et al. 2013) and lipogenesis (Laplante and Sabatini 2012).

In addition to localising within a number of cellular compartments including the nucleus (Zhang, Shu et al. 2002), mitochondria (Paglin, Lee et al. 2005), and plasma membrane (Bridges, Ma et al. 2012), mTOR is known to translocate to the surface of the lysosome in a process that is stimulated by amino acids and dependent on the interacting proteins, Rag and Ragulator (Sancak, Peterson et al. 2008, Heublein, Kazi et al. 2010, Sancak, Bar-Peled et al. 2010, Bar-Peled and Sabatini 2012). Once recruited to the lysosome, mTORC1 is activated by GTP-bound Rheb, and in turn phosphorylates the translation regulators S6K and 4E-BP (Burnett, Barrow et al. 1998, Isotani, Hara et al. 1999, Shigemitsu, Tsujishita et al. 1999). It is through S6K and 4E-BP that mTORC1 signalling regulates protein synthesis and cell growth (Ma and Blenis 2009), although it appears the S6K is likely to be a more potent regulator of cell size than 4E-BP (Dowling, Topisirovic et al. 2010).

It is plausible that the changes in lysosomal morphology seen in SCs with altered BMP signalling will affect the ability of the mTORC1 complex to localise and signal efficiently at the surface of the lysosome, with subsequent impact on translation and cell growth. This offers the intriguing prospect that BMP signalling may modulate endosomal trafficking and lysosome biogenesis to regulate cell growth through mTORC1 signalling activity. Testing this hypothesis in SCs is somewhat hampered by the lack of an appropriate read-out assay for mTORC1 signalling activity. Traditionally, assays, such as Western blots, aimed at determining the phosphorylation status of the target proteins, S6K and 4E-BP, are used to elucidate mTORC1 activity (Burnett, Barrow et al. 1998, Gingras, Gygi et al. 1999, Magnuson, Ekim et al. 2012). This approach, however, is currently unfeasible for assaying SCs given the small number of cells per gland and the difficulties that would be encountered when attempting to sort SCs from MCs. Furthermore, currently available tagged mTOR constructs have not proved useful in localising mTOR to specific subcellular compartments.

4.3.5 The BMP ligand Dpp is expressed in SCs and may signal in an autocrine and/or paracrine fashion

In addition to establishing that BMP signalling regulates multiple aspects of SC cell biology and function, I have presented data that indicate that SCs express, or contain, the BMP ligands, dpp and Gbb. The *dpp*-GAL4 data presented in this chapter indicate that a minority of SCs express *dpp* at a given time point. However, we cannot be certain whether this means that *dpp* expression is confined to just a small subset of SCs which then signal to all other SCs in a paracrine manner. In an alternative model, there could be sporadic, intermittent expression of *dpp* within SCs, which then signal in an autocrine loop. Indeed, both paracrine and autocrine dpp signalling could be involved in SCs, a phenomenon that is not without precedent in fruit fly biology. During *Drosophila* development, BMP signalling activity directs

the spreading and invasiveness of abdominal histoblasts, and aspects of this behaviour depend on both paracrine and autocrine actions of dpp (Ninov, Menezes-Cabral et al. 2010). It is possible then that these autocrine and paracrine functions of dpp associated with developmental processes are recapitulated in adult SCs and act, as in histoblasts, to regulate the migratory behaviour of these cells.

4.3.6 The BMP ligand gbb is localised within SCs, largely inside vacuolar compartments

Antibody staining indicates that the BMP ligand gbb is localised inside SCs, with signal observed largely within compartments adjacent to the large vacuoles in these cells. Interestingly, gbb is a secreted ligand that, most notably at the neuromuscular junction, is endocytosed by (and enriched within) the responding cell type (McCabe, Marques et al. 2003). It is plausible therefore, that *gbb* may be expressed and secreted by another AG cell type, either main cells or muscle, and is then endocytosed by SCs. Indeed, during development of the mature neuromuscular junction, muscle cells secrete gbb before the ligand is ultimately endocytosed by target motoneurons, initiating a signal that stimulates neuronal remodelling (Marques, Haerry et al. 2003, McCabe, Marques et al. 2003). Alternatively, the lack of gbb observed in AG cell types other than SCs may indicate simply that SCs, and no other cell types, express *gbb* and that gbb signals within SCs in an autocrine fashion, or between SCs as a paracrine signal. These models are currently under investigation in the lab, and suggest a complex combinatorial interaction between dpp and gbb signals control the behaviour of secondary cells.

4.4 Conclusions

In this chapter, I have presented evidence demonstrating that age- and mating- dependent growth in adult SCs is regulated by BMP signalling activity. This surprising role for BMP activity in an adult epithelial cell type displays some remarkable parallels the function of this pathway in a range of tissues in *Drosophila* development, including the cells of the wing disc. In addition to this role for the BMP pathway, I have shown that it also regulates the delamination and migration of SCs normally observed in aged, multiply mated males. Interestingly, this also reflects a role that BMP activity plays in a developmental process, where it positively regulates the migratory behaviour of tracheal cells. Given this evidence, it is attractive to postulate that as SCs age they recapitulate aspects of biology normally seen in development, and that this process occurs in a BMP-dependent manner.

Additionally, I have characterised a novel role for BMP activity in SCs as key regulator of the female PMR. Specifically, normal levels of BMP signalling are required in SCs to reduce receptivity in mated females. Collaborative work in our lab has established that BMP signalling in SCs regulates normal exosome biology, with down-regulation resulting in massively reduced levels of exosome secretion. Additionally, suppression of exosome secretion by knockdown of ESCRT components in SCs phenocopies the PMR defects associated with reduced SC BMP activity. Taken together, these data suggest that the mechanisms by which SC BMP activity alters female post-mating physiology involve signals derived from BMP-dependent exosomes.

5 – Chapter five –Ecdysone receptor (EcR) signalling mediates the growth regulatory effects of BMP signalling in secondary cells via a novel post-transcriptional mechanism

5.1 Introduction

Understanding how steroid signalling pathways regulate cell growth is particularly relevant to prostate cancer, a disease where growth of cancer cells is accelerated by activation of Androgen receptor (AR) signalling by circulating androgens (Huggins 1943, Saylor 2013). Indeed, the mainstay of treatment for advanced prostate cancer is androgen ablation therapy, whereby the administration of anti-androgens halts tumour progression (Sharifi, Gulley et al. 2005, Pagliarulo, Bracarda et al. 2012). Unfortunately, treatment failure is inevitable after 12-24 months of therapy, as clones of tumour cells emerge that are hormone-refractory and no longer respond to androgen ablation (Seruga, Ocana et al. 2011). This transition to androgen-independence represents the development of particularly aggressive disease that is not amenable to curative therapy. Although activating mutations in the androgen receptor as well as amplification of the AR gene have been implicated in this phenomenon, the mechanisms underlying escape from hormonal control are poorly understood (Kobayashi, Inoue et al. 2013, Egan, Dong et al. 2014, Yuan, Cai et al. 2014).

In an effort to understand how steroid signalling might regulate cell growth, as AR signalling does in prostate cancer, I studied whether a related signalling system, the ecdysone receptor (EcR) pathway (Koelle, Talbot et al. 1991), was involved in epithelial cell growth in the *Drosophila* accessory gland.

5.1.1 Ecdysone receptor structure and function

There are only 18 genes encoding nuclear receptors in the *Drosophila* genome, compared to 48 in humans, although all 6 subfamilies of nuclear receptor are represented in this number (King-Jones and Thummel 2005). Furthermore, fruit flies possess just 2 physiologically

active steroid hormones, 20-hydroxyecdysone (20-HE) and juvenile hormone (JH) (King-Jones and Thummel 2005). By far, the most important characterised nuclear receptor in flies is the Ecdysone receptor (EcR), with EcR signalling considered a master regulator of many developmental processes, particularly progression through metamorphosis (Ashburner 1990, Thummel 1997, King-Jones and Thummel 2005). To date, EcR is the only fly nuclear receptor that has been shown to initiate downstream signalling activity in response to 20-HE binding, whilst most other nuclear receptors are orphan receptors that either do not interact with ligand at all or their interacting ligand is unknown (King-Jones and Thummel 2005).

Three protein isoforms are encoded by the *EcR* gene and are designated EcR-A, EcR-B1 and EcR-B2. All of these proteins share common DNA- and ligand-binding domains, and differ only in their amino-terminal sequences. EcR-A and EcR-B1 are encoded by overlapping transcription units that contain distinct promoters, which can be separately controlled, whilst EcR-B1 and EcR-B2 derive from a common primary EcR-B transcript that is alternatively spliced (Koelle, Talbot et al. 1991, Mouillet, Henrich et al. 2001).

The ecdysone receptor and mammalian AR share classical structural features common to the majority of nuclear steroid hormone receptors (Koelle, Talbot et al. 1991). Like AR, the C-terminal domain of EcR contains both the DNA- and ligand-binding domains, whilst the N-terminal domain differs between isoforms. In common with AR, each EcR isoform possesses two transactivation domains, an AF2 domain located in the ligand-binding domain that is common to all three isoforms, and an AF1 domain located in the variable N-terminal region that is distinct to each isoform, ranging from 228 amino acids in length for EcR-B1 and only 17 amino acids in EcR-B2 (Talbot, Swyryd et al. 1993) (Figure 5.1).

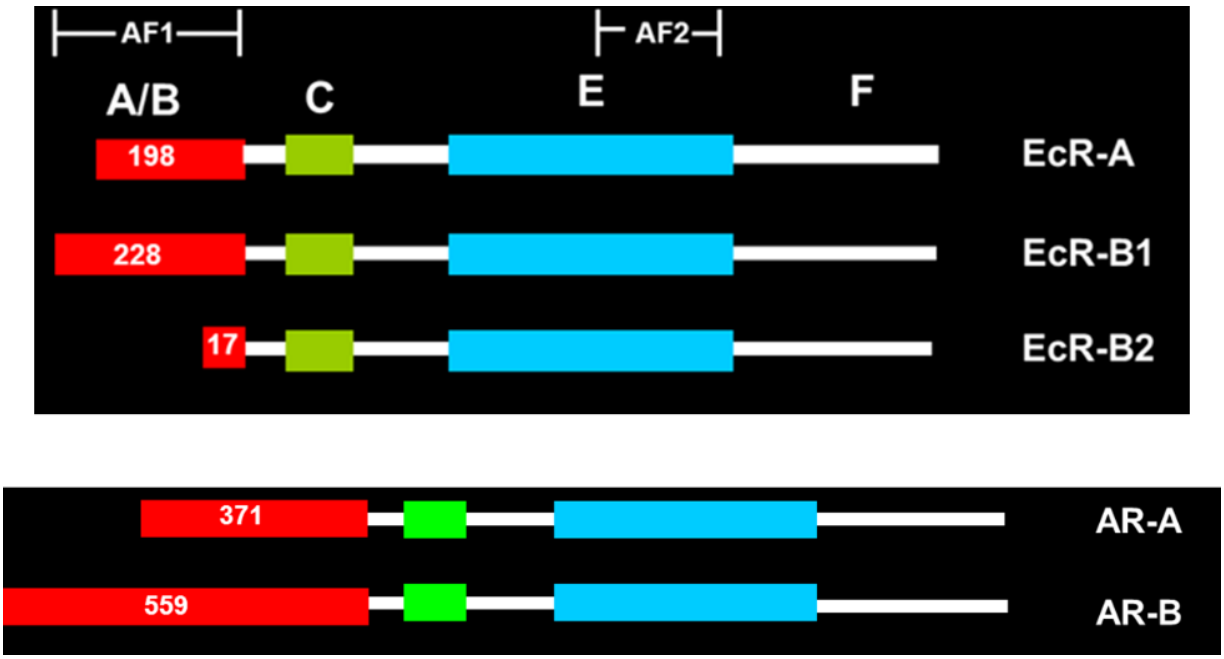


Figure 5.1: The *Drosophila* Ecdysone Receptor (EcR) shares structural similarities with the mammalian androgen receptor (AR). Three isoforms of EcR are made in flies, EcR-A, -B1 and –B2, encoded by the same gene. The C-terminus (F), ligand-binding domain (E), and DNA-binding doman (C) are common to all 3 isoforms, whilst the N-terminal (A/B) sequences differ between all isoforms. These structural features are shared with the two isoforms of AR, AR-A and –B, which also only differ in the sequence of the N-terminus. In common with all classical nuclear steroid hormone receptors, such as AR, EcR contains two transactivation domains: an AF1 region distinct to each isoform located in the N-terminus and an AF2 region common to all three isoforms, located in the ligand-binding domain.

5.1.2 EcR signaling

Functional EcR, like AR, forms a dimer and may complex with another EcR sub-unit, or can hetero-dimerise with the closely related protein, Ultraspiracle (Usp) (Yao, Segraves et al. 1992). The receptor is activated by binding to ecdysteroid hormones, the most abundant of which is 20-hydroxyecdysone (20-HE), promoting translocation of the ligand/receptor complex to the nucleus, where the DNA- binding domain may interact with genomic targets containing Ecdysone Receptor-responsive elements (EcREs) to regulate transcription (Tata 2002) (Figure 5.2). The transcriptional programmes directed by EcR signalling in *Drosophila*

development are well characterised and a handful of target genes have been identified, including *Broad*, *Eip74EF*, and *Eip75B*, most of which encode, in turn, transcription factors themselves that regulate the expression of a broad range of downstream genes, including the cell fate determinants *mef2* and *twist* (Fletcher, Burtis et al. 1995, Fletcher and Thummel 1995, Lovato, Benjamin et al. 2005). In development, EcR sits at the top of a signalling hierarchy that initiates the transition from the larval to pupal stage, prompted by pulses of 20-HE (Thummel 1990, Baehrecke 1996). Thus, EcR signalling is responsible for regulating rather disparate cell biological processes, including cell growth, death, proliferation and axonal remodelling (Lee, Marticke et al. 2000, Lee, Cooksey et al. 2002, Lee, Simon et al. 2002, Kuo, Jan et al. 2005, Zirin, Cheng et al. 2013).

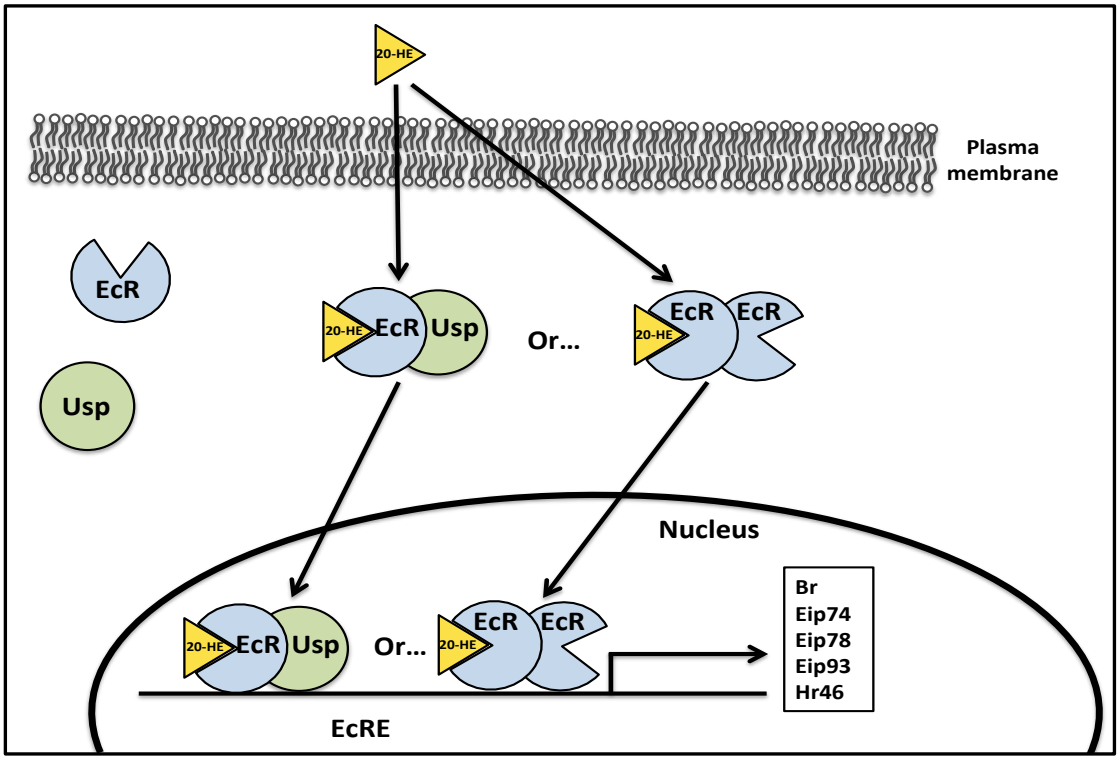


Figure 5.2 *Drosophila* EcR is activated by 20-hydroxyecdysone (20-HE), which promotes nuclear translocation to direct transcription. Circulating steroid hormone, 20-HE, interacts with the EcR receptor complex, which may be composed of an EcR-EcR homodimer or EcR-Usp heterodimer. Ligand-bound EcR complex translocates to the nucleus and binds to genomic elements containing EcRE sequences to direct transcriptional activity of a variety of genes. Target genes in development include *Br*, *Eip73* and *Hr46*.

Despite the relative wealth of research characterising the function of EcR signalling in developmental processes, very little is known regarding the role of this pathway in adult biology (Schwedes and Carney 2012). Intriguingly, in adults, levels of circulating 20-HE can be modulated by socio-sexual behaviour (Harshman, Loeb et al. 1999, Ishimoto, Sakai et al. 2009), leading to altered sexual behaviour in males (Ishimoto, Sakai et al. 2009, Ganter, Panaitiu et al. 2011) and changes in general accessory gland function (Wolfner, Partridge et al. 1997).

Additionally, recent data have implicated accessory gland SCs as a source of 20-HE biosynthesis in adult male *Drosophila*, as evidenced by their expression of the tyrosine kinase Torso (Hentze, Moeller et al. 2013), which is required for ecdysteroidogenesis in the prothoracic gland. Torso is the target receptor of PTTH, a circulating peptide responsible for initiating 20-HE production at the onset of metamorphosis (Rewitz, Yamanaka et al. 2009). Interestingly, administering 20-HE to adult male fruit flies has been shown to increase the synthesis of accessory gland specific proteins, including Acp95EF indicating a role for EcR signalling in gland function (Wolfner, Partridge et al. 1997).

Despite this, no reports exist characterising the expression of EcR in the accessory gland or indeed the role that EcR signalling plays in the cell biology of the AG epithelium. The data presented here characterise for the first time the pattern of expression of EcR in the AG epithelium and demonstrate a role for this receptor in regulating secondary cell growth. Additionally, I highlight a novel and surprising interaction between the EcR and BMP signalling pathways; they co-ordinate together to regulate both cell growth and migration in adult SCs.

5.1.3 *Drosophila* ecdysteroids are synthesised from cholesterol in a series of stepwise reactions and requires the expression of the Halloween genes

During development, the steroid hormone ecdysone (E) is synthesised by the prothoracic gland (Apger-McGlaughon and Wolfner 2013) and is further metabolised to the physiologically active hormone, 20-hydroxyecdysone (20-HE) in target tissues (Gilbert, Rybczynski et al. 2002, Huang, Warren et al. 2008). Steroid synthesis in the PG is initiated by the neurotransmitter prothoracicotropic hormone (PTTH), which is secreted by the larval brain, through its receptor Torso, a receptor tyrosine kinase (Rewitz, Yamanaka et al. 2009). Ecdysteroids are synthesised from cholesterol by a series of stepwise reactions mediated by cytochrome P450 enzymes encoded by a cluster of genes known as the Halloween genes (Huang, Warren et al. 2008). The Halloween genes expressed in the PG include *spook* (*spo*), *phantom* (*phm*), *disembodied* (*dib*) and *shadow* (*sad*), which sequentially catalyse cholesterol into ecdysone (Gilbert, Rybczynski et al. 2002, Rewitz, Rybczynski et al. 2006). In peripheral tissues, Shade (Shd) acts to convert ecdysone into 20-HE (Petryk, Warren et al. 2003) (summarised in Figure 5.3A).

5.1.4 SCs are a putative source of ecdysteroids in the adult male *Drosophila*

Although the mechanisms underpinning ecdysteroidogenesis during *Drosophila* development are well understood, the tissues that synthesise these hormones in adults, which lack PG structures, have historically remained poorly characterised (Schwedes and Carney 2012). However, recent research indicates that SCs may be an important site of ecdysteroid synthesis in the adult male; SCs express the PTTH receptor Torso, and knockdown of this receptor is associated with reduced transcript levels of the EcR target

genes *E74A*, *E74B* and *E75B*, providing good evidence that SCs are an important source of steroid hormones (Hentze, Moeller et al. 2013) (Figure 5.3B).

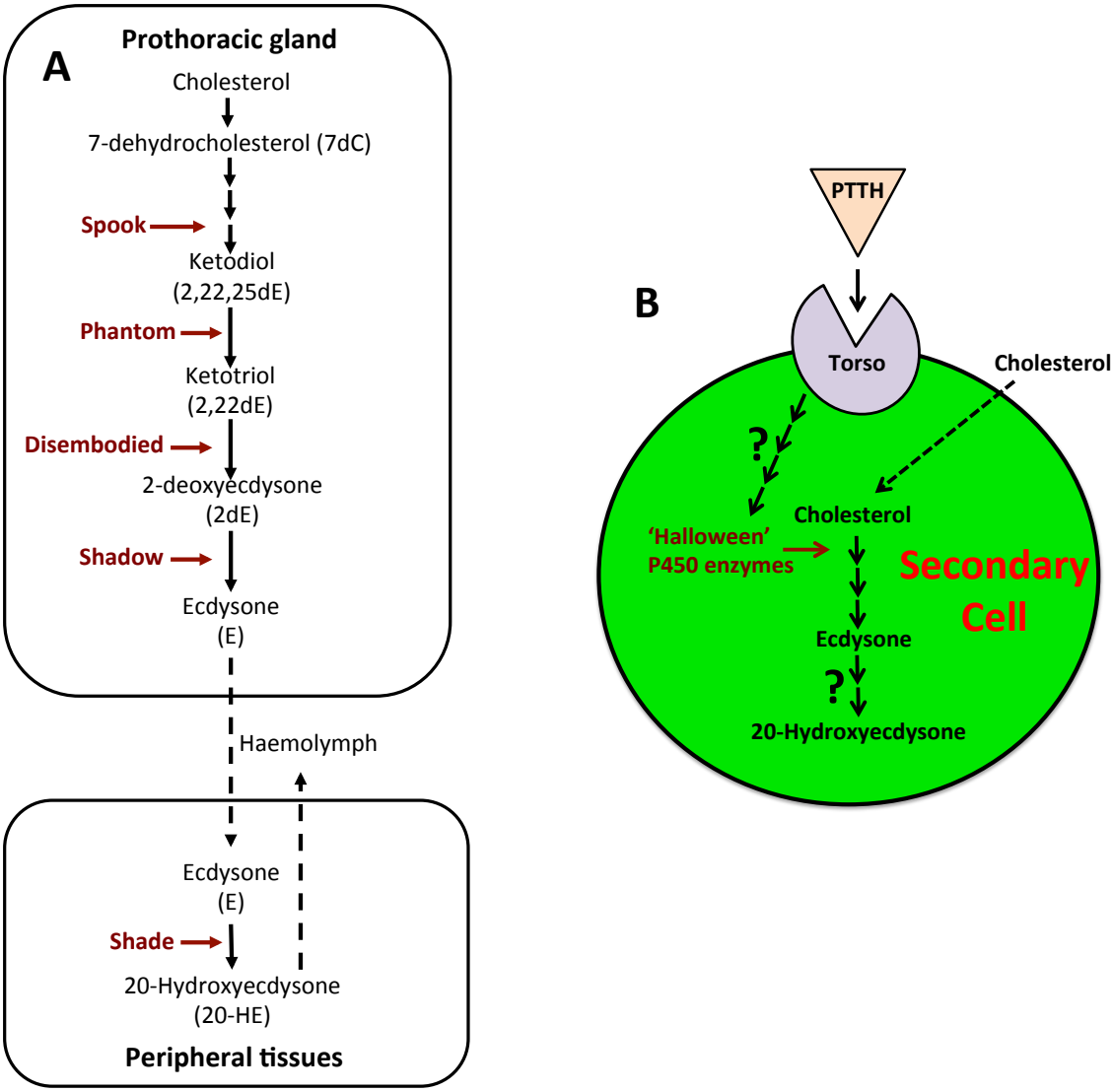


Figure 5.3 The biosynthetic pathway leading to 20-HE. (A) Ecdysone (E) is synthesised in the PG from cholesterol through a series of stepwise reactions catalysed by the P450 enzymes Spook (Spo), Phantom (Phm), Disembodied (Dib) and Shadow (Sad). Secreted E circulates in the haemolymph and is converted in target tissues into biologically active 20-HE by Shade (Shd). 20-HE may the be secreted back into the haemolymph or act directly in the cell it was synthesised in. (B) Recent evidence indicates that SCs are a probable site of ecdysteroid synthesis in the adult, with RNAi mediated knockdown of the PTTH receptor Torso leading to reduced expression of EcR signalling targets.

5.2 Results

5.2.1 The Ecdysone Receptor (EcR) is expressed exclusively by secondary cells in the adult AG epithelium

In order to mark and genetically manipulate SCs, I used the *esg*^{ts} F/O GAL4 system (Jiang, Patel et al. 2009, Leiblich, Marsden et al. 2012). As described in the previous chapter, this system allows secondary cell-specific expression of transgenes under UAS control; transgene expression is activated selectively in adulthood by shifting flies to the permissive temperature of 28.5°C at eclosion.

Staining with a pan-EcR antibody that cross-reacts with all three characterised EcR isoforms (Robinow, Talbot et al. 1993, Talbot, Swyryd et al. 1993), we observed EcR expression in the nuclei of SCs (Figure 5.4A, white arrows) and surrounding muscle cell nuclei (Figure 5.4B, red arrows) of the AG. SC staining was lost in cells expressing an RNAi targeted against all EcR isoforms (Bernardi, Romani et al. 2009) (figure 5.4C). Although antibodies that specifically detect the EcR-A and EcR-B1 isoforms are available (Talbot, Swyryd et al. 1993), I was unable to detect a consistent signal in the AG with them. In an alternative approach, expression of isoform-specific RNAi constructs (Colombani, Bianchini et al. 2005, Bernardi, Romani et al. 2009) was driven in SCs with *esg*^{ts} *F/O*, using a temperature shift in 3-day-old males, which leads to maintained GAL4 expression in only a subset of SCs, producing a mosaic. EcR-A-RNAi did not affect overall EcR levels even when expressed in all SCs from eclosion (Figure 5.4D, white arrows), but little, if any, EcR expression was detected in SCs expressing an EcR-B1-RNAi construct (Figure 5.4E, yellow arrows indicating expression in wild-type SCs), demonstrating that EcR-B1 is the major isoform produced by these cells.

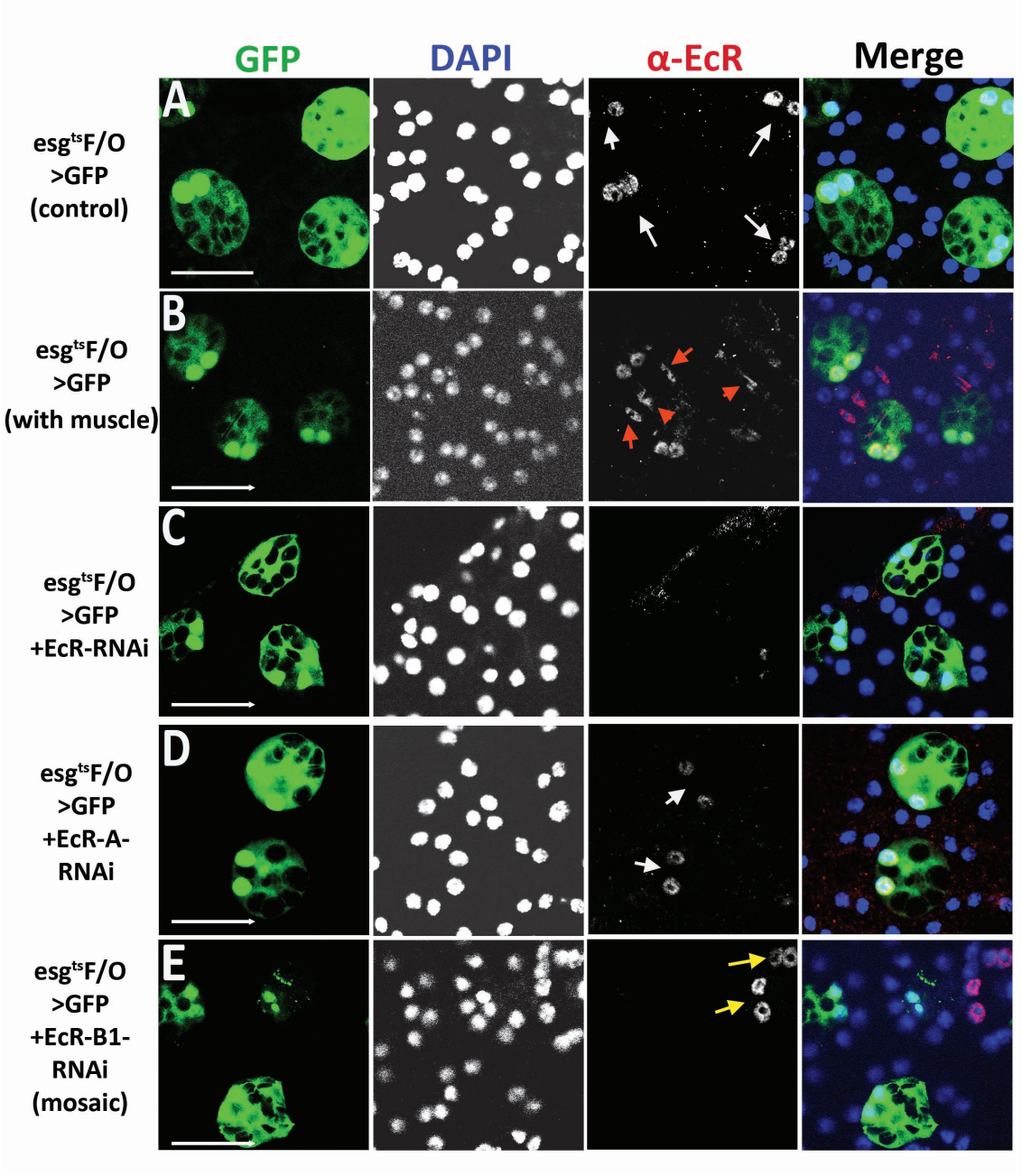


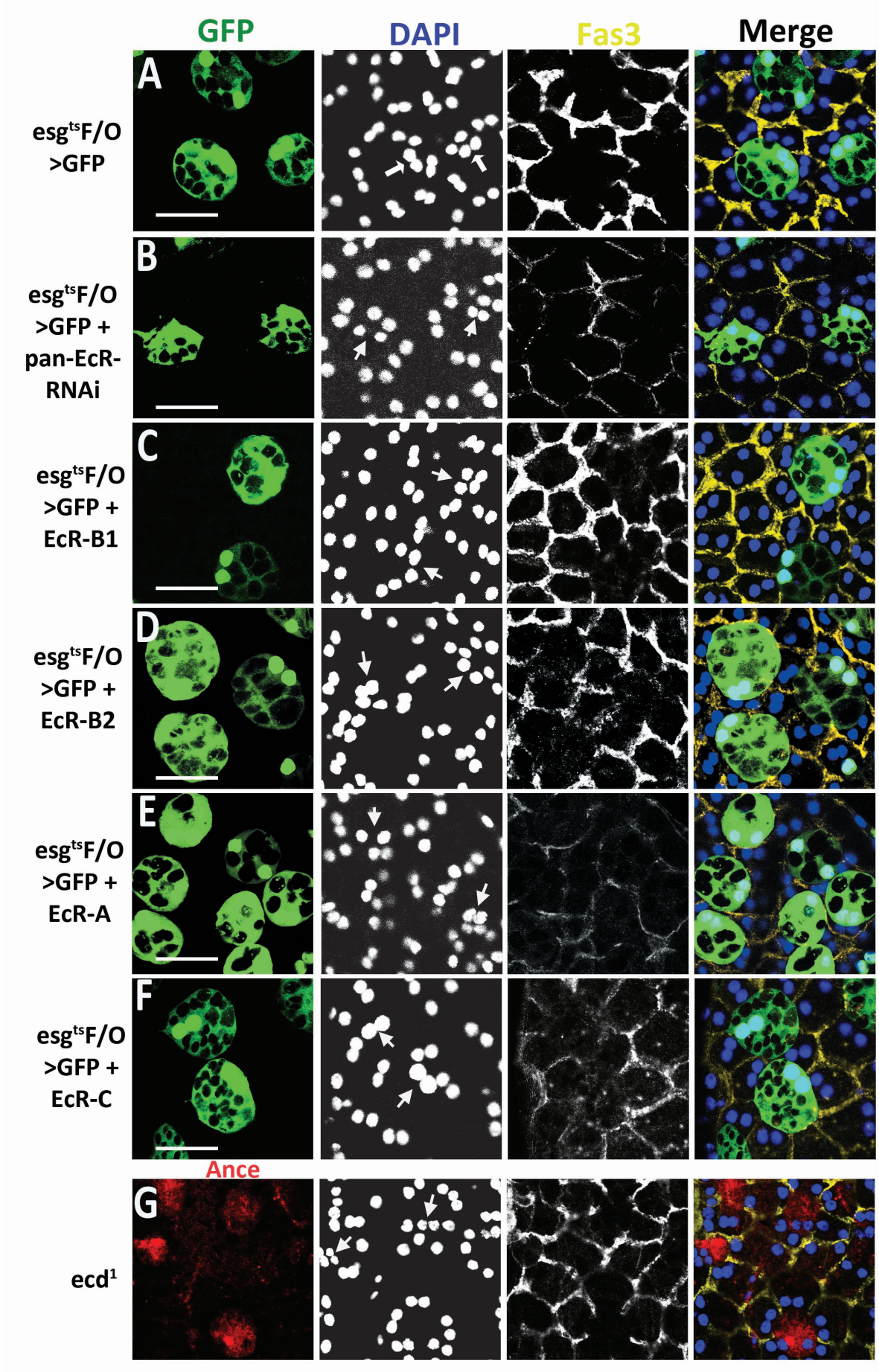
Figure 5.4: EcR is expressed in secondary cell and muscle cell nuclei of the adult male *Drosophila* accessory gland. A. Immunofluorescence analysis of 6d adult virgin male accessory glands using a pan-EcR antibody reveals expression of EcR in secondary cell nuclei (white arrows), but not in main cells. B. Additionally, EcR expression is observed in the flattened and elongated nuclei of muscle cells enshrouding the AG epithelium (red arrows). C. Expression of EcR in SCs is successfully abolished by expressing an RNAi transgene directed against all EcR isoforms selectively in SCs. D. RNAi directed specifically against EcR isoform A does not diminish SC expression of EcR (white arrows). E. Meanwhile, SC mosaic expression of an EcR-RNAi construct directed against the B1 isoform, reveals loss of EcR expression in SCs expressing the transgene (white arrows), whilst expression is maintained in adjacent, non-EcR-B1-RNAi-expressing SCs (yellow arrows). Taken together, these data indicate that the EcR-B1 isoform, and not EcR-A, is expressed in adult SC nuclei. Scale bars = 10µm.

5.2.2 Ecdysone and cell-autonomous EcR signalling is required for SC growth in adult AGs

To test the roles of the EcR in SCs, a previously characterised pan-EcR-RNAi construct (Colombani, Bianchini et al. 2005) was expressed in these cells using the *esg*^{ts}F/O system. As discussed in a previous chapter, SCs and their nuclei continue to grow in adults, unlike other cells of the AG, and this growth can be easily assessed by measuring the absolute size of SC nuclei or the relative size compared to surrounding main cell nuclei (Leiblich, Marsden et al. 2012). The relative and absolute growth of nuclei in SCs was blocked by expression of EcR-RNAi compared to controls (Figure 5.5B vs 5.5A, white arrows and Figure 5.5H), indicating that EcR expression is required for normal SC growth.

Steroid receptors typically modulate gene expression in the presence of their ligands, but in some contexts, it has been proposed that the unliganded EcR complex is repressive, and this repression is released by hormone (Schubiger, Carre et al. 2005). To test whether ecdysone is required to induce growth in SCs or whether it represses the growth-promoting properties of unliganded EcR, we employed a temperature-sensitive allele of ecdysoneless, *ecd*^{1} (Garen, Kauvar et al. 1977), a gene required for ecdysone synthesis in flies. When males were shifted to the non-permissive temperature (28.5^{0}C) directly after eclosion, SCs failed to grow, displaying significantly smaller SC nuclei compared to controls (Figure 5.5G versus 5.5A and Figure 5.5H), suggesting that 20-HE may be required for normal EcR-dependent SC growth.

Since EcR-B1 is the major isoform expressed by SCs, I examined the effect of overexpressing EcR-B1 in these cells. Unexpectedly, SC nuclear size was unaffected by this treatment (Figure 5.5C and 5.5H) or by overexpression of EcR-B2 (Figure 5.5D and 5.5H) or EcR-A (Figure 5.5E and 5.5H). When I analysed the expression of EcR protein in males overexpressing these EcR transgenes under SC-specific GAL4 control, the levels of EcR protein appeared unchanged compared to controls (see Figure 5.7), providing an explanation for the absence of phenotypic effects in SCs. These constructs do induce EcR overexpression in main cells of the AG, showing that they are capable of artificially elevating EcR levels in other cell types. Our data suggest that EcR levels are primarily controlled post-transcriptionally in SCs, so that GAL4-induced overexpression has no obvious effect on receptor levels or growth.


GFP
DAPI
Fas3
Merge
A
esg^tsF/O >GFP
B
esg^tsF/O >GFP + pan-EcR-RNAi
C
esg^tsF/O >GFP + EcR-B1
D
esg^tsF/O >GFP + EcR-B2
E
esg^tsF/O >GFP + EcR-A
F
esg^tsF/O >GFP + EcR-C
Ance
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ecd^1

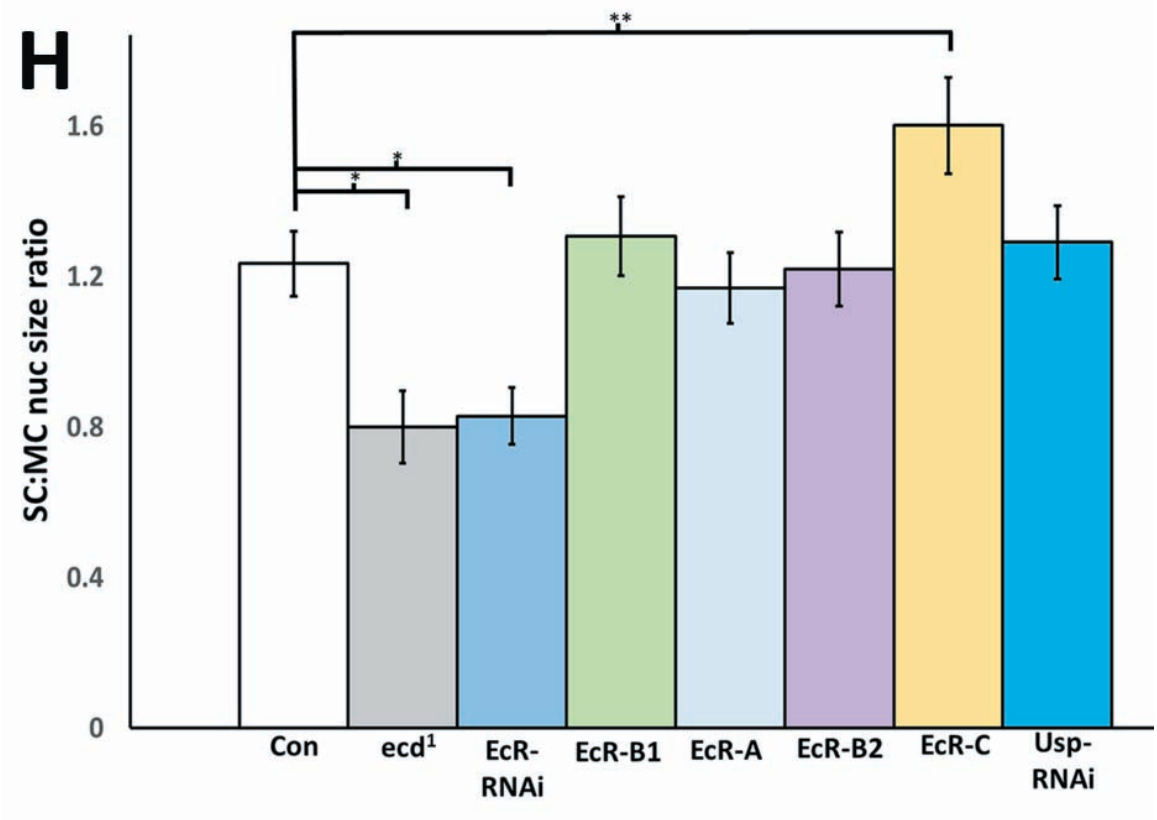


Figure 5.5: Both SC-specific expression of EcR and normal 20-HE production are required for adult SC growth. A, B. SC-specific RNAi knockdown of EcR results in restricted SC growth (B), as evidenced by reduction in SC nuclear size relative to controls (B; white arrows and chart in H). C-F. Over-expression of EcR isoforms B1,B2 and A, has no effect on nuclear size (H). G. Adult *ecd*^{1} mutants with reduced levels of 20-HE also display growth restriction, with small nuclei compared to controls (white arrows and H). SCs are also stained with an antibody against Angiotensin Converting Enzyme I (ANCE), which is highly expressed in SCs. Scale bars =10µm.

5.2.3 Levels of EcR protein are controlled by BMP signaling

As described in the previous chapter, my earlier work has shown that SC growth is positively regulated by BMP signalling (Leiblich et al, 2012). Since EcR signalling is also required for normal SC growth, I investigated the effect of BMP signalling on EcR protein expression. SC-specific expression of a constitutively active form of the Type I BMP receptor Thick veins

(*Tkv*^{act}) induced increased levels of EcR protein, which was primarily localised to the nucleus (compare Figures 5.6B with 5.6A, white arrows), although cytosolic EcR was also detected (red arrows in Fig. 5.6B). When BMP signalling was reduced by inducing, in mosaics, SC-specific knockdown of *Med*, a downstream co-Smad in the BMP signalling pathway, virtually no EcR protein was observed in knockdown cells, while staining was still present in non-expressing cells (Figure 5.6C). Taken together, these data indicate that BMP signalling is a key determinant of EcR protein levels in SCs.

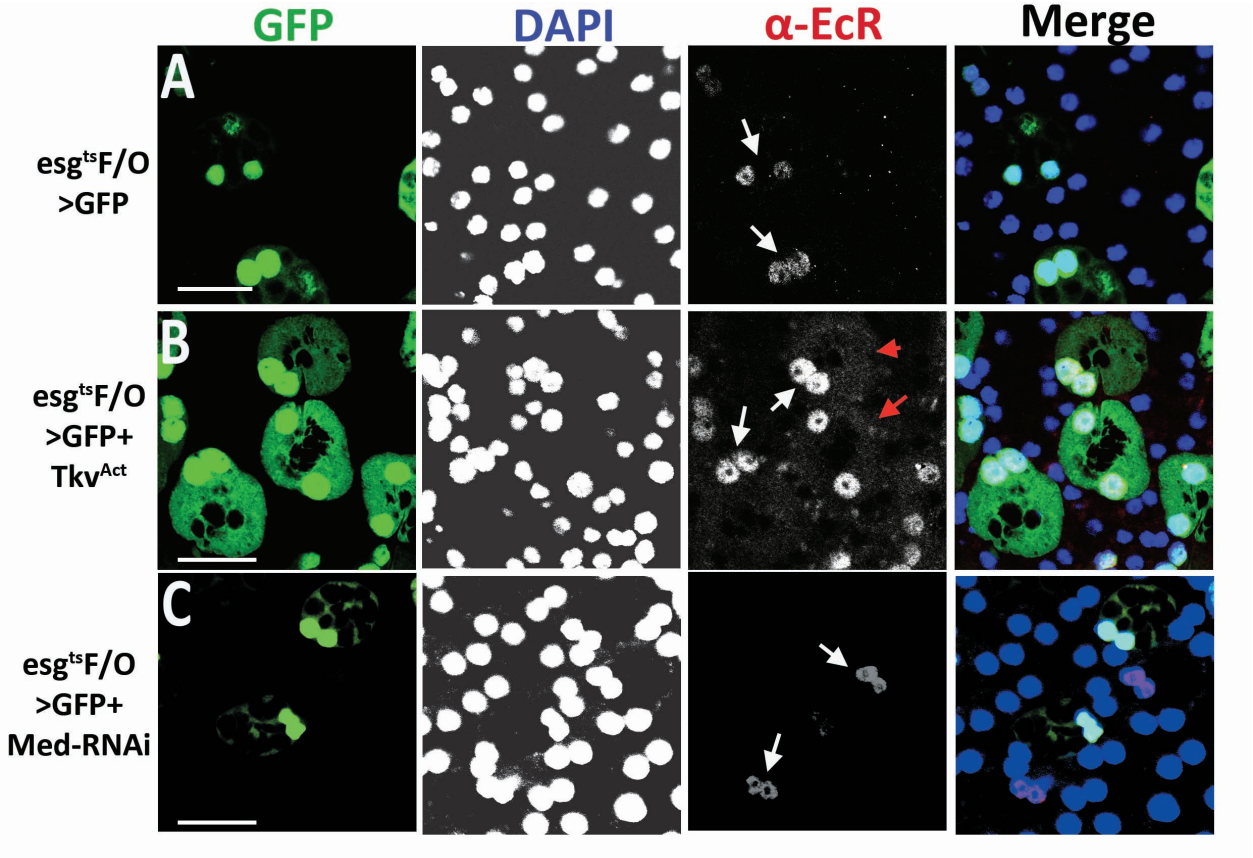


Figure 5.6: BMP signalling activity controls levels of EcR protein in SCs from 6d-old adult males. A, B. Inducing high BMP signalling activity in SCs by expressing Tkv^{act}, a constitutively active form of the BMP type I receptor Tkv, increases levels of EcR protein seen in SC nuclei (B, white arrows) and cytoplasm (red arrows) compared to controls (A). Loss of EcR protein is seen in SCs where the critical co-Smad in *Drosophila* BMP signalling, Medea, is knocked down using RNAi, whilst adjacent non-transgene-expressing SCs maintain EcR levels (5C, white arrows). Scale bars =10µm.

5.2.4 BMP-signalling controls SC EcR protein levels post-transcriptionally

Surprisingly, over-expression of any of the EcR isforms (B1, B2 and A) does not increase levels of EcR protein in SCs as detected by antibody staining (Figure 5.7B, C and D, white arrows, compare with control in Figure 5.7A), implying rapid turnover of expressed EcR protein. In light of this, I sought to test whether BMP signalling regulates EcR levels in a post-transcriptional manner. To do this, I over-expressed each of the EcR isoforms (under UAS control, rather than under their endogenous promoter) in combination with *Tkv*^{act} and analysed the effect on EcR levels by antibody staining. Interestingly, over-expressing any of the EcR isoforms in a background of elevated BMP signalling activity massively increased the levels of EcR protein in SCs, with elevated levels observed in both nuclear and cytoplasmic compartments (Figures 5.7E, F and G). Since over-expression of EcR isoforms in isolation produced no effect on overall EcR protein levels, whilst over-expression in combination with elevated BMP signalling results in much higher EcR protein levels, this indicates that BMP signalling acts post-transcriptionally to control EcR levels in SCs. The different EcR overexpression constructs only share sequences in their open reading frames, suggesting that either these shared sequences contribute to BMP-dependent regulation of transcript levels or the control is probably exerted post-translationally.

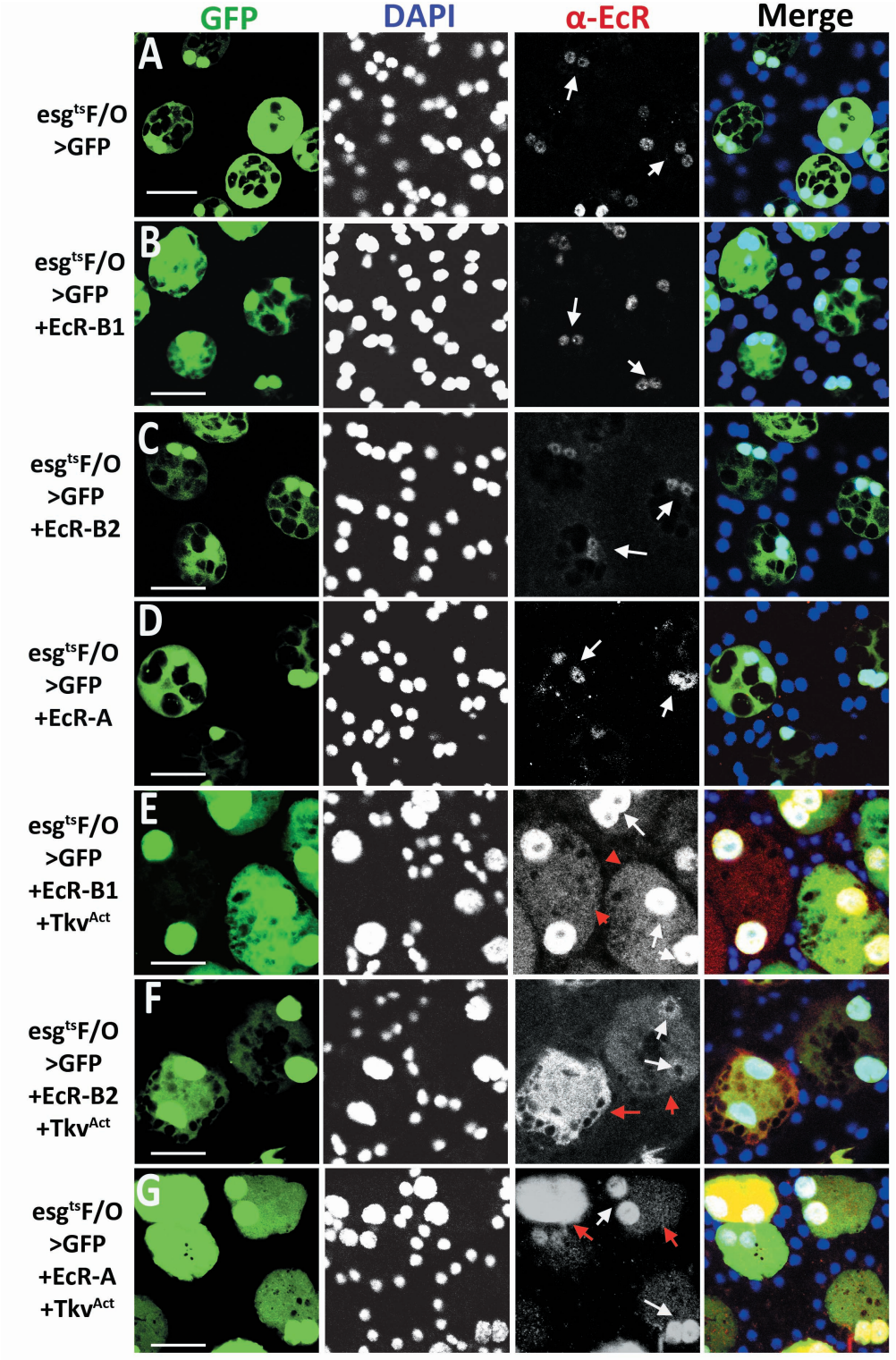


Figure 5.7: EcR protein levels are regulated post-transcriptionally by BMP signalling activity. A-D. Over-expression of each of the EcR isoforms, -B1, -B2 and –A, under UAS control, does not alter levels of EcR protein compared to controls (compare B-D with A, white arrows indicate nuclear EcR). E-G. Meanwhile, over-expression of these isoforms in combination with Tkv^{act} results in massive increases in EcR protein levels, with high levels observed in both the nuclei (white arrows) and cytoplasm (red arrows) of 6d adult SCs. The elevation in EcR levels is much greater than EcR levels in SCs where Tkv^{act} is expressed alone (compare E-G with Figure 5B). Scale bars =10µm.

5.2.4 Some aspects of BMP-dependent EcR control are mediated by sequences in the unique N-terminal AF1 domains of different EcR protein isoforms

Previous *in vitro* studies have revealed that when the *Drosophila* EcR-A and EcR-B1 isoforms are expressed in Chinese Hamster Ovary (CHO) cells, the different N-terminal domains (NTDs) of these proteins, which include the AF1 domain, one of the two transcriptional activation domains in these receptors, partially destabilise these proteins through a ubiquitination mechanism (Schauer, Burster et al. 2012). In this same system, the NTD can interact with the ligand-binding domain of the EcR in the absence of ligand (Tremmel, Schaefer et al. 2011). Although these experiments were performed in a heterologous system and did not reveal similar regulation of the EcR-B2 isoform, which has a much shorter AF1 domain, I tested whether the NTD plays a role in BMP-dependent control of EcR protein levels by expressing an artificial isoform of the protein, EcR-C, that has no NTD, only containing sequences common to all isoforms, and typically displays lower activity than normal isoforms in developmental contexts (Cherbas, Hu et al. 2003). Unlike the normal isoforms of EcR, overexpression of this transgene promoted growth of SCs (compare Figure 5.5F with the control in Figure 5.5A, and with over-expression of the native isoforms in Figures 5.5C-E; white arrows indicate nuclear expression of EcR; Figure 5.5H).

Additionally, over-expression of EcR-C results in much higher levels of EcR protein in SCs than in controls. Crucially, greater protein levels are observed than when the native isoforms are over-expressed in SCs, with strong EcR signal observed in the nuclei and cytoplasm of SCs (contrast Figure 5.8B with Figures 5.8B-D), implying that truncation of the NTD increases stability of the final EcR protein. Co-expressing EcR-C with Tkv^{Act} altered subcellular localisation further, with the strongest EcR signal detected in the nucleus (Figure

5.8C). These findings indicate that BMP signalling activity partly regulates EcR protein stability via a genetic interaction with its NTD, but that other mechanisms are also important in promoting the functional synergy between these two signals.

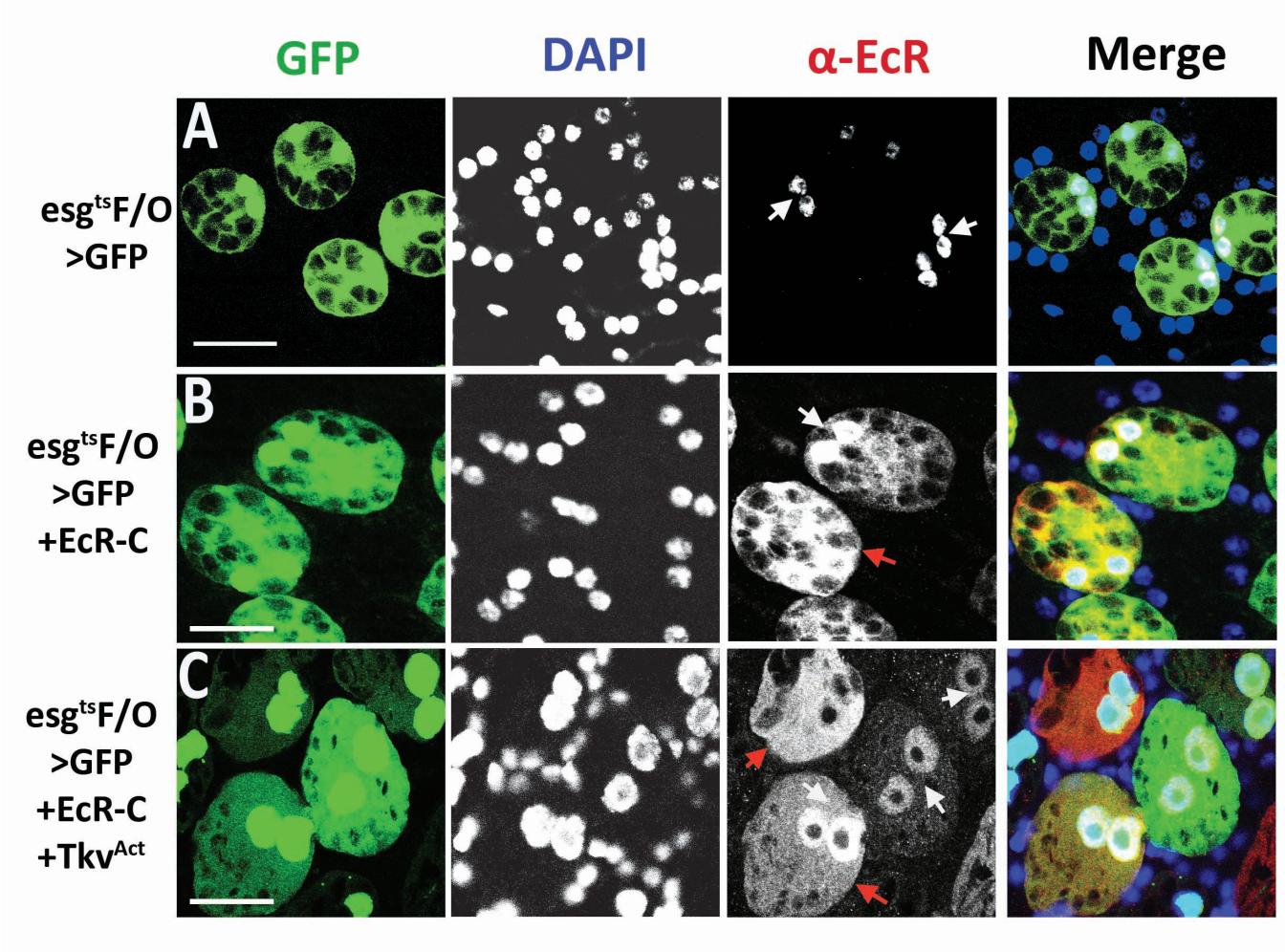


Figure 5.8: Truncation of the NTD increases the stability of EcR protein in SCs. A, B. Expression of EcR-C, a construct lacking the NTD of EcR, results in high levels of nuclear (B, white arrow) and cytosolic (red arrow) EcR as detected by antibody staining compared to control (A), and overexpression of normal EcR isoforms (Figures 6B-D). C. Co-expression of EcR-C with Tkv^{act} also results in particularly high nuclear (white arrows) and lower cytosolic (red arrows) EcR levels. Note the large nuclei of this combination genotype, indicating synergistic growth effects of BMP and EcR signalling (see Figure 5.8). Scale bars =10µm.

5.2.5 EcR and BMP signalling interact to synergistically modulate SC growth

Interestingly, I observed that, in addition to stabilising EcR protein, co-expression of the EcR isoforms with *Tkv*^{Act} appeared to drive massive growth of SC nuclei (note the huge SC nuclei in Figures 5.7E-G and 5.8C). To characterise the effects on growth of expressing EcR isoforms in the context of high BMP signalling activity, employing the methodology described in the previous chapter, I measured the SC:MC nuclear size ratio as a proxy for estimating cell growth. As demonstrated earlier (Figure 5.6H), there was no significant difference in the SC:MC nuclear ratio between controls (Figure 5.10A) and SCs over-expressing EcR-B1, EcR-B2 and EcR-A (Figures 5.10C, E, G respectively). As reported in the previous chapter, the SC:MC nuclear ratio of SCs expressing *Tkv*^{act} was significantly larger than controls (2.03 vs 1.21, $p<0.001$, compare Figures 5.10A and 5.10B; see chart in figure 5.9). Expressing the NTD-deficient EcR-C construct also promoted SC growth with SC:MC nuclear ratio of 1.85 vs 1.21 in controls, $p<0.001$ (compare Figures 5.10A and 5.10I, see chart in Figure 5.9).

Co-expression of each of the EcR isoforms with *Tkv*^{act} promoted massive SC growth. The SC:MC ratio of SCs co-expressing EcR-B1 and *Tkv*^{act} (Figure 5.10D) was 4.11, for SCs co-expressing EcR-B2 and *Tkv*^{Act} (Figure 5.10F), this figure was 4.05 whilst SCs expressing EcR-A and *Tkv*^{Act} (Figure 5.10H) displayed a SC:MC ratio of 3.97, compared to a value of 1.21 for controls ($p<0.0001$ for all genotypes compared to controls, see Figure 5.9). Additionally, the SC:MC nuclear ratio for all of the EcR/*Tkv*^{Act} combination genotypes were significantly larger than the SC:MC nuclear ratio of SCs expressing *Tkv*^{Act} alone ($p<0.0001$). Of interest, co-expressing EcR-C and *Tkv*^{Act} in SCs also drove massive SC growth (Figure 5.10J), with these cells possessing an SC:MC nuclear ratio (4.21) significantly larger than controls (1.21), SCs

expressing just *Tkv*^{Act} (2.03) or EcR-C alone (1.85), p<0.001 for all comparisons. These data imply that mechanisms independent of the NTD are also at least partly responsible for the synergistic growth regulatory effects of BMP- and EcR-signalling.

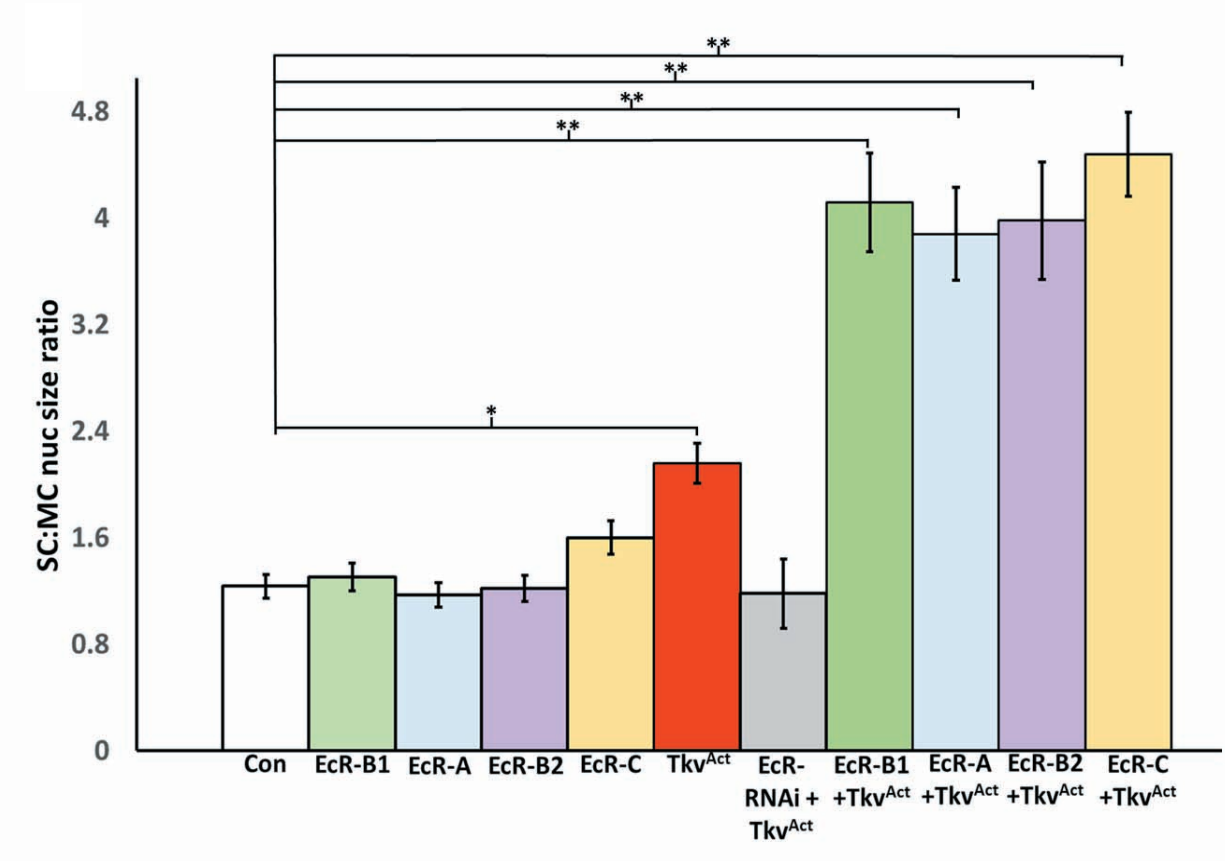


Figure 5.9: EcR- and BMP-signalling pathways interact synergistically to promote SC growth. No increase in SC:MC nuclear ratio is observed in SCs over-expressing the EcR-B1,-B2 or –A isoforms at 6 days. However, SCs co-expressing these isoforms with Tkv^{Act} exhibit massive increases in SC:MC nuclear ratio. *p<0.001, **p<0.0001 one-way ANOVA.

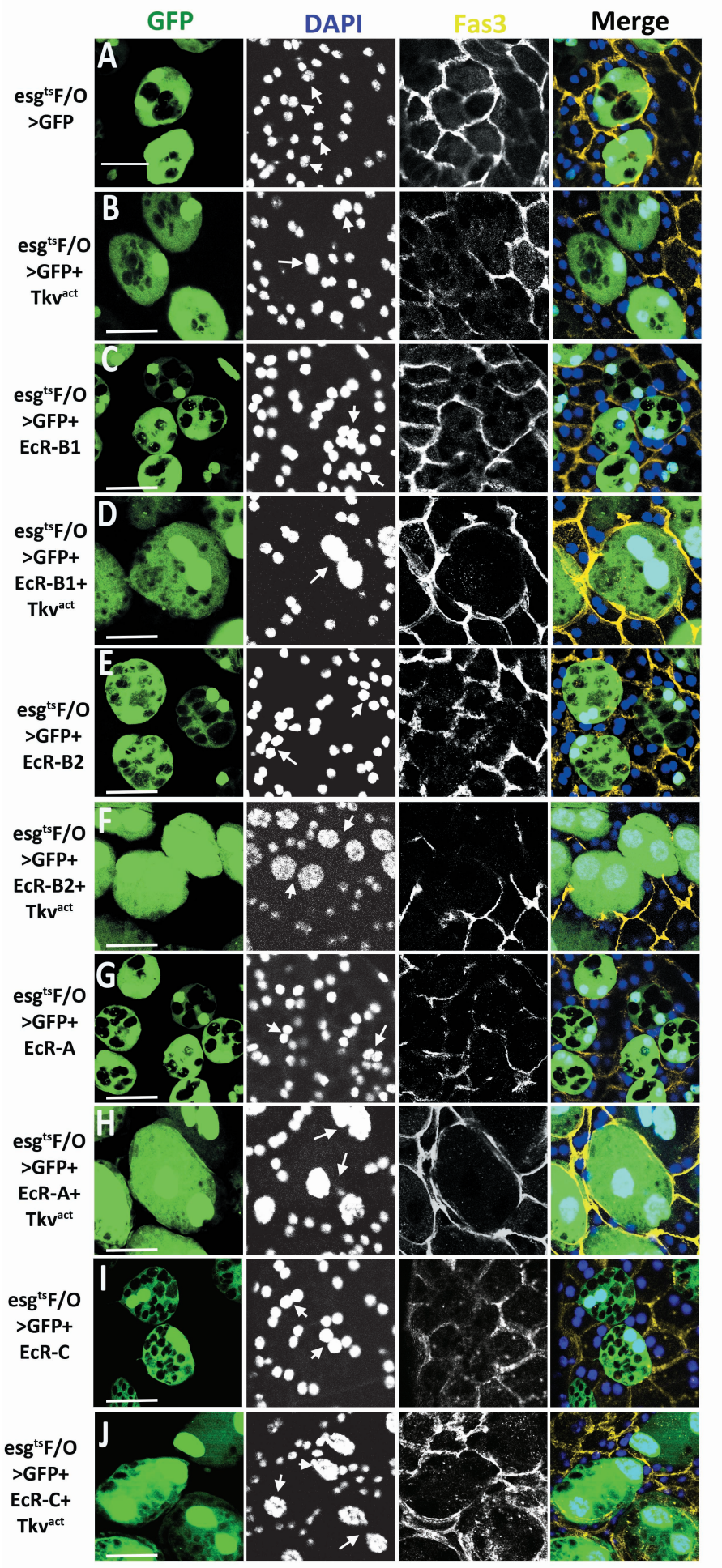


Figure 5.10: EcR- and BMP-signalling pathways interact synergistically to promote SC growth. No effects on growth are observed when over-expressing any of the EcR isoforms, EcR-B1 (C), -B2 (E), and –A (G) in isolation (nuclei indicated by white arrows). Co-expressing these isoforms with Tkv^{Act} promotes massive SC growth (EcR-B1+Tkv^{Act}, D; EcR-B2+Tkv^{Act}, F; and EcR-A+Tkv^{Act}, H), larger than controls (A) and expression of Tkv^{Act} alone (B). Co-expression of EcR-C with Tkv^{Act} also produced synergistic effects on growth (J) compared to expressing Tkv^{Act} or EcR-C (I) alone. Scale bars =10µm.

5.2.6 The synergistic interaction between EcR and BMP signalling pathways is specific to SCs

To test whether the observed genetic interaction between the EcR and BMP signalling pathways was specific to secondary cells in the AG, I used the main cell-specific GAL4 driver, *Acp26Aa*-GAL4 (Chapman, Bangham et al. 2003) to drive expression of Tkv^{act} and EcR isoforms (Figures 5.11B, C, E and G). When GAL4/UAS-induced expression of all three native EcR isoforms (Figures 5.11C,E and G) and the synthetic NTD-deficient EcR-C (Figure 5.11I) construct was induced in main cells, antibody staining using a pan-EcR antibody demonstrated nuclear expression of the constructs in main cell nuclei, which was not observed in controls (Figure 5.11A). In controls and MCs expressing Tkv^{Act}, EcR expression was only seen in SCs, where expression was restricted to the nucleus (Figures 5.11A and B). Over-expression of any of the EcR isoforms or EcR-C in MCs did not affect cell growth (Figure 5.12A-K). Furthermore, unlike in SCs, co-expression of the EcR isoforms or EcR-C with the activated BMP receptor, Tkv^{Act}, in MCs did not increase the stability of the EcR (Figures 5.11D, F, H and J). Again, unlike in SCs, no growth effects were observed in MCs co-expressing Tkv^{Act} with any of the normal EcR isoforms or EcR-C (Figure 5.12K).

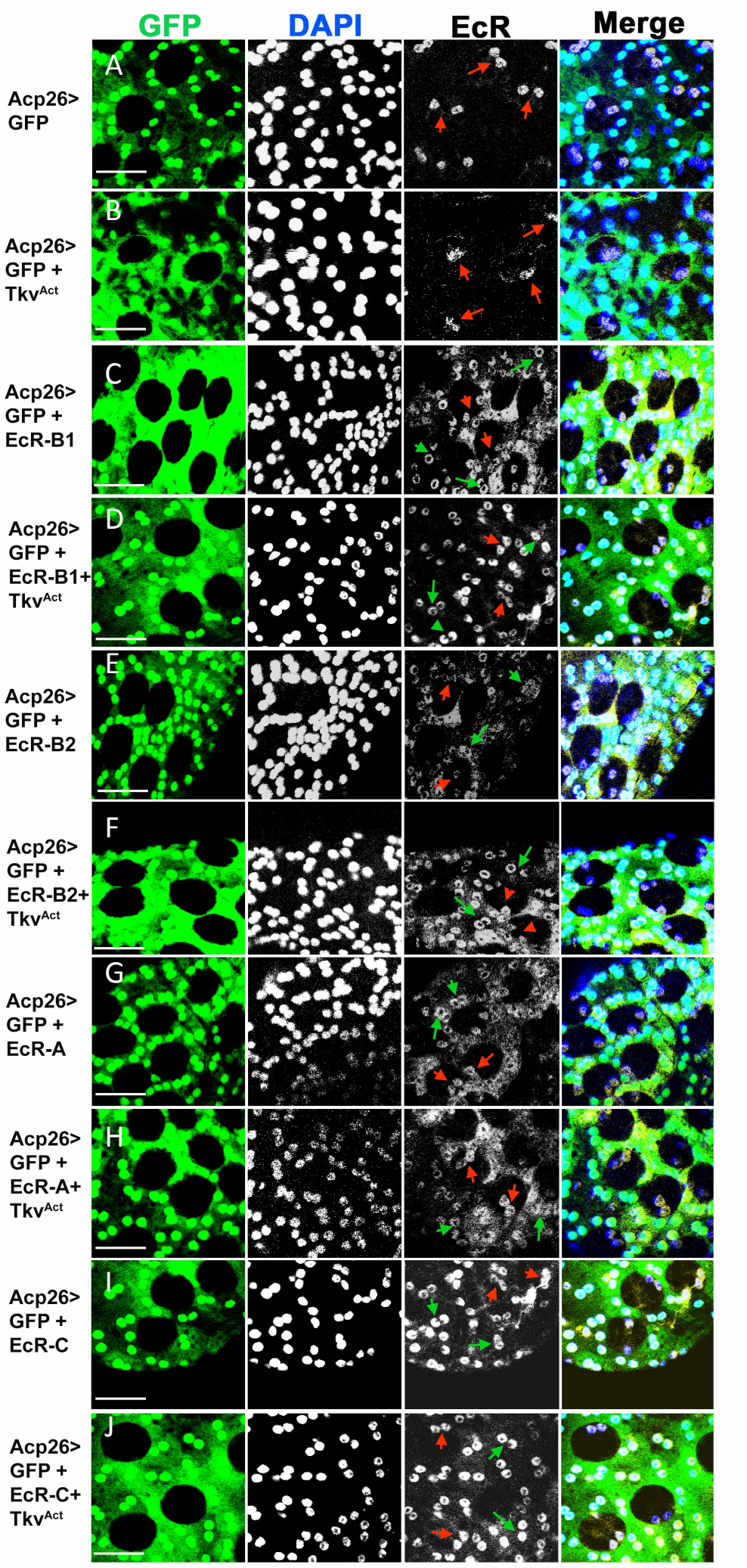
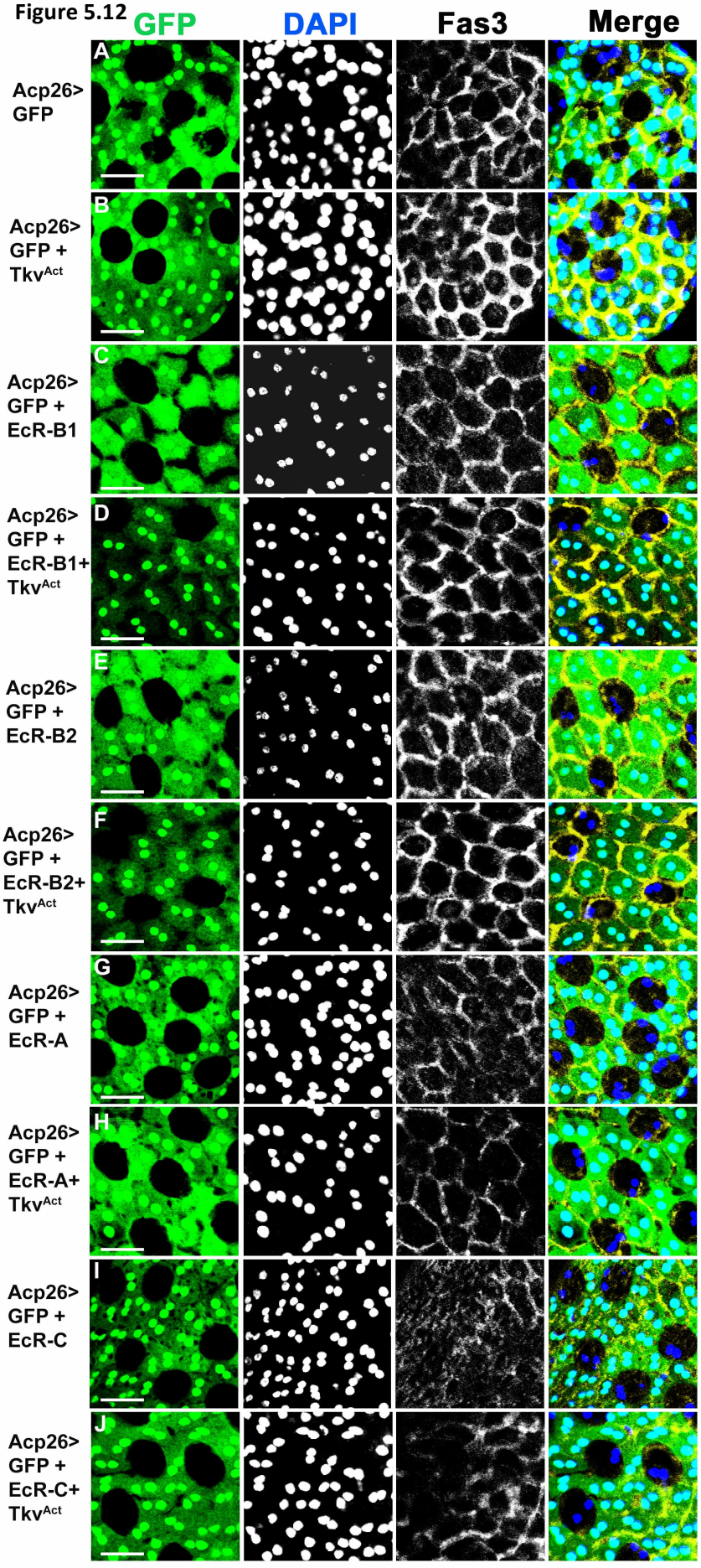


Figure 5.11: BMP signalling does not stabilise EcR in adult male accessory gland main cells. EcR protein was only detected in the nuclei of SCs, and not MCs, in glands where either GFP (A, red arrows) or GFP+Tkv^{Act} (B, red arrows) expression was driven exclusively in MCs using the Acp26Aa-GAL4/Gal80^{ts} driver. In contrast, EcR protein was detected in the nuclei and cytoplasm of MCs expressing EcR-B1 (C), EcR-B2 (E) and EcR-A (G), whilst nuclear expression in SCs was maintained. Co-expressing Tkv^{Act} in MCs with any of the EcR isoforms did not alter levels of EcR detected in MCs (D, F and H, green arrows = MC EcR, red arrows = SC EcR). Relatively greater levels of cytosolic vs nuclear EcR protein were evident in MCs expressing the NTD-deficient EcR-C transgene (I). Co-expression with Tkv^{Act} did not alter protein levels. Scale bars = 15µm.

Figure 5.12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B
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C
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Acp26> GFP + EcR-B1+ TkvAct
E
Acp26> GFP + EcR-B2
F
Acp26> GFP + EcR-B2+ TkvAct
G
Acp26> GFP + EcR-A
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I
Acp26> GFP + EcR-C
J
Acp26> GFP + EcR-C+ TkvAct

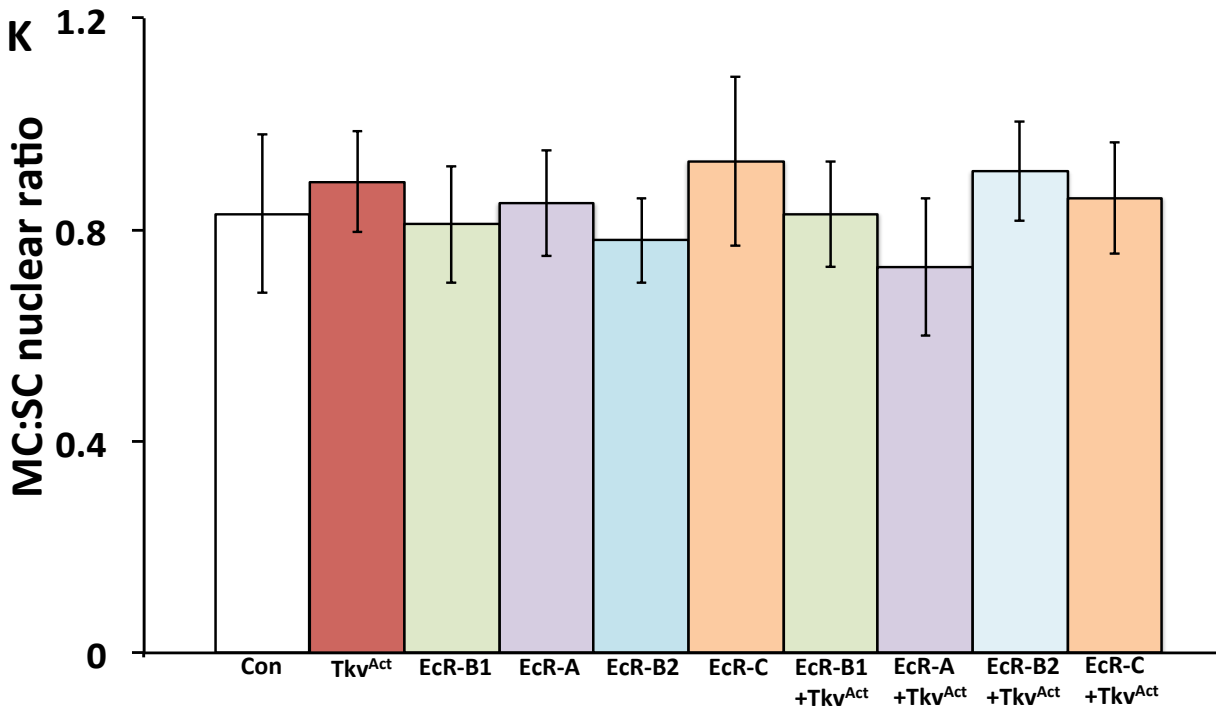
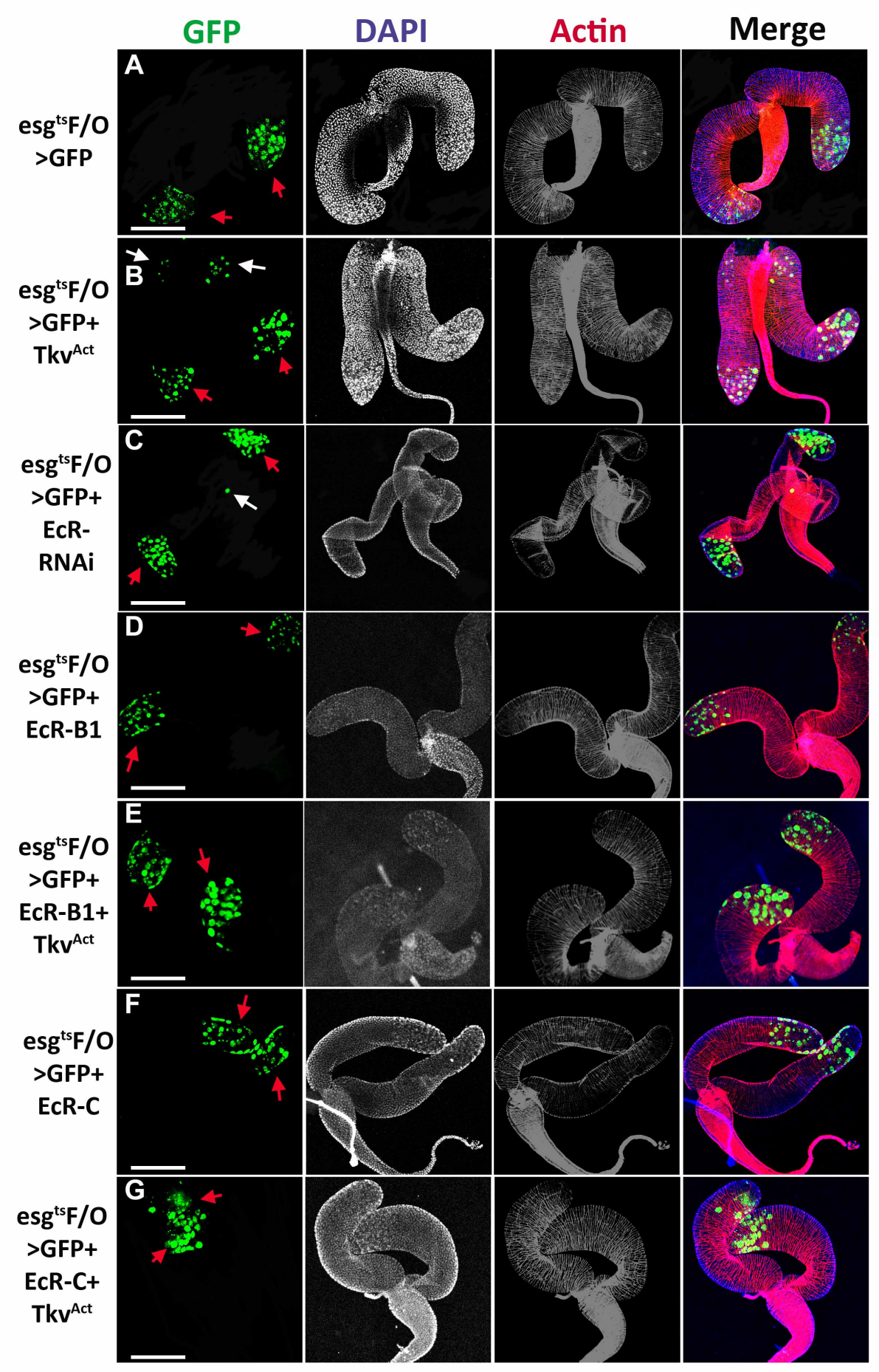


Figure 5.12: Manipulating BMP and EcR signalling in main cells does not alter cell growth. No significant changes in MC growth, as measured by calculating the MC:SC nuclear size ratio, were observed as a result of *Acp26Aa*-GAL4/GAL80^{ts} driven MC expression of *Tkv*^{Act} (B), any of the three EcR isoforms (C,E and G), co-expression of *Tkv*^{Act} with the three EcR isoforms (D, F and H) or expression of EcR-C, alone (I) or together with *Tkv*^{Act} (J). Cell outlines were visualised by staining with anti-Fas3. Chart of MC:SC nuclear ratios for different genetic backgrounds, demonstrating no statistical difference in nuclear ratio across different genotypes (K). Scale bars= 15µm.

5.2.7 EcR and BMP signalling function antagonistically to control SC delamination and migration

As demonstrated in the previous chapter, BMP signalling not only drives SC growth, but also stimulates apical delamination and proximal migration of a subset of these cells (Leiblich, Marsden et al. 2012, Ito, Ueda et al. 2014). Therefore, given the SC-specific interaction between BMP and EcR signalling described above, I hypothesised that this process might also be modulated by EcR signalling. When EcR-B1 was co-expressed with Tkv^{act}, I found that the number of SCs that delaminated and migrated proximally after 16 days in virgin males was significantly reduced compared to flies expressing only Tkv^{act} in SCs. (Figure 15.3E versus Figure 5.13B, Figure 5.13H), Indeed, when Tkv^{act} was co-expressed with EcR-C (Figure 5.13G), which can stimulate growth even in the absence of Tkv^{Act} expression, all migration was suppressed and SCs remained embedded within the distal AG epithelium (Figure 5.13H). Although knockdown of *EcR* expression by RNAi reduced cell size, some of these cells migrated proximally in virgin males, indicating that EcR signalling normally plays a role in preventing premature SC migration. Therefore, not only does BMP signalling tightly control EcR protein levels to determine SC growth rates, but this interaction also allows the EcR to antagonise the effects of BMP signalling on SC migration. This potentially adds a behaviourally modified endocrine input into the regulation of SC transfer from males to females.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D
esg^ts F/O >GFP+ EcR-B1
E
esg^ts F/O >GFP+ EcR-B1+ Tkv^Act
F
esg^ts F/O >GFP+ EcR-C
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esg^ts F/O >GFP+ EcR-C+ Tkv^Act

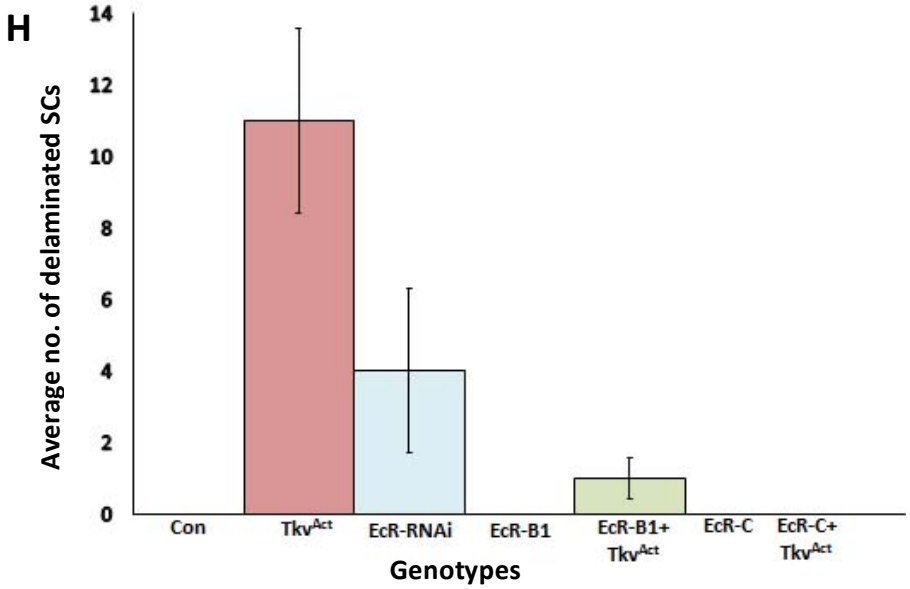
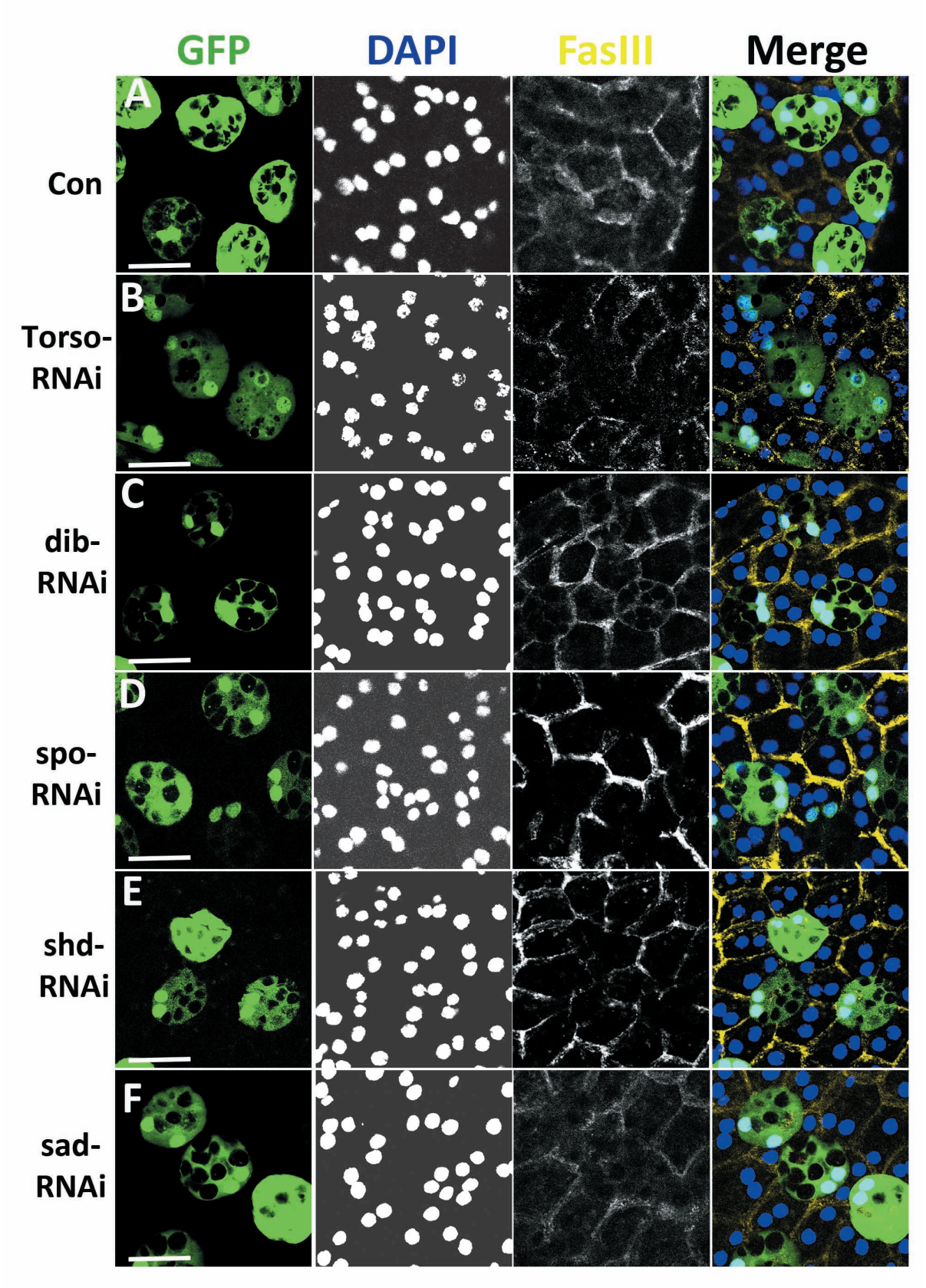


Figure 5.13: EcR antagonises the pro-cell-migratory effects of BMP signalling in adult SCs. In 16d control virgin males, spontaneous migration of SCs is not normally observed (A) (no proximally located SCs observed in 15 males), compared to an average of 11 cells (+/-3) in SCs expressing Tkv^{Act} (B) and 4 cells (+/-1) observed proximally in SCs where *EcR* knockdown is induced with RNAi (C). Over-expression of EcR-B1 (D) has no effect on SC migration (no cells in 15 animals), whilst co-expression of EcR-B1 with Tkv^{Act} (E) results in an average of 1 cell (+/- 0.52) per gland observed proximally. No migratory cells were observed in SCs expressing EcR-C (F) or in SCs co-expressing Tkv^{Act} with EcR-C (G). Data summarised in H (n = 15 for all conditions) (Scale bar = 200μm)

5.2.8 EcR signalling regulates SC growth in paracrine or autocrine manner

Given the postulated role that SCs appear to play in steroid hormone synthesis (see section 5.1.4), I tested whether knockdown of ecdysteroid biosynthetic genes would phenocopy EcR knockdown and lead to defects in SC growth. This was achieved by expressing RNAi constructs directed against the PTTH receptor, Torso (Rewitz, Yamanaka et al. 2009) and the Halloween genes spo , phm, dib, sad and shd. As earlier, measurement of SC:MC nuclear ratio is used as a proxy measure for cell size and growth. Knockdown of the P450 enzymes *spo* and *shd* was associated with a significant decrease in SC:MC nuclear ratio. SCs expressing *spo*-RNAi (Figure 5.14D) had a SC:MC ratio of 0.99+/-0.05 compared to 1.22+/-0.062 for controls; the ratio for SCs expressing *shd*-RNAi (Figure 5.14E) was 0.97 +/- 0.047 ($P<0.001$, n = 5 glands, one-way ANOVA). There were no statistically significant effects on SC growth with knockdown of any of the biosynthetic pathway components *Torso*, *dib* or *sad*.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sad-
RNAi

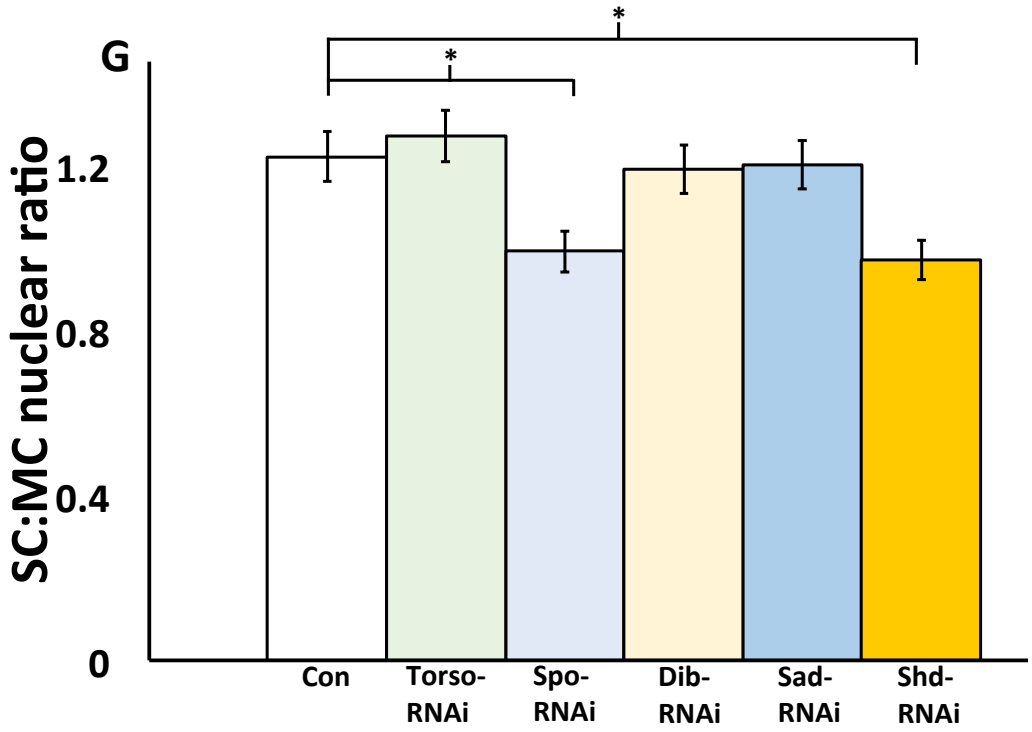


Figure 5.14 RNAi mediated knockdown of the ecdysteroidogenic Halloween genes *spo* (D) and *shd* (E) in SCs restricts SC growth as evidenced by a reduced SC:MC ratio (G). Conversely, neither knockdown of the PTTH receptor Torso (B) nor knockdown of either of the Halloween genes d*ib* (C) and *sad* (F) have any effect on SC growth (G).*=p<0.01, one-way ANOVA. Scale bar = 10 µm

5.2.9 The Rbf/E2F1 cell cycle-regulatory axis controls EcR levels

Loss of the tumour suppressor Rb has been implicated in ligand–independent activity of AR signalling in prostate cancer, a mechanism leading to castrate-resistant prostate cancer (CRPC) (Sharma, Yeow et al. 2010, Dean and Knudsen 2013). Interestingly, *Rb* knockdown in a prostate cancer cell line, LNCaP, results in elevated levels of AR protein (Sharma, Yeow et al. 2010). It is postulated that Rb loss promotes E2F1 recruitment to the AR-regulatory locus, in turn driving transcription of AR (Sharma, Yeow et al. 2010).

I sought to test whether a similar regulatory relationship exists between the Rbf/E2F1 axis and EcR expression in adult SCs, by performing genetic manipulations using the *esg*^{ts} F/O

driver to up- or down-regulate Rbf and E2F1. Normal activity of E2F1 requires expression of the co-transcription factor, Dimerisation partner (Dp), thus up-regulation of E2F1 also necessitates up-regulation of Dp (Neufeld, de la Cruz et al. 1998).

Next, expression of a constitutively active form of *Rbf*, Rbf^{CA} was driven in SCs. This construct contains mutations in four putative phosphorylation sites, where proline or threonine residues have been substituted for alanine. These mutations result in a form of *Rbf* that no longer responds to phosphorylation, and therefore functions permanently in its dephosphorylated, activated state. This resulted in markedly reduced levels of EcR in SCs (Figure 5.15, compare C to control, A; white arrows). Conversely, RNAi-mediated knockdown of *Rbf* (Figure 5.15E, white arrows) resulted in much higher levels of EcR compared to controls (Figure 5.15A), with a strong cytosolic signal observed in addition to nuclear expression.

Similarly, over-expression of E2F1 with Dp induced high levels of EcR expression in SCs (Figure 5.15F, white arrows), with signal observed in the cytoplasm as in addition to the nucleus. Conversely, RNAi-mediated knockdown of E2F1 resulted in weak EcR signal, with expression restricted to the nucleus (Figure 5.15G).

Taken together, these data indicate that the Rbf/E2F1 axis regulates the level of EcR expression in adult SCs. Elevated activity of E2F1, either by over-expression of E2F1 or by loss of *Rbf*, results in a greatly enhanced EcR antibody-detected signal. This neatly echoes observations in human prostate cancer cells, where loss of Rb leads to increased AR protein levels (Sharma, Yeow et al. 2010). Furthermore, I have shown here that reducing E2F1 activity, either directly by RNAi knockdown or indirectly by expressing Rbf^{CA}, results in

reduced EcR levels in SCs, confirming the regulatory role that the Rbf/E2F1 axis plays on EcR levels.

Further observations arose during the course of these experiments that imply important and unexpected cell-biological roles for Rbf/E2F1 activity in SCs. In addition to modulating EcR levels, manipulating Rbf/E2F1 activity in SCs appeared to have major effects on cell growth, for example, the large cells and nuclei in SCs expressing *Rbf*-RNAi (Figure 5.15E) or E2F1/Dp (Figure 5.15F). Even more surprisingly, expression of Rbf^{CA} was associated with the appearance of a single nucleus in SCs, a cell type that is normally binucleate (Figure 5.15C, red arrows).

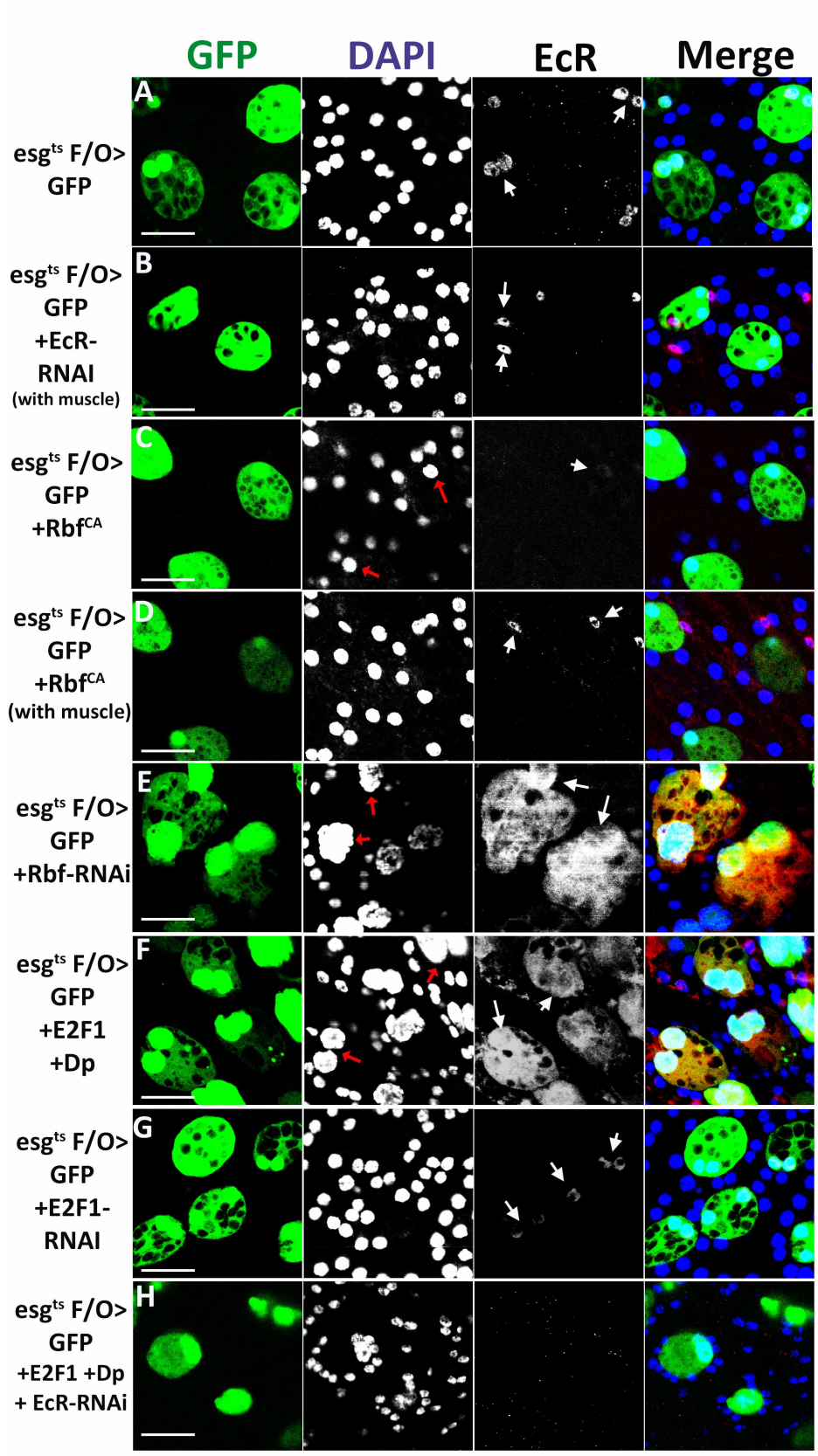


Figure 5.15 Rb/E2F1 modulates EcR levels in SCs. Diminishing E2F1 activity by up-regulation of Rbf (C and D) or RNAi knockdown of *E2F1* (F) in SCs using esg F/O results in reduced EcR levels (white arrows) as detected using a pan-EcR antibody (compare with control, A, white arrows). Knockdown of *Rbf* (E) and over-expressing E2F1 (F) both lead to elevated levels of EcR, with strong cytosplasmic signal detected in addition to nuclear expression (white arrows). Surprisingly, expression of a constitutively active form of Rbf, Rbf^{CA}, results in mono-nucleation of SCs (C, red arrows). Additionally, very large nuclei are observed in SCs where *Rbf* is knocked down (E, red arrows) or E2F1 is over-expressed (F, red arrows). EcR staining of muscle nuclei (white arrows in B [EcR-RNAi knockdown in SCs] and D) confirm staining procedure has worked. Scale bars = 10µm.

5.2.10 The Rbf/E2F1 axis regulates SC growth and maintains bi-nucleation

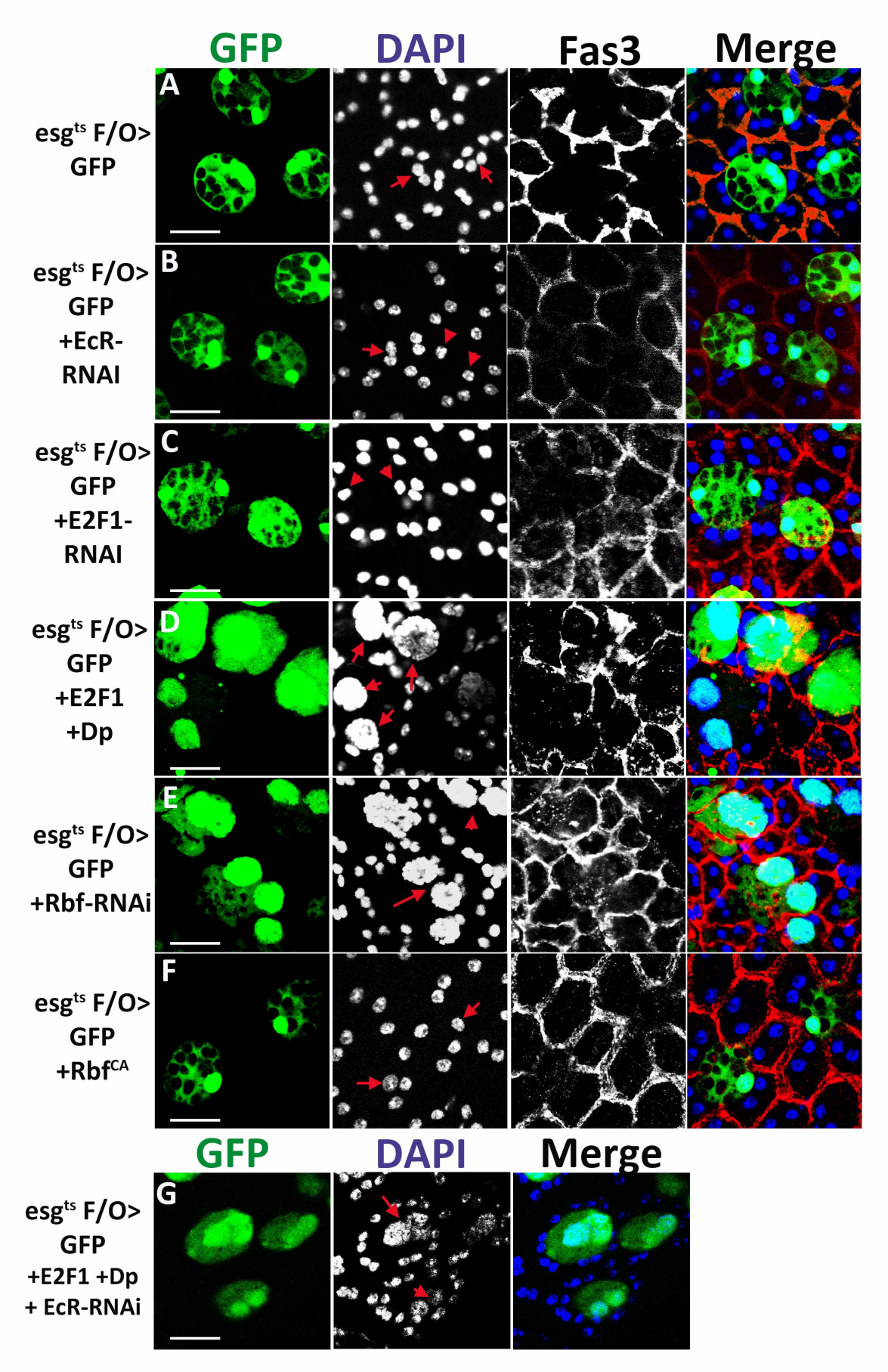
Given the apparent effects on SC nuclear size noted in section 5.2.9, I sought to establish and quantify any role that the Rbf/E2F1 axis plays in regulating SC growth, again employing nuclear size as a proxy for measuring cell growth. Expressing Rbf^{CA} in SCs resulted in significantly reduced SC:MC nuclear area ratio versus controls. The SC:MC nuclear ratio for Rb^{CA} was 0.61 +/-0.27, compared to 1.21+/-0.16 for controls (Figure 5.16, compare A and F, chart in H; n=10, P<0.001, one-way ANOVA). Similarly, RNAi-mediated knockdown of *E2F1* led to reduced SC:MC nuclear ratio (0.87+/-0.12; P<0.001, one-way ANOVA).

Conversely, significant increases in SC:MC nuclear ratio were seen in SCs over-expressing E2F1 with Dp (Figure 5.16 D and H, SC:MC nuclear ratio = 5.71 +/-0.69; P<0.0001, one-way ANOVA) and in SCs where *Rbf* had been knocked down (Figure 5.16 E and H, SC:MC nuclear ratio = 6.51+/- 1;.P<0.0001, one-way ANOVA). It is worthwhile pointing out here that this effect on growth is quite a lot larger than those observed in SCs when BMP signalling is hyper-activated (see Chapter 4).

Significantly, simultaneously up-regulating E2F1 and knocking down EcR (Figure 5.16 G) resulted in a reduced SC:MC nuclear ratio, compared to up-regulating E2F1 alone (Figure 5.16 D) (3.98+/- 0.85 Vs. 5.71+/- 0.69; n=10, P<0.001, one-way ANOVA, Figure 5.16 H). However, these SCs (Figure 5.16 G) display a SC:MC ratio significantly greater than control SCs (Figure 5.16 A) (n=10, P<0.001, one-way ANOVA, Figure 5.16 H).

Taken together these data indicate that at least some, but not all, of the growth effects associated with E2F1 activity are mediated through EcR signalling. Alternatively, it is possible that all of these growth effects occur through EcR, but there was incomplete RNAi knockdown of *EcR* in these experiments.

Interestingly, expression of *Rbf*^{CA} was consistently associated with a mono-nuclear SC phenotype. Of 30 SCs examined (3 SCs per animal, from a total of 10 animals), 28 (96.7%) had a single nucleus in *Rbf*^{CA} expressing cells, compared to 0/30 in control glands. SCs expressing *E2F1*-RNAi also exhibited mono-nucleation, although at a much lower frequency, with 4/30 (13.3%) of these SCs possessing a single nucleus, a phenotype never seen in controls. The relatively low frequency of mono-nucleation seen in *E2F1*-RNAi expressing cells may reflect a variable role for E2F1 in maintaining nucleation, although it is rather more likely that the lower frequency of mono-nucleation is due to the RNAi construct failing to completely knock down *E2F1*.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D
esgts F/O> GFP +E2F1 +Dp
E
esgts F/O> GFP +Rbf-RNAi
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DAPI
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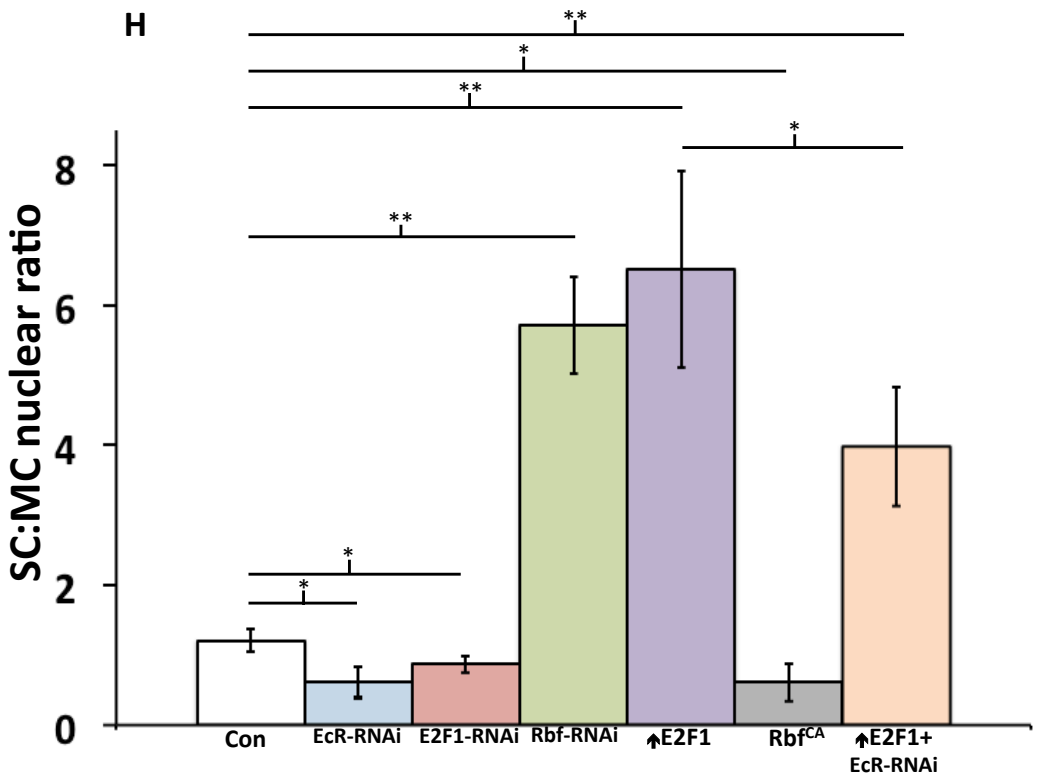

Figure 5.14 E2F1 activation drives SC growth, which is partly mediated through EcR. Activation of E2F1 in SCs, either by RNAi-mediated *Rbf* knockdown (E) or over-expression of E2F1 and Dp (D) using the esg F/O system, results in significant growth with extremely large nuclei (white arrows), compared to control SCs (A). Conversely, reducing E2F1 activity by expressing *E2F1*-RNAi (C) or expression a constitutively active form of Rbf, Rbf^{CA},(F) results in significantly reduced growth, with small nuclei (white arrows). A high frequency of mono-nucleate SCs are also observed in Rbf^{CA}-overexpressing SCs (F, white arrows), implying a role for Rbf/E2F1 activity in maintaining SC binucleation. SCs overexpressing E2F1 with Dp and co-expressing an EcR-RNAi (G) construct display diminished growth compared to SCs overexpressing E2F1 with Dp alone (D), although they remain significantly larger than controls (A) and EcR-RNAi expressing SCs (B). These nuclear size data are summarised in the chart above (H). *P<0.001, **p<0.0001. Scale bar =10µm. All glands dissected from 6-day-old animals.

5.2.11 Increases in SC nuclear size mediated by E2F1 activity do not involve DNA synthesis

To determine whether the increases in SC:MC nuclear ratio observed in SCs where *Rbf* was knocked down or E2F1 was over-expressed were linked to the synthesis of new DNA through endoreplication, flies were cultured on food supplemented with 10mM BrdU from eclosion, dissected at 6 and stained for nuclear BrdU incorporation, indicative of DNA

synthesis. No BrdU incorporation was observed in the nuclei of 6d-old control SCs (Figure 5.15 A), SCs where E2F1 is up-regulated (Figure 5.15 B) or SCs expressing *Rbf*-RNAi (Figure 5.15 C). In total, 5 animals were examined per genotype. However, nuclear BrdU incorporation was observed in mitotic cells located in the testes (Figure 5.15 D) in all animals of all of these genotypes, serving as an internal positive control.

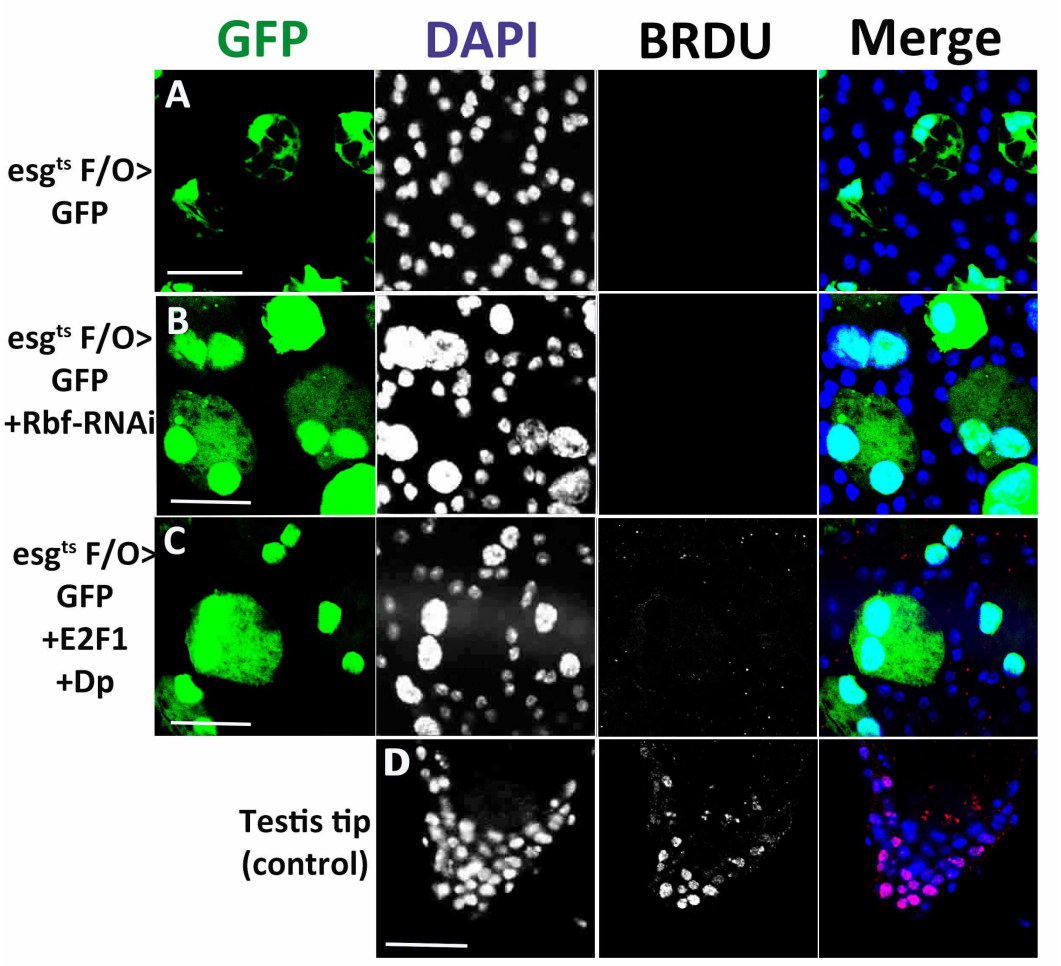

Figure 5.2.15. There is no evidence for new DNA synthesis in SCs with elevated E2F1 activity. No BrdU incorporation is observed in the glands of 6d-old males raised on food from eclosion supplemented with 10 mM BrdU. No detectable BrdU signal was present in control glands (A), glands expressing *Rbf*-RNAi (B) or glands overexpressing E2F1 with Dp (C). BrdU incorporation, however, was observed in the testes of all of these genotypes (example testis in D), indicating that the dietary concentration of BrdU was adequate for incoporation and detection of newly replicated DNA. Scale bar= 10 µm

From these data therefore, I conclude that nuclear growth associated with E2F1 activation in SCs does not occur as a consequence of endoreplication of genetic material, and is more likely a reflection of global increases in SC growth.

5.3 Discussion

In this chapter, I present the first ever characterisation of EcR expression in the adult male *Drosophila* AGs, demonstrating expression of this receptor in the nuclei of SCs and muscle cells, but not in MCs. This observation was of immediate interest to me given the importance of AR signalling in normal human prostate biology and its role as a driver for growth in prostate cancer (Saylor 2013). This led me to the idea that characterising the role of EcR signalling in SCs may highlight some important parallels with the action of AR signalling in prostate epithelial cells.

To this end, I examined the role that EcR expression plays in SC growth and then went on to explore interactions between the EcR and other signalling pathways implicated in growth. In particular, I have shown for the first time important regulatory interactions between the BMP signalling pathway and EcR. In addition, this work highlights a novel role for the Rb/E2F axis as a regulator of EcR protein levels, which may have important implications in understanding cell cycle-mediated cell growth control.

5.3.1 SC growth is regulated by the EcR-B1 receptor

In this chapter, I have shown that the EcR-B1 isoform is expressed specifically in SCs, with expression normally confined to the nucleus. Significantly, RNAi -mediated knockdown of *EcR* in SCs results in diminished cell growth, a phenotype that is also observed in ecd^{1} adults, mutants which are reported to have reduced levels of ecdysone hormone at restrictive

temperatures (Garen, Kauvar et al. 1977, Berreur, Porcheron et al. 1984). Although more recent studies suggest other effects of the *ecd* gene (Claudius, Romani et al. 2014), taken together these observations indicate a role for hormone-dependent EcR signalling activity through EcR-B1, as a regulator of normal SC growth. This neatly parallels observations by others who have shown that EcR signalling controls cell size is in the developing *Drosophila* nervous system (Brown, Cherbas et al. 2006, Zwart, Randlett et al. 2013). In particular, motononeurons undergo significant EcR-dependent remodelling and re-arborisation, a process that necessitates cell growth (Kohsaka, Okusawa et al. 2012). Interestingly, cell-autonomous motoneuron growth requires normal expression of EcR-B2 (Zwart, Randlett et al. 2013). These common roles for EcR across diverse cell types, epithelial SCs and neurons, hint at a conserved role for this steroid signalling pathway in regulation of cell growth across different cell types.

5.3.2 BMP signalling modulates EcR levels to control SC growth

In the previous chapter I provided a detailed characterisation of the role that BMP signalling activity plays as a positive regulator of SC growth. Given that I have shown that EcR also acts to positively regulate SC growth, I sought to establish whether these two pathways interact in SCs to coordinate cell growth. This led to the observation that elevated levels of BMP signalling activity in SCs results in increased levels of EcR protein, with expression seen in the cytoplasm in addition to the nuclei. This EcR-regulatory role for BMP signalling was confirmed by showing that RNAi knockdown of the BMP pathway effector Medea results in loss of EcR expression. Although no other reports in the literature exist describing such an interaction between BMP signalling and EcR expression, an alternative TGF-β pathway, the

activin pathway, positively regulates the expression of EcR-B1 in a subset of developing larval brain neurons (Awasaki, Huang et al. 2011).

The interaction between BMP and EcR signalling pathways acts synergistically to promote SC growth, with massive over-growth observed if EcR isoforms are overexpressed in SCs where BMP activity is hyperactivated. Conversely, BMP-mediated growth effects are abolished in *Tkv*^{Act}-expressing SCs by RNAi knockdown of EcR. These data indicate that the BMP/EcR interaction seen in SCs is necessary for normal growth and that much, if not all, of the BMP-mediated growth effects occur through EcR expression.

Although no other examples of this very specific BMP/EcR interaction have been described in other *Drosophila* tissues, an interesting parallel in human prostate cells has emerged over the past two years. For example, prostate cancer cells co-cultured with macrophages and treated with the BMP ligand BMP-6 exhibit increased AR expression and elevated AR-reporter activity (Lee, Jung et al. 2013). Furthermore, overexpression of BMP-6 induces castration resistance in prostate cancer cells (Lee, Jung et al. 2013). This is supported by additional data showing that prostate cancer bone metastases acquire androgen-independence via induction of BMP-6, a mechanism that requires WNT5A secretion from adjacent stromal cells (Lee, Kang et al. 2014). Recent research shows that BMP-6 may be induced in human prostate stromal cells by the stromal-derived cytokine TGF-β, and then acts on prostate cancer cells to induce and enhance AR signalling activity and may act to maintain AR signalling in the absence of DHT (Yang, Chen et al. 2014).

These remarkable data observed in human prostate cancer cells, considered in the context of my research in the *Drosophila* AG, hint at a conserved steroid receptor regulatory mechanism mediated by BMP signalling activity.

5.3.3 BMP signalling controls EcR levels partly through an interaction with the EcR-NTD

Data in this chapter indicate that the mechanism by which BMP pathway activity increases EcR expression does not occur at the level of transcription. This can be inferred from the observation that driving expression of any of the three EcR isoforms under UAS control does not increase protein levels, implying non-transcriptional regulation of the final protein product, whilst co-expressing with Tkv^{Act} massively increases detectable levels of endogenous and overexpressed EcR protein. As the EcR isoform constructs used here are under UAS control, and not under the control of their endogenous promoter, any increase in EcR levels induced by BMP activity is unlikely to occur as a consequence of increased transcription.

Using the GAL4/UAS system to express EcR-C, an EcR construct lacking the NTD, in SCs leads to cell growth and high levels of nuclear and cytoplasmic EcR, features only seen with native EcR isoforms when they are co-expressed with Tkv^{Act}. This observation indicates that the NTD of EcR plays an important role in controlling the level of expressed EcR protein, potentially by regulating protein stability. This offers the intriguing possibility that one mechanism by which BMP signalling elevates EcR levels occurs at the protein level and involves a direct or indirect interaction with the EcR NTD.

This theory is supported by published research in Chinese Hamster Ovary (CHO) cells that demonstrates that the degradation of the B1 and A isoforms of EcR is modulated by the NTDs of these proteins, which contain isoform-specific ubiquitination sites that mark them for proteasomal degradation. Truncation of the NTD of the EcR-A isoform resulted in high levels of protein due to decreased degradation (Schauer, Burster et al. 2012). Overall, my

findings imply that EcR protein turnover is an important regulatory step, occurring in an NTD-dependent manner. Given that I have shown that BMP-mediated control of EcR occurs post-transcriptionally, an attractive hypothesis is that BMP signalling stabilises EcR at the level of protein, via an interaction involving the NTD, preventing EcR degradation.

Given that turnover of EcR protein can be regulated by the ubiquitination status of the NTD (Schauer, Burster et al. 2012), any BMP pathway interaction with the EcR NTD may alter protein stability via effects on ubiquitination. Indeed, in murine models, BMP signalling controls muscle mass by negatively regulating ubiquitin ligases, including MUSA1, that promote protein degradation in atrophying muscle (Sartori, Schirwis et al. 2013, Winbanks, Chen et al. 2013). Therefore, an attractive hypothesis is that in SCs BMP signalling activity regulates the expression or activity of an as yet unidentified ubiquitin ligase. In this model, BMP signalling activity would negatively regulate the putative ubiquitin ligase, which, if similar to the mechanism observed in mouse models, would occur at the level of transcription (Sartori, Schirwis et al. 2013, Winbanks, Chen et al. 2013). This down-regulation of ubiquitin ligase activity would then reduce the level of ubiquitination status of the EcR NTD, resulting in increased stability of EcR protein (see figure 5.3.1 for a schematic diagram outlining this model).

Interestingly, in mammalian cells the stability of AR protein is regulated by the activity of an E3 ubiquitin ligase, Mdm2, which acts to promote polyubiquitination of AR, marking it for proteasomal degradation (Lin, Wang et al. 2002). Although the AR ubiquitination sequences targeted by Mdm2 are unknown, it is intriguing to speculate that these may, as in EcR, be located in the NTD.

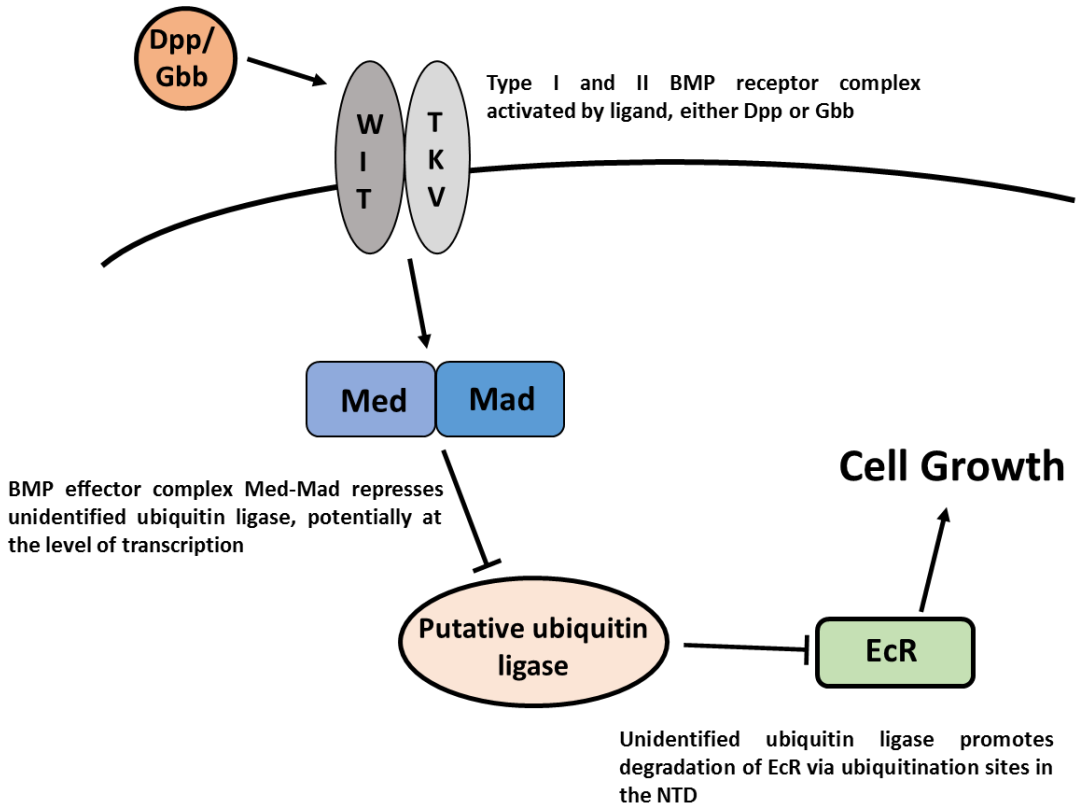

Figure 5.3.1: **Schematic depicting a model of how BMP activity may stabilise EcR protein in SCs.** BMP signalling initiated by the BMP ligands, either dpp or gbb, acting through a BMP receptor complex composed of Tkv and a Type I receptor (eg. Wit) negatively regulates the activity of a putative ubiquitin ligase, normally responsible for EcR turnover via action at the EcR NTD. Therefore, down-regulation of this enzyme by BMP signalling enhances EcR stability, thus promoting EcR mediated cell growth.

5.3.4 The Interaction between BMP signalling and EcR appears to occur selectively in SCs

To test whether the observed role that BMP signalling plays in controlling EcR levels and EcR-mediated growth in SCs occurs in other cell types, I performed genetic manipulations of both of these pathways in the other AG epithelial cell type, MCs. These experiments demonstrated that, unlike SCs, cell growth in MCs cannot be driven by up-regulating BMP signalling or by expressing the N-terminally truncated form of EcR, EcR-C. Furthermore, combinatorial genotypes over-expressing EcR isoforms in a genetic background of BMP hyperactivity were not associated with abnormal levels of MC growth. Additionally, the BMP

up-regulation did not appear to alter the stability of UAS-driven EcR expression. These observations imply that, at least within the AG, this novel BMP/EcR interaction is unique to SCs.

I attempted to determine whether this interaction exists in cell types outside of the AG. Specifically, I used the GMR-GAL4 (Freeman 1996) driver to express BMP and EcR signalling pathway components to examine whether any interaction could be observed in the developing *Drosophila* eye. Unfortunately, although I was able to overexpress and detect the different EcR isoforms in the developing eye, driving Tkv^{Act} with GMR-GAL4 led to embryonic lethality.

5.3.5 BMP and EcR signalling act antagonistically to regulate SC migration

In addition to showing that BMP and EcR signalling pathways interact in SCs to promote cell growth, the data in this chapter demonstrate that these pathways interact antagonistically with respect to effects on SC migration. As detailed in chapters three and four, a subset of SCs normally display migratory behaviour in multiply mated, aged males, and this migratory activity occurs in a BMP-dependent manner. Additionally, delamination and migration can be induced in SCs through hyper-activation of BMP signalling activity by expressing Tkv^{Act} in these cells. However, I have shown in this chapter that elevated expression of EcR in SCs co-expressing Tkv^{Act} reduces the frequency of SC migration compared to SCs expressing Tkv^{Act} alone.

This anti-migratory role for EcR in SCs is perhaps a little surprising given that there is ample evidence that EcR signalling promotes migratory behaviour in a *Drosophila* ovarian follicle cell type known as border cells (Bai, Uehara et al. 2000, Jang, Chang et al. 2009,

Domanitskaya, Anllo et al. 2014). However, it is postulated that EcR controls varied functions in distinct tissue types through interactions with specific cofactors (Davis, SanGil et al. 2011), and it is known that border cell migration specifically requires the expression of the EcR cofactors Taiman (Bai, Uehara et al. 2000) and Abrupt (Jang, Chang et al. 2009). Therefore, it is plausible that the opposite effects of EcR observed in SCs and border cells occur as a result of differentially expressed EcR cofactors, explaining how this pathway can be pro-migratory in one cell type and anti-migratory in another.

Interestingly, the anti-androgen bicalutamide, a competitive AR antagonist used in the management of advanced, hormone-sensitive prostate cancers, has been shown to promote invasion of human prostate cells in *in vitro* migration assays and also in an *in vivo* mouse model of prostate cancer, hinting that, under some circumstances, AR activity may inhibit migratory cell behaviours (Lin, Lee et al. 2013). Again, these data appear to highlight interesting parallels between EcR and AR function.

5.3.6 SCs synthesise 20-HE and knockdown of biosynthetic proteins diminishes SC growth

A number of important roles for EcR signalling have been characterised in adult *Drosophila*, including regulating aspects of sleep (Ishimoto and Kitamoto 2010), memory (Ishimoto, H., T. Sakai et al. 2009) reproduction (Carney and Bender 2000) and courtship (Ganter, Walton et al. 2007, Dalton, Lebo et al. 2009,Ishimoto, Sakai et al. 2009).

Despite the importance of EcR signalling in adult physiology, the post-developmental regulation and location(s) of ecdysone synthesis have, until recently, remained largely elusive (Schwedes and Carney 2012). However, research in the past two years has identified the AGs of adult insects as an important site of ecdysone synthesis (Hentze, Moeller et al.

2013). Specifically, adult *Drosophila* SCs express the receptor for PTTH, Torso. It is known that, through activation of Torso, PTTH initiates ecdysteroidogenesis in the larval prothoracic glands (PGs) (Rewitz, Yamanaka et al. 2009). Furthemore, RNAi knockdown of either *PTTH* or *Torso* in adult male *Drosophila* resulted in significantly decreased expression of the EcR target genes, E74A and E74B, indicating diminished EcR signalling activity in these males (Hentze, Moeller et al. 2013).

My work here shows that several genes in the ecdysone synthesis pathway seem to be required in SCs to control growth. Tools to study the expression of these genes are limited, and typically involve *in situ* hybridisation, which is not straightforward in the AG. However, the fact that knockdown of multiple synthesis genes produces strong growth inhibitory phenotypes, combined with the finding that SCs express the PTTH receptor, strongly indicates that these cells are involved in 20-HE synthesis. In addition to their ecdysone-synthetic function, SCs express the ecdysone receptor, EcR, and are therefore presumably a target for ecdysone signalling. Indeed, reduced levels of 20-HE, as observed in ecd^{1} mutants (Garen, Kauvar et al. 1977, Berreur, Porcheron et al. 1984, Ganter, Walton et al. 2007), results in abnormally small SCs. This phenotype is also observed in SCs where *EcR* has been knocked-down using RNAi constructs directed against EcR (see section 5.2.2), confirming that normal levels of 20-HE and expression of EcR are both required for normal SC growth.

The observation that SCs synthesise ecdysone and also respond to this hormone implies a potential autocrine mechanism of EcR signalling activation in adult SCs. Equally, it is plausible that SC-synthesised ecdysone is secreted and acts in a paracrine or endocrine fashion on other EcR-expressing cell types, including other SCs and AG muscle cells.

Alternatively, EcR activation in SCs may be evoked by a mixture of auto- and paracrine signals.

A model for ecdysone synthesis and action on SCs is depicted in schematic diagram overleaf (Figure 5.3.2). Since the other sources of 20-HE synthesis in adult males are not yet characterised, and the ovary is a key synthesising tissue in females (Tu, Yin et al. 2002), it is possible that SCs are a major regulator of steroid-dependent responses in males.

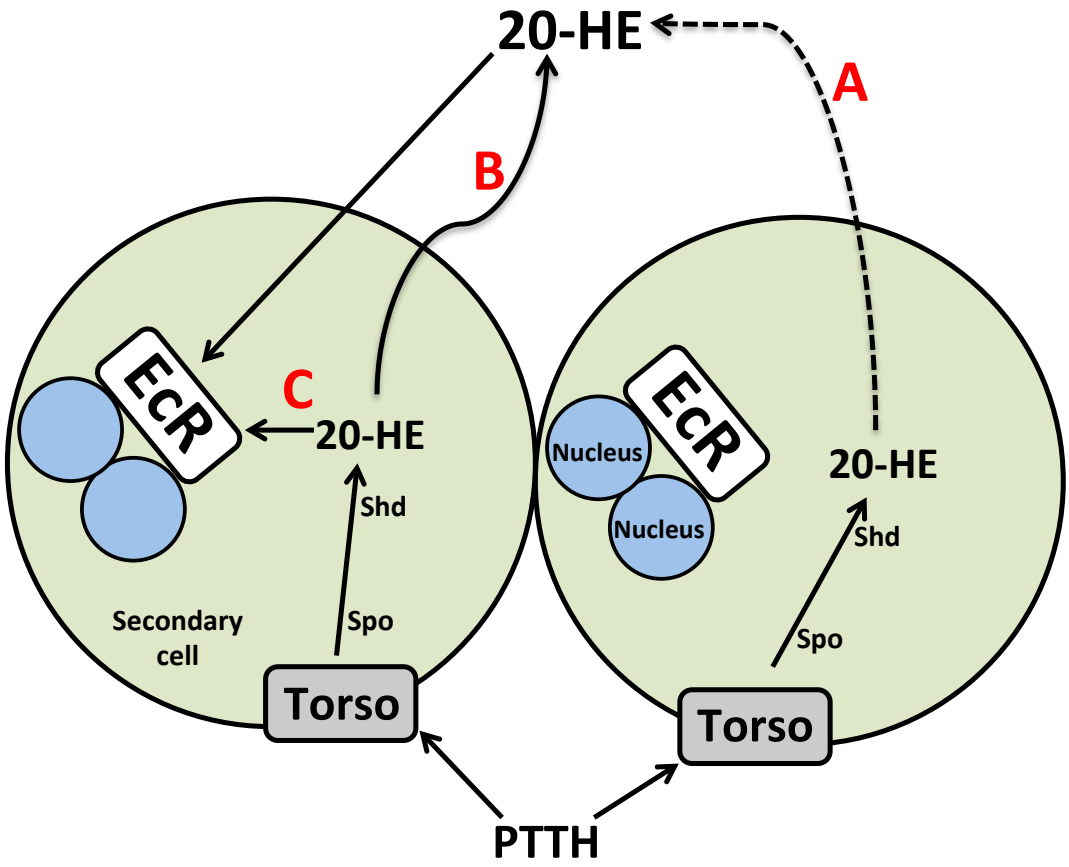

Figure 5.3.2 SCs are a site of ecdysone hormone synthesis and action. SC synthesis of ecdysteroids may be initiated by PTTH/Torso signalling and is mediated by the P450 Halloween genes, including *dib* and *shd* SCs also express the ecdysteroid receptor EcR. EcR activation may involve a paracrine (A), and/or a secretory (B) or non-secretory autocrine mechanism. Secreted 20-HE may also affect other cells in a paracrine or endocrine fashion.

5.3.7. The hormone 20-hydroxyecdysone may modulate AG secretory activity via EcR signalling in SCs

Others have shown that elevated levels of 20-HE can stimulate increased synthesis of accessory gland-specific proteins, including Acp95E, a phenomenon that is also observed in the glands of males that have mated (Wolfner, Partridge et al. 1997). The authors of this work postulate that ecdysone may act in mated males as a signal to initiate synthesis of AG proteins ready for subsequent mating activity, essentially replenishing the seminal fluid cocktail. Indeed, the idea that levels of ecdysteroids in adult males may be influenced by mating activity is supported by the observation that titres of 20-HE spike in males that have their courtship advances rejected by females (Ganter, Panaitiu et al. 2011).

My data suggest the intriguing possibility that 20-HE-mediated induction of AG protein synthesis occurs primarily through SCs, as I have shown that only SCs, and not MCs, express EcR. Interestingly, the synthesis of a number of AG proteins is known to be under 20-HE control, like Acp95E, but they are expressed by MCs and not SCs (Kalb, DiBenedetto et al. 1993, Wolfner, Partridge et al. 1997). This implies that 20-HE, the levels of which fluctuate with socio-sexual experience (Ganter, Panaitiu et al. 2011), may modulate protein synthesis in MCs via activation of EcR signalling in SCs. Presumably, this could occur if EcR in SCs were to control the activity of an as yet unknown signal transferred from SCs to MCs, capable of influencing MC protein synthesis.

Indeed, there is indirect evidence that SCs do control the activity of MC-derived secreted elements. For instance, recent work has shown that SCs express the Hox gene, *Abdominal-B* (*Abd-B*), and loss of function *Abd-B* function in SCs results in defective PMR in females (Gligorov, Sitnik et al. 2013). Specifically, this work shows that one function of SCs is to

regulate the activity of main cell-derived Acps, including Ovulin, a crucial Acp responsible for inducing ovulation and egg-laying in mated females. In this example, SCs appear to modulate Ovulin through a mechanism that involves glycosylation (Gligorov, Sitnik et al. 2013).

5.3.8 Endocrine signals may couple socio-sexual stimuli to AG biology

There is strong evidence that whole animal titres of ecdysone fluctuate dramatically in adult *Drosophila* in response to socio-sexual stimuli (Harshman, Loeb et al. 1999, Ishimoto, Sakai et al. 2009, Schwedes and Carney 2012). A particularly interesting example of this is the observation that levels of ecdysone spike significantly in adult males whose courtship advances are rejected by previously mated females (Ishimoto, Sakai et al. 2009).

Given that ecdysone levels are influenced by socio-sexual experiences, such as courtship interactions (Ishimoto, Sakai et al. 2009), it is likely that these stimuli also modulate EcR signalling-dependent processes in the AG, such as SC growth (section 5.2.2) and MC Acp synthesis (Wolfner, Partridge et al. 1997), an EcR-dependent process that I have already speculated may be regulated indirectly through the action of EcR signalling in SCs (section 5.3.7). Furthermore, given that SCs are a likely site of ecdysone synthesis (Hentze, Moeller et al. 2013), it is probable that these socio-sexual stimuli influence ecdysone levels by modulating steroid hormone biosynthesis in SCs.

We know that ecdysteroidogeneis is initiated by Torso activation in response to PTTH in pupae (Rewitz, Yamanaka et al. 2009), a peptide hormone that is synthesised and secreted by the *Drosophila* central nervous system (CNS) (Zitnan, Sehnal et al. 1993, Ghosh, McBrayer et al. 2010). With this knowledge, it is attractive to speculate that certain socio-sexual

interactions may evoke PTTH secretion from the CNS or PNS in adults, thereby stimulating, via Torso activation, the biosynthesis of ecdysteroids within SCs. In turn, the elevated levels of ecdysone then alter EcR signalling activity within SCs to promote cell growth and stimulate Acp synthesis in MCs. A schematic is depicted on the below summarising this model (Figure 5.3.3).

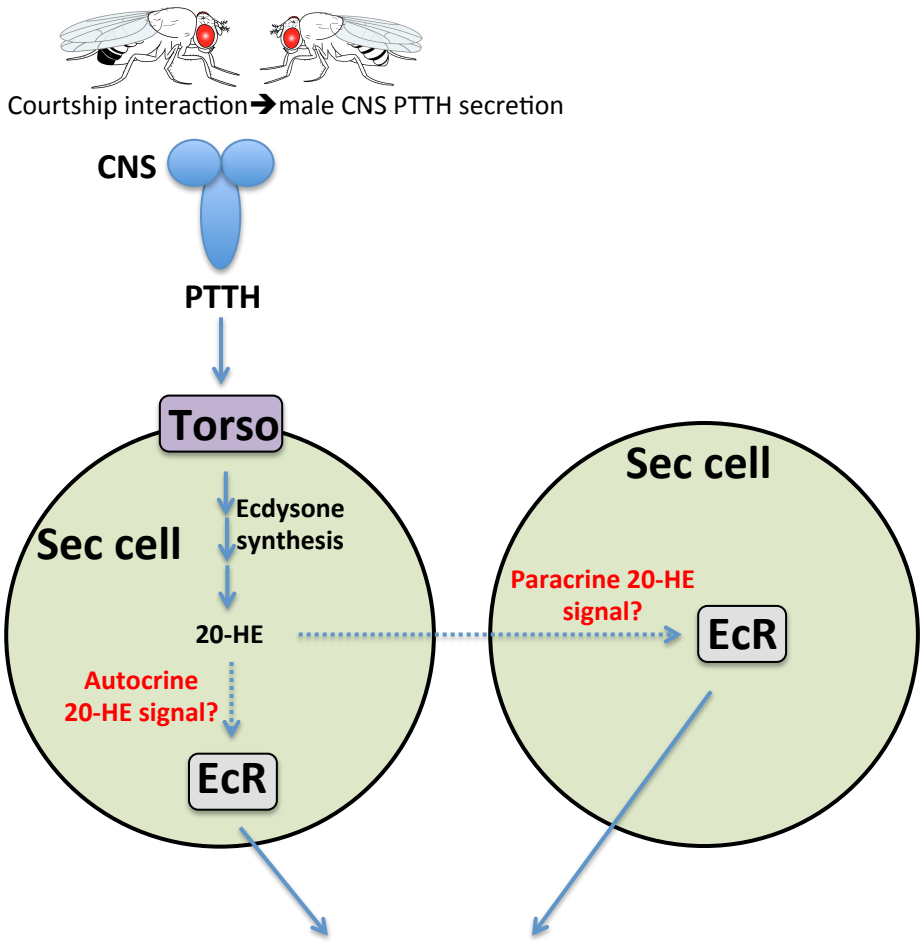

Figure 5.3.3. Model depicting how socio-sexual stimuli might alter AG biology. Behaviours, including courtship interactions, cause ecdysone levels to spike. This may involve CNS/PNS secretion of the Torso ligand, PTTH. Torso activation in SCs would then promote ecdysone biosynthesis, elevating whole animal titres of ecdysone and the active metabolite, 20-HE. These elevated levels may then stimulate, in an autocrine and/or paracrine manner, EcR signalling in SCs. Activation of EcR signalling has growth-promoting and delamination-inhibiting effects on SCs, and may indirectly evoke Acp synthesis in main cells.

5.3.9 Levels of EcR are regulated by E2F1 activity, via a mechanism that may parallel AR regulation in human prostate cells

In this chapter I show that the expression levels of EcR in SCs are modulated by the activity of the Rbf/E2F1 axis. Activation of E2F1, either by *Rbf* knockdown or E2f1 over-expression, is accompanied by very high levels of detectable EcR, with signal observed in the cytoplasmic fraction, as well as the nuclei. Additionally, I demonstrate that E2F1 activation results in massive SC growth, significantly greater than that induced by elevated BMP signalling, which is not accompanied by DNA synthesis. This growth is at least partially mediated by EcR expression. This is the first ever description in *Drosophila* of such an interaction between Rbf/E2F1 and EcR; however, there are remarkable parallels with an analogous interaction in human prostate cancer cells.

Interestingly, unlike many other cancers, loss of *Rb* is a relatively late occurrence in prostate cancer and heralds the development of advanced disease (Macleod 2010). Work has shown that in human prostate cancer cell lines, Rb loss and consequent E2F1 activation leads to increased levels of AR protein and a more aggressive phenotype that, *in vitro*, displays ligand-independent AR signalling activity (Sharma, Yeow et al. 2010). The authors of this work speculate therefore that *Rb* loss may represent an important switch in the development of castrate-resistant prostate cancer (CRPC). Although the authors of this study demonstrate that AR is a direct transcriptional target of E2F1, they do not test the hypothesis that this interaction also occurs at the level of protein and acts by stabilising expressed AR.

Clearly, there are striking parallels between these observations in human prostate cancer cells and the phenomena demonstrated by my work in SCs, as expression levels of both EcR

and AR are regulated by E2F1 and BMP signalling activity, which are both linked to CRPC. It is intriguing to speculate that the nature of this interaction is highly conserved from fly to human, and that studying regulation of EcR expression and activity in SCs could provide mechanistic insights into hormone-independent activity of AR in prostate cancer. These similarities are summarised in the diagram below (Figure 5.3.4)

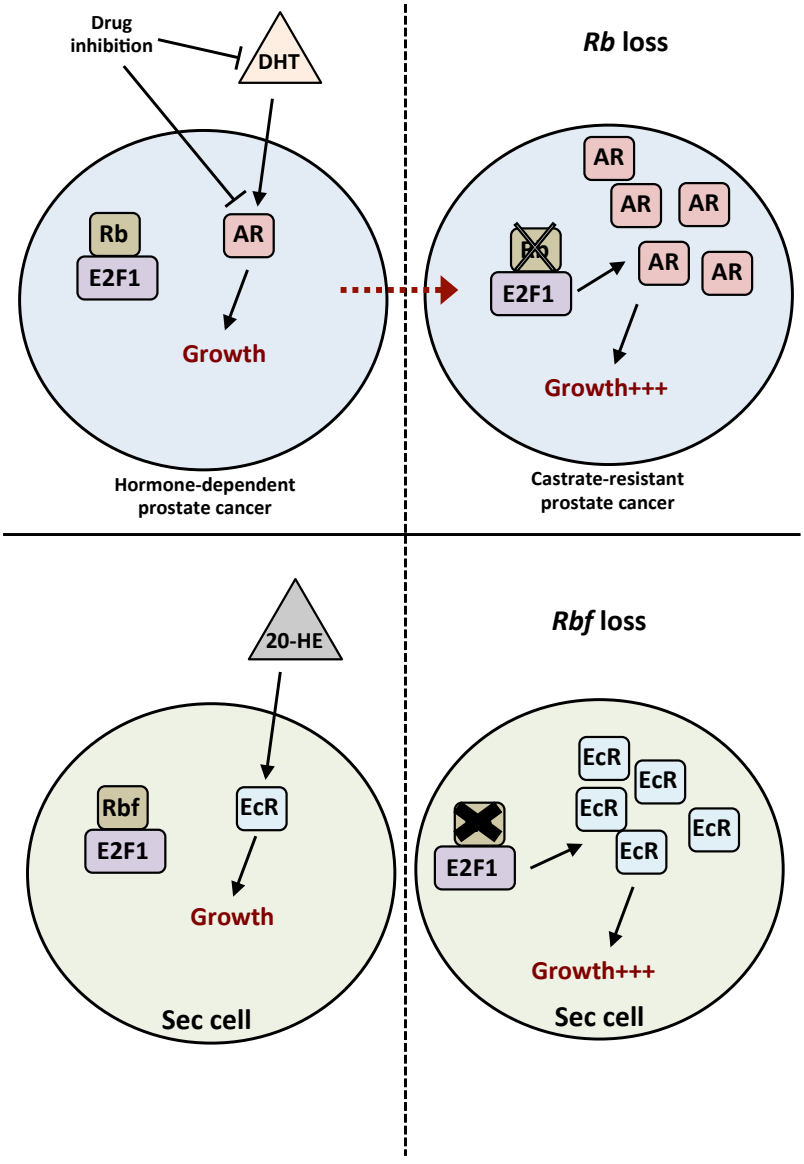

Figure 5.3.4 E2F1 regulates steroid receptor levels in prostate cancer cells and SCs. Rb loss in prostate cancer cells is associated with transition to castrate-resistant prostate cancer and leads to elevated levels of AR protein. Similarly, E2F1 activation in SCs results in very high levels of detectable EcR protein and excessive cell growth.

6 – Chapter Six

Discussion & Concluding Remarks

6.1 Discussion

Much of the discussion of data presented in this thesis is contained within the respective results chapters. Therefore the focus of this chapter will be to provide an overview of my research and refer back to my stated aims in the introduction and address whether these were fulfilled. To save the reader the trouble of having to refer back to the introduction, I will restate those aims below.

- To establish drivers and tools to facilitate study of the AG epithelium
- To develop high resolution fluorescence imaging of these epithelial cells
- To screen for prostate-like activities and to dissect out their genetic regulation
- To start to use the accessory gland system to develop new models to explain prostate biology and disease

6.1.1 Novel tools have been developed to facilitate the study of the AG epithelium

Previous work by others had identified a transcriptional driver, GAL4-*Acp26Aa*, demonstrating selective expression in AG main cells (Chapman, Bangham et al. 2003), but there had been no reports in the literature describing transcriptional drivers displaying selective expression in SCs. Therefore, one of my key early objectives was to perform a screen of GAL4 lines to identify a set of drivers. A personal communication from Professor Stephen Goodwin (University of Oxford) alerted our lab to his observation that SCs express *dsx*-GAL4, which was discovered during the course of experiments conducted in his lab

aimed at characterising the role that *dsx*-expressing neurons in regulating reproductive behaviours in adult male and female *Drosophila* (Rideout, Dornan et al. 2010).

Just prior to this I had discovered and characterised a range of driver lines that displayed selective expression in SCs, including: *esg*-GAL4 (Goto and Hayashi 1999), *spi*-GAL4 (Jiang, Patel et al. 2009), *btl*-GAL4 and *bnl*-GAL4 (Ahmad and Baker 2002). Aside from identifying a series of tools that can be used to drive expression of UAS-transgenic lines, this screen provided some clues that SCs exhibit interesting signalling activity. For instance, the expression of *spi*-GAL4 implies that SCs express the EGF ligand, *spitz*. Furthermore, the observation that SCs express both the FGF ligand *branchless* (*bnl*) and the FGF receptor *breathless* (*btl*), hints at the intriguing prospect of an autocrine role for FGF signalling activity in SCs.

The discovery that *esg*-GAL4 is transiently expressed in SCs was particularly fortuitous, as a related driver line, esg^{ts} F/O was already in circulation (Jiang and Edgar 2009). This line permits sustained GAL4 expression, meaning that transgenes can be induced throughout the lifespan of a SC. A useful feature of this driver line has been its use to create mosaic expression patterns in SCs, essentially allowing me to generate internal controls within a single gland.

Undoubtedly, this characterisation of driver lines has proved to be an important milestone in efforts to define SC biology and these tools have been utilised extensively within our own lab (Leiblich, Marsden et al. 2012, Corrigan, Redhai et al. 2014) and beyond (Ito, Ueda et al. 2014).

6.1.2 These new tools have been combined with high resolution microscopy to define interesting SC behaviours

Once a set of driver lines had been established, I was able to easily mark secondary cells by expressing a GFP transgene, UAS-GFP^{nls} (Davis, Girdham et al. 1995), allowing visualisation of these cells by laser confocal microscopy.

From the outset, I was very keen to identify a stem cell population in the AGs, as this could have allowed the possibility of genetically manipulating these cells to generate tumours, as seen in other *Drosophila* cancer models (Tapon 2003, Vidal and Cagan 2006, Tipping and Perrimon 2014). I was initially intrigued to discover that SCs express *esg*-GAL4, as expression of *esg* has been used as a marker of stem cells in other fly tissues, including the midgut (Jiang and Edgar 2009). This led me to the idea that SCs may have stem-like properties, and could therefore be manipulated to model cancer, although I was ultimately disappointed to discover that this was not the case. Indeed, having studied the AGs for the past three years, I can fairly confidently conclude that the normal epithelium of the adult glands has zero replicative potential.

6.1.3 Secondary cells display behaviours that may be relevant to cancer biology

Despite this setback, I did notice that SCs displayed a set of very interesting behaviours that resonated with my interest in prostate biology and prostate cancer, and I felt that study of these processes could be exploited to inform our understanding of aspects of cell biology relevant to cancer. Early on, I observed that SCs display a pattern of growth, relative to the

rest of the epithelium, which occurs in an age-dependent manner. This mirrors somewhat the situation in the prostate, where the epithelium undergoes age-related overgrowth.

However, this analogy is not entirely accurate as cellular hyperplasia is a far more common feature of benign prostatic enlargement than hypertrophy, although an element of hypertrophy is observed (Timms and Hofkamp 2011).

Perhaps, the most striking behaviour observed in SCs that might prove useful as a tool to study cancer biology is the cell-migratory phenomena that develop in response to mating. This process whereby a subset of SCs delaminate from the epithelium and migrate in a directional manner may provide clues into the genetic mechanisms that underpin the acquisition of invasive phenotypes in cancer, an important feature of malignant progression (Hanahan and Weinberg 2011).

Indeed, my work has stimulated research by others to determine whether parallels can be drawn between the biology that regulates the migratory activity of SCs and the mechanisms controlling invasion in prostate cancer cells (Ito, Ueda et al. 2014). This research entailed a genetic screen to identify modifiers of SC migration and test whether their homologues were involved in prostate cancer metastasis. Interestingly, a number of genes were identified as regulators of SC migration that were previously shown to be implicated in the progression of prostate cancer, including E-Cadherin, N-Cadherin and Paired (Ito, Ueda et al. 2014). In addition to genes already known to play a role in prostate cancer, this screen identified a set of potentially novel regulators of prostate cancer progression, including MRGBP, CNPY2, and MEP1A (Ito, Ueda et al. 2014). Subsequent experiments demonstrate that their homologues were involved in proliferation and invasiveness in human prostate

cancer cells, although the precise roles were not equivalent to those demonstrated in flies (Ito, Ueda et al. 2014).

6.1.4 BMP signalling plays important roles in a diverse range of cell biological processes

Upon discovering that SCs display interesting growth and migratory behaviours, I set about conducting experiments aimed at dissecting out the mechanisms underpinning this biology. To this end, I designed a targeted genetic screen, testing the effects of various signalling pathway previously implicated in growth and/or migration, including the PI3K, RAS/MAPK/ERK and JAK/STAT pathways. Hopefully the reader is aware by now that this screen revealed that BMP signalling activity is a crucial regulator of SC biology, required for promoting both growth and migration in this cell type. Happily (and somewhat boastfully), I can report that many of these data formed the basis of a paper that was published in a well-regarded journal, and has been received with some degree of enthusiasm from members of the *Drosophila* research community working in this (admittedly small) field (Leiblich, Marsden et al. 2012).

For some time I was preoccupied with the notion that this role for BMP signalling in SCs would turn out to be nothing more than of esoteric interest to a rather limited number of researchers absorbed in the arcane field of accessory gland biology. However, several developments in the last couple of years have given me cause to believe that will not be the case. In particular, research in published in the last 12 months by others demonstrates a very pertinent link between BMP signalling and AR activity and castrate resistance in prostate cancer (Lee, Kang et al. 2014, Yang, Chen et al. 2014), closely mirroring my own findings in relation to BMP signalling and its regulation of EcR activity, and I will discuss these parallels in more depth a little later.

An additional breakthrough that endows my own findings significance beyond AG biology was the discovery by a collaborating member of our lab, Laura Corrigan, that BMP-signalling is involved in the regulation of exosome secretion (Corrigan, Redhai et al. 2014). This work showed that SCs secrete exosomes, which are nanoparticles that act as vehicles conveying cell-cell signals (Simons and Raposo 2009, Raposo and Stoorvogel 2013). In another remarkable parallel with human prostate biology, Laura showed that SC derived exosomes are able to fuse with sperm in the female reproductive tract. *Ex vivo* experiments have shown, that human prostate derived exosomes, known as prostasomes, also fuse with sperm. It is postulated that one of the functions of this interaction is to transfer calcium-signalling tools from exosomea to spermatoazoa, modulating sperm motility (Arienti, Carlini et al. 1997, Park, Kim et al. 2011, Aalberts, Sostaric et al. 2013).

One of the functions of SC-derived exosomes is to modulate the female PMR, in a manner almost identical to the role observed for BMP signalling. Specifically, SC-derived exosomes, like BMP signalling, are needed to fully block females from remating to further males (Corrigan, Redhai et al. 2014). This led Laura to investigate whether BMP signalling played any role in exosome biogenesis. Remarkably, these experiments went on to reveal that exosome secretion is tightly regulated by BMP signalling activity, with reduced activity leading to vastly diminished secretion (Corrigan, Redhai et al. 2014). This regulation appears to occur through BMP-mediated effects on endosomal trafficking.

Through collaboration with the Goberdhan lab (University of Oxford), translational work has been initiated to establish whether aspects of SC biology are conserved in human cancer. Happily, I can report that these efforts have already identified populations of exosomes secreted by both human colorectal and prostate cancer cell lines, regulated by some signals

that have been shown to control SC exosome secretion, and an important feature of these ongoing experiments will be to test whether BMP signalling plays an analogous role in these cell types to that observed in *Drosophila* SCs.

6.1.5 Parallels between the biology of SCs and the prostate epithelium

I discuss in the introduction chapter that one of the key ideas stimulating this whole project is the hypothesis that the *Drosophila* accessory gland could serve as a useful model system in which to study biology relevant to the mammalian prostate. During the course of my research I have uncovered several biological parallels between these two organs that lend credence to the notion that multiple features of the biology of SCs is conserved in the prostate.

6.1.5.1 Prostate secretory cells and SCs both display steroid hormone-dependent growth

It has long been known that the major male steroid hormone, testosterone and its active metabolite dihydrotestosterone (DHT), promote the secretory activity of the prostate and positively regulate growth (Huggins, Masina et al. 1939, Berry, Maitland et al. 2008). Following the discovery that SCs display a pattern of growth that is dependent on age and mating, I sought to establish whether, as in the prostate epithelium, growth is under the influence of steroid hormone signalling.

Steroid signalling in flies is much simpler than in mammals, with only a single major signalling pathway described, the EcR signalling pathway. As discussed in sections 5.1.1 and 5.1.2, there are a number of structural and functional parallels between EcR- and AR-

signalling that led me to conclude that studying EcR biology is a useful exercise in attempts to further understand AR signalling.

Researchers interested in prostate cancer have for some time grappled with the question of how advanced castrate-resistant prostate cancers (CRPCs) eventually and inevitably escape hormone control, leading to aggressive, rapidly growing tumours that no longer respond to anti-androgen therapy. A potentially important clue to this problem is the finding that, despite androgen deprivation therapy (ADT), most tumours seen in CRPC display elevated levels of AR signalling activity (Attard, Reid et al. 2009, Marques, Dits et al. 2010). In broad terms, it is speculated that this seemingly ligand-independent activity could arise from several different mechanisms including; constitutive activation of AR (Watson, Chen et al. 2010, Cottard, Asmane et al. 2013), intra-tumoural androgen synthesis (Cai, Chen et al. 2011, Ishizaki, Nishiyama et al. 2013) and elevated AR expression (Koivisto, Kononen et al. 1997, Koivisto, Kolmer et al. 1998, Koivisto and Rantala 1999).

Indeed, there is a wealth of evidence to support the significance of all of these mechanisms as drivers of AR hyper-activation in CRPC, and the likelihood is that all play a role. For instance, mutations affecting AR are extremely rare in early prostate cancer, but are observed in as many as 30% of all CRPCs (Culig, Hobisch et al. 1993, Suzuki, Sato et al. 1993, Gaddipati, McLeod et al. 1994, Taplin, Bubley et al. 1995, Suzuki, Akakura et al. 1996, Taplin, Bubley et al. 1999, Wallen, Linja et al. 1999).

ADT appears to provide selective pressure for the development of AR mutations (Taplin, Bubley et al. 1999, Wallen, Linja et al. 1999). The most frequently observed AR mutations in CRPC arise in the ligand-binding domain (LBD). A number of these mutations have been shown to reduce the specificity of the AR-ligand interaction, allowing AR binding and

activation by a range of ligands including anti-androgens, oestrogen and other steroid hormones, including glucocorticoids (Newmark, Hardy et al. 1992, Suzuki, Sato et al. 1993, Taplin, Bubley et al. 1995)

Of great interest to me was the description of a point mutation in the NTD of AR at a site normally involved in a E3-ubiquitin-ligase interaction (Steinkamp, O'Mahony et al. 2009). Remarkably, this mutation leads to increased AR activity by increasing protein stability, resulting in elevated AR levels. This is extraordinarily similar to biological effects I have observed in SCs when manipulating the EcR. In section 5.2.4, I show an experiment where I have expressed an N-terminally truncated form of EcR under UAS control. The loss of the N-terminus leads to high levels of EcR, presumably due to increased protein stability. Work by others suggests that EcR stability is regulated by activity at ubiquitination sites located in the NTD (Schauer, Callender et al. 2011, Schauer, Burster et al. 2012). Furthermore, I show in section 5.2.5, that this truncated form of EcR drives higher levels of growth than control SCs, presumably reflecting elevated levels of EcR activity. Clearly, this is an example where regulatory mechanisms controlling receptor levels and activity are potentially conserved from fly to human, strengthening the hypothesis that continuing to study EcR biology in SCs can inform our understanding of AR regulation.

6.1.5.2 BMP signalling activity plays important roles in both SC and prostate cancer biology

One of the key findings of my work has been that BMP signalling functions in SCs as a key regulator of growth and migratory behaviour. In short, down-regulation reduces both SC growth and blocks migration, whereas hyper-activation promotes exaggerated overgrowth and stimulates delamination and migration at a high frequency (see Chapter 4).

I then went on to demonstrate that BMP signalling has a positive regulatory effect on EcR. Levels of EcR are massively up-regulated by BMP pathway hyper-activation, with consequent massive cell growth (see Chapter 5). Again, these effects appear to occur at the level of protein rather than transcription.

This interaction represents another example where the biology observed in SCs seems to mirror signalling in prostate epithelial cells. Last year, two papers were published that describe a role for BMP-signalling (specifically BMP-6) as an activator of AR activity in advanced prostate cancer (Lee, Kang et al. 2014, Yang, Chen et al. 2014). Interestingly, one of these papers includes a micro-array analysis that demonstrates up-regulation of several AR-responsive genes, including PSA, although levels of AR transcript appear to be unaffected (Yang, Chen et al. 2014). This implies that BMP-mediated regulation of AR in prostate cancer cells probably does not only occur at the level of transcript. I postulate that it functions to stabilise AR at the level of protein, recapitulating the relationship between BMP and EcR is SCs. This is a hypothesis I hope to test in the future.

Additionally, the progression to castrate resistance is accelerated in a co-culture system where prostate cancer cells are grown together with bone stromal cells (Lee, Kang et al. 2014). The mechanism underpinning this involves BMP-6 induction in the prostate cancer cells, which occurs in response to a secreted bone stromal derived-WNT5A signal. By combining these observations in prostate cancer cell lines, with my own data in the *Drosophila* AG, I am able to synthesise an overarching hypothesis that may explain how BMP signals contribute to growth in CRPC, depicted in the schematic diagram on the next page (Figure 6.1).

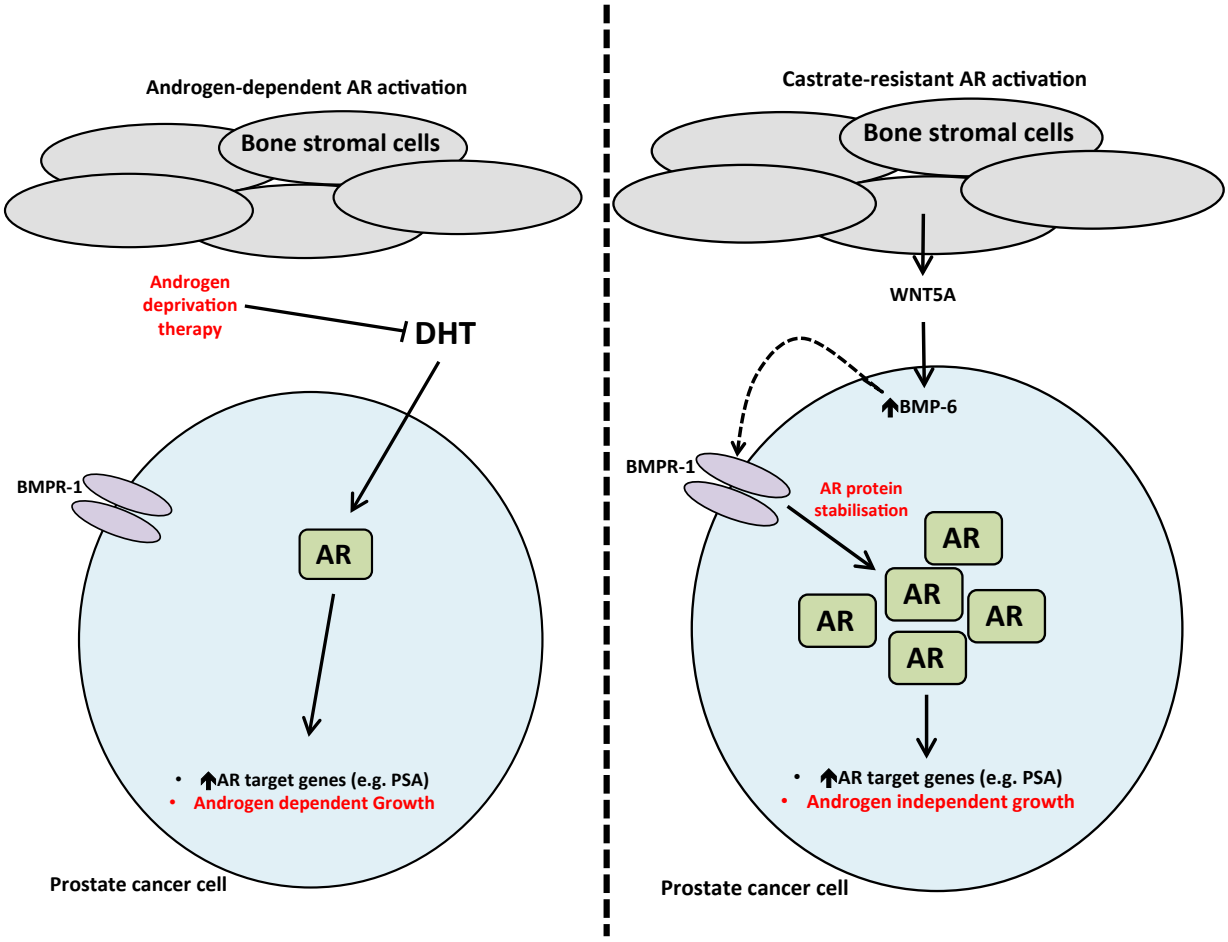

Figure 6.1 Model depicting how BMP signalling may activate AR signalling in CRPC. Based on my own observations that BMP activity potentially stabilises the steroid hormone receptor EcR at the level of protein, and the findings by others that BMP-6 induces AR activation in castrate-resistant prostate cancer, I have developed a hypothesis whereby WNT5A signals originating from bone stromal cells within the metastatic niche, induce BMP-6 within prostate cancer cells. Either by an auto- or para-crine mechanism this activates BMP signalling activity through stimulation of a currently uncharacterised BMP-receptor (BMPR). BMP activity increases AR levels by stabilising AR, possibly involving an interaction in the N-terminal domain of AR. AR signalling activity is observed despite androgen deprivation therapy as a consequence of elevated AR levels, possibly involving androgen-independent ligand binding to other steroid hormones or due to the presence of intra-tumoural androgens.

6.1.5.3 The AG, like the human prostate, may act as a site of steroid synthesis

Work performed by others had previously indicated that adult *Drosophila* SCs possess the upstream signalling machinery to promote ecdysteroid synthesis, namely the PTTH-hormone receptor, Torso. Furthermore, knockdown of either Torso or PTTH results in diminished whole-animal ecdysone titres (Rewitz, Yamanaka et al. 2009, Hentze, Moeller et al. 2013).

My observation that SC knockdown of the ecdysteroidogenic genes, *spo* and *shd*, results in diminished SC growth, a phenotype also associated with *EcR* knockdown, supports the hypothesis that these cells function as a site of ecdysone synthesis. Furthermore, these data suggest that SC-derived ecdysone is responsible, at least partially, for EcR-mediated SC growth, indicating that the mechanism for EcR activation in SCs occurs by either an auto- or para-crine mechanism.

Although in healthy adult men, the testes are the primary source of androgens, work in last decade indicates that the prostate too is a site of androgenic steroid synthesis, which assumes great importance in the context of CRPC (Montgomery, Mostaghel et al. 2008, Cai, Chen et al. 2011). Interestingly, metastatic tumours removed from surgically castrated males with prostate cancer display elevated levels of intra-tumoural testosterone, and at much higher than the levels detected in the primary tumour (Montgomery, Mostaghel et al. 2008). Furthermore, these metastatic lesions have very high expression of steroidogenic P450 enzymes involved in androgen synthesis (Montgomery, Mostaghel et al. 2008). These

enzymes are related to the Halloween genes that appear to be expressed in SCs (Chavez, Marques et al. 2000, Warren, Petryk et al. 2002, Petryk, Warren et al. 2003).

Taken together, these data suggest that steroid synthesis in male accessory reproductive organs is another example of conserved biology from SCs to the human prostate and, given the apparent importance of intratumoural androgens in advanced prostate cancer, studies of the *Drosophila* AG may provide useful insights into this biology.

6.1.5.4 The Rb/E2F1 axis plays important roles in both the accessory gland and the prostate as a regulator of steroid receptor activity

I show in sections 5.2.9 and 5.2.10 that the Rbf/E2F1 axis regulates the level of EcR expression. Activation of E2F1, by *Rbf* knockdown or E2F1 over-expression, results in extremely high levels of detectable EcR, and is accompanied by massive SC growth. The converse treatments, *E2F1* knockdown and *Rbf* up-regulation, result in a reduction of detectable EcR and diminished SC growth.

By now, it should come as no shock to the reader to learn that a similar role for Rb/E2F1 has been observed in human prostate cancer (Libertini, Tepper et al. 2006, Macleod 2010, Sharma, Yeow et al. 2010, Dean and Knudsen 2013). In the LNCaP prostate cancer cell line, over-expression of E2F1 confers resistance to androgen-withdrawal growth arrest, implicating E2F1 in the development of castrate-resistant disease (Libertini, Tepper et al. 2006). Significantly, AR levels detected by western blotting are markedly reduced in LNCaP cells cultured in androgen-depleted media. This contrasts to the observation in E2F1-overexpressing LNCaP cells, which display high AR protein levels in androgen-free media.

Furthermore, PSA, an AR-responsive gene is observed at high levels in the E2F1-overexpressing cells (Libertini, Tepper et al. 2006).

Similarly, a further study demonstrates that, although rare in organ-confined prostate cancer, *Rb* loss is a common hallmark of advanced disease (Sharma, Yeow et al. 2010). Furthermore, *Rb* loss leads to E2F1-mediated deregulation of AR in androgen-depleted conditions, with elevated AR protein levels observed as well as hallmarks of elevated signalling activity, with increased expression of a range of AR-regulated (Sharma, Yeow et al. 2010). The authors also present evidence that Rb loss is associated with recruitment of E2F1 to the AR regulatory locus, implying that the nature of the Rb/E2F1 and AR interaction involves regulation at the level of transcription (Sharma, Yeow et al. 2010), but they do not eliminate the possibility of post-transcriptional control. Two key experiments that now need to be performed are: i. test for post-transcriptional regulation of AR in prostate cancer cell lines using a constitutively expressed tagged AR; ii. test EcR activity in SCs where an ecdysone synthesis gene has been knocked down to determine whether Rbf/E2F1 and BMP signalling can induce hormone-independent signalling.

Again, the studies described above represent another demonstration of the remarkable parallels between the biology observed in human prostate cancer cells and the biology seen in the *Drosophila* accessory gland. From these data, it seems highly likely that some aspects of the interaction between the Rb/E2F1 axis and steroid receptor expression observed here represent a mechanism of growth regulation that is conserved across species. Therefore, these findings are likely to be of general interest to researchers beyond of the field of accessory gland biology, and may assist efforts to understand the genetic regulatory

mechanisms that go awry as prostate tumours progress from a hormone-dependent to castrate-resistant state.

6.2 Concluding remarks

In this thesis I display a body of work that represents a wide-ranging and comprehensive effort at characterising the biology of a hitherto relatively understudied cell type, *Drosophila* accessory gland secondary cells. In doing so, I have uncovered a set of surprising cell biological behaviours that have chimed-in with my long-standing interest in prostate cancer.

For example, secondary cells show a cell growth effect that occurs in an age- and mating-dependent manner, as well as displaying the ability to delaminate and migrate. I have shown here that both of these phenomena require BMP-signalling activity. Furthermore, I demonstrate that, as in the human prostate, steroid receptor signalling activity acts as a potent regulator of cell growth.

Perhaps the most pleasing aspect of this work is the novel finding that EcR levels and activity are tightly regulated by both BMP signalling and E2F1 activity. These observations are particularly interesting as they closely mirror regulatory mechanisms that control AR expression and activity in human prostate cancer cells, and appear important factors in the progression to castrate-resistant disease.

Happily, I can report that the research I have initiated in the *Drosophila* accessory gland has grown beyond all expectation, and generated interest from a number of research groups around the world. Within our own lab, a great deal of innovative research has been stimulated by my initial experiments in the accessory gland. In particular, work done by a fellow doctoral student, Laura Corrigan, was greeted with enthusiasm and interest by

researchers working in the rapidly growing field of exosome biology (Corrigan, Redhai et al. 2014).

Indeed, the flexibility of the genetic tools available to *Drosophila* biologists, combined with the advanced imaging techniques allowing visualisation of SCs pioneered in our lab, uniquely positions the accessory gland as an increasingly powerful model system in which to study exosome dynamics and endosomal trafficking. In fact, the ability to label and identify distinct intracellular compartments in SCs has been an exciting development that has emerged in our lab in the last year or so. As a research group, we are beginning to get a handle on understanding many aspects of how endosomal trafficking events are controlled to regulate a broad range of cellular processes, including growth, secretion and intercellular signalling.

Significantly, our group is now actively collaborating with other labs to test whether the regulation of the many aspects of fundamental cell biology the we have studied in the *Drosophila* accessory gland are conserved in human cell types. Specifically, we work closely with the Goberdhan group (University of Oxford), whose studies in human cancer cell lines broadly overlap with our interests in growth regulation, trafficking dynamics and exosome biology.

Without disclosing the details of the unpublished work of a collaborator, I think I can safely report that a number of our key findings in the accessory gland concerning the regulation of cell growth and exosome dynamics appear to play directly equivalent roles in human cancer cells, including colorectal and prostate cell lines. What seems to be emerging from our collaborative work is a pattern of co-operative research, whereby the fly model serves as an excellent tool to discover and unpick the regulation of an aspect of cell biology of interest,

with the human cell line work serving as a platform to test the relevance to human cell physiology and, if applicable, to perform more detailed biochemical characterisations that are currently beyond our ability in the accessory gland work.

Finally, I would like to close by thanking the reader for taking the time to explore the research within these pages. I do genuinely hope that what is written here has stimulated interest in the work that has absorbed my life these last three years, and I hope that my writing has given justice to the extraordinary biology of these curious cells. I can certainly say that (aside from the thesis writing) I have thoroughly enjoyed every single day of my time as a doctoral student, and I am proud to leave behind a small legacy of research that will hopefully contribute to the on-going studies of others.

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