

Elucidating the role of non-muscle myosin II
in *Caenorhabditis elegans*
stem-like seam cell divisions

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Abstract

Caenorhabditis elegans seam cells (SC) are multipotent neuroectodermal cells that undergo both symmetrical and asymmetrical divisions throughout larval development, thus providing a valuable model system to gain mechanistic insights into the regulation of asymmetric divisions and the switch between the symmetric and asymmetric modes of division. Reiterative SC asymmetric division typically produces a differentiative anterior daughter that moves out of the seam line and joins the hyp7 syncytium and a proliferative posterior daughter that retains seam fate and carries on dividing. Non-muscle myosin II (NMY II) has emerged as a key regulator in the asymmetric divisions of the *C. elegans* zygote, the *C. elegans* Q neuroblast, and the *Drosophila* neuroblast systems. In addition to being an essential player in cytokinesis, *nmy-2*'s roles in cell adhesion and migration processes further underline its potential as a regulator of seam cell asymmetric divisions.

In this thesis work, I investigated the role of NMY-2 in *C. elegans* seam cell divisions. I found that *nmy-2* is expressed in the seam and its protein localization is dynamic during SC divisions. Post-embryonic *nmy-2* knockdown using a combination of temperature sensitive mutants and RNA interference robustly reduces terminal SC number. This reduction is due to progressive SC loss after each asymmetric division as a consequence of aberrant cell fate determination. I identified three classes of cell fate transformation phenotypes following *nmy-2* knockdown, and sought to dissect the cell molecular basis of these phenotypes using a dual-color fate reporter strain. Although prevalent in *nmy-2* knockdown, cytokinesis defects are not the only cause of SC losses. *nmy-2* also does not appear to regulate SC divisions by affecting spindle positioning. In summary, *nmy-2* function is crucial to ensure the proper division and fate specification in post-embryonic SC development.

Thesis supervisor: Prof. Alison Woollard

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I would like to thank Alison for giving me the opportunity to undertake this project in her lab. This work could not have been done without the supervision from Alison and Pete. They showed me my very first worms under the microscope and have been there for me ever since.

I owe my initial interest in biology to my scientist parents. The tiny seeds of curiosity they planted in me have grown with me through all these years of learning and exploring, culminating to this volume of writing at the end of my PhD. Now that I have engaged in active research myself, I understand ever so clearly the circumstances they had gone through to raise me whilst building their own scientific careers. I am eternal grateful for everything they have done for me.

I have always appreciated the wonderful work environment that I am extremely lucky to have during my DPhil years. I am surrounded by the most knowledgeable, caring and supportive colleagues, and I cannot imagine having a better group of people to work with every day. They may not always be my colleagues, but they will always be my friends.

Last but not least, I thank all my other friends for being an important part of my life outside the lab. They have given me a good work-life balance, which is indispensable to my growth both as a scientist and a human being.

List of Abbreviations

A-P: anterior-posterior

AT: annealing temperature

BiFC: bimolecular fluorescence complementation

Cas9: CRISPR associated protein 9

CDK: cyclin-dependent kinase

ChIP: chromatin immunoprecipitation

CK II: casein kinase II

CRISPR: clustered regularly interspaced short palindromic repeat

DIC: differential interference contrast

ELC: (myosin) essential light chain

EMS: endomesoderm

ET: extension time

GFP: green fluorescent protein

GMC: ganglion mother cell

L2.1: L2 first (symmetric) division

L2.2: L2 second (asymmetric) division

miRNA: microRNA

MLCK: myosin light chain kinase

MosSCI: Mos1-mediated single copy insertion

MRCK: myotonic dystrophy kinase-related CDC42-binding kinase

MTOC: microtubule-organizing center

NB: (*Drosophila melanogaster*) neuroblast

NCC: nucleus-centrosomal complex

NGM: nematode growth medium

NLS: nuclear localization signal

NMY: non-muscle myosin

PKC: protein kinase C

PLK: Polo-like kinase

RLC: (myosin) regulatory light chain

RNAi: RNA interference

ROCK: Rho-associated, coiled coil-containing kinase

SEM: standard error of the mean

Semi-qPCR: semi-quantitative PCR

SC: seam cell

TRPM7: transient receptor potential melastatin 7

ts: temperature sensitive

WT: wild-type

ZIPK: leucine zipper interacting kinase

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Chapter 1 General Introduction

The fundamental question developmental biologists ask is how do multicellular organisms arise from a single fertilized cell. Development is under a myriad of complex and precise controls, and involves a fine balance between two processes: proliferation to increase cell numbers, and differentiation to produce different cell types. The cells produced from the proliferation and differentiation processes then form specialized tissues and organs through morphogenesis, a key feature that defines multicellular organisms. The field of developmental biology concerns different aspects of these processes and is a multifaceted field that links genetic and cell molecular regulation to phenotypic outcomes at the organismal level.

The balance between proliferation and differentiation must be precisely controlled during the development of organisms, as failed regulation can lead to disastrous consequences. Cancer, for example, arises from uncontrolled cell proliferation accompanied by aspects of “de-differentiation”. The outcome of a division is often linked to the division symmetry. One of the defining characteristics of stem cells is their ability to divide both symmetrically and asymmetrically. The symmetric division can be proliferative and generate two identical stem daughters to amplify the number of progenitors. This mode of division is frequently used at the early stages of development to increase cell number or to replenish the stem cell population following an injury (Morrison and Kimble, 2006). An asymmetric division, on the other hand, generates two

different daughters. Asymmetric division of a stem cell usually generates one daughter that retains the proliferative ability (i.e. self-renews) and one that differentiates and commits to another fate. Thus for stem cells, symmetric divisions are generally proliferative and asymmetric divisions are usually differentiative. Nevertheless, this link is not absolute, because symmetric divisions can generate daughters that are identical at birth but acquire different fates later on, for example through signaling.

My thesis work presented in this thesis exploited the *Caenorhabditis elegans* seam cells (SC) as a model to study the stem mode of division. These cells execute both symmetric and asymmetric divisions during post-embryonic development. I asked how asymmetric divisions are achieved in SCs and how the pattern of division is regulated at the cellular and molecular level during seam development. I specifically examined the potential involvement of non-muscle myosin II in these processes.

1.1 *C. elegans* seam cells as a model for the stem cell mode of division

C. elegans is a popular model organism for many reasons. *C. elegans* seam cells, also known as lateral hypodermal cells, provide an excellent model to study the stem cell mode of division and the regulation of asymmetric divisions.

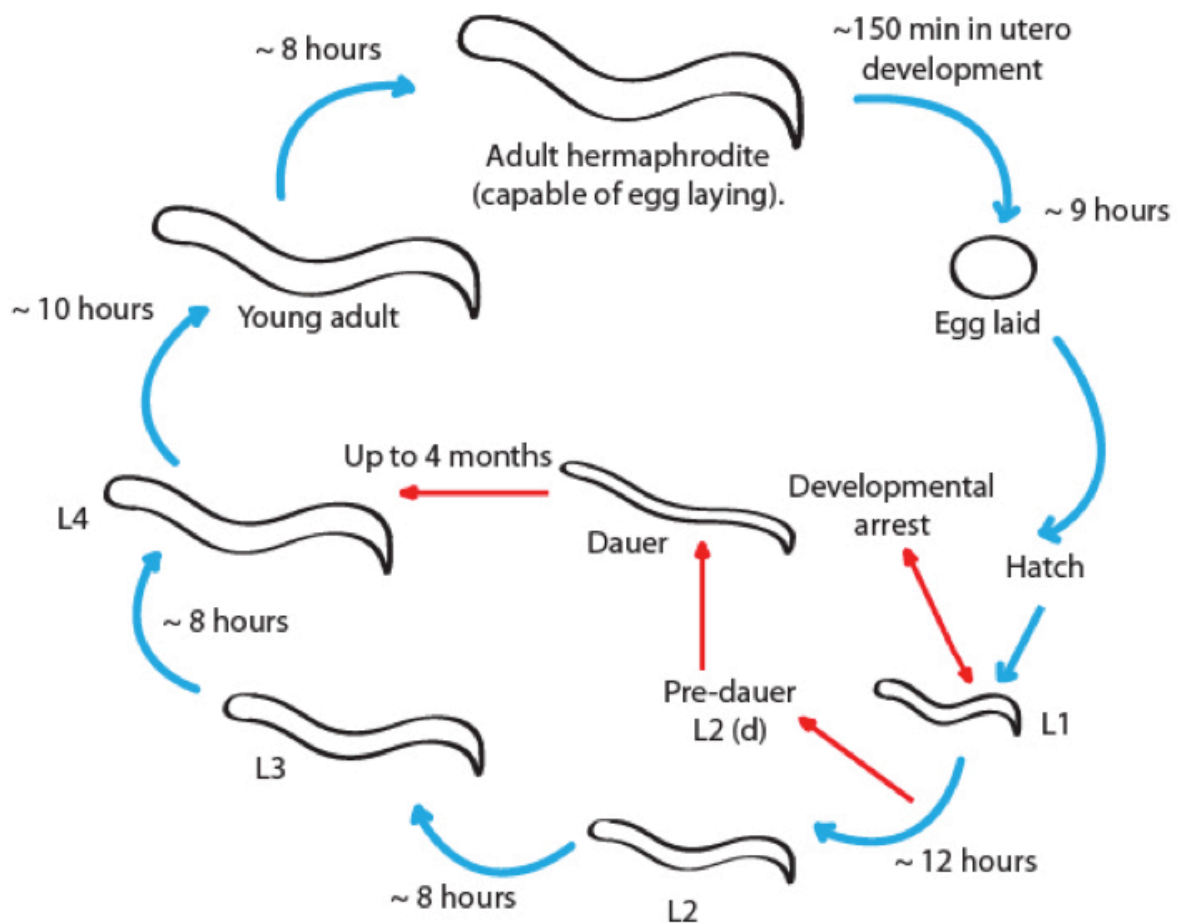
1.1.1 *C. elegans* as a model organism

C. elegans is a well-established model organism for studying a wide range of biological questions. It has a variety of features that make it particularly suitable for the study of development, and the research community has developed a wide range of experimental tools to create ever-expanding opportunities to address complex biological questions using this model organism.

C. elegans is a soil-derived nematode and is naturally found in microbe-rich habitats, such as decaying plant matter (Frezal and Felix, 2015). The worms are very small, with adults being only approximately 1mm in size, and can be easily cultured in a laboratory setting on Nematode Growth Medium (NGM) plates seeded with *E. coli* bacteria. Most natural populations are hermaphrodites (99.5%) (Brenner, 1974), enabling self-fertilization and clonal expansion. Males are rare in nature (0.5%), but can be generated in the laboratory by heat shocking or by crossing with High Incidence of Males (Him) mutants, thus satisfying genetic crossing needs. Due to its prevalence, most of the work presented in this thesis was performed using hermaphrodites unless otherwise indicated.

C. elegans has a short life cycle (Figure 1.1). Eggs hatch within 9 hours of being laid; larvae undergo four molts in about 3 days (at 20°C) before transitioning to adulthood (Byerly et al., 1976). This short generation time (~3.5 days) facilitates rapid genetic crossing. An adult hermaphrodite lives for about 3 weeks and produces 200-300 progeny (Byerly et al., 1976), so the population can expand rapidly to suit large-scale experimental needs. In addition to the

normal life cycle, there are two alternative developmental routes that the worms take in the absence of food. First, newly hatched larvae undergo developmental arrest and enter the L1 diapause if no food is available. Worms can survive as such for up to three weeks (Baugh and Sternberg, 2006) and re-enter the life cycle back at L1 upon feeding. This feature can be experimentally manipulated to synchronize worm populations for post-embryonic studies (Wood, 1988), which is frequently exploited in my thesis work (method described in Section 2.1.5). Alternatively, starvation or crowding during L2 triggers the larvae to enter the “dauer” state (Cassada and Russell, 1975). Dauer larvae undergo extensive physiological and behavioral changes to adapt to unfavorable conditions (Golden and Riddle, 1984) so they can survive up to 4 months. When food source becomes available, dauer larvae re-enter the life cycle at the L4 stage before becoming fertile hermaphrodites.



adapted from WormAtlas

Figure 1.1: *C. elegans* life cycle at 20°C. The normal life cycle is shown by blue arrows. Red arrows indicate two alternative developmental routes triggered by starvation (both routes) or crowding (dauer formation route only).

C. elegans has a relatively simple anatomy to enable easy phenotypic assessments. The worms have transparent bodies, rendering them amenable to microscopy. The complete, invariant lineage of the 959 cells in hermaphrodites (or the 1031 cells in males) has been described (Sulston et al., 1980; Sulston and Horvitz, 1977; Sulston et al., 1983), providing a reference for phenotypic analysis at single cell resolution. Of the 949 *C. elegans* hermaphrodite somatic cell divisions, 807 are asymmetric (Horvitz and

Herskowitz, 1992), making the organism particularly suitable for addressing questions regarding asymmetric divisions.

C. elegans was the first multicellular organism to have its genome completely sequenced (Consortium, 1998), providing an invaluable resource for the discovery of genes and the assessment of their functions. The well-annotated 97Mb *C. elegans* genome contains about 19,000 genes, nearly half of which have identifiable orthologs in humans (Rubin et al., 2000). About 38% of human disease genes have conserved orthologs in *C. elegans*, suggesting that *C. elegans* represents an opportunity to learn about higher organisms in a simple model system. The genomes of *C. elegans* and several other closely related nematode species are easily accessible through a dedicated, user-friendly community database called WormBase (<http://www.wormbase.org/>), alongside a multitude of other information. Forward genetics has generated a tremendous yet ever increasing number of genetic mutants thanks to multiple initiatives (including the *C. elegans* Knockout Consortium, the *C. elegans* National Bioresource Project of Japan, and the Million Mutations Project), as well as a plethora of individual screens. There is now a knockout mutant for nearly every annotated gene in the *C. elegans* genome, allowing the function of most genes to be studied individually and in combination. On the reverse genetics front, RNA interference (RNAi) provides an easy and effective method to silence genes (Fire et al., 1998). RNAi libraries have been constructed to cover a major portion of the *C. elegans* genome, facilitating high-throughput screening and the discovery of novel genetic interactions (Kamath et al., 2003; Rual et al., 2004). Last but not least, genome editing technologies such as

MosSCI (Mos1-mediated single copy insertion) (Frøkjaer-Jensen et al., 2010; Frøkjaer-Jensen et al., 2008; Frøkjær-Jensen et al., 2012; Frøkjær-Jensen et al., 2014; Robert et al., 2009) and CRISPR-Cas9 (clustered regularly interspaced short palindromic repeat - CRISPR associated protein 9) (Chen et al., 2013; Chiu et al., 2013; Cho et al., 2013; Dickinson et al., 2013; Friedland et al., 2013; Lo et al., 2013; Waaijers et al., 2013) have recently been extensively developed and optimized for *C. elegans*. These tools are extremely powerful and provide endless possibilities for precise genome editing, whether it concerns gene deletion, insertion or modification.

1.1.2 Seam cell anatomy

Seam cells are two lateral rows of specialized epithelial cells that line the length of the worm (Figure 1.2A, orange cells). SCs are smoothly tapered in shape, and are embedded in the hyp7 syncytium just underneath the cuticle, which runs along the body of the worm. SCs connect to the hypodermis by small adherens junctions along the apical borders and by small gap junctions on the lateral membranes. Worms are born with ten SCs on each side, and they are named H0, H1-2, V1-6, and T in the anterior to posterior order. Apart from H0, which never divides, all the other SCs undergo multiple divisions throughout larval development to both increase the number of SCs and to generate differentiated hypodermal and neuronal cells. The hypodermal daughter nuclei fuse to the surrounding hyp7 syncytium. In males, V5, V6 and T cells produce sensory rays in the tail through additional lineage divisions (Sulston et al., 1980; Sulston and Horvitz, 1977; Sulston et al., 1983). In

addition, SCs also synthesize alae, a set of longitudinal ridges on the sides of the worm, during L1, dauer, and adulthood (Figure 1.2B) (Cox et al., 1981). At the L4 to adulthood transition, the 16 SCs on each side of the worm terminally exit the cell cycle and undergo homotypic fusion to form a seam syncytium (Podbilewicz and White, 1994). The formation of alae in adulthood is an indication of correct SC terminal differentiation (Frand et al., 2005; George et al., 1998; Koh and Rothman, 2001).

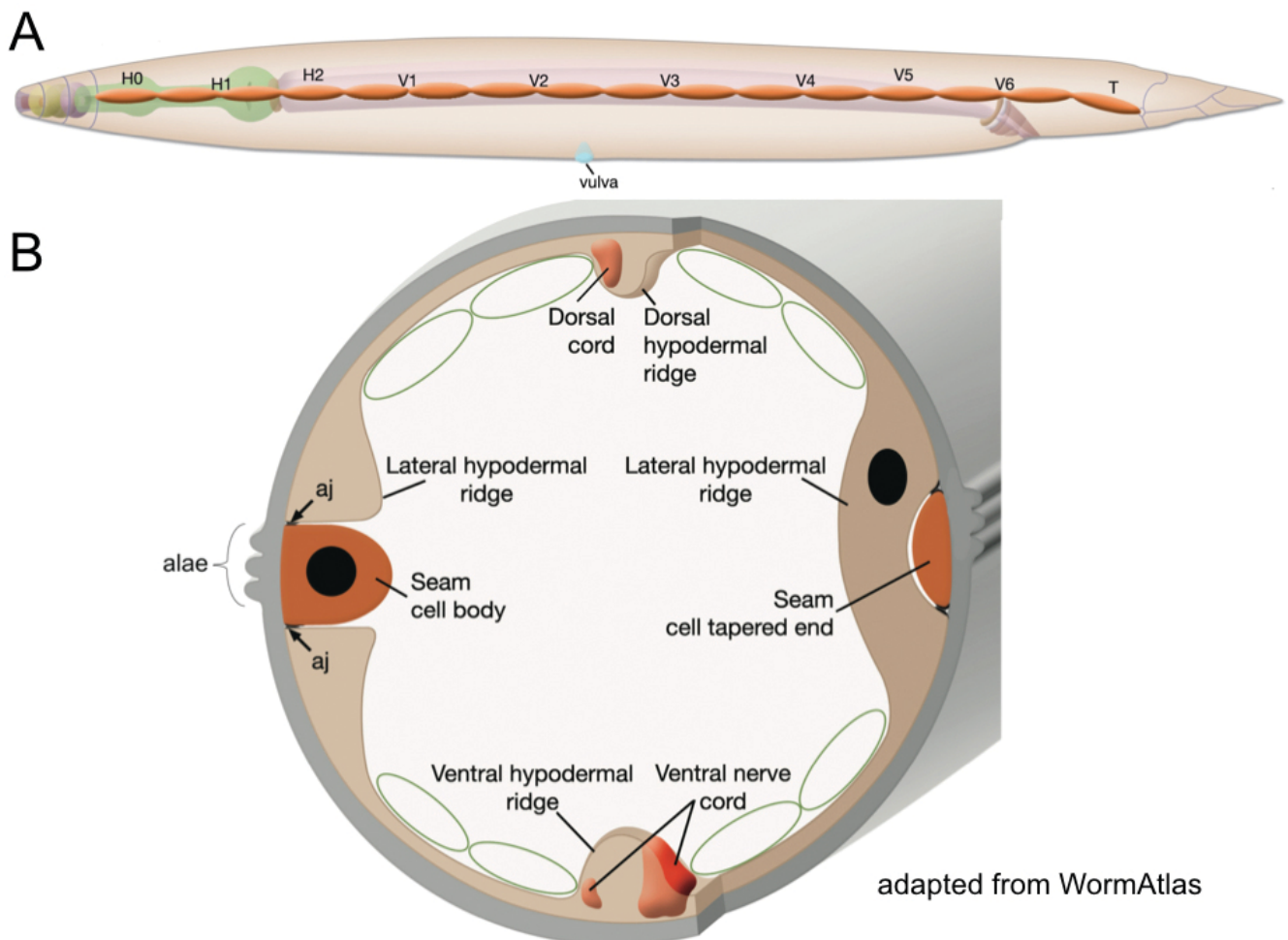


Figure 1.2: Schematic drawings of seam cells in an adult hermaphrodite. A) An overview of the left lateral SCs (orange). Left is anterior, right is posterior, up is dorsal, and down is ventral per developmental biology standard. These orientations will be used throughout the rest of the thesis unless otherwise noted. **B)** Cross section view of the SCs embedded in the hypodermis (brown). Due to the tapered shape, the ends

of the SC are surrounded by medial hypodermis (orange SC on the right side) while the main SC body interrupts the hypodermis completely (orange SC on the left side). In addition, SCs are surrounded by the hypodermis dorsally and ventrally. Cuticle is gray and has alae (sets of cuticular ridges) on both sides, which is secreted by terminally differentiated SCs. Dark green circles are muscle quadrants. aj = apical junction.

1.1.3 Seam cells as stem-like

Seam cells are multipotent stem-like cells that are capable of both symmetric and asymmetric divisions, overall along the anterior-posterior (A-P) orientation. Their complete lineage was determined (Figure 1.3A), allowing studies to be carried out at the single-cell resolution (Sulston and Horvitz, 1977). Shortly after every larval molt that marks the transition between larval stages (except for L1 which is not preceded by molting), the V lineage SCs (except V5) divide once asymmetrically to generate an anterior daughter which typically differentiates and a posterior daughter which retains the proliferative seam fate. After an asymmetric division, the anterior daughters round up and move out of the seam line to join the hyp7 syncytium (Figure 1.3B, ii-iv), while the tapered posterior daughters further elongate to re-form cell-cell contact (Figure 1.3B, iv-v). An additional symmetric division precedes the asymmetric division in the V and T cells at the L2 stage only, generating two seam daughters that are both capable of further divisions (Figure 1.3A). Throughout the rest of this work I will refer to the L2 first (symmetric) division as L2.1 division and the L2 second (asymmetric) division as L2.2 division. The precise lineages vary between different SCs, but the V cells mostly share an identical lineage apart from V5, which produces a sensory structure called the postdeirid. In males, V5, V6 and T also execute different lineages and produce sensory rays.

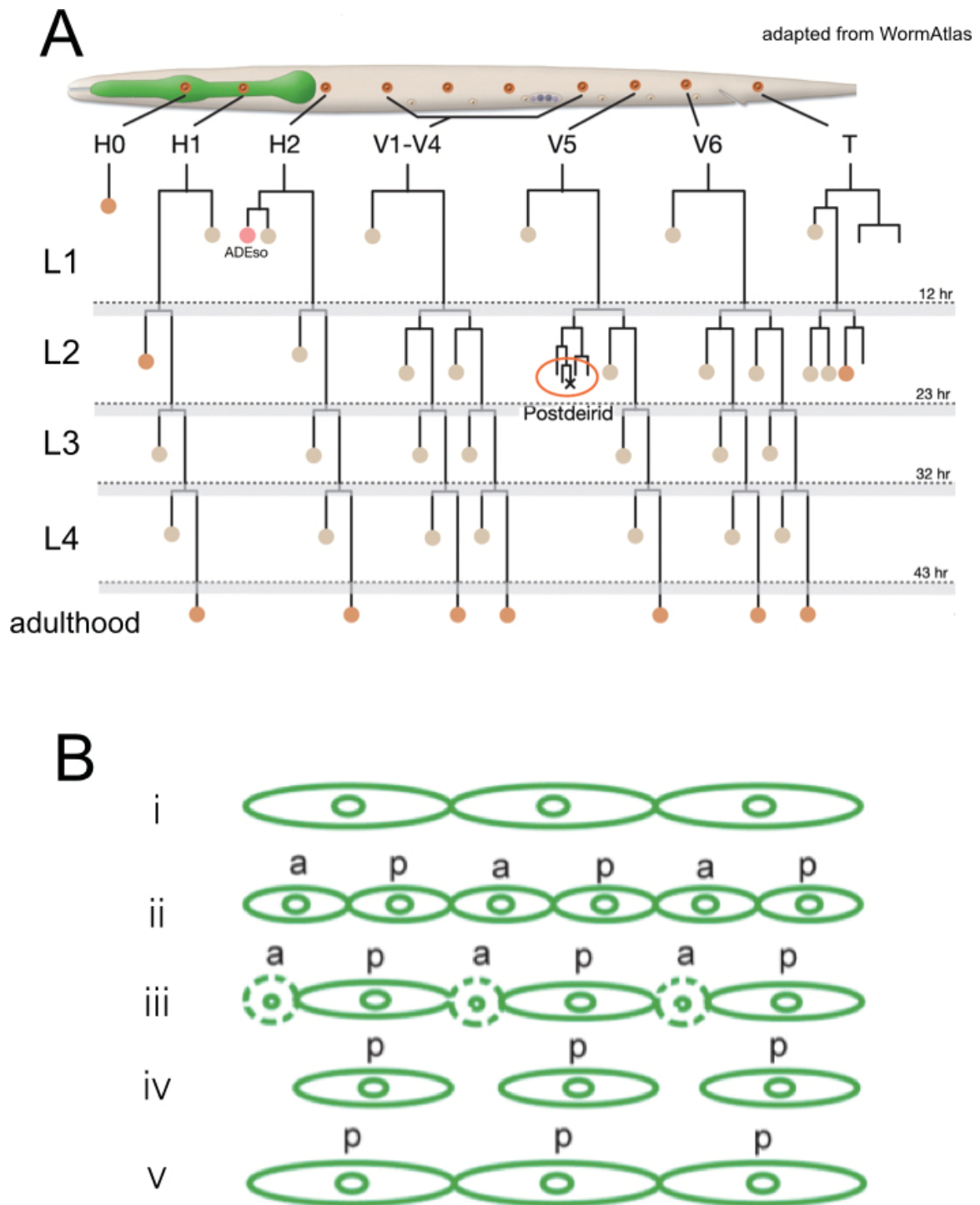


Figure 1.3: Seam cells divide symmetrically and asymmetrically. A) Hermaphrodite seam cell lineage. Dotted lines are transitions between larval stages; shading indicates molting. Time at hatching is 0 hr. Orange circles indicate terminal SCs; beige circles indicate terminal hypodermal cells unless otherwise indicated. **B)** Schematic drawing showing the events following an asymmetric division of three SCs (i). a is the anterior daughter, p is the posterior daughter. After an asymmetric division (ii), the anterior daughters round up and move out of the seam line (iii-iv), while the posterior daughters elongate (iv) to reconstitute cell-cell contact (v). Figure is not drawn to scale in B.

Therefore, SCs are capable of both modes of stem cell divisions: proliferative symmetric division and differentiative asymmetric division. The former increases the number of progenitor cells and the latter enables self-renewal whilst giving rise to multiple cell types, including hypodermal cells, neurons and neuronal support cells. SCs are not true stem cells because they lose the ability to divide indefinitely once they enter adulthood, when they permanently exit the cell cycle and fuse together to form a syncytium. The ability to continue proliferating and self-renewing in adulthood is unnecessary for a short-lived organism like *C. elegans*, so perhaps SCs are best described as transit amplifying cells that increase terminal cell numbers. SCs in *let-7* mutants that fail to undergo larval-to-adult transition continue to divide in adulthood (Reinhart et al., 2000), so perhaps the true stem fate is possible for SCs, however just not normally required by the worm life history, which is not reliant on regenerative capacity.

Nevertheless, SCs are stem-like and their invariant larval division patterns provide a valuable system to study the molecular bases of the stem cell mode of division. Questions such as how asymmetric divisions are controlled, how multiple divisions take place in a defined orientation, and how the decision between different modes of division is made may find answers in this simple yet powerful system.

1.2 How to achieve an asymmetric division

Asymmetric division is a fundamental mechanism for generating diverse cell types in a multicellular organism (Gallagher and Smith, 1997; Inaba and Yamashita, 2012; Knoblich, 1997). Symmetry breaking, separation of cell-fate determinants, chromosome segregation, and cell division must be spatially coordinated in order for a successful asymmetric division to occur, irrespective of context and purpose.

1.2.1 Diverse mechanisms generate asymmetric divisions

Divergent mechanisms are employed to generate asymmetric divisions in different organisms ranging from bacteria to budding yeast to plants and animals. There is no obligatory temporal order in which cell polarity, fate determinant segregation, spindle positioning and division-site specification take place. For example, while in animal cells the site of the cleavage furrow is usually specified by mitotic spindles during anaphase, fungi and plant mitoses have unique morphological features and adopt alternative mechanisms to determine the division site. Specifically, in fission yeast cells, microtubule-dependent placement of the nucleus during interphase dictates the placement of the cytokinesis contractile ring (Bathe and Chang, 2010; Rincon and Paoletti, 2012); budding yeasts specify the division site concomitantly with cell polarization (Bi and Park, 2012); most plant cells determine the cleavage site before mitosis using the preprophase band, a transient microtubule-containing cortical ring that engraves a molecular memory of the future division site to be exploited later on during telophase (Rasmussen et al., 2011). Indeed, each of the subprocesses of an asymmetric division can be the instructive initial event,

or be fully downstream and passive, or exert positive feedback to reinforce the initial asymmetry (reviewed in Li, 2013). Nevertheless cell polarization appears to be the most common initial process that instructs the patterning of asymmetric divisions through spindle orientation and division site placement in multicellular organisms (Li, 2013).

The current paradigm for cell asymmetric divisions is derived from animal development studies, especially of the *C. elegans* early embryonic divisions and the asymmetric divisions of the *Drosophila* neuroblast lineage (Morin and Bellaïche, 2011; Munro and Bowerman, 2009; Prehoda, 2009; Siller and Doe, 2009). A clear hierarchy of the sub-processes is seen in both well-studied systems. Cell polarity is established prior to mitosis and polarized cells have two biochemically and structurally different ends (Li and Gundersen, 2008; Macara and Mili, 2008). Fate determinants are then differentially segregated to the two poles by various mechanisms. Cortical cues dictate the orientation and positioning of mitotic spindles, which in turn specifies the cleavage site.

1.2.2 The paradigm: a closer look at the *C. elegans* zygote and *Drosophila* neuroblast systems

1.2.2.1 *C. elegans* zygotic division

During early embryogenesis, the *C. elegans* zygote undergoes a series of asymmetric divisions to generate different founder cells and establish multiple body axes. The first division has been the most extensively studied and offers

valuable mechanistic insights into the generation of an asymmetric division. The sperm microtubule-organizing center (MTOC) defines the posterior end of the fertilized egg and triggers asymmetric contraction of the cortical actomyosin cytoskeleton (Bienkowska and Cowan, 2012; Munro and Bowerman, 2009). Initially, the actomyosin meshwork was uniformly distributed on the cellular cortex prior to sperm entry, but as the sperm centrosome locally inhibits myosin at the posterior end, the cytoskeleton collapses towards the anterior, leading to actomyosin-mediated cortical ruffling and cytoplasmic streaming towards the anterior (Figure 1.4A) (Hill and Strome, 1988; Hird and White, 1993; Munro and Bowerman, 2009). The resultant polarized cortical flow advectively transports anterior PAR proteins such as PAR-3 and PAR-6 towards the anterior pole (Figure 1.4B) (Goehring et al., 2011; Zonies et al., 2010), leaving posterior PAR proteins behind. The transient PAR protein asymmetry is enhanced by mutual inhibition between anterior (PAR-3, PAR-6, PKC-3) and posterior (PAR-1, PAR-2) PAR proteins, thereby establishing stable anterior (green) versus posterior (red) domains (Goehring et al., 2011).

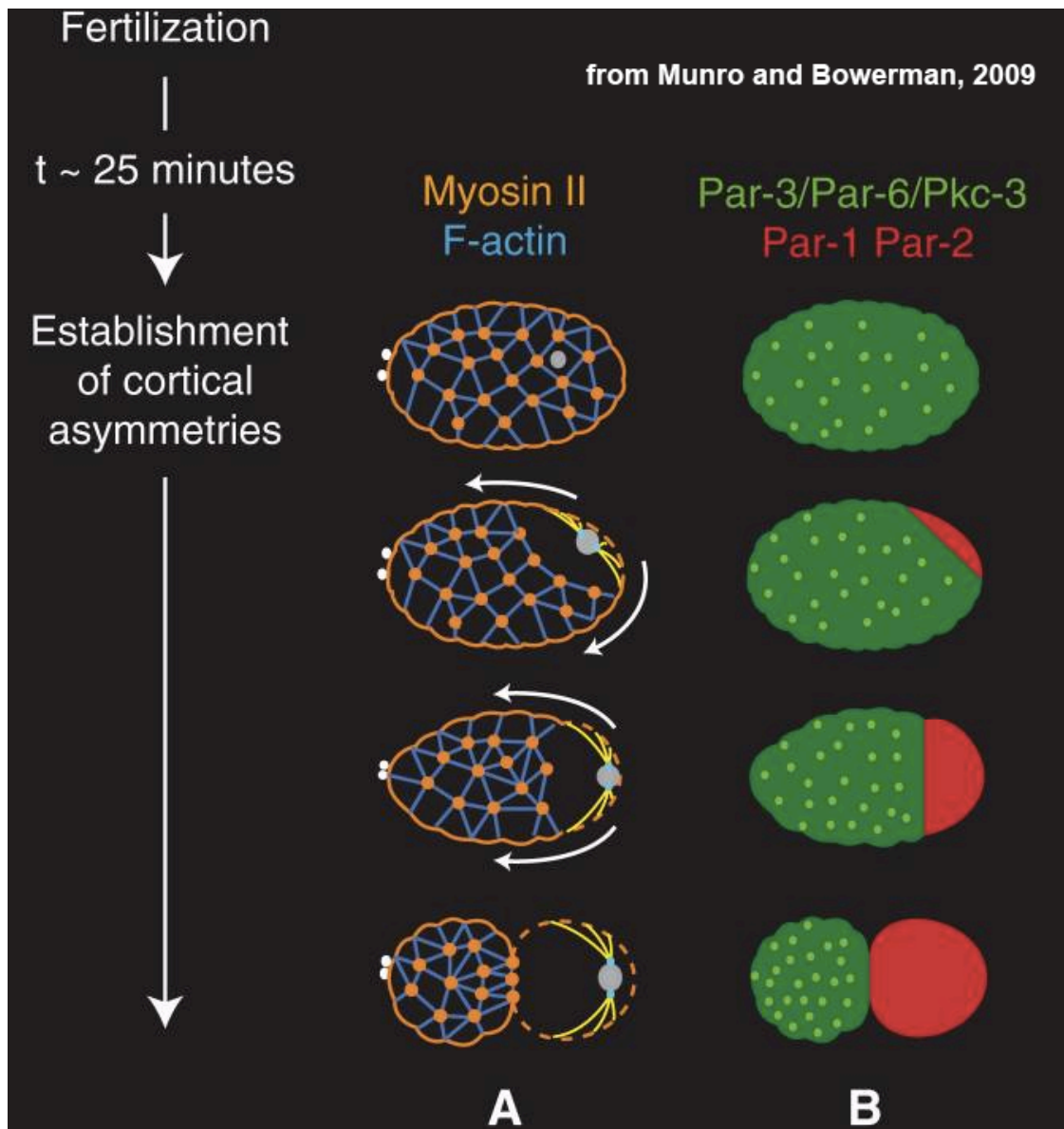


Figure 1.4: Symmetry breaking and polarity establishment in the *C. elegans* zygote. **A)** Sperm entry defines the posterior end of the zygote (sperm MTOC: gray, nucleus; cyan, centrosomes; yellow, microtubules). Actomyosin cortical network (orange, myosin II; blue, F-actin) drives focal contraction and cortical ruffling that transports PAR-3 and PAR-6 to the anterior. Contraction is locally inhibited by the sperm MTOC, thus becoming anteriorly restricted. **B)** PAR-3 and PAR-6 first transiently localize to the anterior by actomyosin-induced cytoplasmic streaming, then PAR proteins establish exclusive anterior (green) versus posterior (red) domains by mutual inhibition. The last frames of the images show the anterior and posterior domains separated by a deep transient furrow called the pseudocleavage (i.e. this is not yet the end of the division).

Next, a process called nucleus/centrosomal complex (NCC) centration occurs, whereby the NCC moves from the posterior cytoplasm, where the male and

female pronuclei first met, to the cell center using cytoplasmically anchored dynein (Figure 1.5A). Cortical asymmetry marked by anterior and posterior PAR proteins (Figure 1.5, red and blue cortical zones) subsequently instructs the orientation and positioning of mitotic spindles (Morin and Bellaïche, 2011; Siller and Doe, 2009). Spindle orientation is achieved by NCC rotation, which aligns the centrosomes in the A-P orientation along which the cell will divide (Figure 1.5B). At first the mitotic spindles form at the center of the zygote, but during early anaphase the elongating mitotic spindle complex moves towards the posterior end of the cell due to a larger net pulling force on the posterior spindle pole (Figure 1.5C). Cortically tethered dynein/dynactin complex (Figure 1.5, pink structures) generates the pulling force for spindle orientation and positioning by tracking along the astral microtubules, and these processes depend on cortical PAR polarity (Figure 1.5, red and blue cortical zones) for cues and Gα/GPR-1/2/Lin-5 heterotrimeric G proteins (Figure 1.5, golden complex) for dynein anchoring (Couwenbergs et al., 2007; Grill et al., 2001; Grill et al., 2003; Nguyen-Ngoc et al., 2007; Pecreaux et al., 2006). Posterior-directed mitotic spindle displacement specifies a displaced division plane, which ultimately generates a larger anterior daughter (AB) and a smaller posterior daughter (P1) from this first zygotic division.

adapted from Siller and Doe, 2009

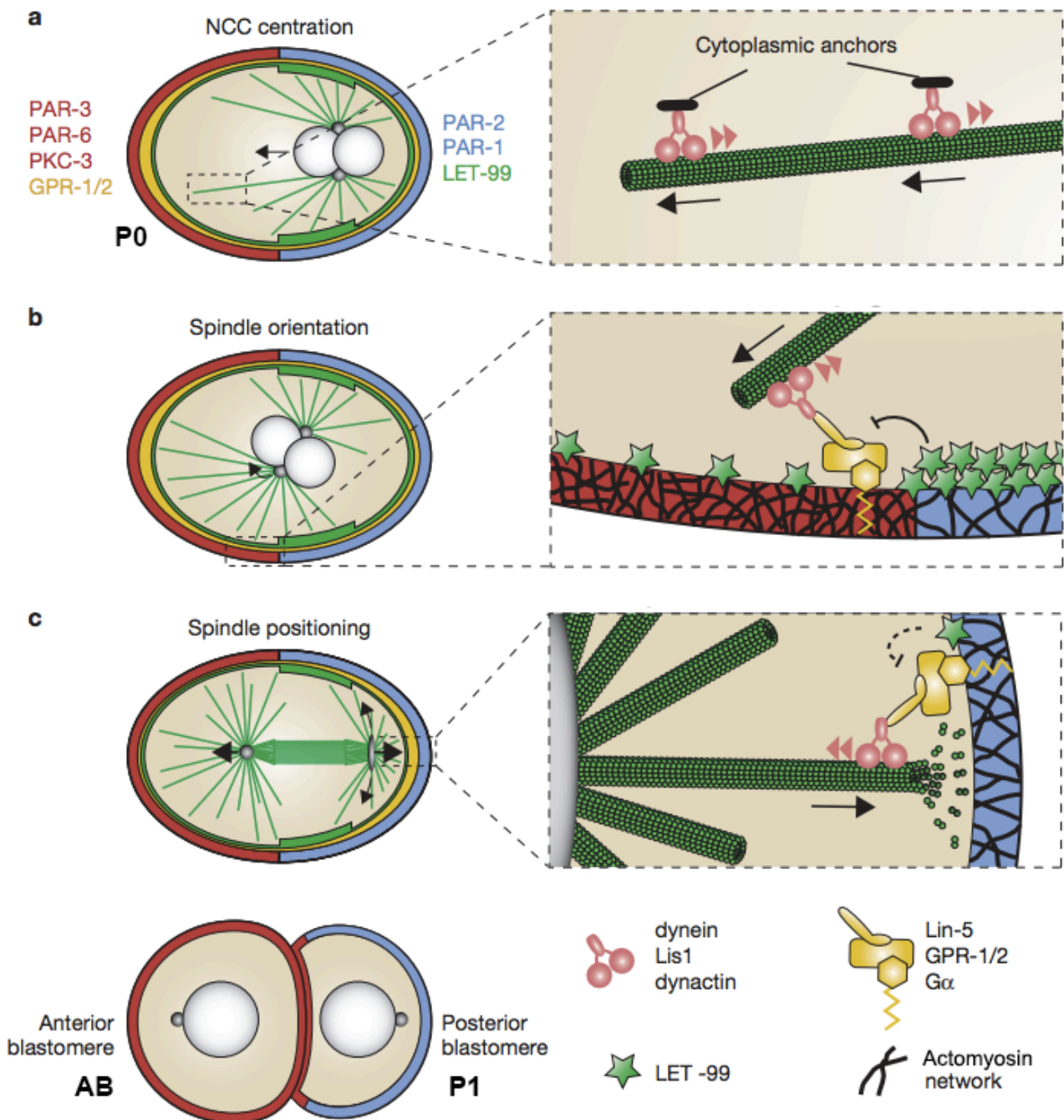
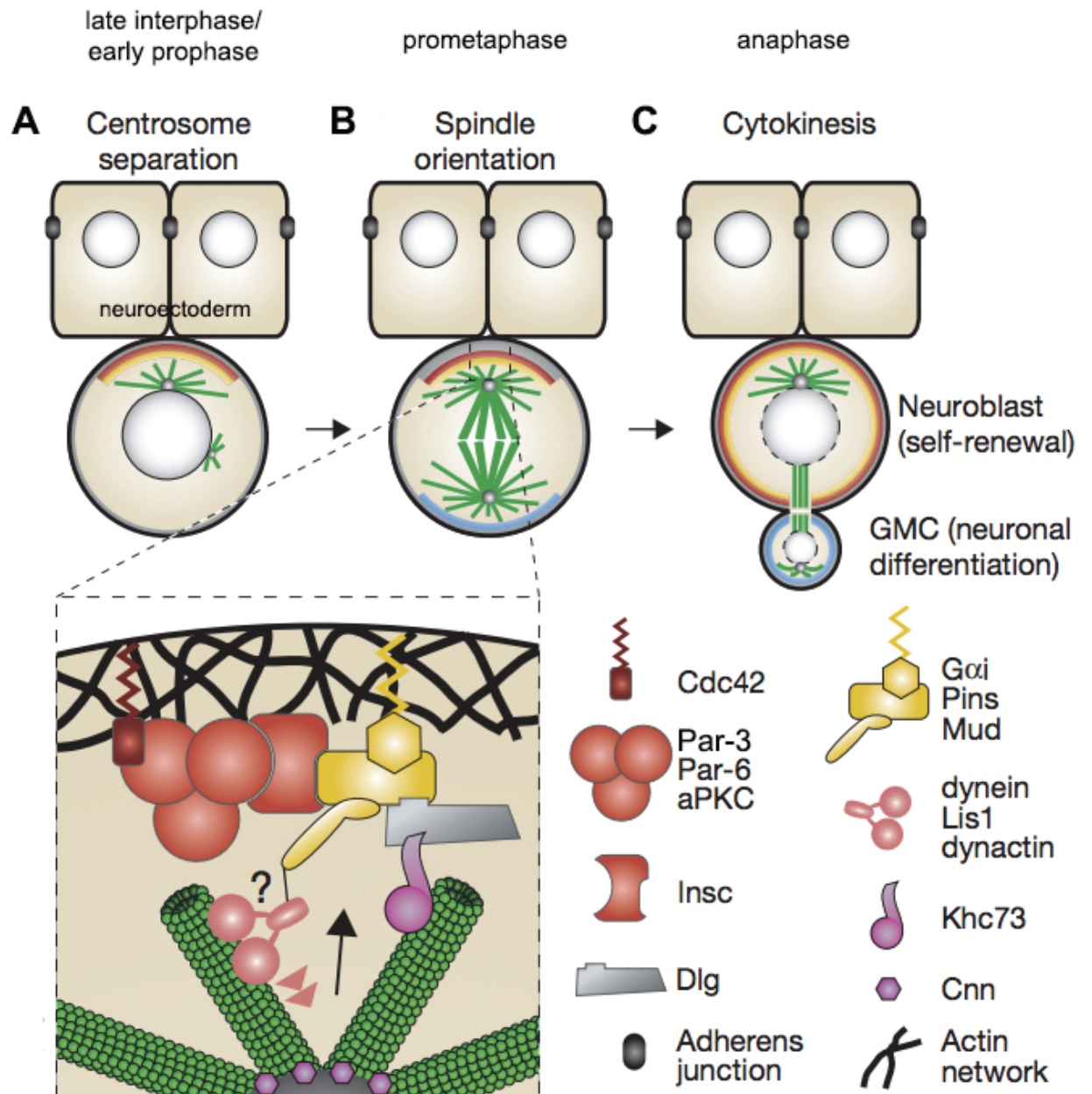


Figure 1.5: Spindle orientation and positioning in the *C. elegans* zygote. **A)** NCC centration occurs via cytoplasmically anchored dynein/dynactin complex (pink). **B)** Cortical PAR proteins provide cues for NCC rotation in order to align the centrosomes in the A-P orientation. **C)** At anaphase, the centrally-located spindle complex is pulled towards the posterior end, so that a larger anterior AB daughter and a smaller posterior P1 daughter are generated from the zygotic division. B and C both depend on dynein/dynactin complex that is cortically anchored by heterotrimeric G proteins (golden complex).

1.2.2.2 *Drosophila* neuroblast asymmetric divisions

The *Drosophila* neuroblast (NB) is an alternative established model, in particular for studying the stem cell mode of divisions. Embryonic NBs delaminate from the neuroectoderm and undergo asymmetric divisions perpendicular to the neuroectoderm to “bud off” a series of ganglion mother cells (GMC). NB asymmetric divisions generate a larger apical daughter that retains the NB progenitor fate and a smaller basal daughter that becomes the GMC, an intermediate neural progenitor to the *Drosophila* central nervous system.



adapted from Siller and Doe, 2009

Figure 1.6: Steps towards *Drosophila* neuroblast asymmetric divisions.

Drosophila NB asymmetric divisions involve three sub-steps. **A)** At late interphase/early prophase, the Par-3/Par-6/aPKC complex and the heterotrimeric G proteins are enriched on the apical cortex. Pins complex anchors the apical centrosome while the other centrosome migrates basally. **B)** Apical-basal spindle orientation takes place during prometaphase and depends on cortical PAR polarity and anchored dynein. **C)** The mitotic spindle becomes asymmetric during anaphase in order to generate a larger, apical NB daughter and a smaller, basal GMC daughter.

In NB divisions, polarity begins to develop in prophase and is established by metaphase. In prophase, the Par-3/Par-6/aPKC complex and Gai/Pins/Mud heterotrimeric G protein complex are already enriched at the apical cortex (Figure 1.6A, red and yellow apical cortical zones) (see Table 1 for a summary of *Drosophila* versus *C. elegans* homologs). The apical centrosome is anchored by Pins while the other centrosome migrates basally. Apical protein aPKC phosphorylates basal determinants to prevent them from apical association, thereby establishing and reinforcing the apical-basal polarity (Prehoda, 2009). Basal determinants include Miranda (an adaptor protein) and its cargos Prospero (a homeo-domain containing transcription factor) and Brat (a component of the RISC pathway), as well as Numb (a repressor of the Notch pathway) and Pon (Partner of Numb, an adaptor protein) (Figure 1.6B, blue basal cortical zone). Studies of cultured embryonic NBs demonstrate that NB polarity can be generated cell autonomously, but extrinsic cues are required to orient the polarity axis and to ensure robust polarization (Siegrist and Doe, 2006). NBs repeatedly break symmetry for each asymmetric division, as they depolarize after cytokinesis and repolarize during prophase of the next division (Prehoda, 2009). Following polarity establishment, mitotic spindles are aligned in an apical PAR protein-dependent manner using pulling force generated from cortically anchored dynein (Figure 1.6B) (Morin and Bellaïche, 2011; Siller and Doe, 2009).

So far there is remarkable mechanistic and molecular conservation between the *C. elegans* zygotic division and *Drosophila* neuroblast asymmetric divisions (Table 1). Both systems utilize orthologous anterior/apical PAR-3/PAR-6/PKC

complex to generate and maintain polarity; both rely on cortical PAR protein asymmetry for spindle orientation through a heterotrimeric G protein and dynein-dependent force generation mechanism.

Table 1: Protein homologs and their localization patterns in the *Drosophila* neuroblast and the *C. elegans* zygote

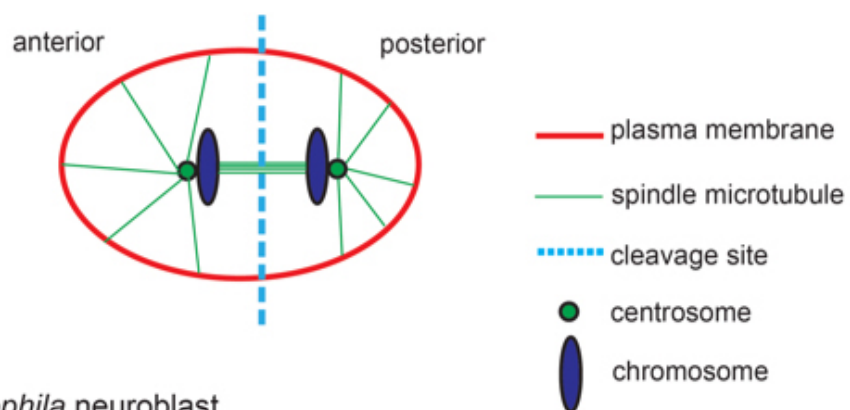
<i>Drosophila</i> neuroblast		<i>C. elegans</i> zygote	
Protein homolog	Localization	Protein homolog	Localization
Bazooka (Par-3)	apical	PAR-3	anterior
Par-6	apical	PAR-6	anterior
aPKC	apical	PKC-3	anterior
Mud	apical	LIN-5	anterior
Pins	apical	GPR-1/2	anterior
Gai	apical	GOA-1, GPA-16	anterior
Prospero	basal	PROS-1*	unknown
Brat	basal	NCL-1, NHL-1, NHL-2, NHL-3, LIN-41**	unknown
Numb	basal	NUM-1***	unknown

All homologs are listed according to Morin and Ballaïche, 2011, except * (Bürglin, 1994), ** (Hyenne et al., 2008), and *** (Nilsson et al., 2008).

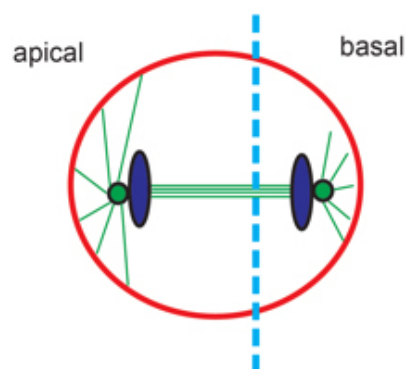
A major difference between the two systems is how the mitotic spindles specify the cleavage site in order to generate two unequal-sized daughters (Figure 1.7A and B). Cleavage sites are usually specified by the spindle midzone and placed so that the two sets of segregating chromosomes are equidistant from the future cleavage site. While in the *C. elegans* zygote the whole mitotic spindle complex migrates towards the posterior during anaphase to generate a smaller posterior daughter (Figure 1.5C, Figure 1.7A), *Drosophila* NBs utilize an unusual mechanism involving two functionally different centrosomes. While the spindles start out aligning in the center of the cell, during anaphase the apical centrosome emanates longer spindles than the basal centrosome does, so that the cleavage plane is shifted basally to generate a smaller GMC

(Figure 1.6C, Figure 1.7B) (Cai et al., 2003; Kaltschmidt et al., 2000; Rebollo et al., 2007; Rusan and Peifer, 2007). In fact *Drosophila* NBs also have a second, spindle-independent mechanism for furrow displacement, which will be introduced in Section 1.4.3.3.

A *C. elegans* zygote



B *Drosophila* neuroblast



C *C. elegans* Q neuroblast

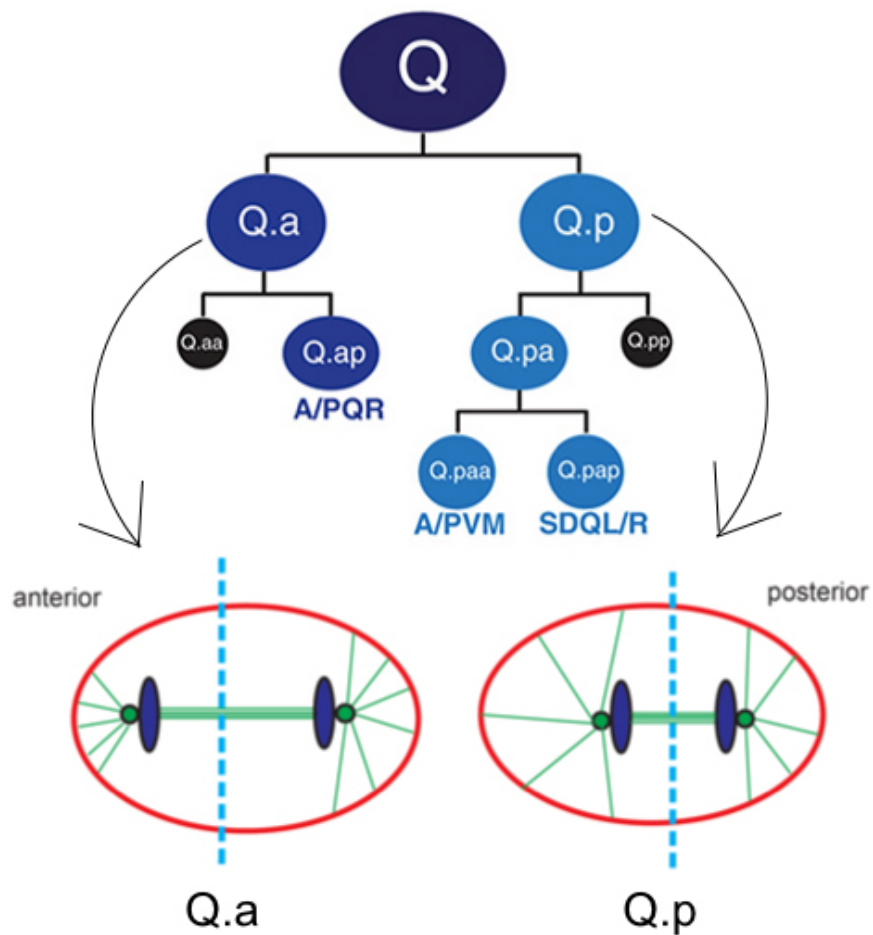


Figure 1.7: Spindle positioning in different systems. A) *C. elegans* zygote displaces the whole mitotic spindle towards the posterior. Cleavage furrow bisects the displaced spindle midzone. **B)** *Drosophila* neuroblasts emanate spindles of unequal lengths from the two centrosomes to basally displace the cleavage furrow. **C)** *C. elegans* Q neuroblast simultaneously employs the *Drosophila* neuroblast mechanism in the Q.a cell and the *C. elegans* zygote mechanism in the Q.p cell. Black filled circles denote apoptotic cells (Q.aa and Q.pp).

1.2.2.3 Spindle positioning in *C. elegans* Q neuroblast

Notably, both spindle-based mechanisms for generating unequal-sized daughters are used simultaneously in a third system, the *C. elegans* Q neuroblast lineage (Figure 1.7C). *C. elegans* Q neuroblasts undergo three rounds of divisions to produce three different neurons and two apoptotic cells. In the second round of division, Q.a generates a smaller anterior apoptotic cell and a larger posterior cell. Conversely, Q.p produces a larger anterior daughter and a smaller posterior apoptotic daughter. Q.a and Q.p exploit different mechanisms to generate daughters of unequal sizes and subsequently, different fates. While Q.p displaces the entire mitotic spindle complex towards the posterior end during anaphase (like the *C. elegans* zygotes), Q.a utilizes spindle arms of unequal lengths (like the *Drosophila* NBs) (Figure 1.7C) (Ou et al., 2010).

1.2.3 The coupling between cell cycle progression and asymmetric divisions

There is an emerging notion that the regulation of proliferation and differentiation can be tightly linked rather than being discrete and binary. Examples of coordination between these two processes in *C. elegans* and *Drosophila* systems have been reviewed (Ishidate et al., 2014; Noatynska et al., 2013). Fundamental cell polarity determinants such as PAR proteins have been shown to influence cell-cycle progression. Conversely, cell cycle regulators such as cyclin/cyclin-dependent kinases (CDKs), Polo/Polo-like kinases (PLKs) and aurora kinases have all been found to regulate cell fate and cell polarity independently of cell cycle progression (Noatynska et al., 2013).

For instance, in *C. elegans* early embryogenesis, polarity determining PAR proteins act via MEX-5/6 to regulate asynchronous mitotic entry at the two-cell stage by controlling the localization of key cell cycle components PLK-1 and CDC-25.1 (Noatynska et al., 2013). On the other hand, the cell cycle regulatory cyclin E/CDK-2 complex not only establishes polarity by regulating centrosome maturation (Cowan and Hyman, 2006), but also modulates the transition from germ cell proliferation to meiotic differentiation (Fox et al., 2011; Jeong et al., 2011). The cyclin B/CDK-1 complex also has at least two roles in embryogenesis. It has been shown to regulate the oocyte to embryo transition by coupling OMA-1 degradation to the first mitotic division (Shirayama et al., 2006), and it cooperates with WNT to promote asymmetric cortical WRM-1 release in the four-cell stage endomesoderm (EMS) blastomere, thereby allowing for spindle rotation and division axis specification in that division. (Ishidate et al., 2013; Kim et al., 2013).

This notion of coordinated cell cycle regulation and cell polarity establishment is relatively new and first emerged from recent genetic screens (Labbé et al., 2006; Noatynska et al., 2010; Tilmann and Kimble, 2005). Rare and subtle mutations generated from forward genetics enable the investigation of developmental roles without affecting cell cycle regulation (Shirayama et al., 2006). It is suggested that this orchestrated crosstalk between the two processes will gain wider experimental support in the future.

1.3 Multiple regulatory pathways and cellular factors regulate seam cell divisions

Various pathways and factors are known to regulate post-embryonic SC development. The WNT pathway sets up the general A-P polarity for SCs, the RNT-1/BRO-1 pathway acts as a major fate-determinant in proliferative seam daughters, and the heterochronic pathway governs stage-specific SC division patterns. Additional factors such as cell shape and cell-cell signaling are also thought to be important in the seam.

1.3.1 WNT signaling

WNT signaling is a conserved pathway impacting on gene transcription during metazoan development. WNT ligand binding transduces WNT signals in a variety of cellular processes such as cell proliferation, differentiation, migration, and polarity (Eisenmann, 2005; Sawa and Korswagen, 2013). There are two

main WNT pathways in *C. elegans*: the canonical pathway and the WNT/ β -catenin asymmetry pathway (schematic drawings of both in Figure 1.8). WNT signaling is responsible for most somatic divisions occurring along the A-P axis in *C. elegans* (Mizumoto and Sawa, 2007b). The WNT/ β -catenin asymmetry pathway, but not the canonical WNT pathway, plays a global role in post-embryonic seam cell development (Gleason and Eisenmann, 2010; Mizumoto and Sawa, 2007b). The canonical WNT pathway, on the other hand, acts through the Hox genes to regulate the formation of the postdeirid from the V5.p seam cell, as well as the development of tail sensory rays in males (Hunter et al., 1999; Maloof et al., 1999; Zhang and Emmons, 2000).

WNT signaling in *C. elegans* utilizes multiple redundant WNT ligands (Yamamoto et al., 2011) and different effector β -catenins. While the canonical WNT pathway functions through the β -catenin BAR-1 as a transcriptional co-activator, the WNT/ β -catenin asymmetry pathway uses both WRM-1 and SYS-1 β -catenins (Eisenmann, 2005). Briefly, in the canonical WNT pathway (Figure 1.8A), WNT ligand binding stabilizes cytoplasmic BAR-1/ β -catenin, which is otherwise ubiquitinated and degraded, and translocates it to the nucleus. Nuclear BAR-1 interacts with POP-1, the sole *C. elegans* TCF family transcription factor, to drive the transcription of WNT target genes. In the WNT/ β -catenin asymmetry pathway (Figure 1.8B), the posterior daughter receives WNT signal whereas the anterior daughter does not. In the unsignaled anterior daughter, high nuclear POP-1 to SYS-1 ratio leads to the transcriptional repression of WNT target genes; in the signaled posterior daughter, low nuclear POP-1 to SYS-1 ratio leads to the activation of WNT

Figure 1.8: WNT signaling. **A)** Canonical WNT signaling pathway uses the β -catenin BAR-1. **B)** WNT/ β -catenin asymmetry pathway uses the β -catenins WRM-1 and SYS-1. The posterior daughter receives WNT signaling while the anterior daughter does not. The rainbow shaped factor in the anterior daughter represents a transcriptional repressor.

The WNT/ β -catenin asymmetry pathway globally regulates seam post-embryonic development along the A-P axis. The pathway components are asymmetrically localized before and after division to ultimately generate different transcriptional activities in the two daughters. In SC asymmetric divisions, the anterior daughter differentiates and the posterior daughter remains seam. Prior to the division, the mother SC is already polarized with more WRM-1/ β -catenin, LIT-1/NLK and APR-1/APC on the anterior cortex (Mizumoto and Sawa, 2007b; Takeshita and Sawa, 2005). This results in more WRM-1, LIT-1 and APR-1 on the anterior daughter cortex following the division. This cortical asymmetry translates into nuclear WRM-1 asymmetry through APR-1 modulation of spindle structures. More astral microtubules are found on the anterior side of the anterior daughter, allowing nuclear WRM-1 to be trafficked to the anterior cortex of that daughter by kinesins (Mizumoto and Sawa, 2007a; Sugioka et al., 2011). This leads to a low level of nuclear WRM-1 in the anterior daughter compared to the high level in the posterior daughter. WRM-1 binds to and activates LIT-1, which in turn phosphorylates POP-1 to facilitate its nuclear export (Rocheleau et al., 1999), so WRM-1 nuclear asymmetry leads to reciprocal POP-1 nuclear asymmetry, with more POP-1 in the anterior daughter nucleus and less in the posterior one (Lo et al., 2004b; Phillips and Kimble, 2009). POP-1 nuclear export in the posterior daughter lowers the ratio between POP-1 and its co-activator SYS-1/ β -catenin in the nucleus. SYS-1 shows reciprocal asymmetry to POP-1 downstream of WNT

signaling (Phillips et al., 2007). Its nuclear asymmetry is regulated independently of WRM-1 and POP-1, directly by the frizzled and dishevelled homologs which also control WRM-1 and POP-1 asymmetry (Phillips et al., 2007). Normally, a high nuclear POP-1 to SYS-1 ratio means POP-1 interacts with transcriptional repressor proteins to inhibit the transcription of WNT target genes. This is the case in the unsignaled anterior daughter, where WNT targets are repressed. By contrast, the signaled posterior daughter has lowered POP-1 to SYS-1 ratio in the nucleus, allowing SYS-1 to interact with POP-1 to activate the transcription of WNT target genes (Lo et al., 2004b; Phillips et al., 2007). A summary of the nuclear asymmetry of key pathway effector molecules is shown in Figure 1.9.

adapted from Munro and Bowerman, 2009

The diagram illustrates the differential expression of a target gene in two nuclei. The left nucleus (Anterior) has a high concentration of POP-1 (blue circles) and low concentrations of SYS-1 (red circles), WRM-1 (green circles), and LIT-1 (yellow circles). The right nucleus (Posterior) has a low concentration of POP-1 and high concentrations of SYS-1, WRM-1, and LIT-1. In both nuclei, a 'Target gene' is shown being transcribed. The diagram is adapted from Munro and Bowerman, 2009.

Anterior nucleus	Posterior nucleus
POP-1 high	POP-1 low
SYS-1 low	SYS-1 high
WRM-1 low	WRM-1 high
LIT-1 low	LIT-1 high

Figure 1.9: Summary of WNT/ β -catenin asymmetry pathway effectors in the anterior and posterior nuclei.

So far it is unknown why only the posterior seam daughter responds to WNT signaling; a number of different models have been proposed to explain the phenomenon, including the establishment of a WNT receptor gradient (Gleason and Eisenmann, 2010; Yamamoto et al., 2011). Ultimately, the WNT-responsive genes are repressed in the anterior daughter of a SC division and activated in the posterior daughter, thereby ensuring that seam fate is specified

in the posterior daughter only. WNT signaling is key for specifying the seam fate. *wrm-1* disruption reduces SC numbers to as low as three per side, and *pop-1* silencing generates up to 67 SCs per side (Gleason and Eisenmann, 2010).

In addition to its role in seam cell asymmetric divisions, the WNT/ β -catenin asymmetry pathway in *C. elegans* is most well-known and best studied in the P2/EMS signaling process at the four-cell stage of embryogenesis. In this very first signal-induced asymmetric division, WNT signaling polarizes EMS to divide into an anterior MS daughter that produces mesodermal tissues and a posterior E daughter that produces endoderm (Eisenmann, 2005). The mechanistic details in this EMS division are strikingly similar to that of the seam cell asymmetric division, even though the latter is a self-renewal division while the former is not. Hence the mechanism operated by the WNT/ β -catenin asymmetry pathway to specify cell fate is not stem-specific.

Several other differences between how seam cells and the EMS uses the WNT/ β -catenin asymmetry pathway to specify differential daughter fates include that 1) the EMS division relies on parallel inputs from the WNT and the SRC pathways at the P2/EMS junction where as SRC signaling appears unimportant for seam divisions. 2) The EMS division involves spindle rotation from the L-R axis to the A-P axis as a result of WRM-1 cortical release from the EMS posterior cortex (Kim et al., 2013), however no spindle rotations occur in seam cell as all divisions take place in the A-P orientation. 3) In the EMS division, WNT and SRC signaling affect POP-1 nuclear localization through

centrosome-dependent asymmetric nuclear anchoring, in addition to POP-1 nuclear export mediated by WRM-1/LIT-1 (Sugioka and Sawa, 2010); this nuclear anchoring has not been reported as the case in seam cells.

1.3.2 RNT-1/BRO-1 pathway

The RUNX family of transcription factors plays an important role in metazoan stem cell proliferation and has been implicated in both cancer biology (Cameron and Neil, 2004) and development (Appleford and Woollard, 2009; Nimmo and Woollard, 2008). The RUNX genes are defined by a highly conserved 128 amino acid Runt domain, which is involved in both DNA binding (using the consensus recognition motif: RACCRCA) and heterodimerization with its CBF β binding partner (Kagoshima et al., 1993; Li and Gergen, 1999; Ogawa et al., 1993). RUNX transcription factors can serve to activate or repress transcription depending on the binding context (Canon and Banerjee, 2003).

RNT-1 and BRO-1 are the sole *C. elegans* homologs of RUNX and CBF β , respectively. The predominant function of RNT-1 and BRO-1 in *C. elegans* is to promote the proliferative fate in the posterior seam daughters (Kagoshima et al., 2007; Kagoshima et al., 2005; Nimmo et al., 2005; Xia et al., 2007). Figure 1.10 provides an overview of the RNT-1/BRO-1 pathway. While *mnt-1* and *bro-1* mutants both have fewer SCs, over-expression of either gene results in seam hyperplasia (Kagoshima et al., 2007; Nimmo et al., 2005). *mnt-1* and *bro-1* mutant males also have defective tail sensory rays due to failed proliferation of

V and T lineages. When ectopically over-expressed, RNT-1 appears to promote SC proliferation in L2 and L3 stages by inhibiting CKI-1, a cyclin E/CDK-2 inhibitor, during G1 phase of the cell cycle (Nimmo et al., 2005). There is some discussion as to whether RNT-1 and BRO-1 function in SC proliferation or fate determination. While Nimmo et al. (2005) concluded that RNT-1 is involved in proliferation rather than fate determination based on their lineage analysis of the V cells, Kagoshima et al. (2005) found that RNT-1 also functions with the WNT pathway to control the asymmetry and fate specification of the T blast cell division. As the lineage outcome in the case of the T division is failed proliferation and thus the subsequent differentiation, it is difficult to distinguish semantically whether *rnt-1* mutation produces a proliferation or differentiation defect in the T blast cell. In other words, if the “fate” is further proliferation, then disruption of either “proliferation” or “fate determination” (if these case can be indeed be separated) will amount to the same thing.

CEH-20/Pbx and UNC-62/Meis TALE-class transcription factors likely function together upstream of the RNT-1/BRO-1 pathway to regulate SC division asymmetry. Their preferential localization in the anterior daughter nucleus suggest that they likely repress RNT-1 and thus the proliferative potential in anterior daughters, thereby allowing them to differentiate (Hughes et al., 2013).

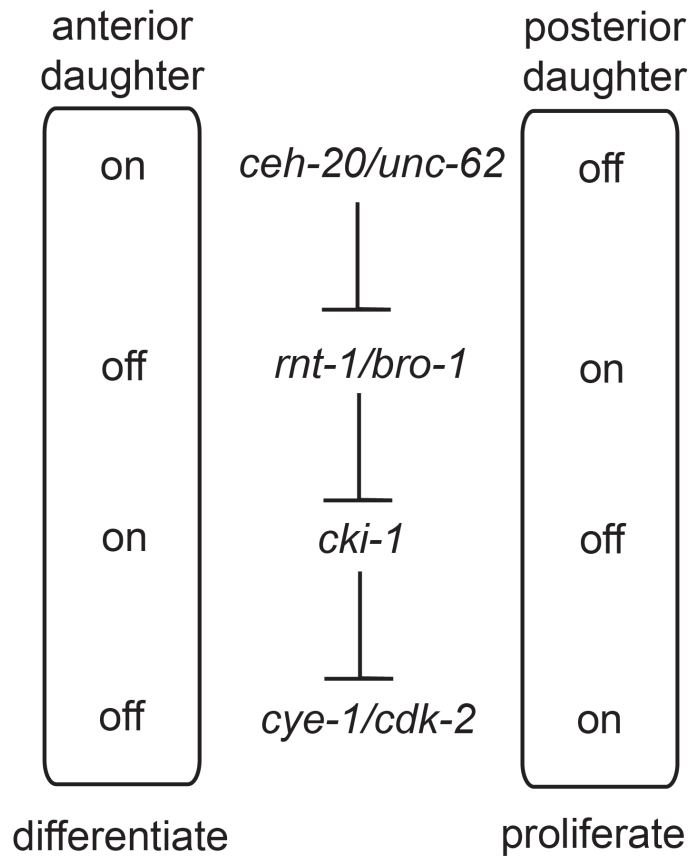


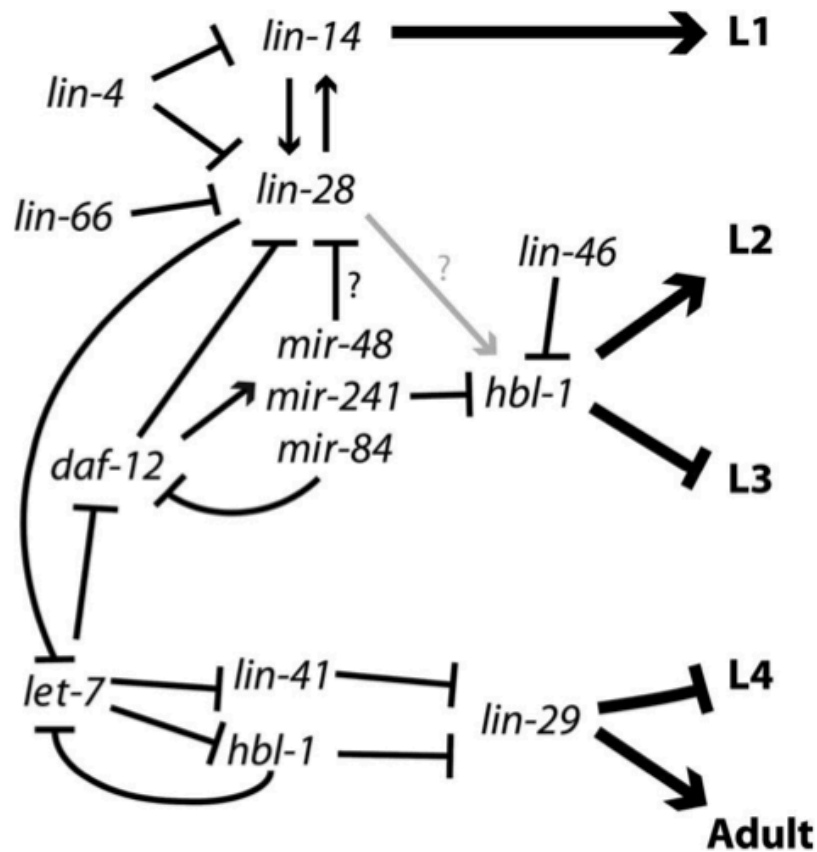
Figure 1.10: *C. elegans* RNT-1/BRO-1 pathway in seam cell asymmetric division. *cye-1* encodes cyclin E.

1.3.3 Heterochronic pathway

Yet another well-studied group of genes that control post-embryonic seam development are the heterochronic genes (Figure 1.11). In *C. elegans* the heterochronic pathway specifies the timing of larval developmental processes in multiple tissues such as the epidermis, neurons, intestine, and muscles (Ambros, 2000; Moss, 2007; Resnick et al., 2010). Heterochronic mutants can have either the precocious or the retarded phenotype, meaning that events specific to a stage are skipped or repeated, respectively. Different developmental events can be affected, including cell division pattern (Ambros

and Horvitz, 1984), differentiation (Ambros, 1989), and cell cycle progression (Ambros and Horvitz, 1984). Heterochronic genes have been most extensively studied in *C. elegans* due to its relatively simple anatomy, short developmental window, and signature stage-specific division patterns. While some heterochronic genes only function in *C. elegans*, others such as *lin-4* and *let-7* have clear orthologs in mammals and other vertebrates that may also have roles in developmental timing (Lagos-Quintana et al., 2001; Lagos-Quintana et al., 2002).

Several heterochronic genes encode microRNAs (miRNAs) to promote transitions between larval stages. *lin-4* is important for the L1 to L2 transition, *let-7* family miRNAs (*mir-48*, *mir-241*, and *mir-84*) for the L2 to L3 transitions, and *let-7* for the L4 to adult transition (Abbott et al., 2005; Lee et al., 1993; Reinhart et al., 2000; Wightman et al., 1993). *lin-4* mutants skip the L2 proliferative division and have a reduced number of SCs; mutants lacking the *let-7* sister miRNAs reiterate the proliferative division to produce extra SCs (Resnick et al., 2010).



adapted from Resnick et al., 2010

Figure 1.11: *C. elegans* heterochronic gene network.

1.3.4 Other factors: cell shape and cell-cell contact

In addition to the three well-defined pathways described above, other factors such as cell shape and cell-cell contact also appear to be important for SC biology. For example, the A-P orientation of SC division axis is redundantly controlled by elongated cell shape, cell junction contacts, and the WNT/ β -catenin asymmetry pathway (Wildwater et al., 2011). Moreover, for the unique seam cell V5, contacts with neighboring SCs are required to produce a neuroblast that gives rise to the postdeirid, supposedly due to cell-cell

signaling via a cell recognition/cell adhesion pathway (Austin and Kenyon, 1994).

In summary, proper SC development is orchestrated by the joint effort of several regulatory pathways and additional cellular factors such as shape and contact.

1.4 Non-muscle myosin (NMY) plays a pivotal role in asymmetric divisions and other biological processes

Myosins are a large family of highly conserved motor proteins that play diverse roles in force generation and movements in the cell (Coluccio, 2008). There are at least 20 structurally and functionally distinct classes. The human genome alone contains nearly 40 myosin genes belonging to 12 of these classes (Krendel and Mooseker, 2005). Alongside actin, myosin is the main contractile protein for cardiac, skeletal and smooth muscles. Crosslinked myosin and actin filaments slide past each other in order to generate the forces necessary to, for example, pump blood and lift objects (Coluccio, 2008). Other important classes of myosin are also found in all non-muscle eukaryotic cells (Clark et al., 2007; Conti and Adelstein, 2008; Krendel and Mooseker, 2005). These are termed non-muscle myosin (NMY) to differentiate them from their muscular counterparts, even though they are similar both in structure and function. Despite the name, NMYs are found both in muscle cells and non-muscle cells. Humans have three NMY heavy chain homologs: NMY IIA (encoded by *MYH9*), NMY IIB (encoded by *MYH10*), and NMY IIC (encoded by

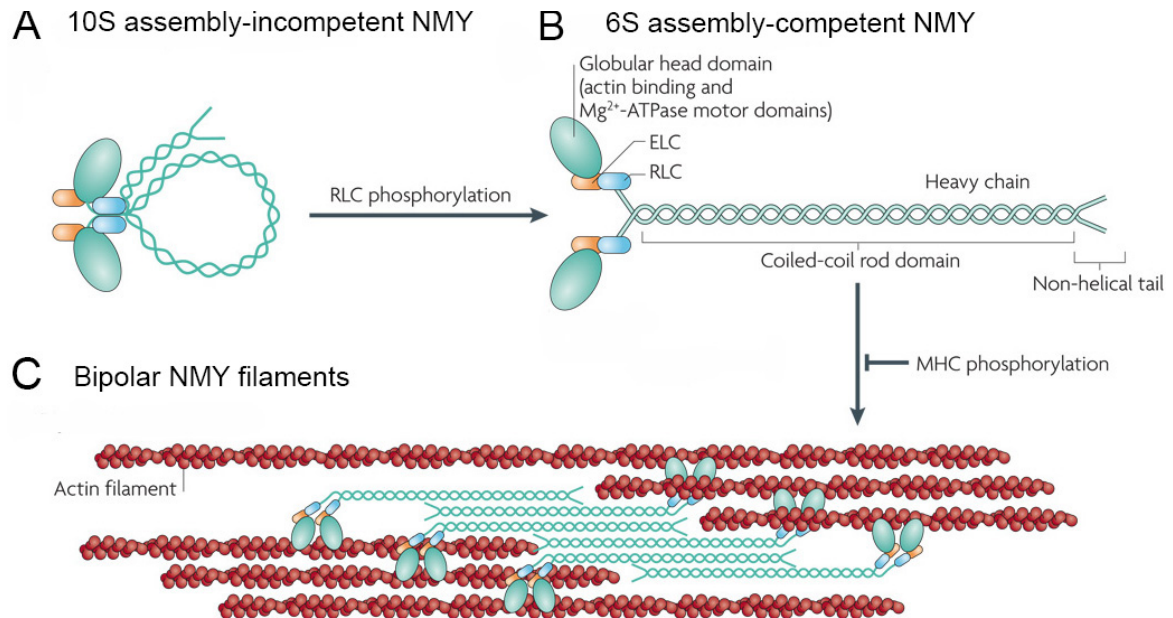
MYH14); *C. elegans* has two: NMY-1 (encoded by *nmy-1*) and NMY-2 (encoded by *nmy-2*).

1.4.1 NMY II structure

Each NMY II molecule is a homodimer of trimers, with the stereotypical two-headed structure (Figure 1.12B). Each trimer consists of a myosin heavy chain, an essential light chain (ELC), and a regulatory light chain (RLC). The myosin heavy chain has a globular head domain, a neck region where the ELC and RLC bind, a coiled-coil rod domain where the two heavy chains associate and intertwine, followed by a non-helical tail region. The energy required for myosin contractility is provided by ATP hydrolysis, which is activated by actin binding and catalyzed by sites in the N-terminal (head) region of the molecule. The C-terminal (tail) regions of some myosins self-associate into filaments, while the tails of other myosin classes bind to and transport cargo (Vicente-Manzanares et al., 2009).

RLC phosphorylation at Ser19 (and sometimes also at Thr18) is essential for NMY II self-assembly, because otherwise the NMY II molecules adopt the 10S form with a head to tail association that renders the molecule compact and assembly-incompetent (Figure 1.12A) (Burgess et al., 2007; Craig and Woodhead, 2006; Jung et al., 2008; Wendt et al., 2001; Woodhead et al., 2005). Upon RLC phosphorylation, the molecules relax to the 6S assembly-competent conformation (Figure 1.12B) and form bipolar filaments through rod domain interactions at physiological ionic strengths. Their head regions bind

thin actin filaments and hydrolyze ATP to move actin filaments in an anti-parallel fashion (Figure 1.12C).

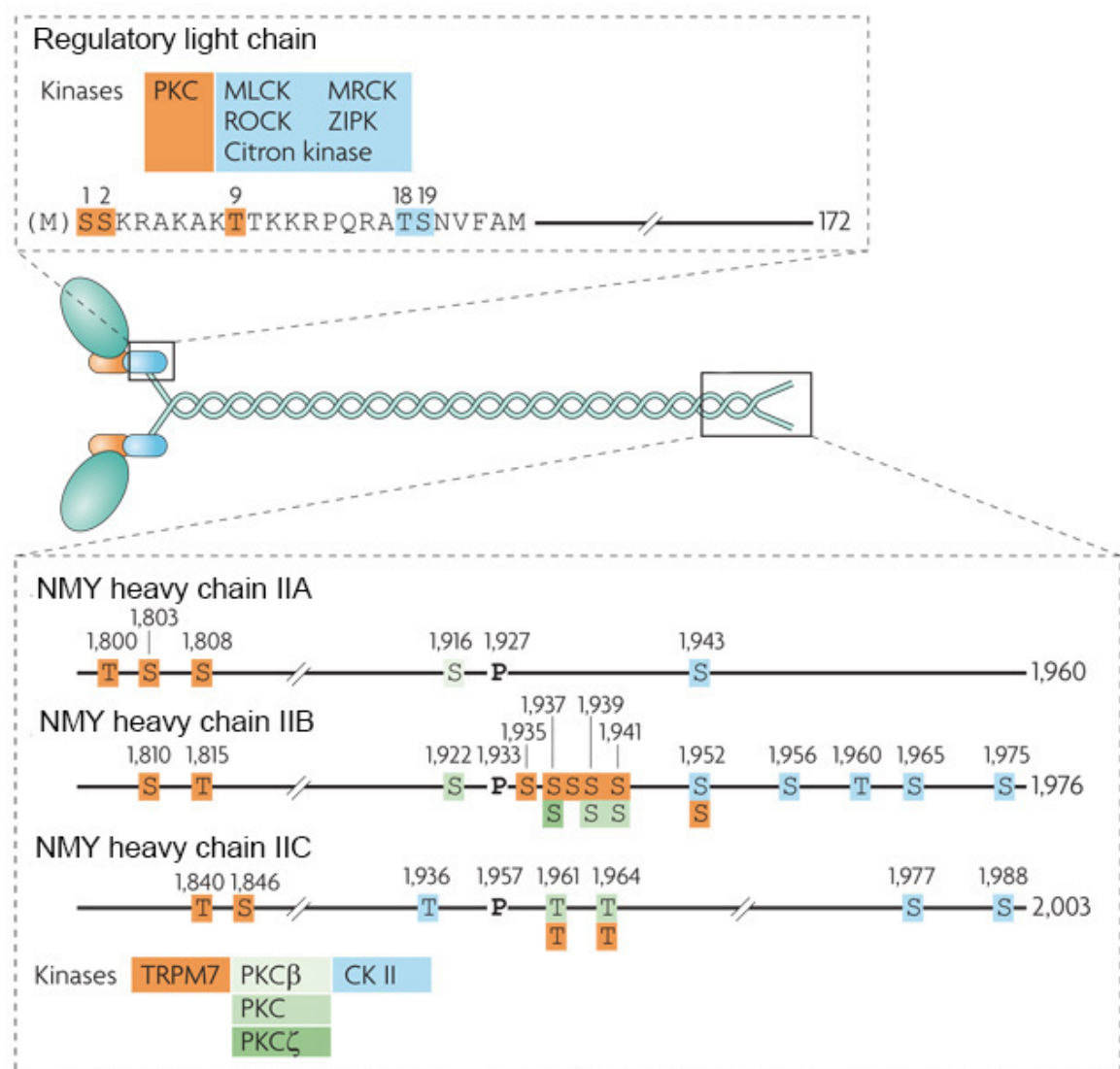


adapted from Vincente-Manzanares et al., 2009

Figure 1.12: NMY II structure. **A)** Without RLC phosphorylation, NMY II molecules adopt the 10S assembly-competent conformation through head to tail interaction. **B)** Phosphorylation of RLC Serine 19 relaxes the molecules into the 6S assembly-competent structure. **C)** NMY II molecules form bipolar filaments through rod domain interactions and bundle actin filaments, which they move in an anti-parallel manner by ATP hydrolysis.

1.4.2 NMY II regulation

NMY II is post-translationally regulated by the reversible phosphorylation of specific amino acids on both the RLCs and the heavy chains. An overview of NMY II RLC and heavy chain phosphorylation is shown in Figure 1.13. The NMY II ELCs function to stabilize the heavy chains and show no evidence of post-translational regulation. Cardiac and skeletal myosins are regulated by a different set of actin-bound proteins.



adapted from Vicente-Manzanares et al., 2009

Figure 1.13: Regulation of NMY II by RLC and heavy chain phosphorylation. The residues phosphorylated by a particular kinase are in accordance with the color-coding scheme for that kinase. Heavy chain phosphorylation occurs on different residues for the three human NMY II heavy chain isoforms A, B and C.

1.4.2.1 RLC phosphorylation

As mentioned, phosphorylation of RLC Ser19 activates NMY II by changing its structure to enable assembly. This phosphorylation also greatly increases the

myosin ATPase activity by altering the myosin head conformation (Somlyo and Somlyo, 2003; Wendt et al., 2001). More than a dozen kinases function downstream of various activating signals to phosphorylate NMY II RLCs on Ser19, Thr18, or both residues (Vicente-Manzanares et al., 2009). Such kinases include the myosin light chain kinase (MLCK), Rho-associated, coiled coil-containing kinase (ROCK), citron kinase, leucine zipper interacting kinase (ZIPK), and myotonic dystrophy kinase-related CDC42-binding kinase (MRCK) (Coluccio, 2008; Somlyo and Somlyo, 2003; Tan et al., 2008; Wendt et al., 2001). Some of these kinases have other substrates in addition to RLCs. ROCK, for example, can activate NMY by directly phosphorylating the RLC, but it also functions to inhibit the major myosin phosphatase, protein phosphatase 1. While most kinases and phosphatases regulate the activation state of NMY II through Ser19/Thr18, protein Kinase C (PKC) decreases NMY II activity by phosphorylating RLCs on Ser1, Ser2 and Thr9, which renders the RLC a poor MLCK substrate. PKCs also phosphorylate the heavy chains in addition to the RLCs.

1.4.2.2 NMY heavy chain phosphorylation

The phosphorylation sites on NMY II heavy chains are located near the C-terminal, including both the coiled-coil domain and the non-helical tail. PKC, casein kinase II (CK II) and transient receptor potential melastatin 7 (TRPM7) phosphorylate different human NMY II heavy chain isoforms (A, B, or C) on different residues, as shown in Figure 1.13 (Clark et al., 2008; Dulyaninova et al., 2005; Even-Faitelson and Ravid, 2006). Heavy chain phosphorylation by

these kinases decreases NMY II activity in vitro either by dissociating myosin filaments or by preventing filament formation.

1.4.2.3 Non-covalent regulations

In addition to RLC and heavy chain phosphorylation, NMY II activity can also be regulated by non-covalent interactions with other proteins in vivo (Bresnick, 1999). Alteration of actin filaments, such as by cofilin binding (McGough et al., 1997), and the binding of other molecules to NMY II, such as during cell locomotion (Svitkina et al., 1997), can both affect NMY II activity.

1.4.3 NMY II in asymmetric divisions

A role for NMY II has been established in various developmental contexts. Here I provide a brief summary of its role in several asymmetric divisions during *C. elegans* embryogenesis (one-cell and four-cell stages), *C. elegans* post-embryonic Q neuroblast development, and *Drosophila* neuroblast development.

1.4.3.1 NMY-2 in *C. elegans* embryogenesis

C. elegans has two NMY II heavy chain homologs, NMY-1 and NMY-2. NMY-1 has been little studied apart from its role in embryo elongation (Piekny et al., 2003), whereas NMY-2 has been shown to play multiple roles in different aspects and stages of *C. elegans* embryogenesis. NMY-2 is required to establish and maintain polarity in the one-cell embryo, as well as to specify

endoderm in the four-cell stage EMS division. Later on in embryonic development, NMY-2 provides the contractile forces necessary for gastrulation and elongation. Finally, NMY-2 is required for cytokinesis (described in Section 1.4.6), including those during embryogenesis.

The *C. elegans* zygotic division was described in Section 1.2.2. Sperm entry defines the posterior end of the cell and initiates actomyosin-mediated cortical ruffling and cytoplasmic streaming (Hill and Strome, 1988; Hird and White, 1993; Munro and Bowerman, 2009). NMY-2 is anteriorly localized and the actomyosin movements bring PAR-3 and PAR-6 to the anterior (Figure 1.4) (Goehring et al., 2011; Zonies et al., 2010), thereby establishing the A-P polarity axis in the zygote. NMY-2 is not only required for embryonic polarity establishment (Cuenca et al., 2003; Guo and Kemphues, 1996), but also for its maintenance. Disrupting NMY-2 by rapid and reversible conditional alleles at the pronuclei-meeting stage or anaphase leads to PAR protein mislocalization (Liu et al., 2010). Subsequently, receptor-independent G α signaling also regulates polarized actomyosin contraction during the zygotic NCC centration process (Goulding et al., 2007).

At the four-cell stage, both the WNT and the SRC signaling pathways are required to polarize EMS, the endoderm precursor cell. SRC signaling involves a family of non-receptor tyrosine kinases called SRC kinases (SRC is short for sarcoma). *nmy-2* mutations enhance the endoderm defects of WNT but not SRC pathway mutants, and cause defective SRC-dependent accumulation of phosphotyrosine at the P2/EMS cell junction (Liu et al., 2010). Taken together,

these results suggest that NMY-2 is required for SRC signaling to specify endoderm in parallel with WNT signaling.

Moreover, NMY-2 functions downstream of various signaling pathways to provide the contractile force necessary for morphogenesis. Gastrulation is the process by which the embryo reorganizes itself into three germ layers. It begins at the 26-cell stage in *C. elegans*, when the endodermal precursor cells Ea and Ep migrate from the ventral outer surface to the interior (Sulston et al., 1983). WNT signaling activates actomyosin contractility to generate the apical constriction necessary for Ea-Ep ingression (Lee et al., 2006). Elongation is another event during *C. elegans* morphogenesis, where the spherical embryo transforms into a long, thin worm. Elongation is driven by actomyosin contractility-dependent seam cell shape changes – as actin filaments in a subset of seam cells shorten, the cells and the embryo lengthen. NMY-1 and NMY-2 function redundantly to control this process downstream of the activating Let-502/Rho-binding kinase pathway or the inhibitory MEL-11/myosin phosphatase pathway (Kaibuchi et al., 1999; Pfitzer, 2001; Piekny et al., 2003; Piekny et al., 2000; Shelton et al., 1999). This “cassette” of regulators including non-muscle myosin, Let-502 and MEL-11 is conserved between *C. elegans* and *Drosophila melanogaster*, and likely used for various developmentally-specific contractile activities during animal development (Piekny et al., 2003).

1.4.3.2 NMY-2 in *C. elegans* post-embryonic development: the Q neuroblast lineage

Beyond embryogenesis, a post-embryonic role of NMY-2 was demonstrated in the asymmetric division of the Q.a neuroblast. As previously described in Section 1.2.2, Q.a generates a smaller anterior daughter that undergoes apoptosis and a larger posterior daughter that survives. Figure 1.14 shows that while during metaphase the spindle is centered and NMY-2 is evenly distributed around the cell cortex in Q.a, during anaphase more NMY-2 (green) concentrates on the anterior cortex. This anterior cortical tension “squeezes” the nascent anterior daughter, causing it to become smaller and the nascent posterior daughter to expand like a balloon. Consequently the smaller anterior daughter undergoes apoptosis whereas the larger posterior daughter survives. Spindle arms are of unequal lengths in this case (Figure 1.7C, Q.a cell), but likely as a passive result of myosin-mediated unequal cortical tension that allows spindles to simply expand in the larger posterior daughter (Ou et al., 2010). This is in contrast to the situation in *Drosophila* NB asymmetric divisions described in the next section, where the apical centrosome actively emanate longer spindles to basally displace the cleavage furrow and generate daughters of different sizes (Cai et al., 2003; Rebollo et al., 2007; Rusan and Peifer, 2007). Partial inactivation of anterior myosin in *C. elegans* Q.a neuroblast allows for a more symmetrical division and the survival and differentiation of the anterior daughter, demonstrating that anterior NMY-2 localization regulates the asymmetry of this division.

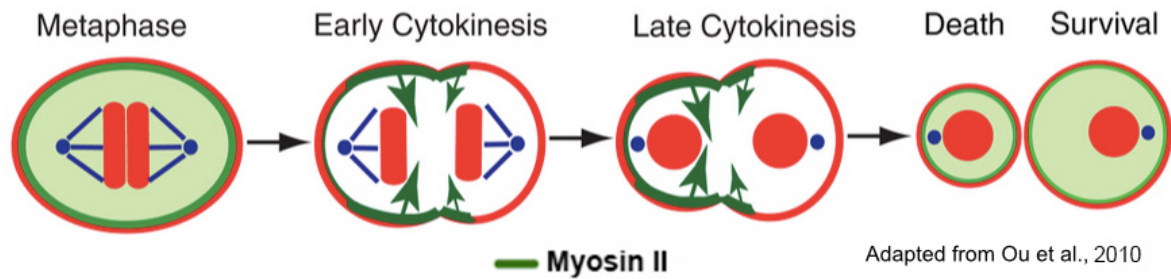


Figure 1.14: NMY-2 plays a key role in the Q.a neuroblast asymmetric division. Schematic drawing of NMY-2 localization during Q.a division. NMY-2 (green); plasma membrane and chromosomes (red); centrosomes and spindles (blue).

1.4.3.3 NMY II in the *Drosophila* neuroblast

Drosophila embryonic and larval NBs undergo reiterative asymmetric divisions to generate smaller basal GMCs while the larger apical daughter self-renews and retains NB fate. NBs specify the cleavage site by employing two spatially and temporally independent cytokinesis pathways (Roth et al., 2015). The canonical doctrine is that the site of cleavage is dictated by mitotic spindles and the centralspindlin complex (described in greater details in Section 1.4.6) (Glotzer, 2004; von Dassow, 2009; White and Glotzer, 2012), which usually specify a cleavage plane equidistant from the segregated chromosomes. The cortical centralspindlin ring recruits NMY II (the heavy chain is encoded by *zipper* in *Drosophila*) and F-actin to the future cleavage site to initiate furrow ingression. As introduced in Section 1.2.2, in *Drosophila* NB divisions, mitotic spindles of unequal lengths emanate from two functionally distinct centrosomes to generate a basally placed furrow (Cai et al., 2003; Rebollo et al., 2007; Rusan and Peifer, 2007).

In addition to this spindle-dependent cytokinesis pathway, which already deviates from the canonical notion by having asymmetric spindle elongation, *Drosophila* NBs have a second, spindle-independent cytokinesis pathway (Cabernard et al., 2010). This pathway relies on cortical tension and precedes the spindle-dependent pathway to generate a basal furrow. In this pathway, Pins-dependent cortical polarity displaces NMY II from the apical cortex and restricts it to the basal cortex at anaphase onset (Figure 1.15) (Cabernard et al., 2010). Polarized cortical NMY II correlates with asymmetric cortical extension in anaphase, as loss of NMY II asymmetry leads to equal-sized daughters. Basally localized NMY II in *Drosophila* NBs is proposed to function in a manner similar to the anteriorly localized NMY-2 in the *C. elegans* Q.a neuroblast, using cortical contractile force to generate a smaller basal daughter while allowing the future apical daughter to expand in response (Connell et al., 2011). Basal cortical NMY II later on restricts to the cleavage site to drive furrow ingression.

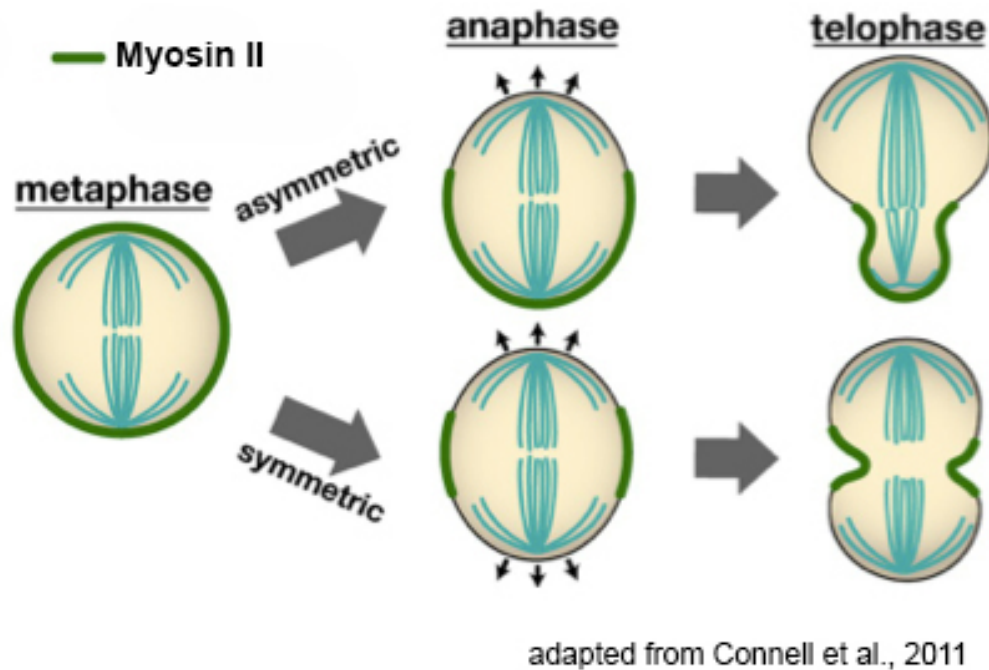


Figure 1.15: NMY II localization changes to produce asymmetric divisions in *Drosophila* neuroblast. Top: NMY II localizes basally at anaphase onset to generate a basally displaced furrow. Bottom: In symmetric divisions, NMY II is normally located on the equatorial cortex at the presumptive cleavage site. Dark green: myosin II; turquoise: spindles.

1.4.4 NMY II in cell adhesion and migration

In addition to its role in asymmetric cell divisions, NMY II also acts as the master integrator of multiple cellular processes owing to its critical convergent position downstream of different signaling pathways. One such role of NMY II is to drive the migration and adhesion in migratory cells, such as fibroblasts, through its actin bundling activity and contractility (reviewed in Vicente-Manzanares et al., 2009). Dynamic actin cytoskeleton remodeling and cellular interaction with its environment are key features of cell adhesion and migration. Different NMY II isoforms function together to coordinate these processes in human cells. First, NMY II limits the net rate of cellular protrusion

in the leading edge of migrating cells (Cai et al., 2006; Ponti et al., 2004; Vicente-Manzanares et al., 2007), both by generating the retrograde flow of actin in the lamellum (Anderson et al., 2008; Ponti et al., 2004) and by promoting the maturation of nascent adhesions inside the lamellipodium via integrins (Choi et al., 2008). Secondly, NMY II is involved in the adhesion disassembly at the trailing edge of a migrating cell, a process that requires coordinated actions between NMY II-dependent contraction, calpain-dependent adhesion component cleavage, and microtubule targeting. Lastly, NMY II determines the polarity of migrating cells by affecting nuclear positioning and the orientation of the microtubule-organizing center and Golgi (Gomes et al., 2005; Lo et al., 2004a; Vicente-Manzanares et al., 2007). Taken together, NMY II is a crucial regulator of cell migration with multiple roles in the process.

1.4.5 NMY II in epithelial cell-cell adhesion and morphogenesis

Apart from its involvement in integrin-mediated adhesion and migration, NMY II also regulates epithelial cell adhesion, polarization, and morphogenesis. Epithelial cells have well-defined cell-cell junctions and use cadherins as the main adhesion receptors. NMY II clusters cadherins and stabilizes cell-cell junction contract (Ivanov et al., 2007; Shewan et al., 2005) by compacting the contact (Yamada and Nelson, 2007) and inhibiting protrusions (Carmona-Fontaine et al., 2008). Additionally, NMY II regulates epithelial tissue organization in three-dimensions, as studies of early embryonic development and organogenesis in *Xenopus laevis*, *Drosophila melanogaster* and *C.*

elegans have revealed (reviewed in Vicente-Manzanares et al., 2009). It is an integral component of the mechanisms by which cells respond to external force and generate internal force. NMY II is also involved in contractile epithelial processes such as gastrulation, trachea formation, ventral furrow formation, dorsal closure, and morphogenesis of the vertebrate neural tube (Vicente-Manzanares et al., 2009). Finally, cells utilize NMY II-mediated tension to form and remodel the extracellular matrix during tissue morphogenesis and wound repair. Examples of this include collagen fiber remodeling (Meshel et al., 2005) and fibronectin fibrillogenesis (Zhang et al., 1997).

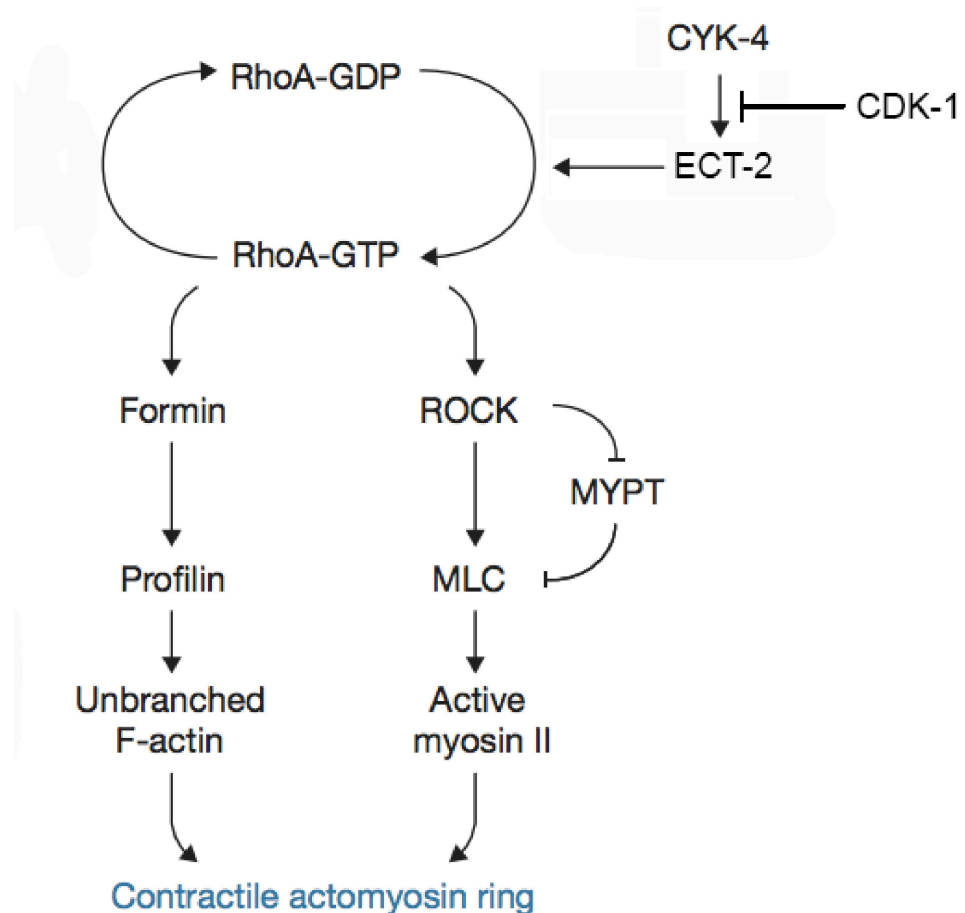
1.4.6 NMY II in cytokinesis

NMY II and actin are the main force generators in cytokinesis, the last step of cell division that physically separates the cytoplasm and produces two genetically identical daughters. The actomyosin cytoskeleton acts downstream of a Rho-A-dependent cytokinesis pathway (Figure 1.16) to provide the contractility that is essential for cytokinesis furrow ingression (Fededa and Gerlich, 2012).

During anaphase, animal cells form a structure called the spindle midzone (also known as the central spindle), the dense bundle of antiparallel microtubules between the two centrosomes. The spindle midzone defines the site of the division via the centralspindlin complex, which binds to overlapping microtubule ends in the midzone. The centralspindlin complex in *C. elegans* is a heterotetramer consisting of a dimeric kinesin motor, ZEN-4/MKLP1, and a

dimeric Rho family GTPase-activating protein, CYK-4/MgcRacGAP (Mishima et al., 2002; Pavicic-Kaltenbrunner et al., 2007). The CYK-4 component of the centralspindlin complex binds and activates ECT-2, a guanine nucleotide exchange factor (GEF), to transduce cues from the central spindle to the equatorial cortex by localized activation of RhoA GTPase at the presumptive cleavage site (Basant et al., 2015; White and Glotzer, 2012). Activated GTP-bound RhoA then further recruits NMY II and F-actin through independent pathways to assemble the contractile ring (White and Glotzer, 2012). The interaction between CYK-4 and ECT-2 is normally inhibited by the cell cycle regulator CDK-1, so the pathway only initiates during anaphase when active CDK-1 level drops due to the destruction of cyclin B (Fededa and Gerlich, 2012; Yüce et al., 2005).

NMY II is required not only to initiate the cytokinesis furrow, but also throughout cytokinesis until contractile ring closure (Davies et al., 2014). Experiments using fast-responding *C. elegans* temperature-sensitive *nmy-2* alleles demonstrated that shifting the mutant embryo to the restrictive temperature caused rapid retraction of deeply ingressed furrows and ultimately cytokinesis failure (Liu et al., 2010). By contrast, formin/linear F-actin is only required for furrow assembly and early constriction, and is dispensable for later stages of cytokinesis (Davies et al., 2014).



adapted from Fededa and Gerlich, 2012

Figure 1.16: The cytokinesis pathway. At anaphase, a decline of active CDK-1 allows the centralspindlin complex to interact with ECT-2 via its CYK-4 component. ECT-2 is a GEF that locally activates RhoA GTPase at the nascent cleavage site on the equatorial cell cortex. GTP-bound active RhoA subsequently recruits F-actin and NMY II to assemble the contractile ring necessary to drive cytokinesis. MLC encodes myosin regulatory light chain.

1.5 Summary

Decisions between the symmetric and asymmetric modes of division are tightly controlled during development to generate a multicellular organism from a single fertilized egg. Asymmetric divisions produce two daughters of distinct fates, and have been most extensively studied in *C. elegans* early embryonic

divisions and *Drosophila* neuroblasts. The current paradigm of animal asymmetric divisions is based on these two systems and involves a strict hierarchy. Cell polarization and the subsequent localization of fate determinants direct mitotic spindle positioning, which in turn specifies the site of the cleavage furrow to generate two daughters of different fates (and often unequal sizes too). However, two important deviations from this paradigm specifically concerning cleavage furrow placement have been also described in the *C. elegans* Q.a neuroblast and *Drosophila* NB asymmetric divisions. Both of these exceptions at least partially involve NMY II-mediated asymmetric cortical tension and the subsequent furrow displacement.

C. elegans seam cells are stem-like. They divide both symmetrically and asymmetrically during larval development, thus providing a valuable system for examining the stem cell mode of division and for investigating how asymmetric divisions are achieved at the cell molecular level. The WNT/ β -catenin asymmetry pathway, the RNT-1/BRO-1 pathway and the heterochronic pathway regulate different aspects of post-embryonic seam cell development. However, little is known about how these pathways interact. Even though past studies of SCs have already revealed a wealth of insights into their regulation, there are still many questions to be answered. One such knowledge gap of SC biology is the mechanism by which mitotic spindles specify the cleavage site in SC asymmetric divisions. Spindle positioning has never been systematically measured in SC asymmetric divisions to determine whether the cytokinesis furrow is centered to generate daughters that are initially identical in size or displaced to produce unequal-sized daughters, so I addressed this in my work.

Non-muscle myosin II has emerged as a key regulator of asymmetric divisions based on studies in the *C. elegans* embryo, *C. elegans* Q neuroblast, and *Drosophila* neuroblast systems. NMY II also functions downstream of multiple converging pathways to provide actomyosin contractile forces in a wide range of biological processes such as cell migration, cell-cell contact, morphogenesis, and cytokinesis. Given that *C. elegans* seam cell biology involves asymmetric divisions as well as cell reshaping and movement after the division, *nmy-2* emerges as a potential regulator for some aspects of seam development. The main question I asked in my thesis, therefore, is whether and how NMY-2 functions in *C. elegans* SC divisions.

1.6 Scope of this project

In this project, I:

- Generated transcriptional and translational reporters to examine the temporal and spatial expression of *nmy-2* (Chapter 3);
- Established a robust system to knock down *nmy-2* post-embryonically and analyzed the seam phenotypes that arose as a result (Chapter 3);
- Constructed a dual fate-reporter strain to examine potential fate changes in asymmetric division daughters. In combination with an apical junction marker, this strain revealed some mechanistic details of NMY-2's involvement in SC divisions (Chapter 4);
- Examined whether NMY-2 functions in the seam by interacting with other known seam regulatory pathways (Chapter 4);

- Measured spindle positioning in symmetric and asymmetric divisions, and then determined whether spindle positioning is changed upon *nmy-2* perturbation (Chapter 5);
- Explored whether the recent conception that cell cycle progression and cell polarity can be coupled applies to the seam (Chapter 6).

Chapter 2 Materials and Methods

2.1 Nematode methods

2.1.1 Maintenance of worms

C. elegans strains were derived from the wild-type Bristol N2 reference strain or CB4856 Hawaiian strain and listed in Table 3 in the Appendix. Worms were maintained by standard methods (Brenner, 1974). Worms were grown on 55 mm NGM plates and fed *E. coli* strain OP50, a uracil-deficient strain that prevents bacterial lawn overgrowth. Strains were propagated by excising small areas of agar with worms onto a fresh plate, or by manual picking using a flattened platinum wire. Strains were usually maintained at room temperature or at 15°C if they were temperature sensitive. Frozen stocks of strains consisted of a 1:1 mixture of freezing solution and starved L1 larvae in M9 buffer, that were slowly frozen in an isopropanol freezing box at -80 °C before being moved into liquid nitrogen for long-term storage.

2.1.2 Generation of males

Males were generated from hermaphrodites for genetic crossing and outcrossing needs. Five plates of six L4 hermaphrodites were heat shocked at 30°C for 6 hours to induce chromosomal non-disjunction, and then allowed to recover at room temperature. Three days later male offspring were isolated and crossed back into the original stock to generate male stocks.

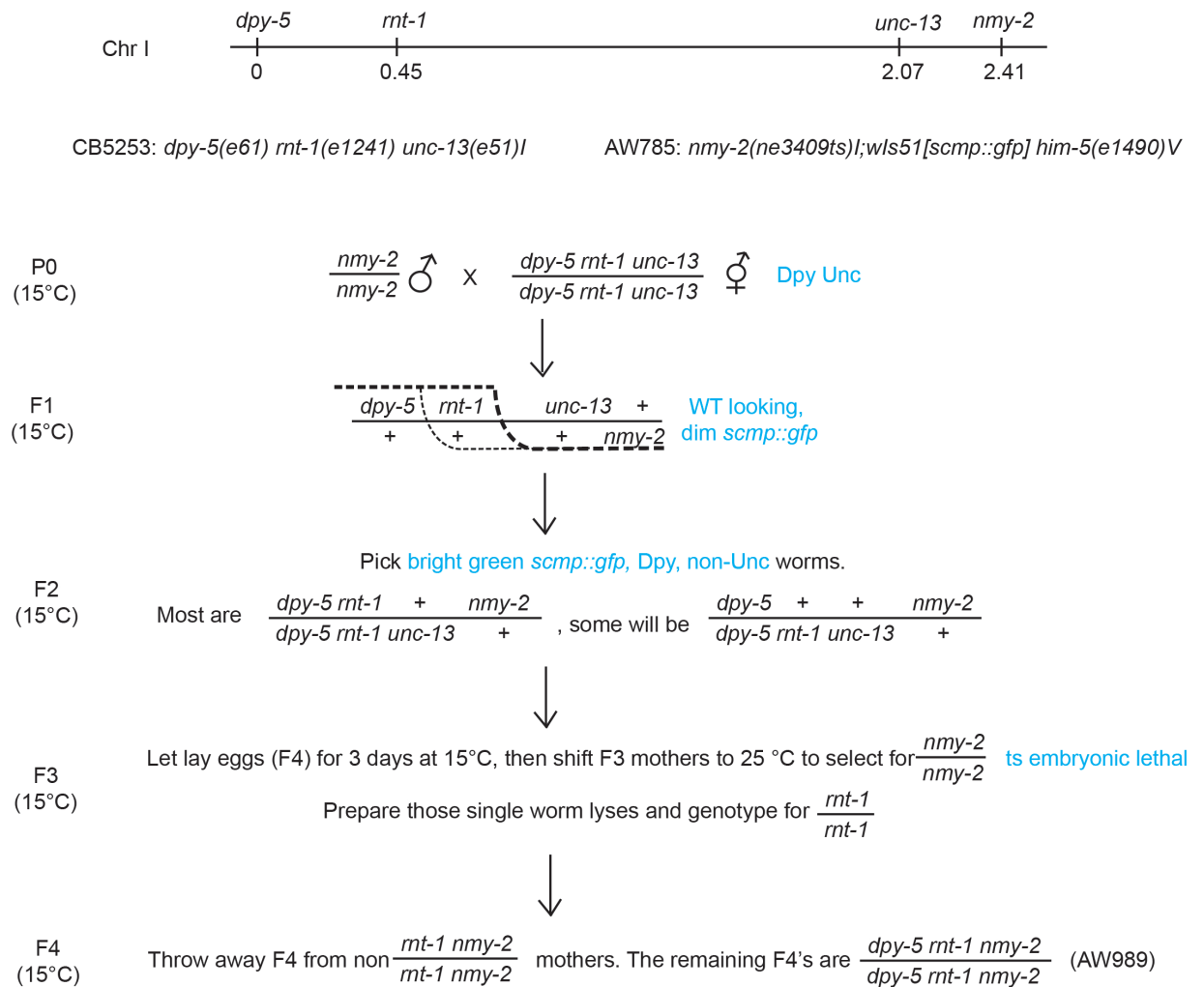
In the case of temperature sensitive mutants where heat shock was not feasible, males were generated by crossing the mutants with N2 or *him-5* males.

2.1.3 Genetic crosses

Genetic crosses were usually set up using 5 males and 2 hermaphrodites, although the optimal ratio varied for specific strains, for example because some males were less successful at mating hence more males would be necessary for the initial set up. Successful sexual reproduction was indicated by a 1:1 ratio between males and hermaphrodites in the F1 generation. Homozygous F2's were picked based on either phenotype or genotype (confirmed by genotyping PCR). This was the method for crossing various integrated cellular markers into desired genetic backgrounds and for generating double mutants.

In the event that two genes were close to each other on the same chromosome, an alternative crossing strategy was designed using visible chromosome markers and the double mutants were confirmed by PCR genotyping. This was the case for the *rnt-1 nmy-2* and *wrm-1 cdk-1* double mutants.

2.1.3.1 Generation of the *rnt-1 nmy-2* double mutant



Note: Since the *rnt-1 nmy-2* double mutant is in the *dpy-5* background, additional strains were generated using similar strategies to put *rnt-1* and *nmy-2* single mutants and WT worms into the *dpy-5* background for later comparison.

AW989: *dpy-5(e61) rnt-1(e1241) nmy-2(ne3409)I;wls51(scmp::gfp] him-5(e1490)V*

AW990: *dpy-5(e61) rnt-1(e1241)I;wls51(scmp::gfp] him-5(e1490)V*

AW991: *dpy-5(e61) nmy-2(ne3409)I;wls51(scmp::gfp] him-5(e1490)V*

AW992: *dpy-5(e61)I;wls51(scmp::gfp] him-5(e1490)V*

Figure 2.1: Crossing strategy to generate the *rnt-1 nmy-2* double mutant. Blue text indicates phenotypes. The *scmp::gfp* marker was used in the cross so seam cells can be counted in the resultant double mutants.

2.1.3.2 Generation of the *wrm-1 cdk-1* double mutant

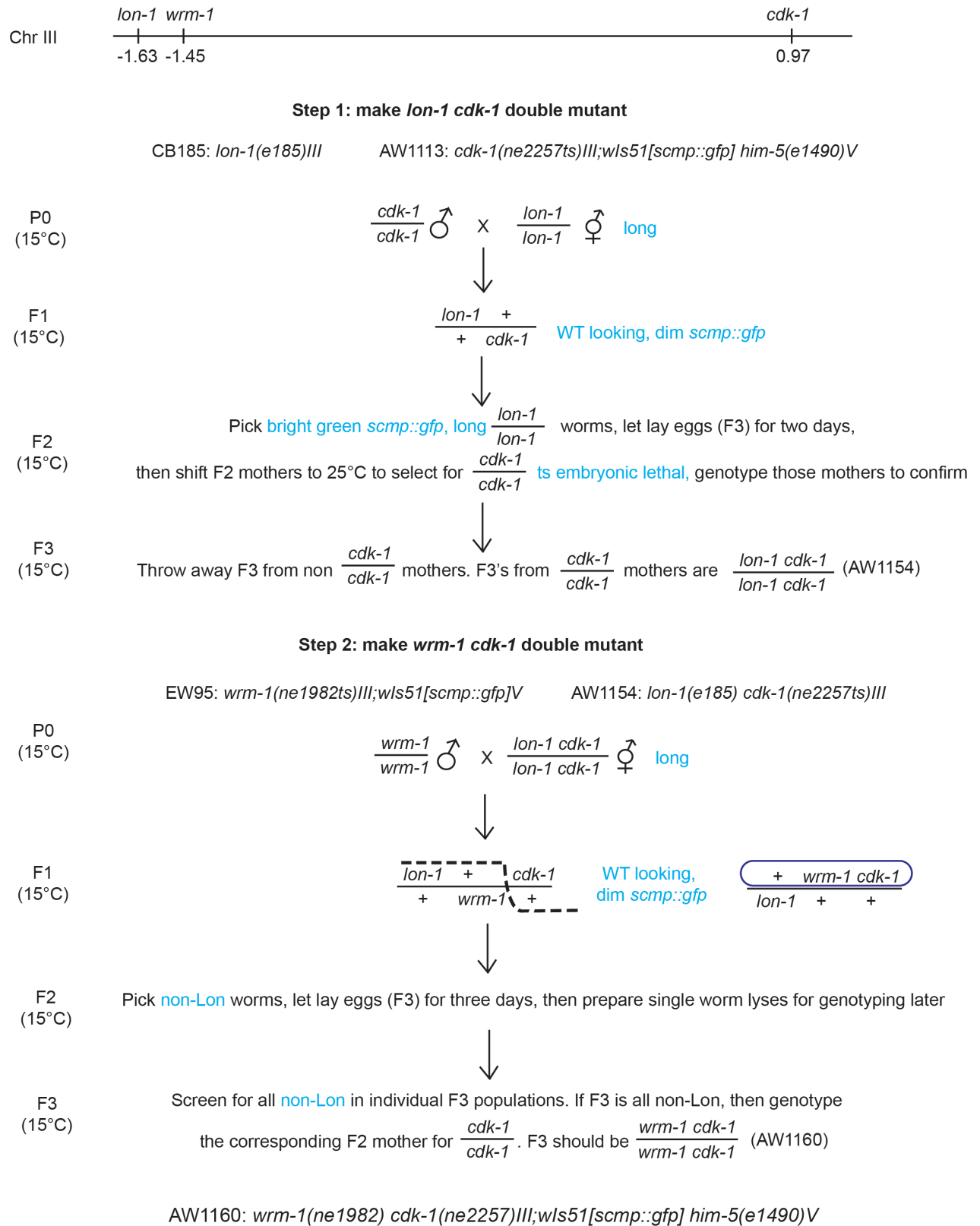


Figure 2.2: Crossing strategy to generate the *wrm-1 cdk-1* double mutant. Blue text indicates phenotypes. The *scmp::gfp* marker was used in the cross so seam cells can be counted in the resultant double mutant.

2.1.4 RNAi by feeding

RNAi was performed by feeding worms HT115 bacteria expressing double-stranded RNA corresponding to the target gene. The Ahringer RNAi library (Kamath et al., 2003) clones were used for most RNAi experiments unless custom RNAi clones were made (see Section 2.2.2.2). The desired clones were taken out of the library, sequenced, and checked for off-target effects (see Section 2.3.1) prior to use. Glycerol stocks of the clone were streaked onto a 2xYT plate supplemented with 50 µg/mL ampicillin, from which a liquid culture was grown in 2 mL of 2xYT Amp broth at 37°C for 6-18 h. RNAi plates (modified from regular NGM to include 1 mM IPTG and 25 ng/mL carbenecillin) were seeded with 100-120 µL of the overnight culture and inoculated at room temperature overnight before worms were placed on. RNAi experiments took place at 25°C unless otherwise noted. *pop-1* L4 RNAi with embryonic lethal phenotype was used as the positive control and HT115 bacteria transformed with empty L4440 vector was used as the negative control.

RNAi experiments were conducted either on L4 larvae for assessing their progeny (L4 RNAi), or on L1 larvae directly for assessment of these worms at a later developmental stage (post-embryonic RNAi). For L4 RNAi, three to five L4 larvae were placed onto seeded RNAi plates and plates were incubated at room temperature or 25°C until their progeny were ready to be examined. To bypass any embryonic effect, post-embryonic RNAi was used instead. This was done by spot bleaching ~30 gravid hermaphrodites onto an RNAi plate to examine a relatively small number of progeny that hatched from the bleach-

resistant eggs. If a larger number of affected worms were desired, as was the usual case for this study, multiple plates of gravid hermaphrodites were bleached and their progeny synchronized to the L1 diapause (see the next section) before being transferred onto RNAi plates.

2.1.5 Bleach synchronization

Worms were synchronized by bursting gravid mothers with bleach to release the more bleach-resistant eggs. Briefly, gravid worms were washed off of two to five 90 mm plates with M9 and washed several times to remove bacteria. 4 mL of 2x bleach solution (1M NaOH, 4.8% sodium hypochlorite) was added to 4 mL of worm pellet in M9 in a 15 mL Falcon tube. The tube was vortexed vigorously for 4 min to burst the gravid worms and topped up with M9 immediately after vortexing. Eggs were pelleted by centrifugation at 560 *g* for 1.5 min. The egg pellet was quickly washed several times to remove any residual bleach and allowed to hatch overnight on a rotator in M9, at room temperature or at 15°C if the strains were temperature sensitive. To re-feed synchronized, starved L1s, worms were pelleted by centrifugation at 560 *g* for 1.5 min and examined in a watch glass before being transferred onto plates containing OP50 or RNAi bacteria and placed at appropriate temperatures.

2.1.6 Single worm lysis

Single worm lysis was used to release genomic DNA from individual worms for genotyping or other PCR purposes. Individual worms were picked into 2.5 μ L of worm lysis buffer (50 μ M KCl, 2.5 μ M MgCl₂, 10 μ M Tris-HCl pH 8.3, 0.45% NP40, 0.45% Tween-20, 0.01% gelatine, 0.1 mg/ml proteinase K) in the lid of a

0.5 mL Eppendorf tube and then briefly spun down to the bottom. The tube was frozen at -80°C for 15 min, followed by incubation in a PCR machine at 60°C for 1 h and then at 95°C for 15 min. This lysis mix was stored at -20°C or used immediately as the DNA template. It was possible to perform two sets of genotyping PCR from the same lysis mix by splitting the 2.5 µL template in half.

2.1.7 Genotyping

Genotyping PCRs were carried out to track mutant alleles that do not produce a unique visible phenotype. Worms were lysed before being used for genotyping PCR. For substitution mutations, tetraARMS PCR primers (Ye et al., 2001) were designed to produce a smaller mutant band or a medium WT band, in addition to a large outer band that would always amplify (i.e. small + large for homozygous mutants, medium + large for WT, small + medium + large for heterozygotes). See Table 4 in the Appendix for all primers.

2.1.7.1 *nmy-2(ne3409)* genotyping

nmy-2(ne3409) genotyping was achieved with *nmy-2*-OF, *nmy-2*-OR, *ne3409*-IF, and *ne3409*-IR primers. Annealing temperature (AT) is 58°C and extension time (ET) is 1 min. Mutant allele gives a 193 bp band, WT allele gives a 829 bp band, and outer band is 967 bp.

2.1.7.2 *nmy-2(ne1490)* genotyping

nmy-2(ne1490) genotyping was achieved with *nmy-2*-OF, *nmy-2*-OR, *ne1490*-IF, and *ne1490*-IR primers. AT is 62°C and ET is 1 min. Mutant allele gives a 447 bp band, WT allele gives a 575 bp band, and outer band is 967 bp.

2.1.7.3 *lit-1(or131)* genotyping

lit-1(or131) genotyping was achieved with *lit-1-OF*, *lit-1-OR*, *lit-1-IF*, and *lit-1-IR* primers. AT is 69°C and ET is 30 s. Mutant allele gives a 210 bp band, WT allele gives a 297 bp band, and outer band is 455 bp.

2.1.7.4 *wrm-1(ne1982)* genotyping

wrm-1(ne1982) genotyping was achieved with *wrm-1-OF*, *wrm-1-OR*, *wrm-1-IF*, and *wrm-1-IR* primers. AT is 57°C and ET is 30 s. Mutant allele gives a 129 bp band, WT allele gives a 231 bp band, and outer band is 307 bp.

2.1.7.5 *rnt-1(e1241)* genotyping

rnt-1(e1241) genotyping was achieved with *rnt-1-OF*, *rnt-1-OR*, *rnt-1-IF*, and *rnt-1-IR* primers. AT is 58°C and ET is 25 s. Mutant allele gives a 257 bp band, WT allele gives a 335 bp band, and outer band is 541 bp.

2.1.7.6 *cdk-1(ne2257)* genotyping

cdk-1(ne2257) genotyping was achieved with *cdk-1-OF*, *cdk-1-OR*, *cdk-1-IF*, and *cdk-1-IR* primers. AT is 63°C and ET is 45 s. Mutant allele gives a 296 bp band, WT allele gives a 396 bp band, and outer band is 635 bp.

2.1.7.7 GFP genotyping

GFP genotyping is a method for tracking GFP-tagged cellular markers during a cross if the markers were invisible on the low power Leica dissecting microscope. GFP genotyping was achieved with *GFP-F* and *GFP-R* primers. AT is 58°C and ET is 1 min. The presence of GFP gives a 844 bp band.

2.1.8 Generation of transgenic lines

Microinjections were performed as previously described (Mello and Fire, 1995) using *unc-119* as a transformation marker. Stable transgenic lines were isolated and maintained at 25°C. Plasmids were injected at 2-20 ng/μL.

2.1.9 Integration of transgenes

Extra-chromosomal arrays were integrated into the *C. elegans* genome by gamma irradiation from a cesium source at the Sir William Dunn School of Pathology, University of Oxford. *unc-119*-rescued lines with medium transmission rate (30-60%) were selected for integration. Seventy L4 worms were exposed to 40 Gy of irradiation and 350 healthy F1's were isolated 3-4 days later. Twenty-five F1's that gave 75-100% transmission rate were further isolated and clonally expanded for 2-4 generations to achieve stable transgene integration that resulted in 100% non-Unc lines. Independent lines were outcrossed 4-6 times to remove background mutations before being used for further analyses.

2.1.10 Bimolecular fluorescence complementation (BiFC) assay

BiFC assay was carried out to test protein-protein interactions in vivo (Hiatt et al., 2008; Shyu et al., 2008). Essentially, the VN173 and VC155 BiFC vectors have the N-terminal or C-terminal half of the Venus molecule in the plasmid backbone, respectively, controlled by a heat shock promoter. Full-length cDNA coding for two potentially interacting proteins were cloned into the two BiFC vectors and the plasmids were co-injected with *unc-119* marker into the *unc-119* worms to generate BiFC strains. BiFC worms were heat shocked at 33°C for 2 h and allowed to recover at room temperature for 20 min before being

imaged. Fluorescence from reconstituted Venus molecule would suggest direct protein-protein interaction in vivo.

2.1.11 Minimal promoter assay

Potential enhancer elements were tested by inserting the sequence of interest into the enhancer assay plasmid pPD107.94 (Addgene #1531). The vector contains the *pes-10* minimal promoter fused to a 15-intron NLS-gfp[S65C]-lacZ reporter with *unc-54* 3' end, which has little or no activity alone but is active in the presence of an enhancer element. The plasmids were injected to generate transgenic lines. If the lines produced tissue-specific nuclear GFP expression, then the inserted sequence would be considered an enhancer.

2.1.12 Brood size assay

OP50 plates were seeded for brood size experiments so that the bacteria form a round lawn in the center of the plate without touching the edge. A single L4 worm was placed onto the plate and incubated at the appropriate temperature. The number of viable progeny was counted twice for each individual when the brood period was over. If the two counts varied by more than 10% then the counts were repeated, otherwise an average between the two counts was used as the brood size of that particular animal. A typical brood size experiment used 5-10 animals for each condition.

2.2 Molecular biology methods

2.2.1 PCR amplification, primer synthesis, and gel electrophoresis

In general, two types of PCR reactions were carried out for this study. One is for genotyping (see Section 2.1.7) and the other for molecular cloning. Primers were synthesized by MWG (<http://www.mwg-biotech.com/>) and listed in Table 4 in the Appendix. Gateway cloning primers were HPLC-purified. PCR products were usually run on a 0.7-2% agarose gel containing ethidium bromide to determine the size of the products, and desired bands were excised for further uses if necessary.

2.2.1.1 PCR for genotyping

Genotyping PCRs were performed in a 25 μ L reaction using *Taq* DNA polymerase (NEB). Single worm lysis or genomic DNA was used as the DNA template. A standard *Taq* reaction contains appropriate concentrations of DNA template, 1X ThermoPol buffer, 200 μ M of each dNTP, 0.4 μ M of each primer, and 2.5 units of *Taq* enzyme. A standard PCR reaction started with the denaturation of DNA for 2 min at 94°C, followed by 25-30 cycles of denaturation (30 s at 94°C), annealing (30 s at annealing temperature) and extension (1 min/kb at 72°C), plus a final extension step at 72°C for 10 min.

2.2.1.2 PCR for molecular cloning

PCR for molecular cloning purposes were performed in a 50 μ L reaction using AccuPrime High Fidelity *Taq* Polymerase (Invitrogen), which has proofreading activity. Plasmids, cDNA, or genomic DNA were used as the DNA template. A standard reaction contained the DNA template, 1X AccuPrime PCR Buffer I (for plasmids or cDNA) or II (for genomic DNA), 0.2 μ M of each primer, and 2.5 units of AccuPrime enzyme. A standard PCR reaction started with the denaturation of DNA for 30 s at 94°C, followed by 25-30 cycles of denaturation

(30 s at 94°C), annealing (30 s at the appropriate annealing temperature) and extension (1 min/kb at 68°C), plus a final extension step at 68°C for 5 min.

2.2.2 Molecular Cloning

Various plasmids were constructed for this study using both Gateway and conventional cloning methods. All the plasmids are listed in

Table 5 in the Appendix. AccuPrime *Taq* Polymerase was used for all cloning PCRs. PCR fragments were usually run on an agarose gel and desired fragments were excised and gel purified using GeneClean (MP Biomedicals) or QIAEX II (Qiagen) kits. TOPO XL Cloning Kit or pCRII-TOPO Kit (Invitrogen) was used for intermediate sub-cloning purposes. Electro-competent *E. coli* cells and a MicroPulser Electroporator (Bio-Rad) were used for all bacterial transformations. Gateway cloning plasmids were transformed into Mach1 cells, RNAi feeding plasmids into HT115 cells, and all other plasmids into XL1-Blue cells. All plasmids were sequenced by Source BioScience, (<http://www.lifesciences.sourcebioscience.com/>).

2.2.2.1 *bro-1CNE+pes-10p::nmy-2::gfp* (pAW749)

bro-1CNE+pes-10p::nmy-2::gfp plasmid construct was made using the MultiSite Gateway Pro kit (Invitrogen) following a 3-fragment cloning strategy. Individual fragments were amplified using primers flanked with appropriate *att* recombination sites. First, *bro-1CNE+pes-10p*, *nmy-2* and *gfp* fragments were amplified from pAW582, *C. elegans* genomic DNA and pPD95.75, respectively, and cloned into pDONR P1-P4, pDONR P4r-P3r and pDONR P3-P2 vectors via BP reactions to generate pAW746, pAW747 and pAW748, respectively. Then the three entry clones were used to deposit the desired fragments into the pcDNA6.2v5PL-DEST vector via the LR reaction in order to produce the final plasmid construct, pAW749.

2.2.2.2 Custom *nmy-1* and *nmy-2* RNAi feeding clones (pAW744 and pAW745)

Because the *nmy-2* RNAi clone from the Ahringer library may have a knock-on effect on *nmy-1*, custom *nmy-1* and *nmy-2* RNAi feeding clones were designed against the sequences coding for the less conserved myosin tail region. A 475 bp fragment was amplified from the genomic DNA for *nmy-1* using the primer pair SDnmy-1-F and SDnmy-1-R, and a 419 bp fragment amplified for *nmy-2* using the primer pair SDnmy-2-F and SDnmy-2-R (both using 57°C AT and 35 s ET). The PCR products were cloned into pCRII-TOPO vector (Invitrogen) in the reverse orientation to produce pAW742 for *nmy-1* and pAW743 for *nmy-2*. Next, pAW742, pAW743 and the L4440 RNAi feeding vector were digested with XbaI and HindIII. Appropriate fragments were excised from the gel and ligated together to produce pAW744 and pAW745, which contain *nmy-1* and *nmy-2* RNAi sequences, respectively, inserted between the XbaI and HindIII sites of the L4440 feeding vector.

2.2.2.3 *nmy-2* cDNA (pAW808)

nmy-2 full-length cDNA was amplified using a three-fragment overlapping strategy due to the large size of the cDNA (~6 kb). N2 cDNA primed with random hexamers was used as the template and individual fragments were amplified. Fragment 1 (2290 bp), 2 (1620 bp) and 3 (2331 bp) were amplified using nmy-2CDS1-F and nmy-2CDS1-R primer pair, nmy-2CDS2-F and nmy-2CDS2-R primer pair, and nmy-2CDS3-F and nmy-2CDS3-R primer pair, respectively, all using 58°C AT and 2.5 min ET. Fragments 1, 2 and 3 were individually cloned into TOPO XL to generate pAW804, pAW805 and pAW806.

Next, fragments 1 and 2 were combined. This was done by first individually re-amplifying the fragment 1 from pAW804 using M13-R and nmy-2CDS1-R primer pair, and fragment 2 from pAW805 using nmy-2CDS2-F and M13-F primer pair. Then 1 μ L was taken from each re-amplification PCR product and mixed together to serve as the template, from which the joined 1+2 fragment (3821 bp) was amplified using nesting primers nmy-2CDS1-F and nmy-2qCDS2-R (58°C AT and 4 min ET) and cloned into TOPO XL to generate pAW807. A similar approach was used to join fragments 1+2 with fragment 3, first amplified using M13-R and nmy-2CDS2-R primer pair and nmy-2CDS3-F and M13-F primer pair, respectively, and then mixed together and nested with nmy-2CDS1-F and nmy-2CDS3-R primer pair (58°C AT and 6.5 min ET). The resultant full-length *nmy-2* cDNA (joined fragments 1+2+3) was cloned into TOPO XL to produce pAW808.

2.2.2.4 *nmy-2* cDNA in pCE-VN173 BiFC vector (pAW811)

nmy-2 full-length cDNA was cloned into the AgeI site of pCE-VN173 BiFC vector for BiFC experiment. *nmy-2* full-length cDNA was first amplified from pAW808 using nmy-2CDS.AgeI-F and nmy-2CDS.AgeI-R primers (58°C AT and 6.5 min ET) to produce a full-length cDNA fragment with AgeI tags on both ends. The primers were designed so that *nmy-2* cDNA would be translated in frame with the N-terminal half of the Venus molecule in the BiFC vector backbone. This tagged cDNA was then cloned into TOPO XL to produce pAW809. Next, pAW809 was digested with AgeI and MscI (NEB), so AgeI could release the cDNA fragment and MscI would break up the vector backbone. pCE-VN173 vector was digested with AgeI and treated with

Antarctic phosphatase (NEB) to prevent self-ligation. Lastly, treated pAW809 and pCE-VN173 vector were ligated together to produce pAW811, which contains the full-length *nmy-2* cDNA inserted into pCE-VN173's *AgeI* site in the forward orientation.

2.2.2.5 *wrm-1* cDNA in pCE-VC155 BiFC vector (pAW812)

wrm-1 full-length cDNA was cloned into the *NcoI* and *XhoI* sites of pCE-VC155 BiFC vector for BiFC experiment. Luckily *wrm-1* full-length cDNA was already available in pAW501 so it was amplified from there with *wrm-1*CDS.*NcoI*-F and *wrm-1*CDS.*XhoI*-R primers (58°C AT and 3 min ET) to produce a full-length cDNA fragment with a 5' *NcoI* tag and a 3' *XhoI* tag. The primers were designed so that *wrm-1* cDNA would be translated in frame with the C-terminal half of the Venus molecule in the BiFC vector backbone. This tagged cDNA was then cloned into TOPO XL to produce pAW810. Next, pAW810 and pCE-VC155 were both digested with *NcoI* and *XhoI* (NEB). Appropriate fragments were isolated from an agarose gel and ligated together to produce pAW812, which contains the full-length *wrm-1* cDNA inserted between pCE-VN155's *NcoI* and *XhoI* sites.

2.2.2.6 *nmy-2* full-length promoter in pPD107.94 (pAW850)

nmy-2 promoter was cloned into pPD107.94, so that transgenic worms were made later to see when and where the promoter drives transcription. *nmy-2* full-length promoter was defined as the 1723 bp region between the end of the last gene preceding *nmy-2* and the *nmy-2* gene start codon. The promoter was amplified from an N2 worm lysis using *nmy-2*p-F and *nmy-2*p-R primers (58°C

AT and 2 min ET) and cloned into TOPO XL in the forward orientation to generate pAW849. pAW849 and pPD107.94 vector were digested with XbaI and HindIII. Appropriate fragments were isolated and ligated together to produce pAW850, which contains the *nmy-2* full-length promoter inserted between the HindIII and XbaI sites of pPD107.94. Unfortunately a small region of the promoter could not be sequenced, probably due to the formation of a hairpin structure.

2.3 Bioinformatics methods

2.3.1 RNAi off-target effect search

Potential off-target RNAi effects were checked using Clone Mapper (Thakur et al., 2014) (<http://bioinformatics.lif.univ-mrs.fr/RNAiMap/target.php>).

2.3.2 Protein structure search

The sequences targeted by various *nmy-1* and *nmy-2* RNAi clones were searched against known protein structure families using Pfam (Finn et al., 2014) (<http://pfam.xfam.org>).

2.3.3 DNA sequence alignment

nmy-1 and *nmy-2* sequences were aligned using the EMBOSS Water pairwise nucleotide sequence local alignment tool (http://www.ebi.ac.uk/Tools/psa/emboss_water/nucleotide.html).

nmy-2 promoter regions from various *Caenorhabditis* species were extracted from Wormbase (<http://www.wormbase.org>) and aligned using ClustalW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2>).

2.3.4 Transcription factor binding site search

Potential RNT-1 binding sites were identified using the JASPAR Core Vertebrata transcription factor binding profile database Version 5.0_ALPHA (<http://jaspar.genereg.net>). Mouse RUNX1 (MA0002.2) and human RUNX2 (MA0511.1) were selected as matrix models and *nmy-2*'s 5' UTR, 3' UTR and intron sequences were scanned for potential binding sites.

2.4 Imaging methods

2.4.1 Light and fluorescence microscopy

A low power Leica dissecting microscope (1-6.3x objective) was used for routine worm maintenance and for examining obvious phenotypes such as Lon, Unc and Dpy without the need for mounting the worms.

A low power Leica MZIII fluorescent dissecting microscope (1-10x objective) was used to visualize bright fluorescence without the need for mounting, as was necessary for crossing in fluorescent cellular markers, for example.

To visualize phenotypes in greater detail, a Zeiss Axiophot DIC/fluorescent microscope or a Leica SP5 laser scanning confocal microscope (10-63x objective) was necessary. Worms were mounted onto 2% agarose pads and

anesthetized in 0.5% phenoxypropanol (Aldrich) before being visualized. The former microscope is equipped with an AxioCam camera and AxioVision software for image acquisition.

2.4.2 Spindle position image analysis

Worms of the desired stage were mounted in 0.5% phenoxypropanol on 2% agarose pads. Two sets of images for each desired cell were taken on GFP and Rhodamine channels on the Zeiss Axiophot fluorescent microscope, one set with *wrt-2p::gfp::H2B* in focus and the other set with *ajm-1p::mCherry* in focus. Post-acquisition analysis involved using ImageJ to overlay the GFP channel focusing on *wrt-2p::gfp::H2B* with the Rhodamine channel focusing on *ajm-1p::mCherry*. This was done because of the tapered shape of the SCs. Cell size as outlined by the PH plasma membrane marker would vary depending on the Z plane, so instead *ajm-1p::mCherry* on the apical surface was used to show the “true” cellular boundary. After cell identity and orientation were determined by eye, distance measurements were made in ImageJ. V1-4 lineages were pooled for the analysis because they share the same division patterns. V5 has a different lineage and V6, while sharing the V1-4 lineage, was obscured by the *lin-48p::mCherry* co-injection marker in *hels63* so they were excluded from the analysis. For interphase through metaphase, distances between anterior and posterior ends of the cell, and between ends of the cell and the DNA were measured. For late anaphase, additional distances between the two sets of daughter chromosomes and between the chromosome and the cleavage furrow were measured. All measurements within a cell were normalized to the full A-P length of that cell, which was assigned a value of

100. Two-tailed T tests were performed to determine statistical differences – paired T test for anterior versus posterior measurements within the same cell, and unpaired T test assuming unequal variances for measurements comparing different cells belonging to the anterior versus posterior lineage branches.

Chapter 3 *nmy-2* is expressed in the seam and

nmy-2 knockdown results in progressive seam cell loss after asymmetric divisions

3.1 Introduction

Non-muscle myosin II (NMY II) participates in a wide range of cellular processes such as adhesion, movement and polarization by means of generating actomyosin contractility forces. The *C. elegans* homolog NMY-2 has been extensively studied in embryogenesis (see Section 1.4.3.1), where its diverse role ranges from polarizing the one-cell embryo to promoting endoderm specification to morphogenesis, all the while being absolutely critical for all cytokinesis events.

Beyond *C. elegans* embryogenesis, polarized cortical NMY II has been shown to regulate the asymmetric divisions of both the *C. elegans* post-embryonic Q.a neuroblast (Section 1.4.3.2) and the *Drosophila* embryonic and larval neuroblasts (Section 1.4.3.3). In both cases the half of the cell with preferential NMY localization becomes the smaller daughter with a “stiffer” cortex, while the other half becomes the larger daughter.

Given the established role of NMY II in various developmental contexts involving asymmetric divisions, and the observation that cellular resizing, reshaping, and movement take place after an asymmetric SC division, I asked

whether NMY-2 also plays a role in *C. elegans* SC asymmetric divisions and/or the subsequent cell fate allocation. In this chapter, I examined *nmy-2* gene expression and protein localization, as well as seam phenotypes that arose from *nmy-2* silencing and over-expression.

3.1.1 Aims and hypotheses for this chapter

The aims of the work presented in this chapter were:

- To visualize spatial and temporal *nmy-2* gene expression in *C. elegans* SC development;
- To examine NMY-2 localization in SC asymmetric divisions;
- To devise methods for over-expressing and for silencing *nmy-2* in the seam;
- To study seam phenotypes that arises from altered *nmy-2* expression.

I hypothesized that:

- In an asymmetric division, NMY-2 may preferentially localize to the anterior or the posterior half of a dividing seam cell to generate asymmetry. It may also be found in the daughters post-division to help the anterior daughter round up and move out of the seam line and/or help the posterior daughter elongate. This potential NMY-2 polarity may not be the case in the L2.1 symmetric division.
- *nmy-2* over-expression or depletion may produce visible phenotypes in the seam.

3.2 Results

3.2.1 *nmy-2* full-length promoter drives expression in the seam and in other tissues

In order to see when and where *nmy-2* is expressed in development, *nmy-2* full-length promoter was cloned into the pPD107.94 enhancer assay plasmid (to generate pAW850) and injected alongside *unc-119* to produce independent transgenic lines. This *nmy-2* transcriptional reporter was expressed strongly in the seam in a 3-fold embryo (Figure 3.1A), consistent with NMY regulatory light chain (MLC-4) localization in a previous finding (Shelton et al., 1999) and its role in seam-dependent embryonic elongation (Gally et al., 2009; Piekny et al., 2003; Shelton et al., 1999). *nmy-2* was also expressed in SCs later on in L1 and L2 larvae (Figure 3.1B-C, white arrows). Seam-specific expression seemed to decrease from embryo to larvae. It became difficult to capture *nmy-2* expression in the seam in later stage larvae from any of the lines, especially because of the high background autofluorescence associated with these lines. Additionally, in late L4 and adult worms *nmy-2* was expressed strongly in different tissues, including the vulva (Figure 3.1D, possibly the vulB1 cell), the head neurons (Figure 3.1E, possibly the VLM and VLL neurons), and the posterior intestine and the rectal gland (Figure 3.1F).

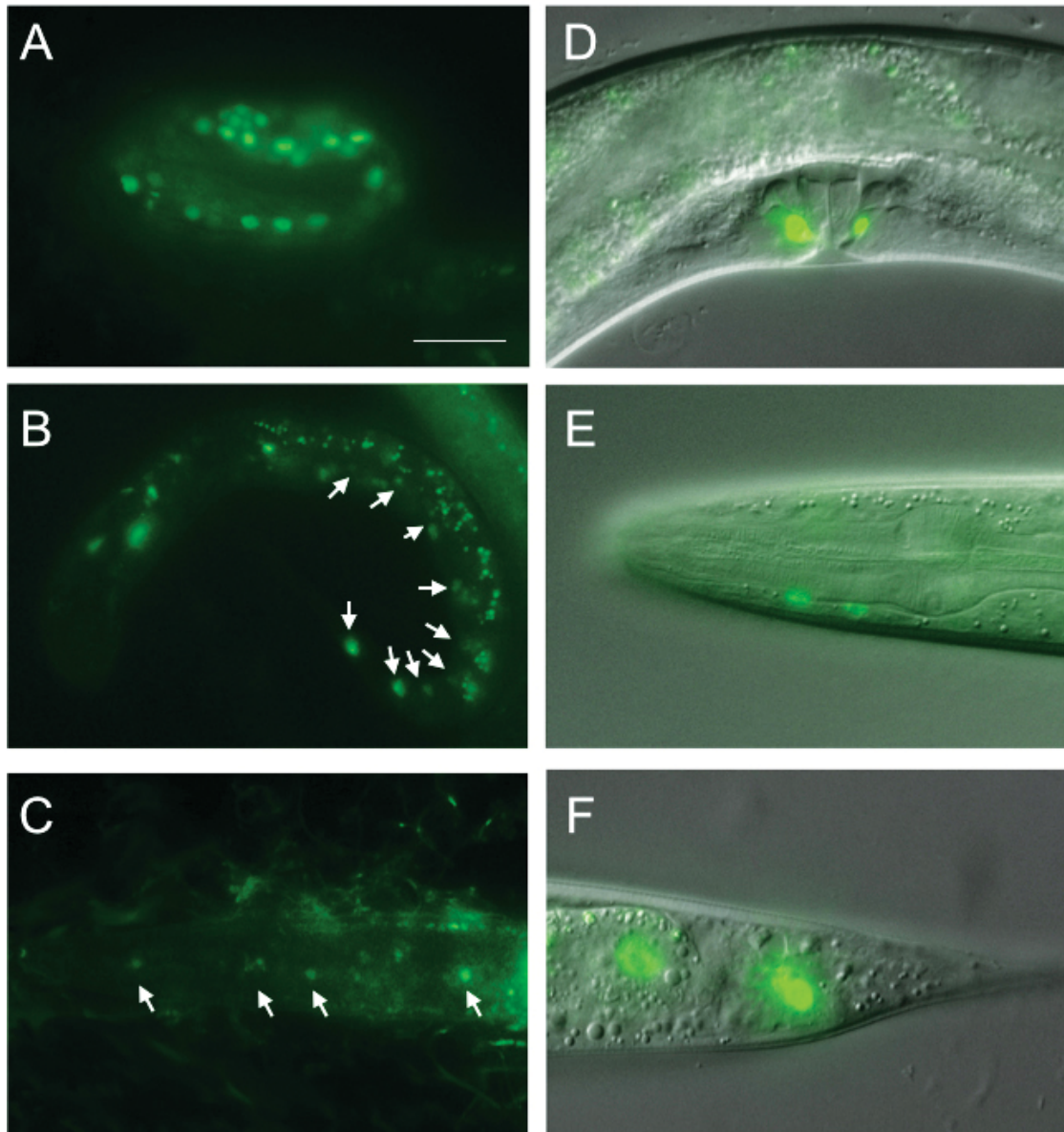


Figure 3.1: *nmy-2* transcriptional reporter drives expression in the seam and other tissues. A-C show seam cells (white arrows) in a (A) three-fold embryo, (B) L1, and (C) L2 larvae of strain AW1118. D-F show adult tissues including (D) the vulva, (E) head neurons, and (F) the posterior intestine and the rectal gland of strain AW1120. Scale bar = 50 μ m.

3.2.2 NMY-2 protein localization is dynamic during SC divisions

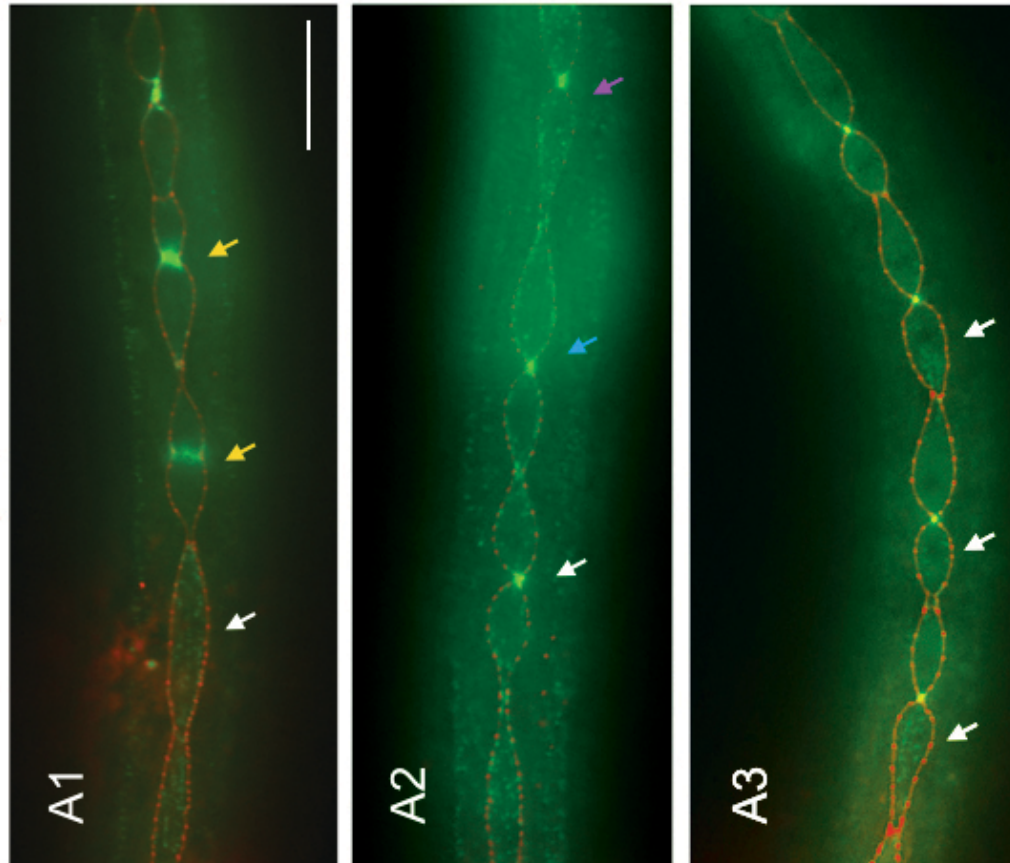
NMY-2 protein localization has been studied in the asymmetric zygotic division and in the Q.a neuroblast in *C. elegans*. In both cases NMY-2 is found to be

preferentially localized to the anterior side of the cell to establish division asymmetry, although the contexts of these patterns are different and NMY-2 localization is not correlated with the size of the daughter generated (i.e. the anterior daughter is the bigger one in the zygotic division, but is the smaller one in Q.a neuroblast division). In the case of the first embryonic division, anterior localization of NMY-2 establishes the A-P axis by further localizing PAR proteins in exclusive anterior versus posterior domains (Goehring et al., 2011; Zonies et al., 2010). In the Q.a neuroblast, more NMY-2-based contractility on the anterior cortex produces a smaller anterior daughter that undergoes apoptosis whereas the larger posterior daughter carries on dividing (Ou et al., 2010). Hence it appeared important to examine whether NMY-2 localization is also biased in SC asymmetric divisions. The localization pattern both during and after SC divisions needed to be examined, because NMY-2 may have polarized localization to generate asymmetry during the division, and/or it can be involved in SC daughter movements and shape changes after division.

zuls45[nmy-2p::nmy-2::gfp] was the most commonly used array for NMY-2 localization studies at the onset of this study. Although this array is sufficiently bright for embryonic studies, it is much weaker in post-embryonic development, consistent with the expression levels seen with my *nmy-2* transcriptional reporter in the previous section. We lacked the appropriate microscope facility necessary to visualize the dim GFP in *zuls45* during seam development, hence I set out to generate a seam-specific *nmy-2::gfp* over-expression strain. The *bro-1CNE+pes-10p::nmy-2::gfp* plasmid (pAW749) was

generated using Gateway cloning and co-injected with *ajm-1p::mCherry* apical junction marker to produce the AW876 strain. The 122-bp long *bro-1CNE* was previously identified in our lab as a seam-specific enhancer in a *pes-10* minimal promoter assay; the *bro-1CNE+pes-10p* sequence drives strong expression in the seam (Brabin et al., 2011) (see Table 2 for a list of tissue-specific promoters). Over-expressed NMY-2::GFP was visible with our microscopes, albeit being mosaic. As I was in the process of integrating AW876 by gamma irradiation to generate AW1094, the advent of CRISPR technology enabled the construction of an *nmy-2::gfp* knock-in strain, LP162 (Dickinson et al., 2013). This is a superior strain because it is brighter than *zuls45* and visible on our microscopes, and it has the endogenous expression from the native *nmy-2* promoter without the need for artificial over-expression. Thus I crossed integrated *ajm-1p::mCherry* into LP162 to mark the SCs and used the resultant AW1092 strain to analyze NMY-2::GFP localization in SCs alongside the over-expression transgene.

CRISPR GFP knock-in
with *ajm-1p::mCherry*



seam over-expression transgene

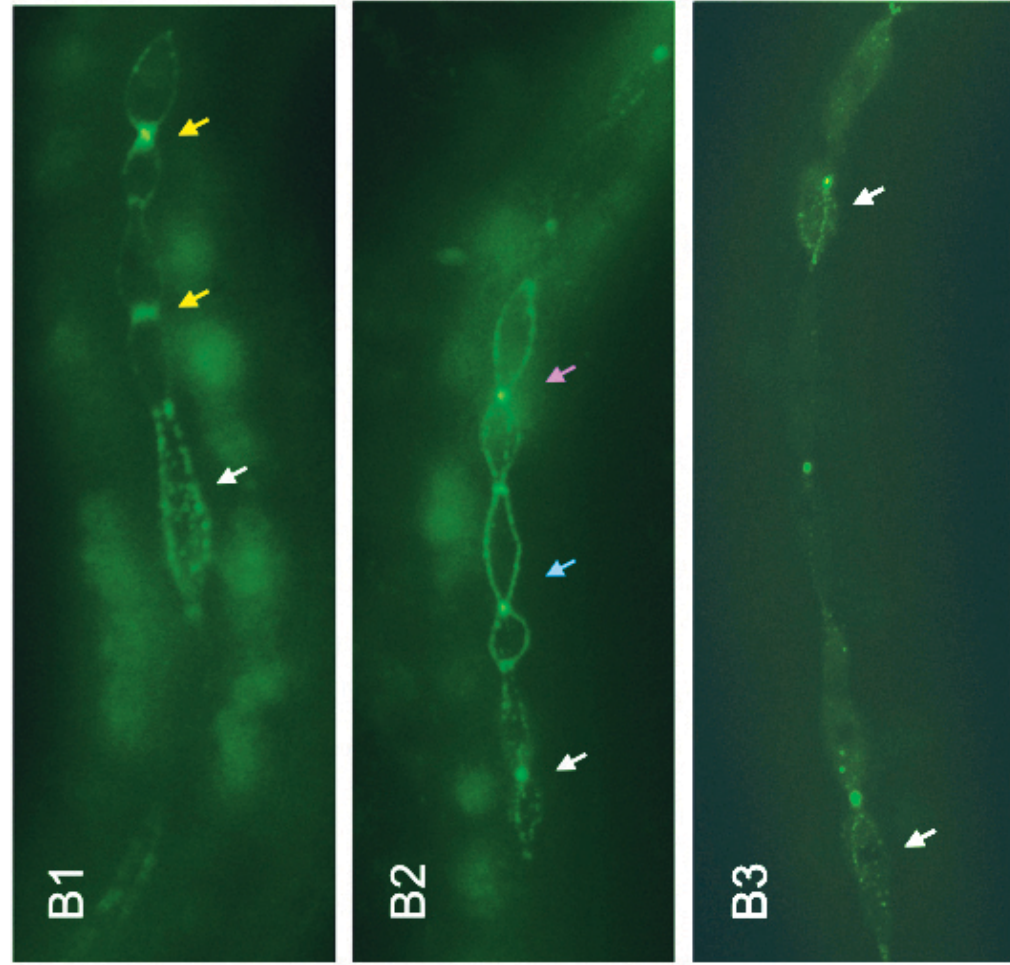


Figure 3.2: NMY-2::GFP localization is dynamic. A1-3 are from the CRISPR knock-in strain AW1092; **B1-3** are from the seam over-expression strain AW876, showing the GFP channel only. Larvae shown here are undergoing or have just completed L2.2 asymmetric SC divisions. Yellow arrows: localization at the cleavage furrow; white arrows: cytoplasmic localization; blue arrows: cortical localization; purple arrows: cytoplasmic and cortical localization. Scale bar = 50 μ m.

Figure 3.2 shows larvae at or after the L2.2 asymmetric division, however they are also representative of the L3 and L4 asymmetric divisions. AW1092 (Figure 3.2 A1-3) and AW876 (Figure 3.2 B1-3) showed very similar localization patterns, suggesting that over-expressing NMY-2 in the seam does not produce artifacts that obscure localization. A1 and B1 show that NMY-2 puncta formed fibers along the A-P axis (white arrows), which has not been reported in other systems previously. Without a DNA marker it was not possible to tell at what stage of cell division these fibers formed (see Section 3.3.1 for discussion). These novel fibers can potentially be involved in trafficking molecules in the A-P orientation, or be exerting A-P contractile forces, or be associated with mitotic spindles. Yellow arrows in A1 and B1 show that NMY-2 localized to the cleavage furrow during cytokinesis as expected. A2 and B2 show that NMY-2 localization was very dynamic. For three adjacent V lineage daughter pairs which had all recently divided, NMY-2 localization varied between being mainly cytoplasmic (white arrows), mainly cortical (blue arrows), or both cytoplasmic and cortical (purple arrows), presumably depending on exactly how long ago the division was completed. Because the V cells shown here share the same lineage and divide more or less synchronously, this is an indication that NMY-2 is highly dynamic after SC division. A3 and B3 show that after SC divisions, occasionally one can catch more NMY-2 in the anterior daughters (white arrows) than the posterior

daughters. Perhaps NMY-2 in the anterior daughter coordinates the rounding up and/or the movement out of the seam line. Most of the time it was not possible to confidently conclude by eye that one daughter had more NMY-2 than the other, and computer-based GFP intensity quantification was not performed. Thus at this point NMY-2 localization early on in division has not been properly examined and I am not able to conclude whether NMY-2 localization is biased in the anterior or posterior daughter after division. In the future it will be very useful to incorporate a DNA marker to examine NMY-2 localization before and during SC division (see Section 3.3.1 for discussion).

3.2.3 Post-embryonic *nmy-2* knockdown is achieved using a combination of RNAi and temperature shift treatments

RNA interference is a popular reverse genetics method whereby gene function is determined by silencing the gene and studying the resultant phenotype. RNAi by feeding is easy in *C. elegans*, especially thanks to a commercially available RNAi library that covers a large portion of the *C. elegans* genome. *nmy-2* RNAi clone (F20G4.3) was obtained from the library. *nmy-2* RNAi was performed on L4 larvae and terminal SC numbers were counted in their progeny at the young adult stage. There was a significant reduction of SC numbers from 16 per side in control RNAi animals to 13 per side in *nmy-2* RNAi animals (Figure 3.3B, left two bars). Note that all the terminal SC numbers in this study were counted in just one side of the worm, and hereon any reference to SC numbers pertains to the number per side, not per animal (which would presumably be double the number per side, however the

numbers sometimes vary between the two sides). In addition to the SC reduction, I observed fewer progeny, suggesting a degree of embryonic lethality, and some very sick animals in the F1 generation (such as Dpy or bursting through the vulva) following *nmy-2* RNAi on L4 larvae. This is not unexpected given the multiple key roles *nmy-2* plays in embryogenesis and its more general role in cytokinesis. Hence to bypass the embryonic effects I decided to perform RNAi post-embryonically. This produced ~0.5 fewer SC on average (Figure 3.3B, right two bars). This mild reduction is statistically significant, however the biological relevance is questionable.

Next, I looked into *nmy-2* mutants and found two temperature sensitive (ts) alleles *nmy-2(ne3409)* and *(ne1490)*. Both are loss-of-function substitution alleles predicted to disrupt the S2 region of the myosin molecule (Figure 3.3A), which is involved in dimerization and head-head coupling (Tama et al., 2005). Mutants with these alleles were maintained at the permissive temperature of 15°C, as the alleles respond rapidly (within seconds) and reversibly to temperature shifts (Liu et al., 2010). L4 larvae shifted to the restrictive temperature of 25°C showed 100% embryonic lethality (n>50). Temperature-sensitive mutants containing the *scmp::gfp* marker were bleach synchronized to L1 diapause at 15°C before being transferred to 25°C. This post-embryonic temperature shift treatment did not produce biologically significant changes in terminal SC numbers (Figure 3.3C, solid bars).

Failing to produce a strong SC phenotype by either post-embryonic RNAi or temperature shift, I proceeded to combine the treatments. Essentially, *nmy-2* ts

mutants were synchronized to L1 before being transferred onto *nmy-2* RNAi plates and incubated at 25°C. This combination of treatments produced a robust reduction of SC numbers from 16 in WT animals to 11-12.5 in *nmy-2* knockdown animals (Figure 3.3C, hashed bars).

For the rest of the thesis work I used this combined RNAi and temperature shift treatment scheme to abrogate *nmy-2* function entirely post-embryonically. Whenever “*nmy-2* knockdown” is referred to, it means worms in either *nmy-2* ts mutant background were bleach synchronized to L1 at 15°C, and then subjected to *nmy-2* RNAi and incubated at 25°C to achieve the knockdown.

Figure 3.3: A combination of RNAi and temperature sensitive alleles is required to achieve robust post-embryonic *nmy-2* knockdown. **A)** Schematic drawing showing the part of the NMY molecule that the RNAi clone and ts alleles target. **B)** A comparison between L4 (purple borders) versus post-embryonic (black borders) *nmy-2* RNAi on SC numbers. A strain with the *wls51[scmp::gfp]* marker (JR667) was used. **C)** A comparison between WT, *nmy-2(ne3409)* and *(ne1490)* genetic backgrounds in combination with control RNAi (solid bars) or *nmy-2* post-embryonic RNAi (hashed bars). The Y-axes in B and C begin at 10 because the animals hatch with 10 SCs and gain additional SCs during post-embryonic development. The strains used in this experiment were AW335, AW785 and AW786. All experiments were conducted at 25°C. n>50 for each sample. Dashed lines denote the 10 SCs that the worms hatch with. Error bars are the standard errors of the mean (SEM). Unpaired T-test was used to determine significance (**: p<0.01; ****: p<0.0001). At least three independent replicates of the experiments were performed.

3.2.4 *nmy-2* knockdown results in fewer terminal SCs

In the combined RNAi/ts experiment, *nmy-2* knockdown led to fewer terminal SCs in young adults as indicated by the integrated *scmp::gfp* marker. Loss of the SC marker could occur at any point along the length of the worm (Figure 3.4B) except in the H0 and H1 lineages. No particular lineage appeared to lose the *scmp::gfp* marker more frequently than others. A previous study reported that the SC marker may become uncoupled from the seam fate when certain genes become perturbed (Brabin et al., 2011), so that a SC may not express the *scmp::gfp* marker but still retains seam fate, dividing as normal. Therefore DIC optics were used to examine regions where *scmp::gfp* was lost in *nmy-2* knockdown animals, and in no case was a SC visible on DIC but not expressing *scmp::gfp* marker (n>50). Thus, in this situation, the loss of *scmp::gfp* correlates with the loss of the seam fated-cell itself. Normally, terminally differentiated SCs fuse to form a seam syncytium that secretes alae, a set of raised cuticle ridges on the worm surface (Figure 3.4C, DIC channel, between dotted lines). Where SCs were lost due to *nmy-2* knockdown (Figure 3.4D, white arrows), alae ridges were always absent (Figure 3.4D, brackets) or

broken (black arrows) (n>50), supporting that the lack of *scmp::gfp* marker in *nmy-2* knockdown young adults was indicative of SC loss prior to terminal differentiation. This is in contrast to the situation in *rnt-1* mutants, where a similar reduction in seam cell numbers has no effect on the alae (Nimmo et al., 2005).

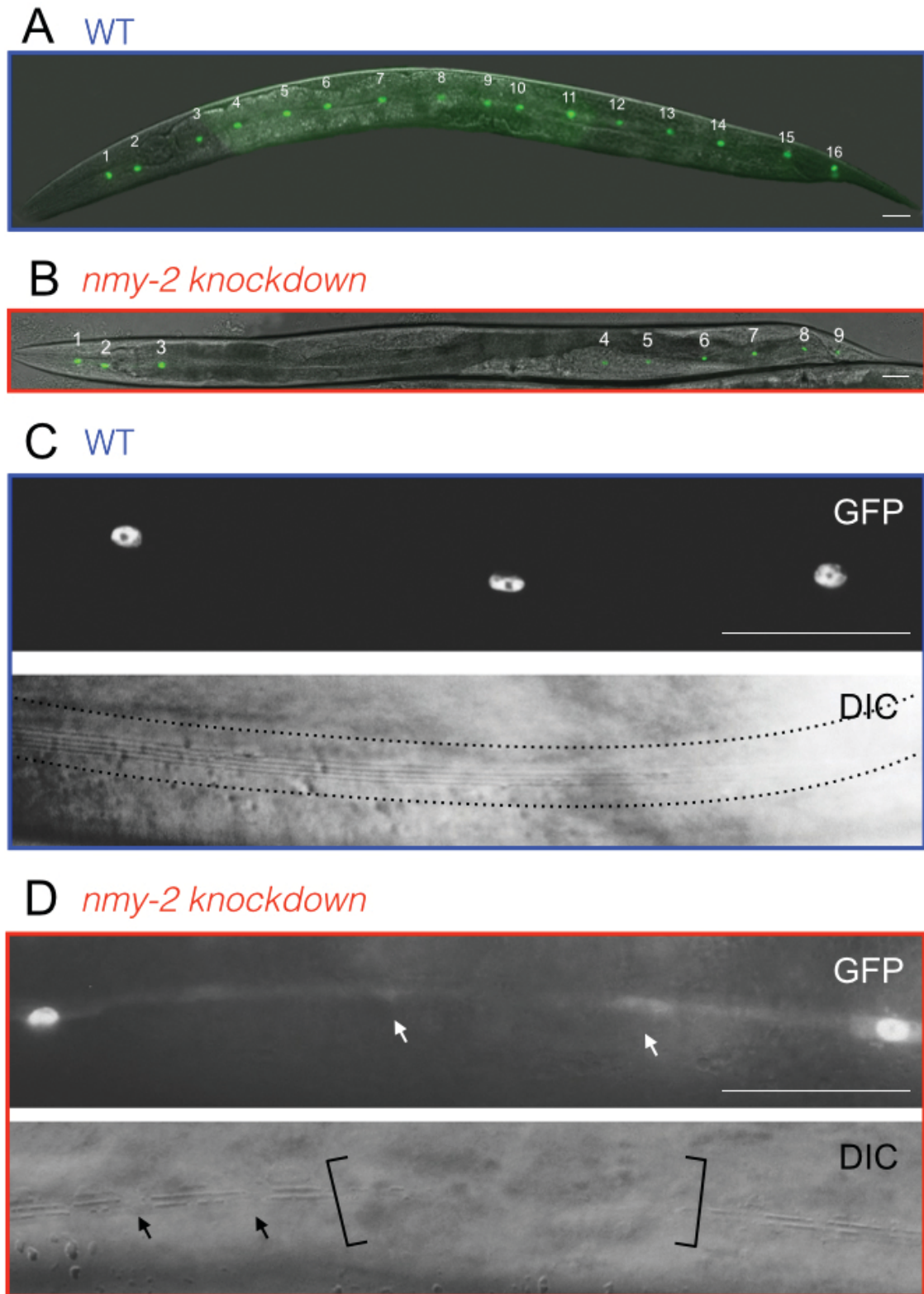


Figure 3.4: *nmy-2* knockdown animals have fewer terminally differentiated SCs. **A)** WT young adult with the *scmp::gfp* marker (JR667). **B)** An example of a *nmy-2* knockdown young adult with the *scmp::gfp* marker (AW785). **C)** Terminally differentiated SCs produce alae in WT young adults. The alae ridges are between dotted lines. **D)** *nmy-2* knockdown animals have none or compromised alae structure corresponding to where SCs are lost. White arrows: lost SCs; black brackets: alae ridges absent; black arrows: alae ridges broken. Scale bars = 20 μ m.

3.2.5 *nmy-2* knockdown leads to progressive SC loss and leaves gaps in the seam

Having fewer terminally differentiated seam cells left gaps in the seam syncytium and the alae. I crossed *hels63[wrt-2p::gfp::PH;wrt-2p::gfp::H2B;lin-48p::mCherry]* (Wildwater et al., 2011), an array that marks both the seam plasma membrane (using GFP-tagged PH domain) and the seam DNA (using GFP-tagged histone H2B), into both *nmy-2* mutant backgrounds and subjected the strains to *nmy-2* knockdown. In WT adults the *wrt-2p::gfp::PH* marker forms a continuous outline of the fused seam syncytium (Figure 3.5A). In *nmy-2* knockdown worms, however, this outline was gapped due to SC loss (Figure 3.5B, dotted brackets). It was possible to have a single gap or multiple gaps; the example shown here had two gaps with a single SC in between. The phenotype was seen in both *nmy-2* mutant backgrounds to a similar extent.

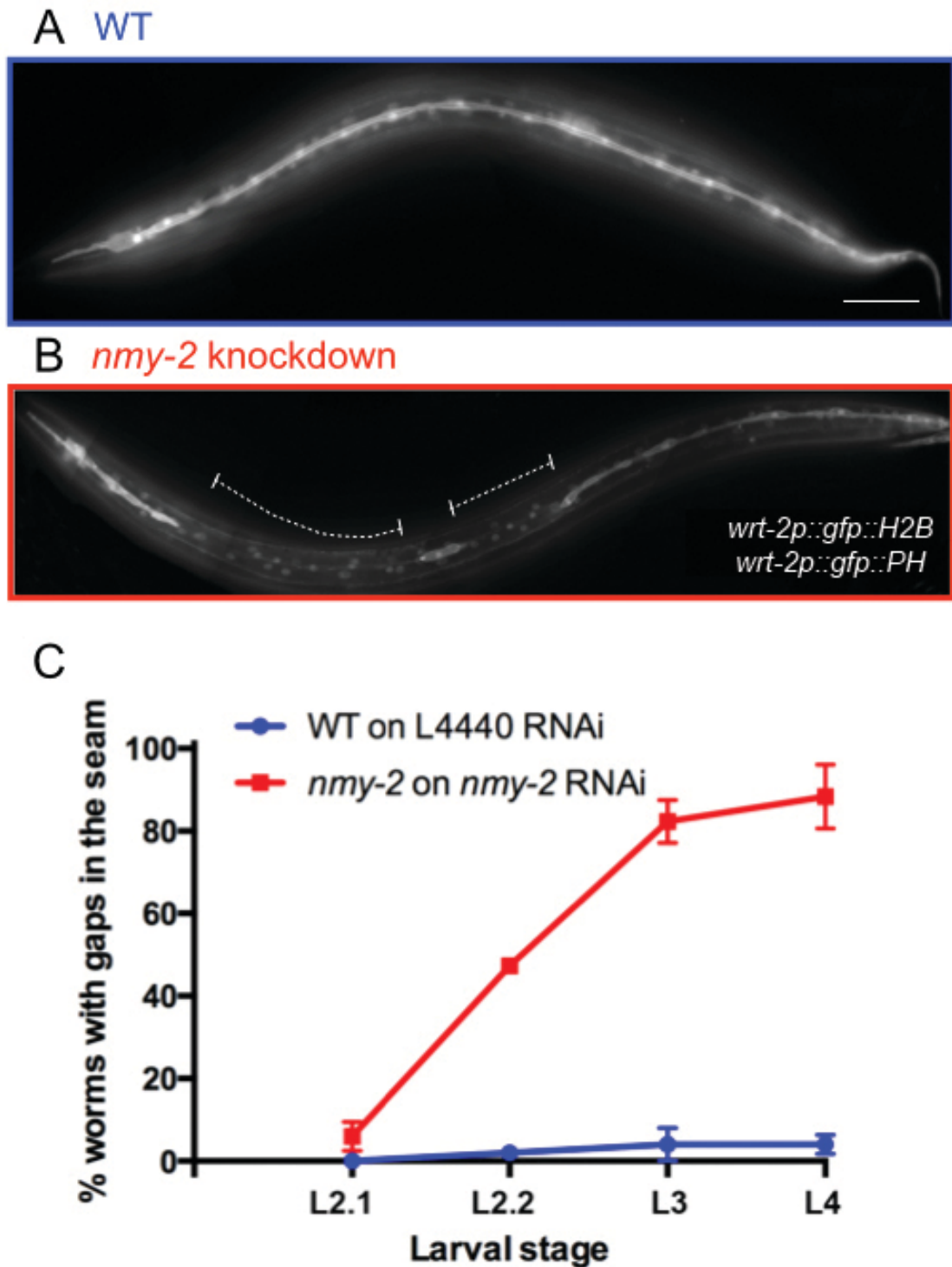


Figure 3.5: *nmy-2* knockdown causes gaps in the seam due to progressive SC loss. *hels63[wrt-2p::gfp::PH;wrt-2p::gfp::H2B;lin-48p::mCherry]* in WT, *nmy-2(ne3409)* and *(ne1490)* backgrounds are strains SV1009, AW788 and AW789, respectively. **A)** A WT young adult worm (SV1009) showing continuous seam syncytial membrane. **B)** An *nmy-2* knockdown young adult (AW788) showing gapped seam syncytial membrane (white dotted brackets). **C)** Percentages of worms with at least one gap in the seam were quantified after each indicated larval division in WT or *nmy-2* knockdown animals. Quantification was performed for *nmy-2(ne3409)* only and not for *nmy-2(ne1490)*. The strains used here were SV1009 and AW788. $n > 50$ per strain per

stage. Two independent replicates of the experiments were performed. Scale bar = 50 μ m.

I next sought to quantify the penetrance of seam gaps using the WT and *nmy-2(ne3409)* strains. Although SCs only fuse to form a syncytium after the L4 division, after each earlier larval division they form end-to-end contact throughout the length of the worm, so it was clear to see and quantify the gaps after each division. Figure 3.5C shows that in *nmy-2* knockdown, seam gaps became more frequent after each asymmetric division (L2.2, L3, L4). After the last SC division in L4, 88% of the worms had at least one gap in the seam, indicating that SC loss is highly penetrant when *nmy-2* is compromised. It is not possible to conclude whether *nmy-2*-related SC loss occurred in L2.1 symmetric division from these data, because the RNAi silencing effect can take some time to initiate in the feeding method (Kamath et al., 2001). So even though gaps were not seen after the L2.1 division, it may be that *nmy-2* does not affect the symmetric division, or it may also just be a result of delayed RNAi effect, since the *ts* mutation alone without RNAi would not be sufficient to generate SC loss.

3.2.6 *nmy-2* knockdown results in cytokinesis failures and multi-nucleated cells

Given the key role *nmy-2* plays in cytokinesis, it was not surprising to observe frequent cytokinesis failures when *nmy-2* function was compromised. The *hels63* array was useful for visualizing this phenomenon. *nmy-2* knockdown worms displayed multi-nucleated cells that arose from failed cytokinesis. Bi-

nucleated cells (Figure 3.6A, white arrow) were the most common, but cells with additional nuclei were also seen (Figure 3.6A, yellow arrow), presumably as a result of multiple rounds of failed cytokinesis across different larval divisions. Figure 3.6B shows a tri-nucleated cell where the middle nucleus (white arrow) is misshapen. Last but not least, failed cytokinesis was occasionally seen together with other phenotypes such as rotated division axis (Figure 3.6C, white arrow). It is unclear whether this spindle rotation derived from or coincided with cytokinesis failure, as spindle rotation had also been seen to occur independently of multi-nucleated cells (data not shown). Either way rotated spindles were very rare (<1%) so they were not followed up.

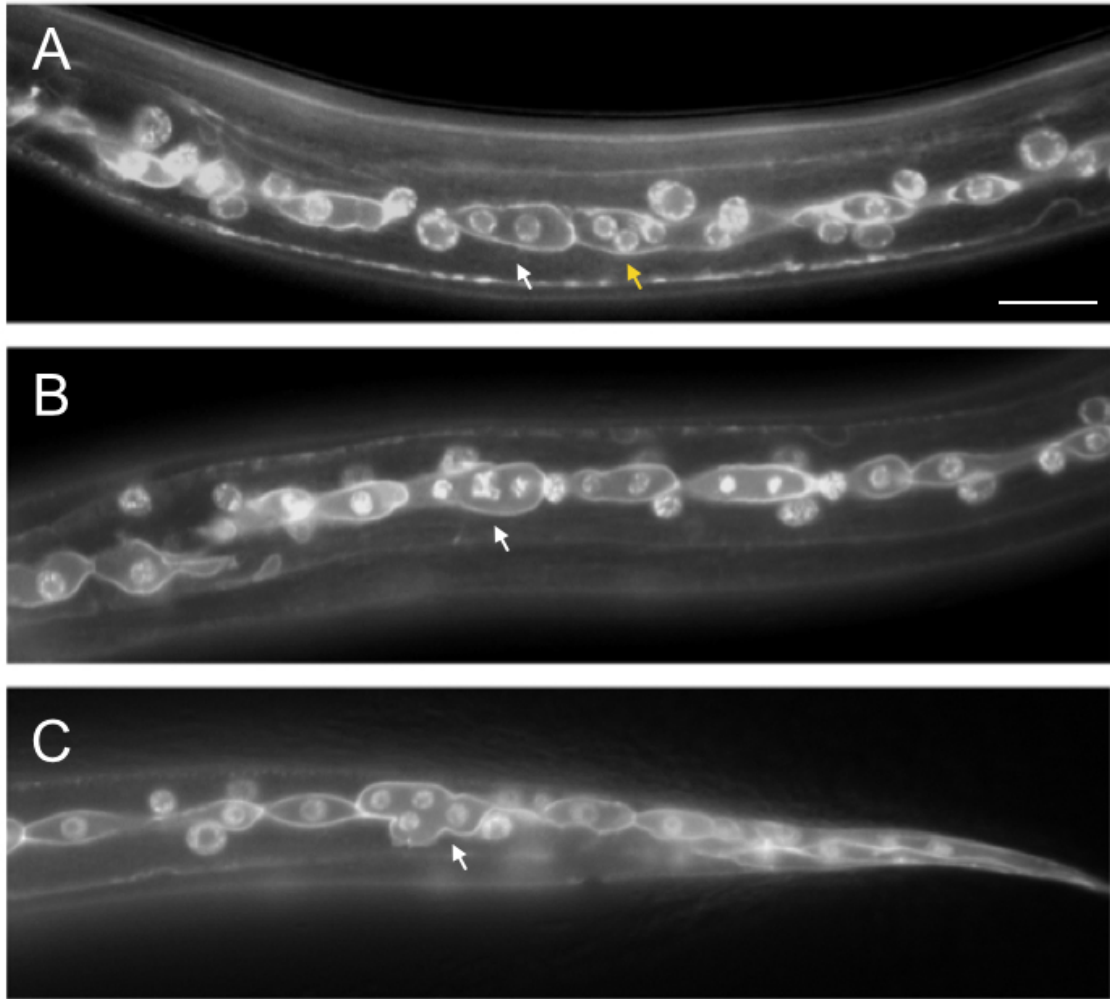


Figure 3.6: *nmy-2* knockdown results in cytokinesis failure and multi-nucleated cells. Both *nmy-2(ne3409)* and *(ne1490)* backgrounds in combination with *nmy-2* RNAi were used to examine cytokinesis failure. Strains used were AW788 and AW789. **A)** White arrow: bi-nucleated cell; yellow arrow: multi-nucleated cell. **B)** White arrow: misshapen nucleus. **C)** White arrow: multi-nucleated cell involving axis rotation. Scale bar = 50 μ m.

It should be mentioned that cytokinesis failure was observed with the *scmp::gfp* marker too in the previous experiment. It exemplified itself as two or more very closely positioned GFP positive nuclei. Thus whenever clusters of nuclei were seen when counting SC numbers using the *scmp::gfp*, DIC optics was used to examine the SC boundary in order to decide whether the nuclei

belonged to the same or different SCs. A single SC with two or more GFP positive nuclei was counted as one SC.

3.2.7 The two ts alleles behave similarly

Up until this point, most *nmy-2* knockdown studies were carried out using both *ne3409* and *ne1490* ts alleles (Sections 3.2.3 through 3.2.6, and later on in Section 4.2.8). Because the results were very similar between the two alleles, plus the mutations are close together in the same region of the NMY molecule, this point onwards *ne3409* was used exclusively as the *nmy-2* mutation for all studies (except in Section 4.2.8, where both alleles were used).

3.2.8 Seam-specific *nmy-2* over-expression does not alter SC numbers at the permissive temperature but may trigger *nmy-2* down-regulation at the restrictive temperature

A seam-specific *nmy-2* over-expression strain was previously made in order to study NMY-2::GFP localization pattern (see Section 3.2.2). This high-copy number extra-chromosomal array was integrated into the *C. elegans* genome by gamma irradiation and crossed into the *nmy-2* mutant background. A set of experiments at 15°C or 25°C, with or without *nmy-2* RNAi, revealed some curious consequences of over-expressing *nmy-2* in the seam.

At the permissive temperature of 15°C, WT and *nmy-2* mutant worms with or without the integrated transgene all had 16 SCs (Figure 3.7, solid bars on the

left), suggesting over-expressing *nmy-2* in the seam does not alter SC numbers.

At the restrictive temperature of 25°C, having the array in the *nmy-2* background reduced SC number by 1 (Figure 3.7, red vs. green solid bars in the middle, $p < 0.0001$) on control RNAi. Perhaps this is because the repetitive sequences carried on the extrachromosomal array triggered the worm's intrinsic RNAi mechanism, thus causing a mild reduction of SC number that is resembles extrinsic *nmy-2* RNAi as previously seen. It is not known why the array triggers the reduction only in the *nmy-2* background but not the wild-type background.

When *nmy-2* RNAi was added to the strains at 25°C (Figure 3.7, hashed bars on the right), WT and *nmy-2* mutant strains with the array both saw a significant reduction of SC numbers similar to the reduction in the *nmy-2* mutant on *nmy-2* RNAi (i.e. a typical *nmy-2* knockdown). This is not surprising for *nmy-2*+array on *nmy-2* RNAi, since the RNAi probably silenced all of the extra *nmy-2* produced by the array so that *nmy-2*+array on *nmy-2* RNAi was similar to *nmy-2* without array on *nmy-2* RNAi (compare hashed green bar to hashed red bar on the right). By comparison, the significant reduction by 4 SCs in WT+array on *nmy-2* RNAi was a curious one (compare hashed yellow bar to hashed blue bar on the right). Perhaps this represents the result of having the array triggering the intrinsic RNAi mechanism, combined with the extrinsic implementation of *nmy-2* RNAi.

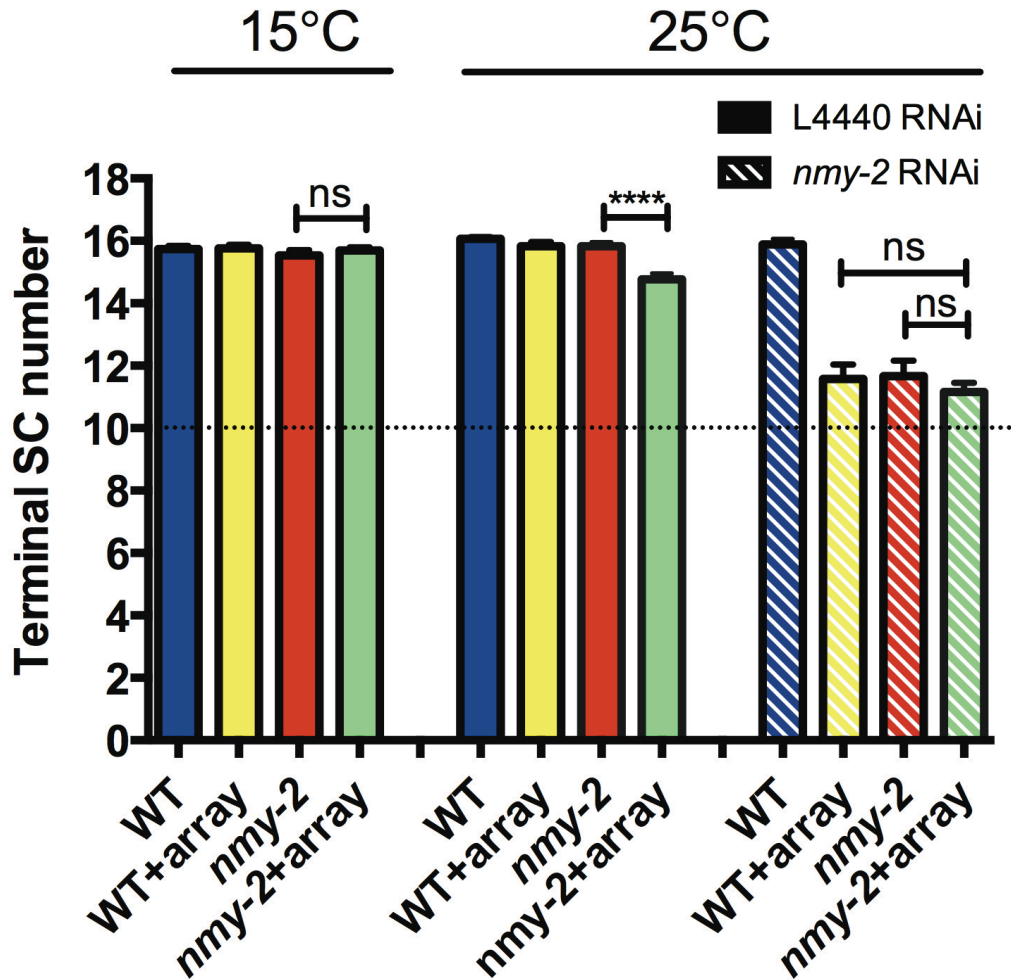


Figure 3.7: Seam-specific *nmy-2* over-expression does not alter SC numbers at the permissive temperature. Worms in WT or *nmy-2* background, with or without *ouls18[bro-1CNE+pes-10p::nmy-2::gfp;ajm-1p::mCherry]* over-expression array were synchronized to L1, hatched onto control (solid bars) or *nmy-2* (hashed bars) RNAi plates, and incubated at 15°C or 25°C as indicated. Terminal SC numbers were counted in young adults. The strains used here were JR667, AW1050, AW785, AW1052. $n > 50$ for each sample. Dashed line denotes the 10 SCs that the worms hatch with. Error bars are the SEM. Unpaired T-test was used to determine significance (ns: $p > 0.05$; ****: $p < 0.0001$). Two independent replicates of the experiments were performed.

3.2.9 Possible involvement of *nmy-1* in SC loss

When the *nmy-2* RNAi clone was initially taken out of the Ahringer library, it was checked in Clone Mapper (Thakur et al., 2014) and there was no indication of potential off-target effects. However, a search of the targeting

sequence in PFAM later on indicated that the RNAi clone targets the highly conserved myosin head domain (63% amino acid identity between NMY-1 and NMY-2). This finding raised a concern that *nmy-2* RNAi may have off-target effect on *nmy-1*, the other *C. elegans* non-muscle myosin homolog. *nmy-1* is much less studied compared to *nmy-2* but has been shown to function redundantly with *nmy-2* during embryo elongation and morphology establishment (Piekny et al., 2003). An alignment between the *nmy-1* genomic sequence and the *nmy-2* RNAi targeting sequence revealed a 20 bp region with 100% nucleotide identity (Figure 3.8A, orange box). This is at the limit for effective siRNA silencing (~21 nt being the optimal length most found *in vivo*) (Qiu et al., 2005), thus it is possible that *nmy-1* was silenced by *nmy-2* RNAi.

Thus, the relative contributions of *nmy-1* and *nmy-2* in producing SC loss were assessed. The Ahringer library contains a clone for *nmy-1* (F52B10.1), but it had no effect on SC number when fed to L4 larvae (Figure 3.8B). Feeding L4 larvae a 1:1 mixture of *nmy-1* and *nmy-2* RNAi culture abolished the SC reduction effect that *nmy-2* L4 RNAi alone had, probably because *nmy-2* RNAi strength was diluted. This was confirmed by using a 1:1 mixture of L4440 and *nmy-2* RNAi culture, which also produced no SC loss. These results suggest that either silencing *nmy-1* alone does not have an effect on the SC number, or that *nmy-1* was refractory to the particular RNAi clone.

I then constructed my own *nmy-2* and *nmy-1* RNAi clones and named them SD*nmy-2* and SD*nmy-1*, respectively. Both clones target the less conserved tail region (36% amino acid identity between NMY-1 and NMY-2) in their

respective myosin molecule. The RNAi targeting sequences were confirmed to be much more divergent between the two molecules. *nmy-2* ts mutant was treated with library *nmy-2*, *SDnmy-2* or *SDnmy-1* RNAi post-embryonically and terminal SC numbers were counted. The SC loss phenotype at 25°C seen with Ahringer library *nmy-2* RNAi was partially reproduced with *SDnmy-2* but not with *SDnmy-1* RNAi (Figure 3.8C). This still did not exclude the possibility that *SDnmy-1* RNAi failed to silence *nmy-1*, so I could not conclude that *nmy-1* is not important for the seam. The role of *nmy-2*, however, is firmly established by this experiment, because the post-embryonic combination of *SDnmy-2* RNAi and *nmy-2* ts allele, with no suspected knock-on effect on *nmy-1*, was able to trigger a loss of terminal SCs. This regime led to a smaller extent of SC loss (a loss of 4 SCs) compared to the Ahringer library *nmy-2* plus ts mutation treatment combination (a loss of 6 SCs). This may be attributed to the RNAi clones' different effectiveness in silencing *nmy-2*, and/or perhaps because *SDnmy-2* RNAi does not target *nmy-1* whereas Ahringer library *nmy-2* RNAi does. At any rate, since the role of *nmy-2* in the seam was reaffirmed by this experiment, I was less concerned about the partial redundancy between *nmy-1* and *nmy-2*. The Ahringer library *nmy-2* RNAi clone was used for further studies as it produces a stronger effect on the seam.

A

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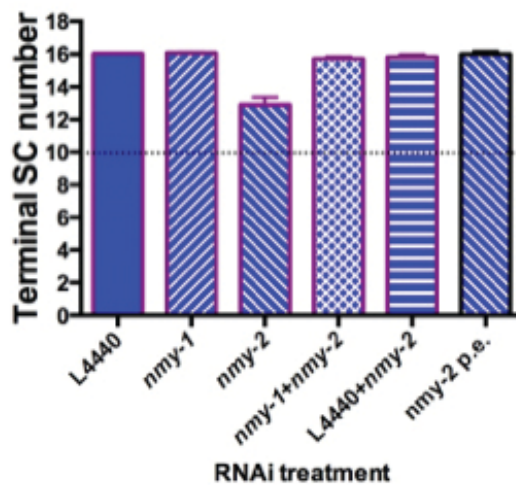
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      |||||.|||.|||||.|||.|||||.|||||.|||||.|||||.
nmy-2  CCAATCTACACGGATACAATTG-CTGAACAGTTCAAATGCAAAAAGAGAA

      ACGAATGCCACCACACATATTTGCAATTGCTGATACTGCGTACAGAAGT
      |||.|||||.|||||.|||||.|||||.|||||.|||||.|||||.
      AGGAGATGCCACCACACATATTTGCTGTTGCCGATGAAGCCTACCGCAGC

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      |||||.
      ATGCTTCA-----

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B



C

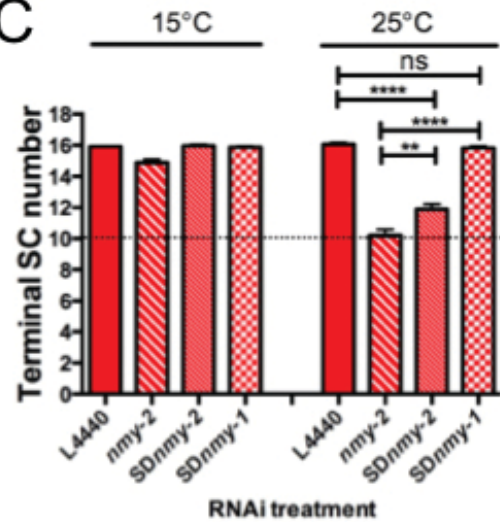


Figure 3.8: *nmy-2*, and possibly *nmy-1*, is responsible for SC loss. **A)** Alignment between the *nmy-1* genomic sequence and the *nmy-2* RNAi targeting sequence was performed using EMBOSS Water pairwise nucleotide sequence local alignment tool. The orange box denoted the region with 100% amino acid identity. **B)** WT L4 larvae with the *scmp::gfp* marker (JR667) were subjected to various RNAi treatments as indicated (except for the last bar) and terminal SC numbers were counted in their progeny. Purple border denotes L4 RNAi treatment. The last bar (black border) represents post-embryonic (p.e.) *nmy-2* RNAi treatment. **C)** *nmy-2* ts mutant with the *scmp::gfp* marker (AW785) was subjected to various post-embryonic RNAi treatments as indicated. Terminal SC numbers were counted in young adults. $n > 50$ for each sample. Dashed lines denote the 10 SCs that the worms hatch with. Error bars are the SEM; unpaired T-test was used to determine significance (ns: $p > 0.05$; **: $p < 0.01$; ****: $p < 0.0001$). Two independent replicates of the experiments were performed.

3.3 Discussion

3.3.1 *nmy-2* is expressed in the seam and shows dynamic protein localization in SC divisions

In this chapter *nmy-2* gene expression and protein localization patterns were assessed using transcriptional and translational GFP reporters, respectively. *nmy-2* was found to be expressed in the seam in the embryo and in young larvae (Figure 3.1), but little transcription was seen in the seam in older larvae and adults. Strong *nmy-2* expression in the three-fold embryo SCs is consistent with its role in SC-dependent embryonic elongation, and the decreasing level of *nmy-2* transcription during larval development may suggest that the regulation of NMY later on in development occurs primarily at the post-translational level by the activities of kinases and phosphatases, rather than at the transcriptional level. This is supported by my observation that while *nmy-2* RNAi at the L4 stage caused embryonic lethality and a penetrant decrease of SC numbers in the next generation, post-embryonic silencing of *nmy-2* transcription by RNAi produced little effect on SC numbers (Figure 3.3B). However, the requirement of RNAi treatment as part of the post-embryonic *nmy-2* knockdown regime suggests at least some regulation at the transcriptional level during post-embryonic development.

The NMY-2 protein expression reporter showed dynamic NMY-2 localization during late stages of SC asymmetric divisions and after the divisions. NMY-2 formed fibers of unknown function along the A-P axis in mother SCs, and can

rapidly localize both to the cell cortex and in the cytoplasm. NMY-2 localization at the cleavage furrow is consistent with its general role in cytokinesis.

Additionally, it was reassuring to ascertain that my seam-specific over-expression strain showed similar NMY-2 localization patterns to the CRISPR-generated GFP knock-in strain. However, observations of NMY-2 localization polarity in SC divisions were inconclusive due to the lack of a DNA marker and a suitable imaging device (further discussed in the next section).

3.3.2 NMY-2 protein localization study before and during SC division requires a DNA marker and better imaging device

While the CRISPR-generated *nmy-2::gfp* knock-in strain revealed dynamic NMY-2 localization in SC divisions, there is an urgent need for a DNA marker to be built into the strain to take the most advantage out of it. Without the DNA marker, only late mitotic stages from furrow ingression to abscission can be identified by the *ajm-1p::mCherry* cell shape marker. Thus vital information regarding NMY-2 localization dynamics prior to SC division and during early stages of the division is lacking, simply because it is not possible to tell the exact stage of mitosis based on cell shape alone. In the case of the *C. elegans* zygote, NMY-2 localization is highly dynamic early on and is critical for establishing the division axis, so if only anaphase through cytokinesis was looked at then all the key early information would have been lost. In my case, for example in Figure 3.2 A1 and B1, long fiber-like structures were seen with NMY-2::GFP puncta prior to cleavage furrow ingression as if NMY-2 associated with the spindles. It will be fruitful to know exactly which stage

these cells were at in order to infer *nmy-2* function. The addition of a *histone::mCherry* marker, for example, can show chromosome dynamics without obscuring NMY-2::GFP on the green channel.

The other limitation I had with my NMY-2 localization analysis was the lack of an appropriate imaging device. All the images shown in Figure 3.2 were captured on a Zeiss Axiophot fluorescent microscope. A spinning disc confocal laser microscope would allow better visualization of highly dynamic molecules with low expression levels. In order to capture NMY-2::GFP on the Zeiss fluorescent microscope, an acquisition time of 2 seconds or more was used for each snapshot (plus another 1.5 seconds for *ajm-1p::mCherry* in order to outline the seam cells). Such high UV exposure quickly bleaches the fluorescence and is highly toxic to the animals, so only 1-2 snapshots could ever be taken of the same region in a particular animal. This renders time-lapse imaging of NMY-2 dynamics impossible. We have a Leica SP5 confocal laser scanning microscope which is more sensitive for detecting fluorescence, but scanning confocal microscopes are also sub-optimal for capturing dynamic molecules when both high resolution and high speed are required. Acquisition of a typical image stack for visualizing NMY-2::GFP takes about a minute, so the total photobleaching and phototoxicity levels are still too high. The most ideal type of microscope for my purpose will be a spinning disc confocal laser microscope, which accommodates both fast imaging and high sensitivity to enable the possibility of capturing NMY-2 dynamics in quick succession across multiple points of time, so that for example a kymograph can be generated. Unfortunately none of the spinning disc confocal microscopes in the

Department of Biochemistry has the correct set up for imaging *C. elegans*, so the localization study was not pursued further.

3.3.3 A method for post-embryonic *nmy-2* knockdown is established

This chapter contains the groundwork for establishing a method to silence *nmy-2* entirely post-embryonically. The scheme utilizes a combination of *nmy-2* RNAi (Ahringer library clone F20G4.3) and *nmy-2* ts mutation (*ne3409*), which robustly produced progressive cell loss after each asymmetric SC division. It is interesting that the ts mutation or the RNAi on its own both produced very strong embryonic lethality but little post-embryonic seam phenotype until the two treatments were combined. This suggests that post-embryonic seam development is a fairly robust system that can withstand certain genetic fluctuations. However, in the light of the fact that the ts alleles are fast-acting on the order of seconds (Guo and Kemphues, 1996), all the experiments would have carried much more merit if performed using a temperature-controlled chamber, or any other method to quickly equilibrate the slide stage temperature to 25°C.

In the combined regime using both RNAi and the ts mutation, *nmy-2* (possibly some *nmy-1* too) was sufficiently perturbed to cause seam cell loss. However the necessity for both treatments made further studies more challenging. All the cellular markers must be crossed into the mutant background before being examined. Genetic crosses must take place at the permissive temperature of 15°C, which meant approximately double the length of generation time than at

room temperature. For the subsequent epistasis analysis (presented in Chapter 4 and Chapter 6), it was not possible to use a mutant for one gene and RNAi for the other. Undiluted *nmy-2* RNAi and the *nmy-2* ts mutation must both be used simultaneously, necessitating the construction of double mutants between *nmy-2* and the gene of interest, which were then subjected to *nmy-2* RNAi. This was laborious and time-consuming, and potentially limiting if no suitable mutation for the gene of interest exists.

3.3.4 There is a potential conundrum concerning NMY-2 preferential localization and SC loss

The SC loss observed by perturbing *nmy-2* is likely due to inappropriate cell fate allocation in the seam asymmetric divisions. Perhaps the posterior seam daughters whose fate is to remain seam and continue dividing inappropriately acquired a differentiative fate instead. I hypothesized that NMY-2 may exhibit polarized localization during or after a wild-type SC asymmetric division to specify daughter fates, so maybe *nmy-2* perturbation affected this localization pattern and led to mis-specified daughter fate and the subsequent SC loss. Unfortunately, my hypothesis was neither supported nor rejected by my limited localization analysis. Even though I occasionally caught more NMY-2 in the anterior daughter than the posterior daughter after a WT SC asymmetric division, I was not able to conclude that it was a general pattern. Whether there is any preferential localization earlier in SC asymmetric divisions was not assessed due to the lack of a DNA marker and appropriate imaging devices as

discussed in Section 3.3.1, and the localization pattern was not investigated in the L2.1 symmetric division to offer comparison to the asymmetric divisions.

Nevertheless, if later on NMY-2 is discovered to localize preferentially to the anterior, as in the case of the zygote and the Q neuroblast, then we face a conundrum. Such a result would suggest that silencing *nmy-2* should lead to more, not fewer SCs. If more NMY-2 in the anterior is important for specifying the differentiative fate of that daughter, then an overall depletion of NMY-2 should render the anterior daughter more similar to the posterior one, meaning the anterior daughter would adopt the seam fate and the overall SC number would increase. However when I knocked down *nmy-2* I saw the opposite effect, i.e. a SC loss rather than a SC gain, so it would not fit with the model. Since I am yet to establish whether NMY-2 localization is biased towards either the anterior or the posterior in SC asymmetric divisions, I will leave this hypothetical issue behind for the rest of my thesis. However, in the future if NMY-2 is indeed shown to polarize along the A-P axis in these divisions, then it will definitely be worth revisiting this problem to find a suitable resolution.

Chapter 4 *nmy-2* knockdown results in cell fate transformations

4.1 Introduction

The work described in the previous chapter established a robust method for post-embryonic *nmy-2* knockdown, which caused SC loss after each round of SC asymmetric division. The work contained in this chapter sought to investigate how SC loss occurred when *nmy-2* function was compromised.

SCs could be lost for different reasons. The most likely explanation is that an asymmetric division that normally produces a differentiative anterior daughter and a proliferative posterior daughter had symmetrized towards conferring the differentiative fate to both daughters, leading to an increase in the number of hypodermal cells at the expense of SCs. Alternatively, maybe instead of becoming a hypodermal cell, the posterior daughter which normally remains seam adopted yet another cell fate such as becoming a neuronal cell or undergoing apoptosis. To this end, I constructed a seam-hypodermal dual color fate-reporter strain to identify and quantify potential cell fate transformations in *nmy-2* knockdown. Based on the ensuing phenotypic analysis, I investigated whether cytokinesis or elongation defects could account for the SC loss seen in *nmy-2* knockdown.

Considering other mutants representing different molecular pathways are known to reduce terminal SC numbers, insight would be gained by creating double mutants between these mutants and *nmy-2*. For example, mutations in *wrm-1*, a gene encoding the WNT/ β -catenin asymmetry pathway effector β -catenin (introduced in Section 1.3.1), lower the terminal SC to about 7 (Gleason and Eisenmann, 2010). Given that the WNT/ β -catenin asymmetry pathway governs divisions along the A-P axis and that I suspected NMY-2 may have A-P polarity in SC asymmetric divisions (although the results in Section 3.2.2 were inconclusive), the WNT/ β -catenin asymmetry pathway emerges as a prominent candidate that *nmy-2* may interact with to regulate the seam. The main effectors of the WNT/ β -catenin asymmetry pathway are WRM-1/ β -catenin, LIT-1/NLK, and POP-1/TCF, all of which show polar localization patterns in an asymmetric division. I set out to examine whether *nmy-2* interacts with these effector molecules by epistasis (further introduced below) and localization analyses. Moreover, mutations in both *mt-1* and *bro-1* lower terminal SC numbers to about 13 (Kagoshima et al., 2007; Nimmo et al., 2005). The RNT-1/BRO-1 pathway is predominantly involved in promoting SC proliferation (Kagoshima et al., 2007; Kagoshima et al., 2005; Nimmo et al., 2005; Xia et al., 2007), so it also constitutes a potential candidate for *nmy-2* interaction. The heterochronic pathway, however, is unlikely to interact with *nmy-2* based on *nmy-2* knockdown phenotypes. Heterochronic genes specify stage-specific division patterns during larval development. *nmy-2* knockdown resulted in neither stage-specific nor lineage-specific SC phenotypes, so it is unlikely to interact with heterochronic genes during SC development.

4.1.1 Epistasis analysis

Epistasis analysis was carried out in this chapter to assess potential genetic interactions between *nmy-2* and other candidate genes in regulating seam development. Epistasis is a term broadly used to refer to interactions between two genes, first coined by Bateson in 1909 to refer to the ability of one gene locus to “mask” over the mutational effect of another gene locus (Bateson, 1909). However, over time the term has also been adapted to describe the much more general phenomena concerning deviations from the additive phenotypic outcome of two genes. To differentiate the usage of this term, Phillips (2008) proposed that “epistasis” may refer to 1) functional epistasis, describing the protein (or other genetic elements) interactions on the molecular level, whether they function within the same pathway or form a direct complex with one another (Boone et al., 2007); 2) compositional epistasis, describing Bateson’s original usage of the term where one allelic effect is blocked by an allele at a different locus; or 3) statistical epistasis, in a population genetics sense, describing how the multi-locus phenotype statistically deviates from the additive phenotypic outcome of each mutation (Fisher, 1918).

For the purpose of my thesis, epistasis analysis pertains to the first two “biological/physiological” categories of term usage (as opposed to statistical). In practice it involves comparing the phenotypic outcome of a double mutant to those of the single mutants, from which potential genetic interactions and the order of genes within a regulatory pathway may be revealed. This technique has been widely used to construct multiple genetic pathways in *Drosophila*,

yeast, and *C. elegans* (Phillips, 2008). For example, the elucidation of the sex determination (Goodwin and Ellis, 2002), vulva development (Sternberg and Horvitz, 1989), and dauer entry pathways (Thomas et al., 1993) in *C. elegans* all owe much to elegant epistasis analyses.

The interpretation of epistasis data has been extensively reviewed (Avery and Wasserman, 1992; Boone et al., 2007; Gao et al., 2010; Huang and Sternberg, 2006; Phillips, 2008). In general, if mutations in gene A or gene B each have a phenotypic effect, and the total effect on the double mutant is simply additive, then gene A and B are considered independent. If the total effect on the double mutant differs from the additive outcome, then it suggests genetic interaction between A and B. In the event that both mutations cause similar phenotypes, and that the phenotype of the double mutant is more severe than expected under independence, then the epistasis is negative (also described as synergistic or synthetic). Conversely, if the double mutant shows a less severe phenotype than expected under independence, then the epistasis is positive (also described as antagonistic or alleviating) (Gao et al., 2010). In the event that mutations in the two genes cause opposite phenotypes and the double mutant shows only the phenotype of gene A, then gene A is epistatic to gene B and the two genes likely function in the same pathway. The epistatic gene can be either upstream or downstream of the other, depending on the nature of the mutation and the type of regulation (Avery and Wasserman, 1992).

The interpretation of epistasis data can be complicated and greatly depends on the assumptions, the nature of the alleles (null, partial loss of function, gain of

function), and the property of the pathway (switch regulatory, substrate-dependent, or complex (Huang and Sternberg, 2006)). For example, synthetic lethality in yeast refers to the situation where, while a single mutation in either gene A or B has little effect on viability, the double mutant for both gene A and B is unviable, i.e., synthetically lethal. Synthetic lethality can be interpreted differently with regard to the interaction and function of the two genes depending on the context. It may suggest that protein products of genes A and B physically associate to function together in the same pathway, so that only the double mutation can sufficiently disrupt their interaction and function to cause lethality. Alternatively, it may suggest that gene A and B function in parallel pathways controlling a common process, whereby mutations in A is partially compensated by the pathway involving B, and vice versa; but disrupting both pathways is devastating and leads to lethality.

Such a situation is well-illustrated by experiments involving mutations in *CDC28*, the yeast cyclin-dependent cell cycle kinase, and *CLB1* and *CLB2*, which both encode the G2 cyclin. The temperature sensitive *cdc28-1N* mutation is lethal at the high temperature and causes G2 arrest (Piggott et al., 1982). At the permissive temperature, while neither *cdc28-1N* mutation nor *clb2* deletion has any pronounced effects on its own, the combination of the two is lethal (Surana et al., 1991). Additionally, *clb1* deletion is only lethal in the presence of *clb2* deletion (Surana et al., 1991). While *cdc28-1N* and *clb1* both exhibit synthetic lethality in combination with *clb2*, the results mean different things. The former is because the protein products of *CDC28* and *CLB2* function in the same pathway and physically form a CDK/cyclin complex to

drive cell cycle progression, while the latter is because *CLB1* and *CLB2* function in parallel pathways to encode the G2 cyclin (Guarente, 1993). Such interpretation could not have been achieved using epistasis data alone without any knowledge about the gene products. Therefore, epistasis analysis results need to be interpreted carefully with contextual considerations if possible, and inferred genetic interactions should be confirmed by additional biochemical or imaging methods.

4.1.2 Aims and hypotheses for this chapter

The aims of the work presented in this chapter were:

- To construct a dual fate-reporter strain that marks hypodermal and seam fates with different fluorophores, in order to identify any potential transformation between those two fates in *nmy-2* knockdown;
- To examine whether SC loss in *nmy-2* knockdown is attributable to cytokinesis or elongation defects;
- To investigate whether *nmy-2* interacts with one of the known seam regulatory pathways by constructing double mutants and carrying out epistasis analyses of seam phenotypes;
- If epistasis data suggest genetic interaction, to further validate the interactions and place *nmy-2* within the known network.

I hypothesized that:

- SCs were lost after asymmetric divisions because some seam daughters inappropriately adopted a different fate. They would most

likely have become hypodermal cells, but could possibly take on yet a different cell fate as a result of *nmy-2* knockdown;

- Cytokinesis or elongation defects may partially contribute to the SC loss phenotype seen in *nmy-2* knockdown, but *nmy-2* has additional roles in regulating seam development;
- *nmy-2* may act through one of the known SC regulatory networks to ensure proper SC divisions. The most likely candidate is the WNT/ β -catenin asymmetry pathway, followed by the RNT-1/BRO-1 pathway.

4.2 Results

4.2.1 A dual fate-reporter strain reveals three classes of cell fate changes as a result of *nmy-2* knockdown

The most obvious explanation for SC loss in *nmy-2* knockdown is that following an asymmetric division, some SCs adopted the hypodermal fate instead of remaining seam. Although SCs are multipotent and give rise to hypodermal cells, neurons and neuronal support cells, I am mostly concerned about the V1-4 lineages, which only beget hypodermal cells. Thus assuming a binary choice between hypodermal fate versus seam fate, I constructed a dual fate-reporter strain marking the two fates with different-colored fluorophores. I co-injected a *scmp::NLS::tdTomato* plasmid that marks seam nuclei in red, a *dpy-7p::2xNLS::yfp* plasmid that marks hypodermal nuclei in yellow (appears green through the GFP filter so will be referred to as green now on), and a *wrt-2p::gfp::PH* plasmid that marks SC membranes (see Table 2 below for a list of

tissue-specific promoters). The resultant extrachromosomal array was integrated by gamma irradiation to produce *ouls10* (Figure 4.1A). The red marker expressed in SCs bound by the PH marker, and the green marker expressed in hypodermal nuclei outside of the seam boundaries. With my imaging settings, the *scmp::NLS::tdTomato* marker did not bleed over to the green channel and the *dpy-7p::2xNLS::yfp* marker was bright on the green channel and weak on the red channel.

Table 2: Tissue-specific promoters used in this study

Promoter	Tissue-specific expression
<i>scmp</i>	seam
<i>bro-1CNE+pes-10p</i>	seam
<i>wrt-2p</i>	seam
<i>dpy-7p</i>	hypodermis
<i>ajm-1p</i>	apical junction

Using the dual fate-reporter strain, three distinct classes of fate changes were identified from SC asymmetric divisions in *nmy-2* knockdown worms. Class I was a straightforward seam to hypodermal fate change (Figure 4.1B). Cells that should normally express the red seam marker inappropriately expressed the green hypodermal marker (white arrows). Class II and III fate changes were both associated with cytokinesis failures, which was commonly seen in *nmy-2* knockdown animals. Class II fate change was defined as having both daughter nuclei in a bi-nucleated cell express the green hypodermal marker (Figure 4.1C, white arrow), and Class III was the opposite situation where both daughter nuclei in a bi-nucleated cell expressed the red seam marker (Figure

4.1D, white arrow). Occasionally cells were caught in the process of acquiring a different fate, thus expressing both markers (Figure 4.1 B-C, yellow arrows).

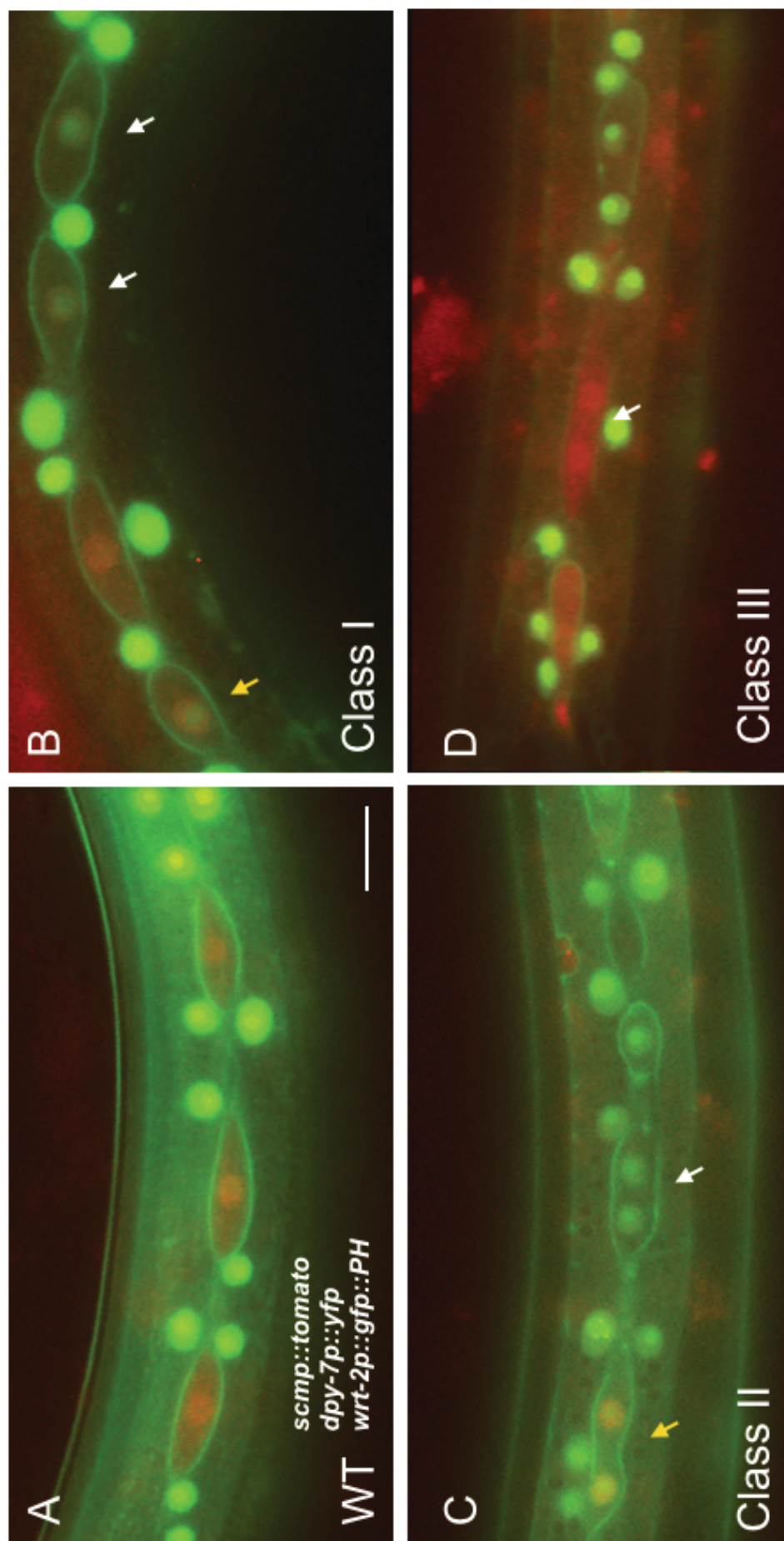


Figure 4.1: *nmy-2* knockdown produces three distinct classes of cell fate changes. **A)** WT worm with *ouls10* dual color fate-reporters (AW1015). **B-D)** Worms with *ouls10* in the *nmy-2* background (AW1018) were subjected to *nmy-2* knockdown to produce distinct classes of cell fate changes. SCs imaged here had completed their L2.2 asymmetric divisions. White arrows: representative Class I, II, and III cells; yellow arrows: transitioning cell expressing both fate markers. Scale bar = 20 μ m.

Cells that should normally express the red seam marker inappropriately expressed the green hypodermal marker (white arrows). Class II and III fate changes were both associated with cytokinesis failures, which was commonly seen in *nmy-2* knockdown animals. Class II fate change was defined as having both daughter nuclei in a bi-nucleated cell express the green hypodermal marker (Figure 4.1C, white arrow), and Class III was the opposite situation where both daughter nuclei in a bi-nucleated cell expressed the red seam marker (Figure 4.1D, white arrow). Occasionally cells were caught in the process of acquiring a different fate, thus expressing both markers (Figure 4.1 B-C, yellow arrows).

4.2.2 The three classes of fate changes are moderately penetrant and account for overall seam cell loss

Next, I quantified the penetrance of those three fate change phenotypes after the L2.2 (Figure 4.2, top two graphs) and L3 asymmetric divisions (bottom two graphs). I expressed phenotypic penetrance both as a percentage of total V lineage cells (left two graphs) and as a percent of worms (right two graphs). Because it was possible for a single worm to have multiple classes of phenotypes and/or multiple instances of the same phenotype, the two measures might tell slightly different stories. However the overall similar

shapes between the left and right graphs indicate that both outputs ended up showing similar relative penetrance of the three phenotypes. Quantification results were also largely similar between the L2.2 and L3 divisions (compare top versus bottom graphs), except for Class III phenotype, which was more penetrant following the L3 division than after the L2.2 division.

In the control group (WT background, 15°C on control RNAi), none of the phenotypes were observed except a baseline level of Class I, which physically exhibited itself as a mild hue of green over the red seam marker in the seam line. With the implementation of *nmy-2* knockdown (*nmy-2* mutant background, 25°C on *nmy-2* RNAi), about 5%, 10-13%, and 2-8% of the total V lineage cells exhibited Class I, II or III phenotype, respectively. In terms of the percentage of worms having each phenotype, about 40%, 60%, and 20-55% of the *nmy-2* knockdown worms had at least one instance of the Class I, II or III phenotype, respectively.

Therefore all three classes of fate change phenotypes were moderately penetrant as a result of *nmy-2* knockdown. Class III was the most rare after the L2.2 division but became more frequent after the L3 division. Because Class I and II phenotypes both led to SC loss while Class III potentially led to a gain of SCs, the quantification data were consistent with an overall loss of SCs when *nmy-2* is perturbed.

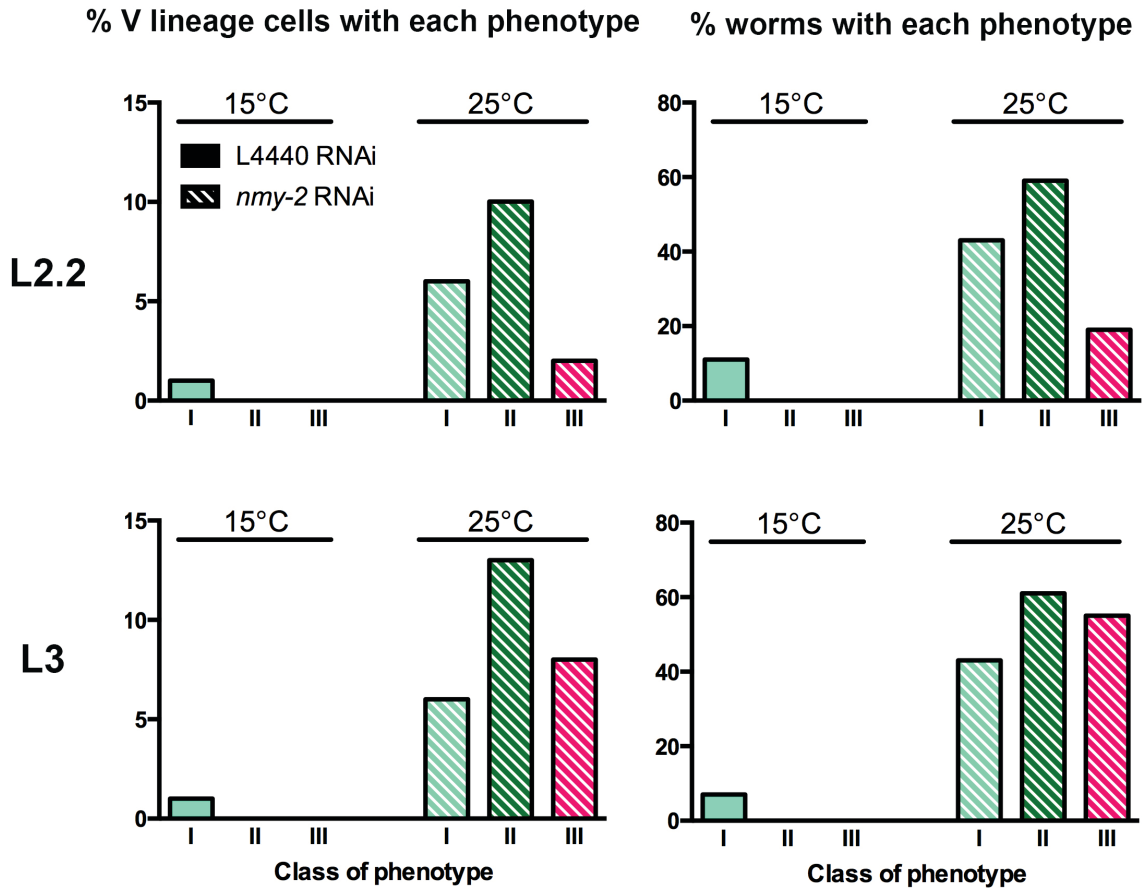


Figure 4.2: Quantification of *nmy-2* knockdown fate change phenotypes. *ouls10* dual fate-reporters in WT and *nmy-2* mutant background (AW1015 and AW1018 respectively) were bleached synchronized. AW1015 was fed L4440 RNAi at 15°C and AW1018 was fed *nmy-2* RNAi at 25°C. The three classes of fate change phenotypes were quantified after L2.2 and L3 divisions, both as a percentage of total V lineage cells (V1-V4 only) and as a percentage of worms.

4.2.3 The dual fate-reporter strain lacks a good SC marker

It is worth noting that the *scmp::NLS::tdTomato* marker (from pAW584) used in *ouls10* was not optimal. The expression was cytoplasmic despite having a nuclear localization signal. The marker was the strongest in between divisions, but during division the marker was often lost despite being integrated.

Therefore the Class III phenotype could be underrepresented and potential

new phenotypes that involve gaining SCs could be missed due to the lack of a reliable seam marker. Even though *nmy-2* knockdown produced an overall loss of terminal SCs as assayed by the *scmp:gfp* marker, there could possibly be some SC gains to mitigate the overall loss. However such potential SC gain could not be identified without a reliable seam marker in the dual-reporter strain. This sub-optimal dual-reporter strain also hindered the downstream analyses necessary to elucidate the molecular basis of fate change phenotypes. For example it was difficult to tell when exactly the seam fate disappeared for Class I and II and whether it was concomitant with hypodermal fate acquisition. Class III was essentially impossible to analyze further, because the process of acquiring seam fate could not be visualized and only the end result could sometimes be seen with the seam marker.

A *bro-1CNE+pes-10p::NLS::tdTomato* construct was available (pAW582) as an alternative to the *scmp::NLS::tdTomato* but it had exactly the same problem (Figure 4.3). The marker did not behave consistently despite being integrated in *ouls22[bro-1CNE+pes-10p::NLS::tdTomato(pAW582)+wrt-2p::gfp::PH(pAW561)+dpy-7p::2xNLS::yfp(pAW516)+ajm-1p::mCherry(pAW373)]*. It could be bright and nuclear in some SCs (yellow arrows) but completely absent in the adjacent cells (white arrows), which were at the same stage and clearly showed SC nuclei on the DIC channel.

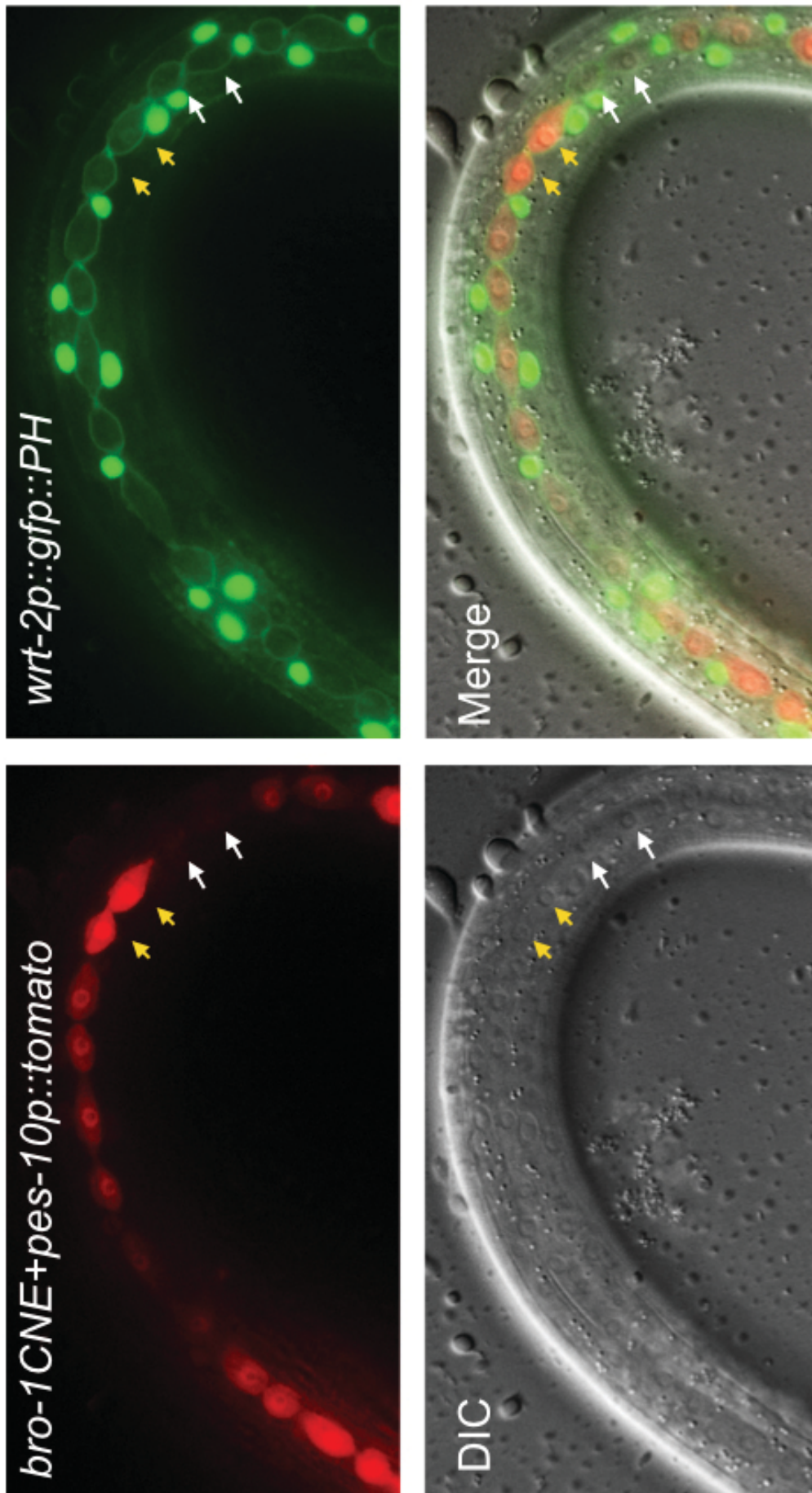


Figure 4.3: The red seam marker does not express consistently. This is an AW1140 worm (*ouls22* transgene in the WT background), just after the L2.2 asymmetric division. Yellow arrows: nuclei with bright nuclear marker; white arrows: nuclei with no marker. Scale bar = 20 μ m.

Lots of effort had been put into generating a better red seam marker construct, but in vain. I tried to substitute *mCherry* for *tdTomato* and add nuclear localization signal (NLS) repeats, but the constructs never worked so the cloning is not described here in this thesis. A red hypodermal marker (*stls10166 [dpy-7p::HIS-24::mCherry]*) became available shortly before this thesis was written. I tried to take advantage of it to generate a “reverse dual fate-reporter” strain by using this red marker for hypodermal nuclei and the more reliable green *wls51[scmp::gfp]* marker for seam nuclei. However the red hypodermal marker bleeds heavily through the green channel (data not shown), so it was not very useful for indicating fate changes in the end.

4.2.4 Hypodermal cell fate is normally specified after seam cell division is complete, while rounded anterior daughters are still in the seam line

Despite having a sub-optimal seam marker, the dual fate-reporter strain proved useful for improving the general understanding of seam biology as well as illuminating the mechanism for some *nmy-2* knockdown fate changes.

For example, I asked when the anterior daughter starts to express the hypodermal marker in a wild-type asymmetric division. Figure 4.4 illustrates a sequence of events after an asymmetric division. Though individual panels are

snapshots of different worms, the situation shown here is typical of anterior daughter differentiation.

In Figure 4.4A, the anterior daughters (white arrows) all still expressed the red SC marker after the division, even when the two daughters on the left had already rounded up. In Figure 4.4B, while some of the rounded anterior daughters still expressed *scmp::NLS::tdTomato* (white arrows), others expressed *dpy-7p::2xNLS::yfp* instead (yellow arrows). Without the fate markers it would not have been possible to tell when the fate switched from seam to hypodermal in these cells. Here the dual fate-reporter highlighted a gradual acquisition of the hypodermal fate on a cellular level. Even though all these anterior daughters were supposedly more or less at the same stage after the division, the minor temporal difference was enough to specify hypodermal fate in some cells but not the others. Anterior daughters acquired the hypodermal fate in what seems a random order with respect to the A-P axis. In Figure 4.4C, all anterior daughters now expressed the *dpy-7* hypodermal marker (yellow arrows), and then in Figure 4.4D some anterior daughters moved dorsally (purple arrows) while others moved ventrally (yellow arrows) out of the seam line in order to join the hyp7 syncytium. Eventually, all the anterior daughters fused with the hypodermal syncytium and the posterior seam daughters elongated to reconstitute the seam line (Figure 4.4E).

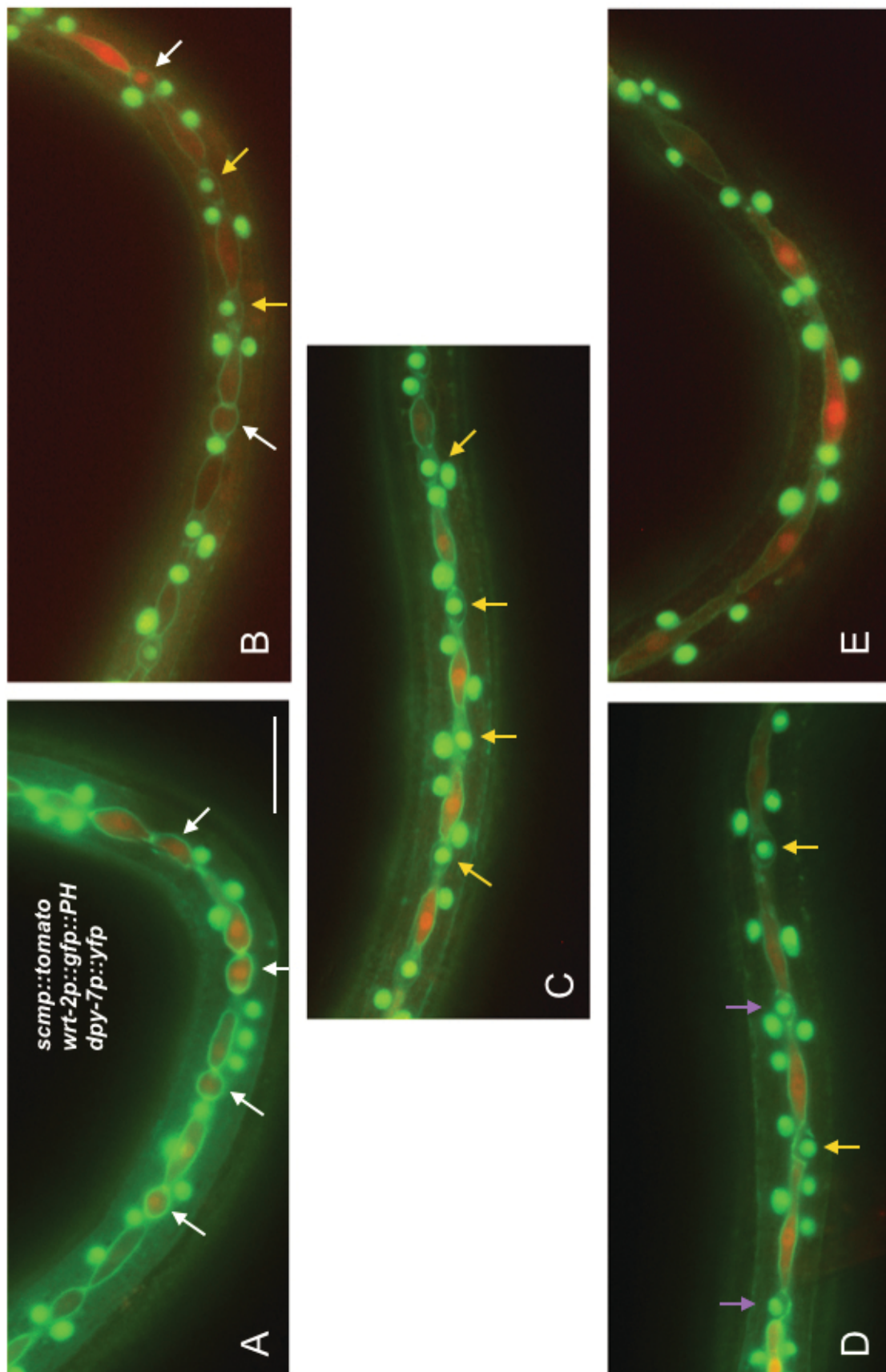


Figure 4.4: Hypodermal fate is expressed after the anterior daughter rounds up in the seam line. A-E are snapshots of the AW1015 dual fate-reporter strain after L2.2 asymmetric division. A-C) White arrows: anterior daughters expressing the seam marker; yellow arrows: anterior daughters expressing the hypodermal marker. D) Yellow arrows: anterior daughters moving ventrally; purple arrows: anterior daughters moving dorsally. Scale bar = 50 μ m.

Brabin et al. (2011) reported that the acquisition of hypodermal fate in the anterior daughter only occurs after the apical junction disintegrates, a process that depends on the fusogen EFF-1. EFF-1 promotes the fusion of hypodermal seam daughters to the hyp7 syncytium by forming pores in their plasma membrane, so they can receive differentiation signal from the hypodermis (Mohler et al., 1998). I crossed the integrated *ajm-1p::mCherry* marker into the dual fate-reporter strain (thereby generating AW1097) and found that partial disintegration of AJM-1 indeed usually preceded *dpy-7p::2xNLS::yfp* expression in the anterior daughters (data not shown). Where the apical junction might appear intact whilst the anterior daughter expressed the hypodermal marker, it was probably because the punctures in the apical junction boundary were too small to be seen as a clear gap. The fusion aperture always originate as a unique primary pore before slowly widening (Mohler et al., 1998), so the early stage fusion pores can facilitate hypodermal differentiation without producing a visible gap in the apical junction just yet. There are some examples of this in the next two sections (Figure 4.5A, yellow arrow; Figure 4.6B, yellow arrow).

4.2.5 Class II fate change phenotype is a result of improper segregation of daughter cytoplasm

The dual fate-reporters and apical junction marker were crossed into the *nmy-2* background and used to examine *nmy-2* knockdown phenotypes. It offered a convincing explanation for the Class II phenotype, where both daughter nuclei of a bi-nucleated SC express the *dpy-7* hypodermal marker. Essentially, Class II cells were often found with a broken apical junction (Figure 4.5 A-B, white arrows), which presumably allowed hypodermal signaling to affect the whole cell and both of its nuclei, due to the lack of proper cytoplasm segregation. Additionally, sometimes cells were found in the transitional stage where the anterior nucleus expressed *dpy-7* marker more strongly than the posterior one (Figure 4.5A, yellow arrow). The reverse case, where the posterior nucleus expresses more *dpy-7* than the anterior one, has never been seen.

Based on these observations, I propose the following model to explain Class II fate change. *nmy-2* knockdown leads to cytokinesis failures and produces bi-nucleated cells. After an asymmetric division, the anterior half of the bi-nucleated cell proceeds to differentiate as normal by losing apical junction integrity and receiving hypodermal signaling. Due to failed cytokinesis and the lack of adequate cytoplasm segregation, the hypodermal signal that enters from the anterior half of the bi-nucleated cell travels down towards the posterior, turning the posterior nucleus hypodermal with a slight delay compared to the anterior nucleus.

The anterior differentiation despite failed cytokinesis suggests that SC polarity, which is established prior to division, was not altered by *nmy-2* knockdown, at least in Class II bi-nucleated cells. Class III phenotype was not extensively

investigated in this study owing to the lack of a reliable seam marker, so it is not known why Class III phenotype was much less common than Class II when both involve cytokinesis failures and polyploid cells. Perhaps the differentiation signal from *hyp7* is highly potent, rendering Class II phenotypes more prevalent.

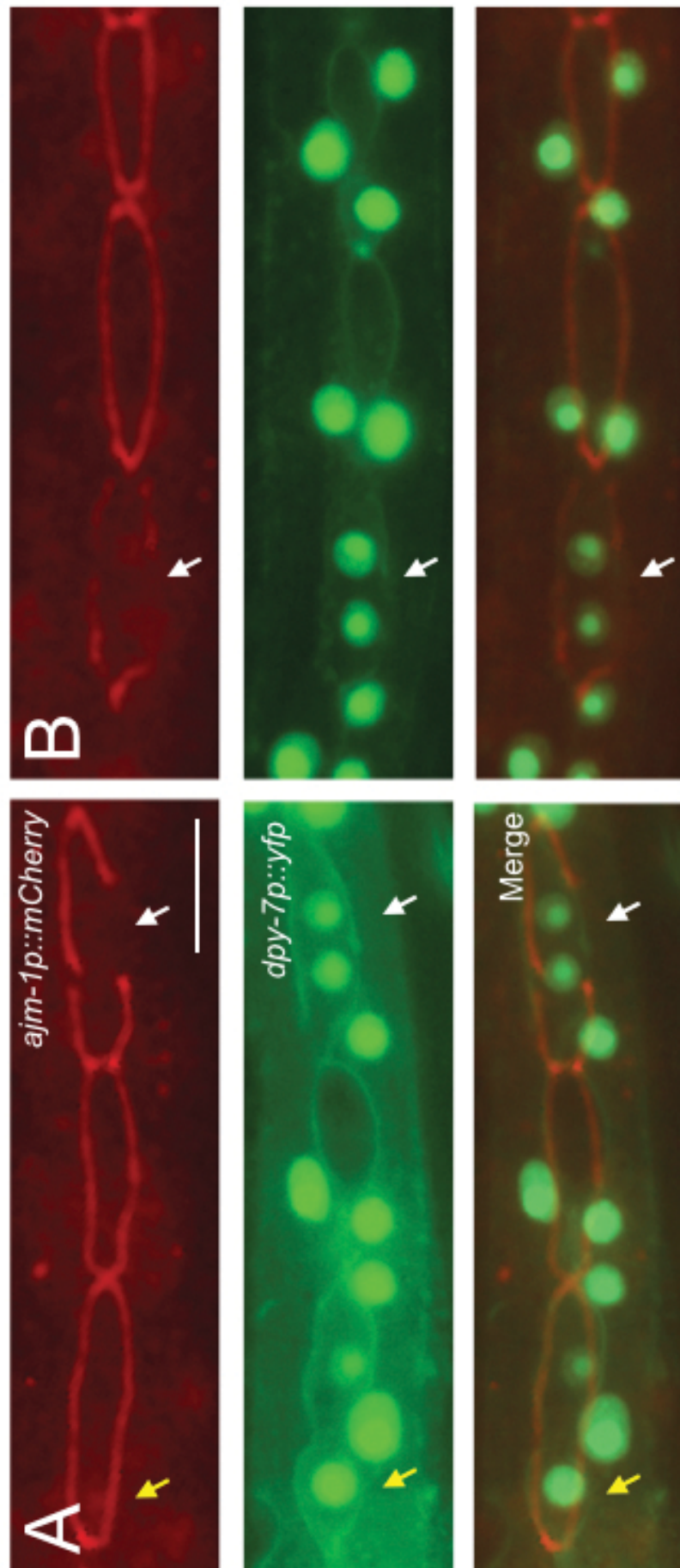


Figure 4.5: Class II *nmy-2* knockdown fate change is likely a result of cytokinesis failure. AW1106 (dual fate-reporters and the apical junction marker in *nmy-2* background) worms were bleach synchronized and fed *nmy-2* RNAi bacteria at 25°C. **A** and **B** are snapshots taken after the L2.2 division. White arrows: Class II cells with a broken apical junction; yellow arrow: a transitioning cell with stronger *dpy-7* expression in the anterior nucleus than the posterior one. Scale bar = 20 µm.

4.2.6 Class I fate change may or may not involve cytokinesis defects

Having understood how Class II fate transformation occurs, I examined the Class I phenotype using the same approach. Class I phenotype describes a direct seam to hypodermal fate change within the seam line, independent of cytokinesis failure. It was possible for Class I and Class II phenotypes to occur in the same worm (Figure 4.6, white and purple arrows, respectively).

Some Class I cells had compromised apical junctions while strongly expressing *dpy-7p::2xNLS::yfp* in the seam line (Figure 4.6 A-B, white arrows). Even though these cells were mono-nucleated thus initially appeared independent of cytokinesis failures, one cannot rule out the involvement of possible cytokinesis defects in triggering their inappropriate differentiation. Suppose that cytokinesis was completed with a slight delay, then perhaps some hypodermal signal in the differentiative anterior daughter would have traveled to the posterior before the daughter cytoplasms completely separated. Delayed, but successful cytokinesis completion would eventually generate two distinct mono-nucleated daughters. The anterior daughter would differentiate as normal, but the fate of the posterior daughter could eventually be altered towards differentiation thanks to the leak-through of hypodermal signal. While this situation is purely hypothetical with no experimental support at this point, if

the delayed cytokinesis was indeed the case here, then the Class I phenotype is actually rather similar to the Class II phenotype. In both cases, cytokinesis failure (Class II) or delay (Class I) allows the differentiative hypodermal signal to travel from the anterior to the posterior, thereby triggering inappropriate differentiation of the posterior daughter nucleus.

Some Class I cells that strongly expressed *dpy-7* had intact-looking apical junctions (Figure 4.6B, yellow arrow). As mentioned in Section 4.2.4, EFF-1-dependent apical junction disintegration is a pre-requisite for seam cells to fuse with the hyp7 syncytium, so this is probably because the small membrane pores in these differentiating cells had not yet produced a visible break in the apical junction outline (see where the yellow arrow points to in the *ajm-1p::mCherry* boundary in Figure 4.5A).

Therefore, while it is possible that Class I phenotype arise from mild cytokinesis defects such as a slight delay in cytokinesis completion, it is also entirely possible that a completely different, cytokinesis-independent mechanism drives Class I fate changes. The broken apical junction could in fact be a secondary effect rather than the cause of a Class I fate change, or be a part of the cause but without the involvement of cytokinesis. Moreover, Class I fate change might come from both cytokinesis-independent and cytokinesis-associated mechanisms. Further studies are necessary to determine exactly how the Class I phenotype arises.

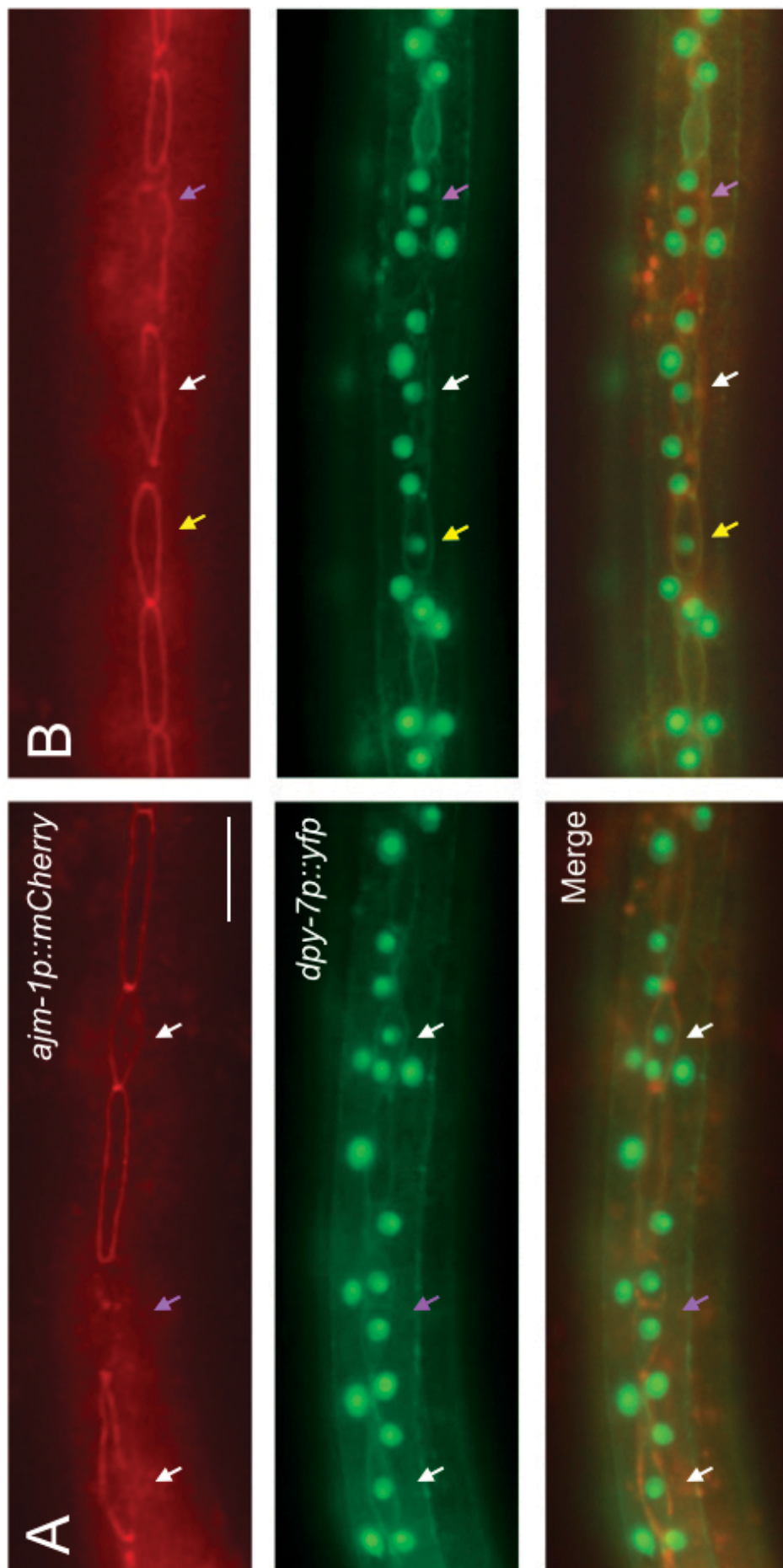


Figure 4.6: Class I *nmy-2* knockdown fate change may or may not be a result of cytokinesis failure. AW1106 (dual fate-reporters and the apical junction marker in *nmy-2* background) worms were bleach synchronized and fed *nmy-2* RNAi bacteria at 25°C. **A** and **B** are snapshots taken after the L2.2 division. White arrows: Class I cells with compromised apical junctions; purple arrows: Class II cells; yellow arrow: Class I cell with intact-looking apical junction. Scale bar = 20 µm.

4.2.7 The *nmy-2* knockdown phenotype is not simply attributable to cytokinesis failures, and is unlikely a result of SC elongation defects

At least two *nmy-2* knockdown fate change phenotypes arose in bi-nucleated cells, thus I assessed how much of the overall *nmy-2* SC loss phenotypes were directly attributable to cytokinesis failures. I obtained Ahringer library RNAi clones for two key players involved in cytokinesis: *cyk-4*, which encodes a component of the centralspindlin complex, and *ect-2*, which encodes the RhoA GEF (see Figure 1.16 for a schematic drawing of the cytokinesis pathway). Strikingly, post-embryonic RNAi of these two cytokinesis genes produced the complete opposite effect to *nmy-2* knockdown. Whereas *nmy-2* knockdown caused an overall reduction of GFP positive seam nuclei, silencing other cytokinesis pathway components led to an overall increase. *cyk-4* and *ect-2* RNAi produced 20 and 22 terminal GFP positive seam nuclei, respectively (Figure 4.7, left two bars). Some of these nuclei might belong to the same polyploid SC that had failed cytokinesis, although DIC optics was not checked to confirm this. The main conclusion here is that *nmy-2* knockdown does not phenocopy the knockdown of other cytokinesis pathway components. These results indicate that *nmy-2* knockdown phenotypes were not a simple result of cytokinesis failures, and that additional mechanisms must have been

disrupted to cause those fate change phenotypes. Importantly, because more GFP positive seam nuclei were seen when cells simply failed cytokinesis, the effect of knocking down *nmy-2* is ever more dramatic because the overall reduction of seam nuclei would start from an increased baseline seam nuclei numbers, taking into account the cytokinesis failure effect, instead of the normal number of 16 seen in WT worms. In other words, RNAi silencing of *cyk-4* and *ect-2* allowed us to uncouple the cytokinesis involvement of *nmy-2* from its other functions. Disrupting *nmy-2* likely led to a small increase of GFP positive seam nuclei based on cytokinesis effects alone, concurrently with a large decrease of seam nuclei due to non-cytokinesis associated effects, overall resulting in a robust decrease of SC numbers. The one caveat here is that the cytokinesis pathway activates NMY-2 and actin separately downstream of GTP-bound active RhoA (Figure 1.16), so silencing the upstream *cyk-4* and *ect-2* would reflect the combined effect of disrupting both NMY-2 and actin instead of NMY-2 alone, with regard to cytokinesis failures. Nevertheless, this is unlikely to change the conclusion that *nmy-2* has cytokinesis-independent roles in regulating seam development.

In addition, I performed post-embryonic RNAi on *let-502* and *mel-11*, the two genes known to function upstream of NMY-2 to control seam-dependent embryonic elongation (Gally et al., 2009). It is not known whether the embryonic elongation pathway is involved in post-embryonic SC elongation, which occurs repeatedly in posterior seam daughters after asymmetric divisions in order reconstitute the seam line. Perhaps knocking down an elongation pathway component prevents the seam daughter from reforming

cell-cell contact, which can be necessary for seam fate specification. However, RNAi of these elongation pathway components did not produce any obvious post-embryonic seam phenotype and the terminal SC count is 16 as in WT (Figure 4.7, right two bars). This suggests that the embryonic elongation pathway involving *let-502* and *mel-11* is not responsible for producing the *nmy-2* phenotypes. In contrast to the embryonic elongation pathway, a nuclear receptor NHR-25 has been shown to regulate seam cell elongation after asymmetric divisions. Post-embryonic RNAi of *nhr-25* produced gaps in the seam due to improper cell elongation and an increase of GFP positive seam nuclei up to 20, presumably because the fate of the anterior daughters were mis-specified in the absence of proper cell-cell contact so they retain the seam fate and occasionally divide (Silhánková et al., 2005). Even though the mechanism of *nhr-25*-dependent SC elongation is unknown, *nhr-25* RNAi and *nmy-2* knockdown have opposite effects on terminal SC numbers, suggesting that *nmy-2* phenotype is not a result of improper SC elongation following asymmetric divisions.

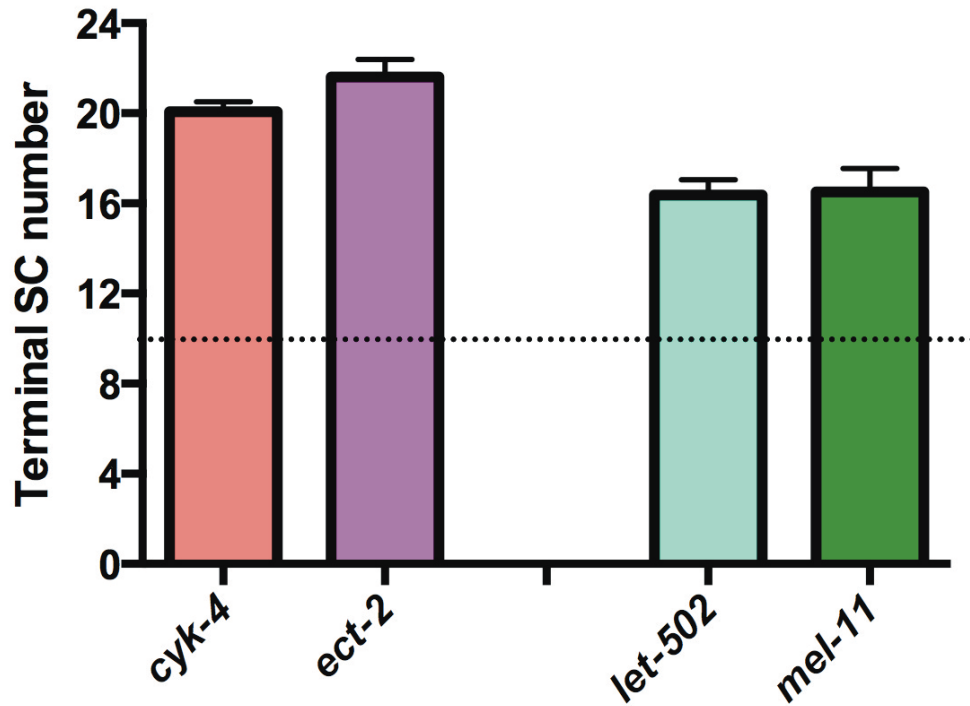


Figure 4.7: Post-embryonic RNAi of cytokinesis pathway and elongation pathway components show different phenotypes to *nmy-2* knockdown. All four RNAi clones were obtained from the Ahringer library and gravid JR667 worms were spot-bleached onto RNAi plates to achieve post-embryonic RNAi. RNAi took place at 25°C and terminal SC numbers were counted in young adults. $n > 50$ for each sample. Dashed line denotes the 10 SCs that the worms hatch with. Error bars are the SEM. Two independent replicates of the experiments were performed.

4.2.8 *nmy-2;wrm-1* double mutant shows synthetic effects in post-embryonic seam development and in embryogenesis

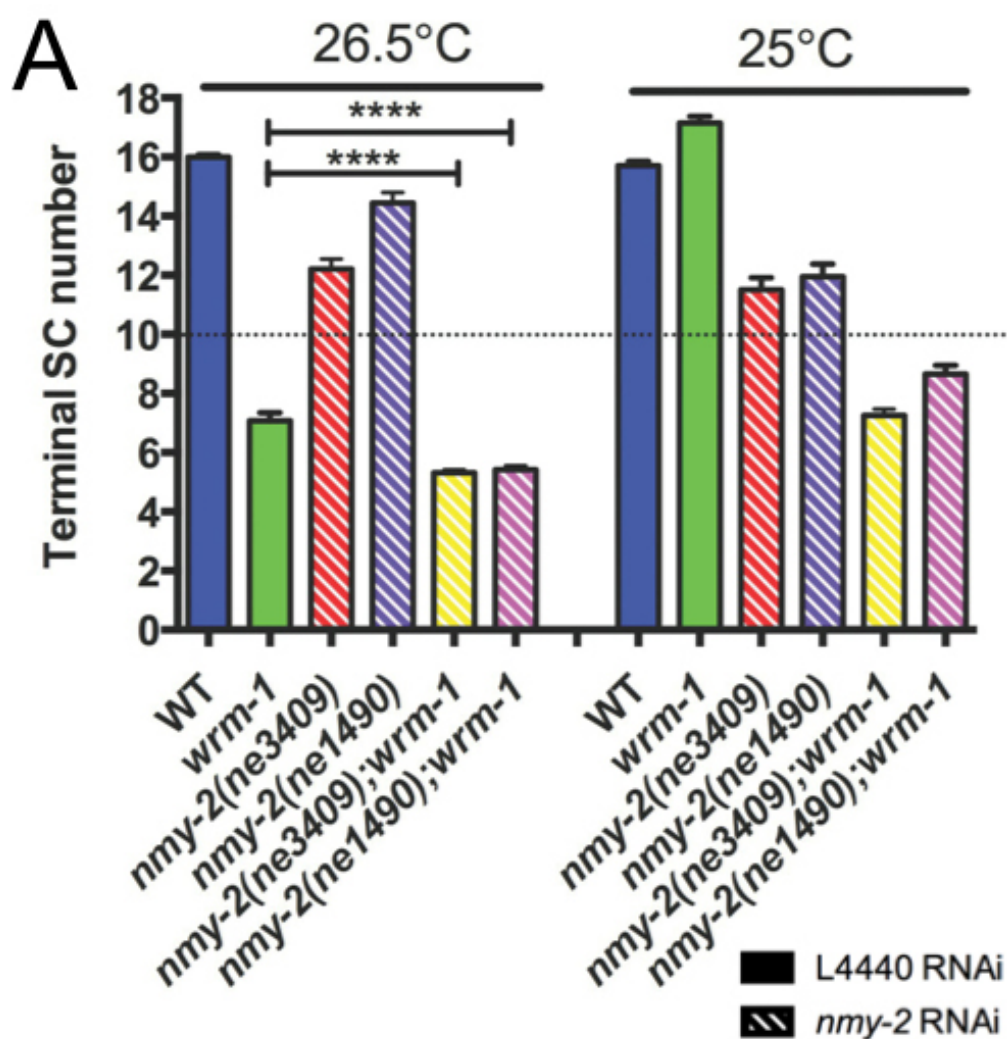
The WNT/ β -catenin asymmetry pathway plays a key role in SC fate specification so it emerged as the prime candidate for testing whether *nmy-2* regulates seam development through a known network. Double mutants were constructed between the *wrm-1* ts allele (*ne1982*) and both *nmy-2* ts alleles (*ne3409*) and (*ne1490*). As mentioned in Section 3.2.7, most of the work described in this thesis used only *nmy-2(ne3409)*, but this was one of the early

pieces of work where both alleles were tested. Both *nmy-2* alleles indeed behaved similarly for the experiments in this section.

WT worms, *wrm-1* and *nmy-2* single mutants, and the *nmy-2;wrm-1* double mutant were subjected to temperature shift and RNAi at the L1 stage (Figure 4.8A). The *wrm-1* ts allele has been shown to produce little post-embryonic effect at 25°C but significantly reduces SC number at 26.5°C (Gleason and Eisenmann, 2010). As expected, at 26.5°C the *wrm-1* single mutant displayed a dramatic decrease of SC numbers from 16 in WT down to 7. This decrease was very significant because the worms started out with 10 SCs at hatching, so more SCs were lost than gained during *wrm-1* mutant post-embryonic development, pushing the final SC number below the initial 10. *nmy-2* single mutants also had reduced SC numbers – 12 or 15 depending on the allele. Terminal SC numbers were further decreased in the *nmy-2;wrm-1* double mutants to about 5. Whether the SC reduction effect from each mutation was additive or synergistic in the double mutants could not be appropriately assessed, because a major number of SCs were already lost due to *wrm-1* alone. Hence the experiments were repeated at 25°C where the *wrm-1* mutation had little effect on terminal SC numbers. At 25°C, the double mutants showed a significant reduction of terminal SC numbers compared to the single mutants. The *wrm-1* single mutant had 17 SCs at 25°C and the *nmy-2* single mutant had 11-12. The double mutants had 7-9 SCs, indicating that the reduction of SCs was synergistic rather than additive. This suggests that NMY-2 potentially works in a protein complex with WRM-1 or in parallel to the WNT/ β -catenin asymmetry pathway. Of course it is also possible that *nmy-2*

inactivation simply renders the seam cells less fit to execute its normal functions, such as divisions under the control of the WNT, so that the synergistic reduction of seam cell numbers in fact does not suggest any interaction between *nmy-2* and *wrm-1*.

The synergistic effect between *wrm-1* and *nmy-2* was not only seen in post-embryonic seam development but also in embryogenesis. Figure 4.8B shows that *wrm-1* and *nmy-2* single mutations were both embryonic lethal at 25°C. The single mutants produced viable progeny at lower temperatures such as 20°C, 17°C, and 15°C. However, *nmy-2;wrm-1* double mutants were embryonic lethal at 20°C and only produce viable progeny at 17°C and 15°C. Thus the permissive temperature for embryonic viability was lowered from 20°C to 17°C in the double mutant. The synthetic lethality at 20°C suggests that NMY-2 and WRM-1 may work in parallel pathways during embryogenesis, consistent with a previous report stating that NMY-2 functions in parallel to WNT to regulate the EMS division in the four-cell stage embryo (Liu et al., 2010). Once again, the two *nmy-2* ts alleles behaved in the same way, so *ne1490* was no longer used in subsequent experiments.



B

	15°C	17°C	20°C	25°C
WT	✓	✓	✓	✓
<i>wrm-1</i>	✓	✓	✓	×
<i>nmy-2(ne3409)</i>	✓	✓	✓	×
<i>nmy-2(ne1490)</i>	✓	✓	✓	×
<i>nmy-2(ne3409);wrm-1</i>	✓	✓	×	×
<i>nmy-2(ne1490);wrm-1</i>	✓	✓	×	×

Figure 4.8: *nmy-2; wrm-1* double mutant has synergistic effects on post-embryonic seam development and on embryogenesis. **A)** WT, single mutants and double mutants, all with the *scmp::gfp* marker, were synchronized to L1 and subjected to temperature shift and RNAi treatments as indicated. Terminal SC numbers were counted in young adults. $n > 50$ for each sample. Dashed line denotes the 10 SCs that the worms hatch with. Error bars are the SEM. Unpaired T-test was used to determine significance (****: $p < 0.0001$). **B)** A single WT, single mutant or double mutant L4 animal was placed at indicated temperatures, and its embryonic viability was assessed. A tick mark means the L4 animal produced viable progeny and a cross means no viable progeny was produced. $n = 15$ for each sample. The strains used in both A and B were AW335, EW95, AW785, AW786, AW861, AW862. Three independent replicates of the experiments were performed.

4.2.9 *nmy-2; lit-1* double mutant analysis is consistent with the *nmy-2; wrm-1* analysis

A similar epistasis experiment was conducted between *nmy-2* and *lit-1*. *wrm-1(ne1982)* ts allele has dramatically different effects on SCs at 25°C versus 26.5°C, so I sought to repeat the epistasis analysis with another WNT effector for confirmation and clarity. LIT-1 is the Nemo-like kinase that stably associates with WRM-1 to phosphorylate POP-1, leading to POP-1 nuclear export (Lo et al., 2004b; Rocheleau et al., 1999). *lit-1(or131)* is the temperature sensitive allele used in this experiment. Post-embryonic incubation of the *lit-1* mutant at 25°C (or 26.5°C – data not shown) did not have an effect on terminal SC numbers (Figure 4.9). Consistent with previous experiments, *nmy-2* mutant had 11 SCs at 25°C. The *nmy-2; lit-1* double mutant had 8 SCs at 25°C, which is a synergistic reduction compared to the combined effects of the single mutants. Once again, the terminal SC number in the double mutant was very significantly reduced because it dropped below the 10 SCs that the worms had at the beginning of larval development. These results were very similar to the 25°C data in the *nmy-2; wrm-1* analysis.

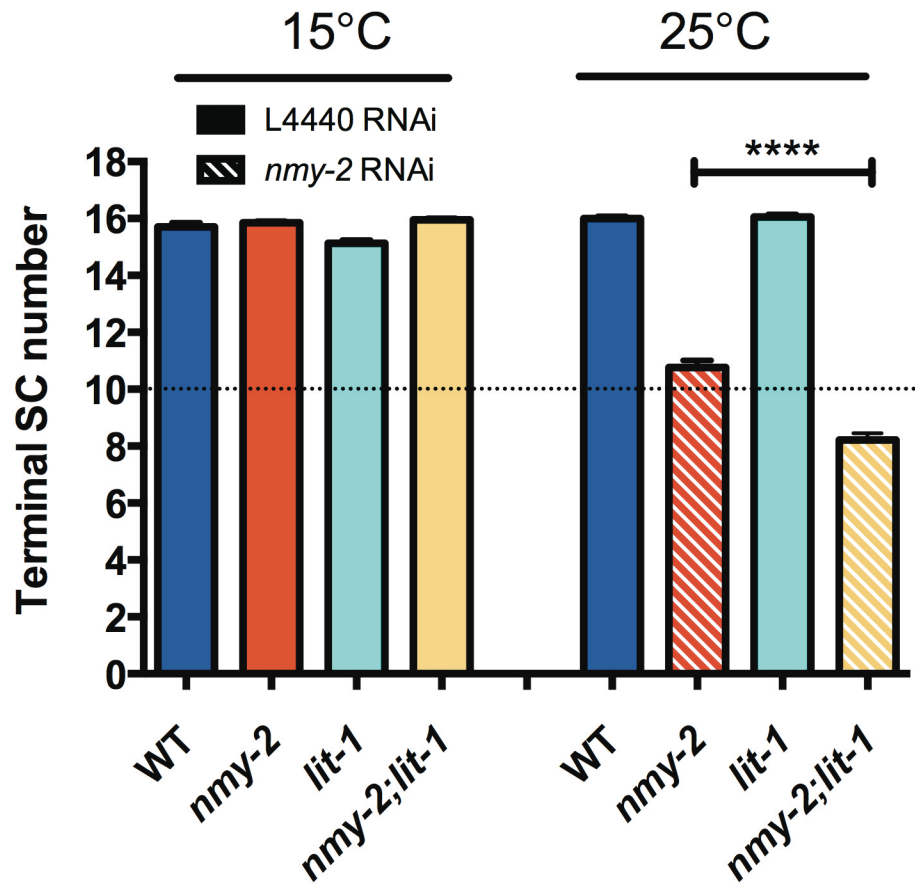


Figure 4.9: *nmy-2;lit-1* double mutant has synergistic effects on post-embryonic seam development. WT, single mutants and the double mutant, all with the *scmp::gfp* marker, were synchronized to L1 and subjected to temperature shift and RNAi treatments as indicated. Terminal SC numbers were counted in young adults. The strains used here were AW335, AW785, AW943, and AW983. $n > 50$ for each sample. Dashed line denotes the 10 SCs that the worms hatch with. Error bars are the SEM. Unpaired T-test was used to determine significance (****: $p < 0.0001$). Three independent replicates of the experiments were performed.

4.2.10 *nmy-2* is unlikely to function as part of the WNT/ β -catenin

asymmetry pathway

In order to determine whether *nmy-2* functions as part of the WNT/ β -catenin asymmetry pathway, I next examined whether POP-1 localization was

changed upon *nmy-2* perturbation. POP-1/TCF is downstream of WRM-1 so if knocking down *nmy-2* changes POP-1 localization pattern, then it supports that NMY-2 works in a protein complex with WRM-1/LIT-1 upstream of POP-1. An integrated POP-1 reporter *qls74[sys-1p::pop-1::gfp]* was used for this purpose.

Following a WT SC asymmetric division (Figure 4.10, left two bars), in most daughter pairs there was more POP-1 in the anterior daughter nucleus than in the posterior daughter nucleus (>90% of the time). Occasionally (6-7% of the time) POP-1 levels were equal in the two daughters, and in rare cases (1%) reversed polarity was seen where there was more POP-1 in the posterior daughter nucleus than in its anterior counterpart. The quantification results for POP-1 localization were similar between WT L2.2 and L3 asymmetric divisions.

nmy-2 knockdown did not alter POP-1 localization after the L2.2 division (Figure 4.10). POP-1 localization pattern was only slightly changed after the L3 division when *nmy-2* was perturbed, with 85% of the daughter pairs showing normal polarity, 12% having POP-1 symmetry, and 3% showing reversed polarity. Having high levels of POP-1 in both daughter nuclei can lead to symmetrization of the division towards the differentiative fate and cause SC loss. However, altered POP-1 localization only occurred in L3 but not L2.2 division and to a limited extent, so it did not fit with the highly penetrant SC loss seen across multiple divisions in *nmy-2* knockdown. It is possible, however highly unlikely, that there are different mechanisms at work and *nmy-2* only

acts through the WNT pathway in the L3 division to specify fate in some daughters, and function through some other mechanisms in other divisions to specify other cells. The more likely explanation here is that rather than being a direct consequence, altered POP-1 localization pattern was secondary to some other effects produced by *nmy-2* knockdown.

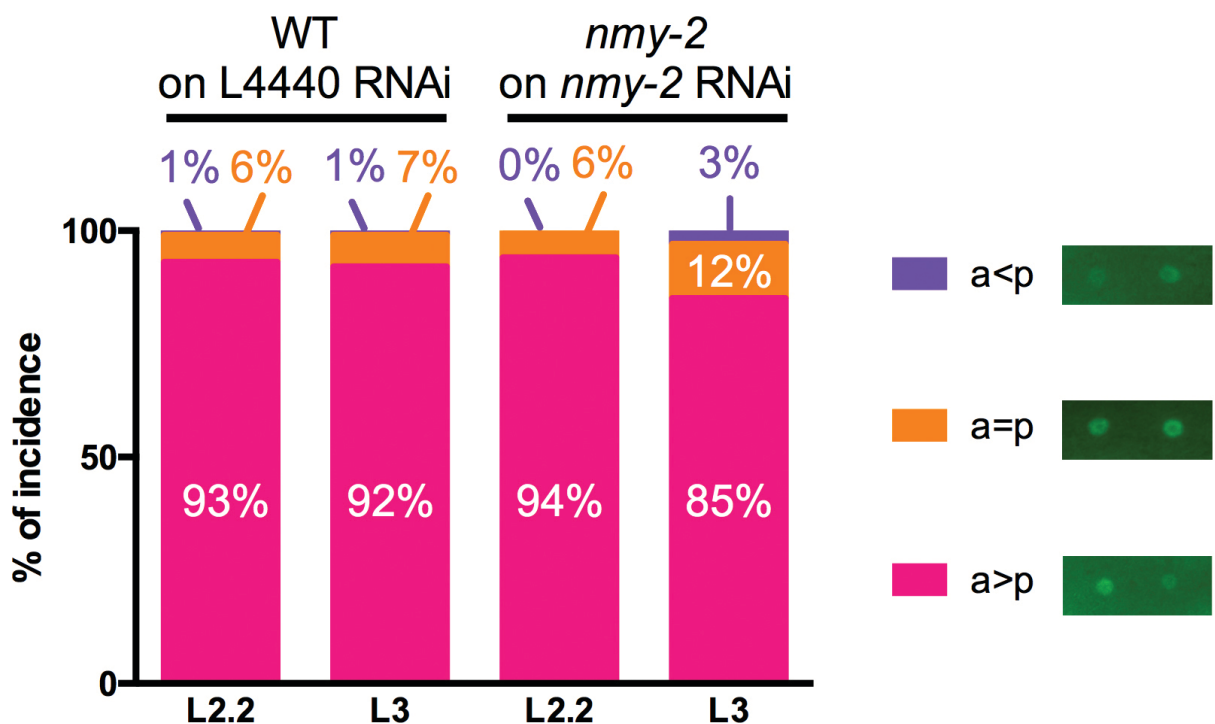


Figure 4.10: POP-1 localization is only slightly altered in *nmy-2* knockdown. WT and *nmy-2* worms with *qls74[sys-1p::pop-1::gfp]* were bleach synchronized and placed at 25°C with the appropriate RNAi bacteria. POP-1 localization was assessed in the nuclei of daughter pairs shortly after the L2.2 and L3 asymmetric divisions, and each daughter pair was assigned into one of the three polarity categories. a>p: regular polarity; a=p: loss of polarity; a<p: reversed polarity. Only V1-4 lineage daughter pairs were considered for this analysis. The strains used here were JK3437 and AW950. n>50 daughter pairs. All experiments were conducted at 25°C.

It is important to note that additional mechanisms can override the effects of WNT signaling, so POP-1 localization alone cannot be used to predict SC daughter fates. For example POP-1 and APR-1 are asymmetrically localized in

SCs in the L2.1 symmetric division, just as they are in other asymmetric divisions (Wildwater et al., 2011). Additional mechanisms, probably through the heterochronic pathway, ensure that the symmetric division takes place then despite WNT asymmetry. Therefore just because POP-1 localization was only slightly altered in *nmy-2* knockdown does not mean the daughter fates were mostly specified as normal. However it does suggest that *nmy-2* is unlikely to function as part of the WNT/ β -catenin asymmetry pathway itself.

Lastly, bimolecular fluorescence complementation assay (BiFC) was carried out to assess whether NMY-2 and WRM-1 proteins interact directly in vivo. Essentially, full-length *nmy-2* and *wrm-1* cDNA were each fused to one half of the VENUS molecule under the control of a heat shock promoter. The resultant vectors were co-injected with *unc-119* to produce multiple transgenic lines. If heat shocking induces fluorescence from reconstituted VENUS molecule, then it suggests direct protein-protein interaction between NMY-2 and WRM-1. While the positive control experiment using RNT-1 and BRO-1 showed bright fluorescence, no fluorescence was seen in any of the NMY-2 and WRM-1 transgenic lines (AW951-959) following a variety of heat shock and recovery times (data not shown). Therefore, it is unlikely that NMY-2 interacts directly with WRM-1 in vivo.

4.2.11 *rnt-1 nmy-2* double mutant analysis was insufficient to reveal the nature (or lack) of interaction between the two genes

Since *nmy-2* does not seem to function through the WNT/ β -catenin asymmetry pathway to specify cell fate, I next turned to the RNT-1/BRO-1 pathway. I decided to construct a double mutant between *nmy-2* and *rnt-1*, rather than between *nmy-2* and *bro-1*, for two reasons. First, in addition to playing a role in SC development as the RNT-1 binding partner, *bro-1* appears to also have some *rnt-1*-independent roles in vulva and/or uterine morphogenesis (Kagoshima et al., 2007). Second, *rnt-1*, *bro-1* and *nmy-2* are all closely linked on Chromosome I. A *dpy-5 rnt-1(e1241) unc-13* strain with visible markers already exists (strain name CB5253), so I took advantage of it to construct the *rnt-1 nmy-2* double mutant (see Section 2.1.3.1 for detailed crossing strategy). This resulted in *dpy-5* in the background (so effectively a *dpy-5 rnt-1 nmy-2* triple mutant), so I also placed the single mutants in the *dpy-5* background for comparison (thereby generating *dpy-5 rnt-1* and *dpy-5 nmy-2* double mutants), and used the resultant strains for the epistasis analysis between *rnt-1* and *nmy-2*.

rnt-1(e1241) is a null allele. It causes a decrease of SC number from 15 in *dpy-5* to 10-11 in *dpy-5 rnt-1*, at both 15°C and 25°C (Figure 4.11). At 15°C the *dpy-5 rnt-1 nmy-2* triple mutant had 11 SCs due to the *rnt-1* effect only, because no *nmy-2* effect should occur at 15°C on control RNAi. At 25°C, despite the activation of *nmy-2* knockdown, the *dpy-5 rnt-1 nmy-2* triple mutant had the same 11 SCs just like the *dpy-5 rnt-1* double mutant. This potentially suggest that *rnt-1* is epistatic to *nmy-2* and that the two genes may function in the same pathway. However, the interpretation of these epistasis data is tricky because *nmy-2* mutation is not null and the two mutations show the same

phenotype (i.e. a reduction of seam cell numbers), just with differing severity, so similar results may be obtained in the absence of an epistatic interaction too. Thus based on this analysis was alone we were unable to determine whether *nmy-2* and *rnt-1* interact. It will be much more informative to repeat the experiment using a RNT-1 over-expression strain with the seam hyperplasia phenotype in combination with *nmy-2* knockdown (further discussed in Section 4.3.6).

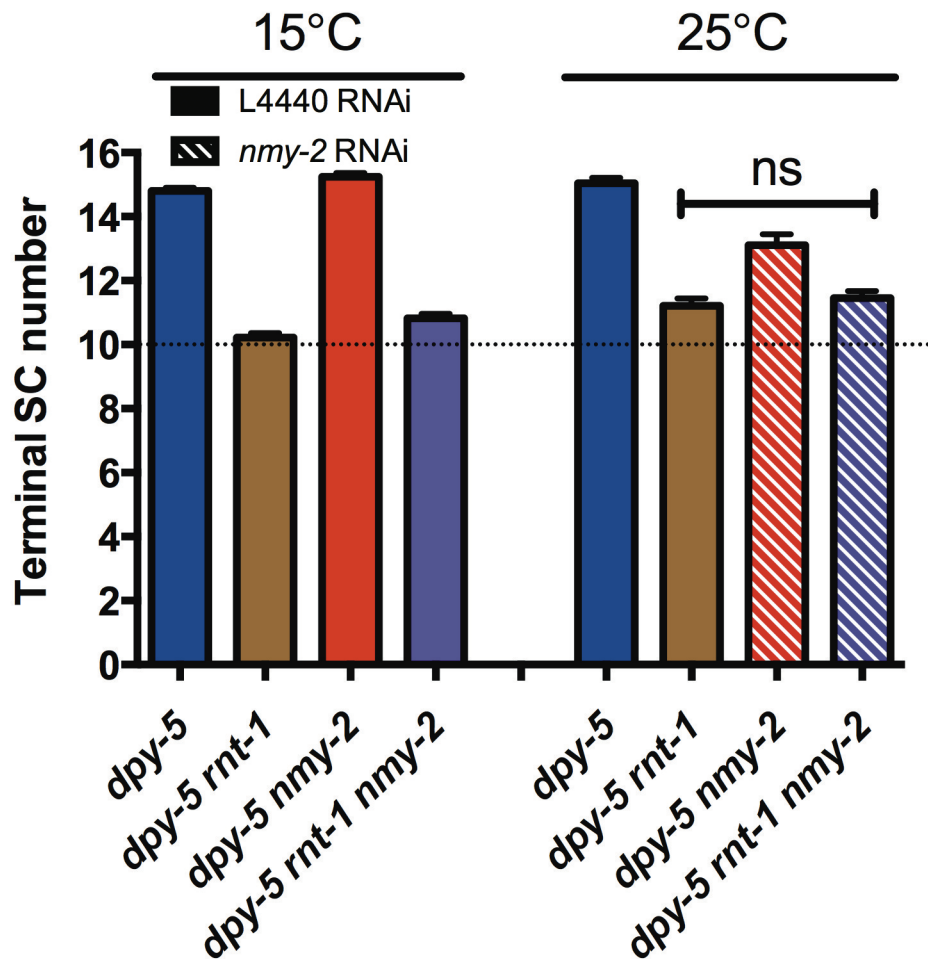


Figure 4.11: *rnt-1* and *nmy-2* may function in the same pathway to control seam development. All four strains with above indicated genotypes, all with the *scmp::gfp* marker, were synchronized to L1 and subjected to temperature shift and RNAi treatments as indicated. Terminal SC numbers were counted in young adults. The strains used here were AW989, AW990, AW991, AW992. $n > 50$ for each sample. Dashed line denotes the 10 SCs that the worms hatch with. Error bars are the SEM.

Unpaired T-test was used to determine significance (ns: $p > 0.05$). Three independent replicates of the experiments were performed.

One piece of evidence that supports that *nmy-2* and *rnt-1* potentially function in the same pathway is the similar expression profiles between *rnt-1* (Nimmo et al., 2005) and *nmy-2* (Figure 3.1). Both genes are strongly expressed in the seam from embryogenesis to L2, but the expression level decreases thereafter. Another support is an unpublished RNT-1 chromatin immunoprecipitation (ChIP) study in Valerie Reinke's lab (Yale University), which identified *nmy-1* as a RNT-1 target (personal communication). Although *nmy-2* was not pulled down by RNT-1 ChIP, its partial redundancy with *nmy-1* suggests that it could also be a potential RNT-1 target.

4.2.12 RNT-1 transcription factor is unlikely to directly regulate *nmy-2* expression

Given our interest that *nmy-2* and *rnt-1* may function in the same pathway and the insights from the RNT-1 ChIP data, I tested whether the RNT-1 transcription factor directly regulates *nmy-2* expression. I used the JASPAR Core Vertebrata transcription factor binding profile database to search for potential RNT-1 binding sites in *nmy-2* regulatory sequences. Mouse RUNX1 (MA0002.2) and human RUNX2 (MA0511.1) were selected as the matrix models and the 5' UTR, 3' UTR and intron sequences of the *nmy-2* gene were scanned for potential binding sites. Several potential RNT-1 binding sites were identified in the *nmy-2* promoter region (data not shown). The full-length *nmy-2* promoter cloned into the enhancer assay plasmid drove fluorescence in the

seam (Figure 3.1), thus it does harbor enhancer elements, as expected of a full promoter. However, individual potential sites identified by JASPAR within the promoter were not further tested for enhancer activity, because I performed an alignment of *nmy-2* promoter regions from various *Caenorhabditis* species using ClustalW2 and found no conservation of any of the sites. Therefore, it is unlikely that RNT-1 binds to *nmy-2* regulatory sequences to directly control its gene expression. The next steps for determining whether and how *nmy-2* and *rnt-1* may function in the same pathway are discussed in Section 4.3.6.

4.3 Discussion

4.3.1 The hypodermal-seam dual fate-reporter is a useful resource for studying fate allocation in the seam

The AW1015 dual fate-reporter strain was proven integral for the work described in this chapter. The differential coloring associated with hypodermal versus seam fate enabled the identification and quantification of three distinct classes of *nmy-2* fate change phenotypes. Both seam to hypodermal (Class I and II) and hypodermal to seam fate (Class III) changes were possible, and some were associated with cytokinesis failures (Class II and III). The addition of an apical junction marker further enabled the understanding of the Class II phenotype, where polyploid SC nuclei all become hypodermal, following anterior hypodermal differentiation and cytokinesis failure that lets the differentiation signal travel through the cytoplasm to the posterior. Moreover, the strain improved our general understanding of fate allocation to seam

division daughters, such as by showing what appears to be a gradual acquisition of hypodermal fate in the rounded anterior daughters before they move out of the seam line to join the *hyp7* syncytium; this phenomenon should certainly be validated by time-lapse imaging using this existing resource. Finally, the dual fate-reporter strain has already been used in a recent publication from our lab to demonstrate seam to hypodermal fate changes in *trd-1* RNAi (Hughes et al., 2014).

4.3.2 There is a need for a better seam cell marker in the dual fate-reporter strain

Despite the apparent usefulness of the dual fate-reporter strain, the merit of this strain is limited by the lack of a good seam marker. As demonstrated in Section 4.2.3, the red seam marker was diffuse and cytoplasmic despite the NLS, and appeared mosaic despite being integrated. This probably caused an underestimation of the Class III phenotype where both nuclei in a bi-nucleated cell express the seam marker, and definitely presents a challenge for studying this particular phenotype when the seam marker is unreliable for visualizing seam fate acquisition. This is a pity because Class III phenotype is in fact very curious as it produces additional seam nuclei, similar to the outcome of silencing other cytokinesis components. Class II and Class III phenotypes both entail bi-nucleated cells in the seam line, with the former having two hypodermal nuclei and the latter having two seam nuclei. I formulated an explanation for Class II but do not yet understand Class III. The comparison between Class II and Class III phenotypes inevitably prompted the question of

how being bi-nucleated could lead to opposite consequences. Was the choice random and I had simply underestimated Class III due to the problem with the seam marker, or was Class II truly much more likely to occur than Class III, as my quantification data in Section 4.2.2 suggest? Why was Class III more frequently seen after the L3 division than the L2.2 division, when this was not the case for the other two classes of phenotypes? Most importantly, how did the Class III phenotype arise and did it lead to more SC in the end?

In addition, I might have missed additional *nmy-2* knockdown phenotypes due to the unreliable seam marker. I had very occasionally observed hypodermal to seam fate change in mono-nucleated cells within the seam line (data not shown). However, it did not constitute a phenotype because the seam marker was very dim and this was so rarely observed. With a better seam marker it is possible that I find more of these cases to establish a fourth class of *nmy-2* knockdown phenotype, which would indeed be an intriguing class to study.

As explained in Section 4.2.3, I had multiple failed attempts to clone a better red seam marker and to improve the strain. It is also possible to try switching the colors in the future, i.e. using the existing *scmp::gfp* marker and a new red hypodermal marker that does not bleed through to the green channel. The *scmp::gfp* marker can appear cytoplasmic mid-division as well, but it definitely works better than the red seam markers I used in this chapter. An improved dual fate-reporter strain with more reliable fate markers will be extremely valuable for thoroughly examining fate changes in the seam.

Last but not least, my reporter strain only has hypodermal and seam markers. As previously mentioned, it is possible that some SCs adopt neuronal or apoptotic fates instead of becoming hypodermal when *nmy-2* is perturbed. These situations are less likely and thus not examined in my work, but perhaps they are still worth looking into in the future.

4.3.3 Time-lapse imaging is the next step

This chapter contains some cellular level analyses of the *nmy-2* knockdown-associated fate change phenotypes. The animals were examined after SC divisions and snapshots of cell fate changes were taken and quantified. Inferences were made as to how some fate changes arose, but time-lapse imaging will of course be much more superior and unequivocal than snapshots. For example it will be fruitful to follow through a Class II phenotypic change to prove that indeed the anterior daughter nucleus goes green first, followed by the posterior daughter nucleus turning green and the whole cell moving out of the seam line and joining the hypodermis.

However I had not yet pursued this more advanced analysis due to some limitations. The first problem is the lack of a good seam marker, as discussed in the previous section. It is hardly worth the effort of imaging the Class III phenotype, for example, if the red seam marker is unreliable. The second and main problem with time-lapse imaging is the lack of an appropriate microscope set-up in my lab. For live imaging of *nmy-2* treated animals, a temperature control chamber is required to maintain the ambient temperature at 25°C,

because the *nmy-2* ts alleles respond rapidly and reversibly to temperature changes on the order of seconds (Liu et al., 2010). The microscopes I had access to in this study were not equipped with a temperature control chamber, so live imaging always involved minimizing the time between taking the worms out of the 25°C incubator for mounting and the actual imaging. Microscope slides were freshly prepared and never used for more than 5-10 min, but even during that time the ambient temperature was probably mostly below 25°C so that the full extent of *nmy-2* knockdown was not reached. My set-up worked fine for taking snapshots of knockdown consequences, but without a way to maintain the temperature at 25°C I could not image *nmy-2* knockdown phenomena over time to capture the full process, because *nmy-2* will not be sufficiently perturbed.

4.3.4 Cytokinesis defects complicate fate specification analyses but are not solely responsible for the *nmy-2* phenotypes

Out of the three *nmy-2* knockdown fate change phenotypes identified in this chapter, both Class II and Class III are associated with cytokinesis defects. While the Class I phenotype is observed in mono-nucleated cells, one cannot rule out the possibility that a subtle timing difference in cytokinesis completion may have caused or contributed to it. This illustrates the challenge of studying essential genes with key roles in the cell. Knocking down *nmy-2* interferes with cytokinesis, which complicates fate determination analyses, especially for post-division fate acquisition and changes as was the case for most of this study. In the end I am interested in the role of *nmy-2* in specifying cell fate insofar as

whether it generates molecular and/or physical asymmetry. Unfortunately cytokinesis defects can strongly mask over other subtle phenomena, and the possibility alone undermines the merit of potentially interesting observations. For example, the Class I phenotype may have absolutely nothing to do with cytokinesis defects, yet one cannot rule out the possibility at this point.

Nevertheless, cytokinesis defects are not fully responsible for the *nmy-2* knockdown phenotypes. Post-embryonic RNAi of key cytokinesis pathway components generated promising results to demonstrate that *nmy-2* has important cytokinesis-independent roles in regulating seam development. Quite strikingly, while *nmy-2* knockdown led to fewer terminal seam cells, silencing cytokinesis genes *cyk-4* or *ect-2* generated more GFP positive seam nuclei. These opposite phenotypes further emphasize the importance of *nmy-2*'s cytokinesis-independent roles in seam development. The overall SC loss seen with *nmy-2* knockdown is actually more dramatic than it originally appeared, since it may work against a background increase of SC nuclei due to cytokinesis complications. The terminal SC number in *nmy-2* knockdown therefore represents the combined effect of a massive cytokinesis-independent SC decrease and a relatively small cytokinesis-related SC increase, and it is this considerable cytokinesis-independent SC loss that I am mainly interested in. This experiment to a certain extent enabled the uncoupling of cytokinesis defects from other *nmy-2* roles, but only in terms of seam nuclei numbers. Unfortunately there is currently no way to circumvent the complications of cytokinesis defects and only look at fate-specific roles of *nmy-2* in the seam at the molecular level and for imaging purposes. Perhaps one day forward

genetics will gift us with some new mutant *nmy-2* alleles to enable further uncoupling.

4.3.5 *nmy-2* does not function as part of the WNT/ β -catenin asymmetry pathway in post-embryonic seam development

The epistasis, localization and BiFC analyses conducted in this chapter suggested that *nmy-2* does not function as part of the WNT/ β -catenin asymmetry pathway to regulate seam development.

Double mutants between *nmy-2* and the WNT pathway components *wrm-1* or *lit-1* showed synergistic reduction of terminal SC numbers. According to the general guidelines of epistasis data interpretation (introduced in Section 4.1.1), these functional epistasis results suggested that NMY-2 potentially works 1) in parallel to the WNT/ β -catenin asymmetry pathway, or 2) as a component of the pathway by forming a physical complex with WRM-1 and LIT-1. However, there is also the possibility that 3) the synthetic effect has little to do with genetic interactions, but rather shows the enhanced phenotype of WNT disruption when the animal is already unfit due to *nmy-2* inactivation.

Additional experiments were required to determine which of the three scenarios is the case, hence POP-1 asymmetry was analyzed and BiFC analysis was performed between NMY-2 and WRM-1 proteins. POP-1 asymmetry in the seam was not significantly altered by *nmy-2* knockdown, and BiFC demonstrated that NMY-2 and WRM-1 did not physically interact in vivo. These results suggested that NMY-2 does not directly interact with WRM-1

upstream of POP-1 as part of the WNT/ β -catenin asymmetry pathway during seam development. However, without additional data we are unable to conclude whether NMY-2 functions in parallel to WNT or has no genetic interaction with this pathway.

4.3.6 Further experiments are required to determine whether and how *nmy-2* may function as a component of the RNT-1/BRO-1 pathway to regulate seam development

The epistasis analysis presented in this chapter was insufficient to conclude the nature (or the lack) of genetic interaction between *rnt-1* and *nmy-2*. A much better experiment is to utilize the RNT-1 over-expression strain (AW189) with the seam hyperplasia phenotype (Kagoshima et al., 2007) and see whether the hyperplasia is suppressed by *nmy-2* knockdown. Mutations (or over-expression strains in the absence of a suitable mutant (Prelich, 2012)) causing opposite phenotypes are much more useful for determining the linear order of a switch regulatory pathway than mutations causing the same phenotype (Huang and Sternberg, 2006). While *nmy-2* knockdown causes SC loss, the integrated *rnt-1::gfp* transgene (*msls114*) with constitutive RNT-1 over-expression causes an increase of the terminal SC number from 16 to 20 in AW189 (Kagoshima et al., 2007). *msls114* could be crossed into the *nmy-2* background and subjected to *nmy-2* knockdown treatments to determine what the terminal SC numbers are like, thus shedding light on whether the two genes interact and if so, the functional order between *nmy-2* and *rnt-1*. More

SCs will suggest that *rnt-1* is further downstream, and fewer SCs will suggest that *nmy-2* is further downstream.

Another method to assess the nature of the interactions is to examine whether the localization pattern of one gene's protein product is changed when the other gene is perturbed (i.e. NMY-2 localization in the *rnt-1* mutant, and RNT-1 localization in *nmy-2* knockdown). The necessary strains had been constructed, but these experiment have not yet been carried out.

My bioinformatics results had shown that the nature of *nmy-2* and *rnt-1* interaction is unlikely to involve the binding of the RNT-1 transcription factor to *nmy-2* regulatory sequences. Thus if further epistasis experiments do establishment that *nmy-2* in fact function as part of the RNT-1/BRO-1 pathway, then there are several possibilities as to how they may interact. On the one hand, perhaps NMY-2 functions upstream of RNT-1 by trafficking RNT-1/BRO-1 pathway components. NMY-2 has previously been shown to localize fate determinants in the *C. elegans* zygote by generating asymmetric cytoplasmic flow to transport PAR proteins (Goehring et al., 2011; Zonies et al., 2010), so it can have a similar function in the seam by localizing RNT-1 pathway components such as RNT-1 itself or CEH-20/Pbx and UNC-62/Meis, two transcription factors that normally repress RNT-1/BRO-1 in the anterior seam daughters to promote differentiation at the expense of proliferation (Hughes et al., 2013). One of my previous result showed that NMY-2 forms fibers along the A-P axis in SCs (Figure 3.2A), so perhaps this is how it traffics these molecules. On the other hand, NMY-2 may function downstream of RNT-1

instead. The precise mechanism through which RNT-1/BRO-1 promote cell proliferation is yet to be understood, but there were indications that they work by inhibiting CKI-1 (Nimmo et al., 2005), a protein that normally limits mitotic activities by inhibiting cyclin E/CDK-2. Owing to NMY-2's position downstream of converging pathways in a wide range of biological processes (see Section 1.4), it is conceivable that it acts as a RNT-1/BRO-1 downstream effector.

4.3.7 *nmy-2* and *rnt-1* perturbations have different effects on the adult alae

One difference between the *nmy-2* and *rnt-1* mutant phenotypes is the integrity of the adult alae associated with reduced SC numbers. While *nmy-2* mutants had gaps in their alae, which correlated with gaps in the seam (Figure 3.5), *rnt-1* mutants have intact wild-type alae despite having fewer SCs (Brabin et al., 2011). Perhaps the difference lies in the pleiotropic effects of *nmy-2* knockdown. Maybe *rnt-1* mutant adults have normal alae because they only experience proliferation defects, so the existing SCs, albeit being fewer, still fuse together to form a seam syncytium and secrete normal alae. By contrast, *nmy-2* knockdown led to not only potential proliferation abnormality via the RNT-1 pathway, but also other phenotypes such as SC loss via the Class II cytokinesis-associated fate change. Perhaps it was the “active” SC loss via fate transformation, as opposed to the “passive” failure to proliferate, that led to alae gaps in *nmy-2* knockdown. In the future it will be interesting to test this hypothesis against the *rnt-1 nmy-2* double mutant and other mutants with fewer SCs, such as the *wrm-1* mutant.

Chapter 5 Mitotic spindle positioning may hold clues to seam cell biology

5.1 Introduction

In the previous chapter I identified distinct classes of cell fate change phenotypes as a result of *nmy-2* knockdown, and examined potential *nmy-2* interactions with known regulatory networks to control seam development. In this chapter, I sought to further explore the molecular mechanisms of *nmy-2* function, specifically by examining its role in SC spindle positioning.

Mitotic spindle positioning plays a vital role in asymmetric divisions by specifying the division axis orientation and the future cleavage site (Section 1.2). Mitotic spindles are orientated along the polarity axis using cortical cues to ensure the correct partitioning of genetic material into two future daughters. The overlapping microtubule ends in the spindle midzone also specify the future site of cytokinesis to generate daughters of the correct sizes. Spindle positioning was studied in great detail in several model systems, however it has not been examined in SCs. Thus I first systematically measured spindle positioning in WT SC divisions, and then asked whether *nmy-2* knockdown affects spindles. Perhaps the cell fate transformation phenotypes seen in *nmy-2* knockdown were due to changes in spindle positioning, which might subsequently lead to mis-specified daughter sizes and fates. For example, perhaps an asymmetric division was symmetrized by changing spindle

positioning; or maybe key cellular components were incorrectly segregated into the two daughters, because altered spindle structure mis-specified the appropriate cleavage site.

5.1.1 Aims and hypotheses for this chapter

The aims of the work presented in this chapter were:

- To establish a method for examining spindle positioning across different stages of the SC symmetric and asymmetric divisions;
- To investigate whether mitotic spindle positioning is changed in *nmy-2* knockdown.

I hypothesized that:

- It will be fruitful to systematically measure the position of spindles in wild-type SC divisions. This has not been done before and may shed light on fundamental SC biology;
- *nmy-2* knockdown may alter spindle positioning, which could potentially contribute to the observed cell fate transformations.

5.2 Results

5.2.1 A method was established for examining spindle positioning in the wild-type symmetric and asymmetric divisions

Correct spindle orientation and positioning is thought to be vital for successful asymmetric divisions. As spindle positioning has not been previously investigated in SCs, I set out to examine it across different stages of SC divisions. The AW1095 strain was used for the analysis. It has an integrated *ajm-1p::mCherry* apical junction marker in addition to the previously used *hels63* seam DNA and membrane markers. The detailed image analysis methodology is described in Section 2.4.2. Essentially, images of V1-4 lineage cells undergoing the pertinent SC divisions were captured (Figure 5.1) and various distances between the ends of the cell (marked by *ajm-1p::mCherry*) and the chromosome (marked by *wrt-2p::gfp::H2B*) were measured. For anaphase, the distances between daughter chromosomes and the site of cleavage furrow ingression were also measured. The DNA marker was chosen because no suitable spindle marker was available in the seam. Seam-expressed centrosome markers (such as *γ-tubulin::gfp*) were not used, but provide a suitable alternative to the DNA marker for the purpose of this experiment.

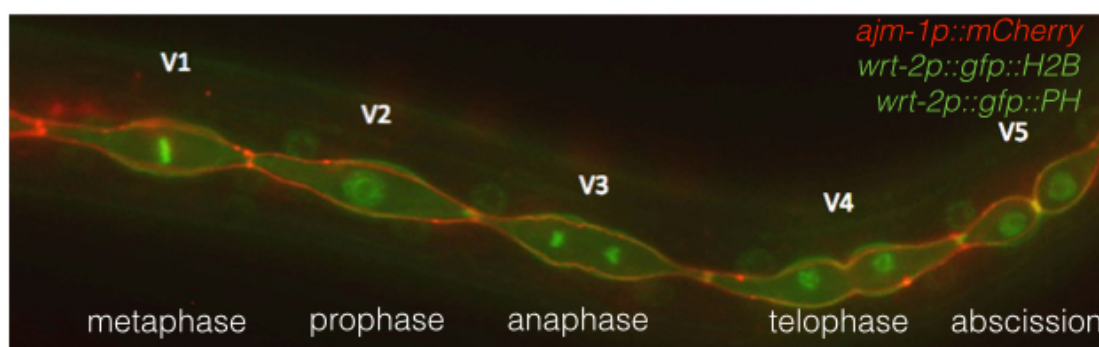


Figure 5.1: Representative image of the various stages measured in the spindle dataset. V-lineage cells undergoing interphase (not pictured), prophase, metaphase, and anaphase were analyzed for spindle positioning, using overlaid images focusing on both the apical junction and the chromosome. The worm shown is undergoing the L1 asymmetric division. The strain used here was AW1095. Scale bar = 10 μ m.

Initially I had chosen to focus on the L2.1 symmetric division and the L2.2 asymmetric division, because this offers a comparison between the symmetric and asymmetric modes of SC division within a short time window. However exactly because these two divisions happen in such quick succession (~30 min at 25°C) and with spatial constraint (i.e. no molting between the two divisions for the larvae to grow larger), spindle positioning in the L2.2 asymmetric division may be different to the other more “stereotypical” SC asymmetric divisions that take place at the beginning of a larval stage just after molting. Hence I decided to also include the L3 asymmetric division in my analysis, as this may, alongside the L2.1 symmetric division data, facilitate a better comparison between the two modes of SC division. An overview of spindle positioning for all three divisions is shown in Figure 5.2.

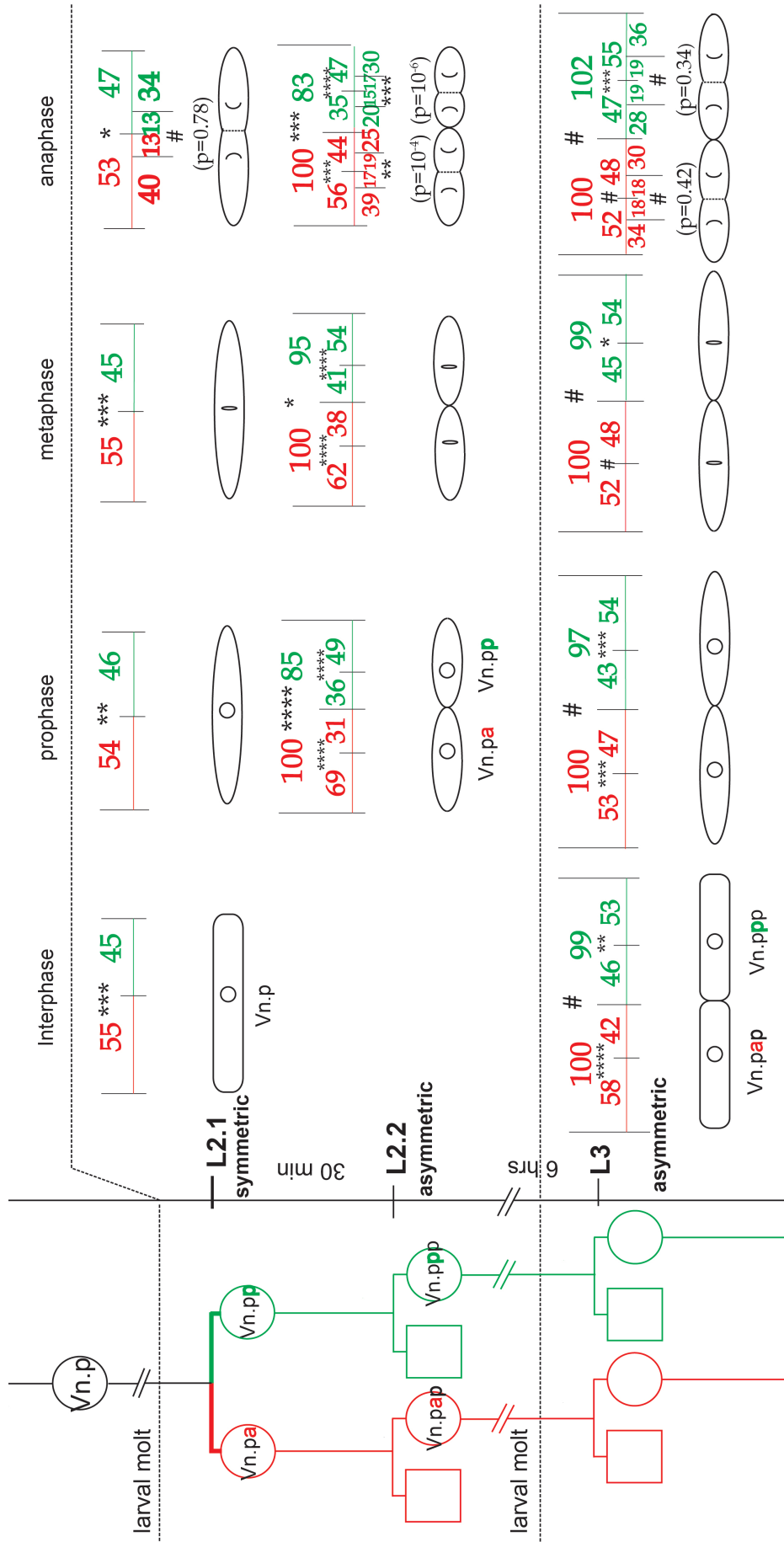


Figure 5.2: Summary of wild-type spindle measurements during the L2.1 symmetric, L2.2 asymmetric, and L3 asymmetric divisions. Figures are drawn to scale along the A-P axis within each division. AW1095 worms (strain with *hels63[wrt-2p:gfp::PH;wrt-2p::gfp::H2B;lin-48p::mCherry]* and *ouls21[ajm-1p::mCherry]* in the WT background) were bleach synchronized to L1 and transferred to OP50 plates at 25°C. Quantitative measurements were performed as described in Section 2.4.2. Only V1-4 lineages were considered for the analysis so for example “Vn.p” includes pooled data from “V1/2/3/4.p” and “Vn.pa” includes V1/2/3/4.pa. Squares represent hypodermal cells and circles represent seam cells. All measurements within a cell are normalized to the full A-P length of that cell, which is assigned a value of 100. Vertical timeline indicates the approximate time between each division at 25°C. n>45 for each stage of each division. T tests were performed to determine differences – paired T test for anterior versus posterior measurements within the same cell, and unpaired T test assuming unequal variances for measurements between different cells belonging to the anterior versus posterior lineage branches. #: p>0.05; *: p<0.05; **: p<0.01; ***: p<10⁻⁵; ****: p<10⁻¹⁰.

5.2.2 WT nuclear positioning data reveal two aspects of seam cell

biology

For the wild-type L2.1 symmetric division, it is notable that the nucleus was placed slightly off-center throughout the division (see Figure 5.3A for a cartoon drawing). The term “nucleus” is broadly used in this section to refer to the chromosomal mass between interphase and metaphase (even after nuclear envelope breakdown), as opposed to the segregating daughter chromosomes in anaphase, which concerns the next section. The nucleus was closer to the posterior end of the cell than the anterior end, as the distance between the nucleus and the posterior end was only 82% of the distance between the nucleus and the anterior end. This off-center placement of the chromosomes carried on through anaphase and the symmetric division produced a slightly larger anterior daughter (Vn.pa, shown in red) and a slightly smaller posterior daughter (Vn.pp, shown in green) (Figure 5.3B). The posterior daughter was 85% the size of the anterior daughter as determined by A-P cell length, but both daughters would retain the seam fate and carry on dividing.

Throughout the L2.2 asymmetric division, the anterior branch (red) daughter Vn.pa remained larger than the posterior branch (green) daughter Vn.pp. The placement of the nucleus was closer to the posterior in Vn.pa and closer to the anterior in Vn.pp (Figure 5.3B). Thus, the two seam daughters derived from the L2.1 division behave as a pair rather than two identical individual cells. The nucleus of each daughter was placed closer to the end of the cell that bordered its sister, than to the other end that bordered a different pair of daughters. In other words, if one were to imagine the L2.1-derived seam daughters as a pair of eyes and their nuclei as the irises, then those eyes would be mildly “crossed”. This is quite remarkable, because irrespective of the differential initial nuclear displacement along the A-P axis, Vn.pa and Vn.pp would each generate an anterior daughter that differentiates and a posterior daughter that remains seam in the subsequent L2.2 division (Figure 5.3C). Therefore, the L2.2 asymmetrical division does not require specific nuclear placement prior to division to determine its division outcome.

The L3 asymmetric division data were somewhat unexpected. I originally included this division in my analysis because the L2 to L3 larval molt presumably allows the L3 division mechanism to “reset” from external influences such as the spatial constraint. The Vn.pap and Vn.ppp cells derived from L2.2 would have time and space to reorganize and resize, so that they would be independent and identical to each other in how they divide next. However this was not entirely the case. Prior to the L3 division the size difference between Vn.pap and Vn.ppp cells had indeed vanished and sizes

remained equal throughout the L3 division (Figure 5.3D). However, the pairing of the anterior (red) and posterior (green) branch-derived seam daughters, seen in L2.2, had persisted through the rest of the L2 stage and was retained in the L3 division. The nucleus of Vn.pap (red) was closer to the posterior end until metaphase, and the nucleus of Vn.ppp's was closer to the anterior end throughout L3 division (Figure 5.3D). Therefore the “cross-eye” phenomenon observed in L2.2 was not simply because it took place so soon after L2.1 that the daughters organized themselves in pairs. It was retained for the L3 division after the long quiescent period following the L2.2 division. This remarkable retention of branch identity may require positional adjustments of some seam nuclei along the A-P axis. For example, Vn.pap would likely have an anteriorly-displaced nucleus after the L2.2 division (this was speculated but not actually imaged, hence the dashed box) (Figure 5.3C). After Vn.paa left the seam line to fuse with the hyp7 syncytium, Vn.pap would need to shift its nucleus slightly towards the posterior in order to reconstitute the “cross-eyed” nuclear placement with its Vn.ppp counterpart prior to the L3 division (Figure 5.3D).

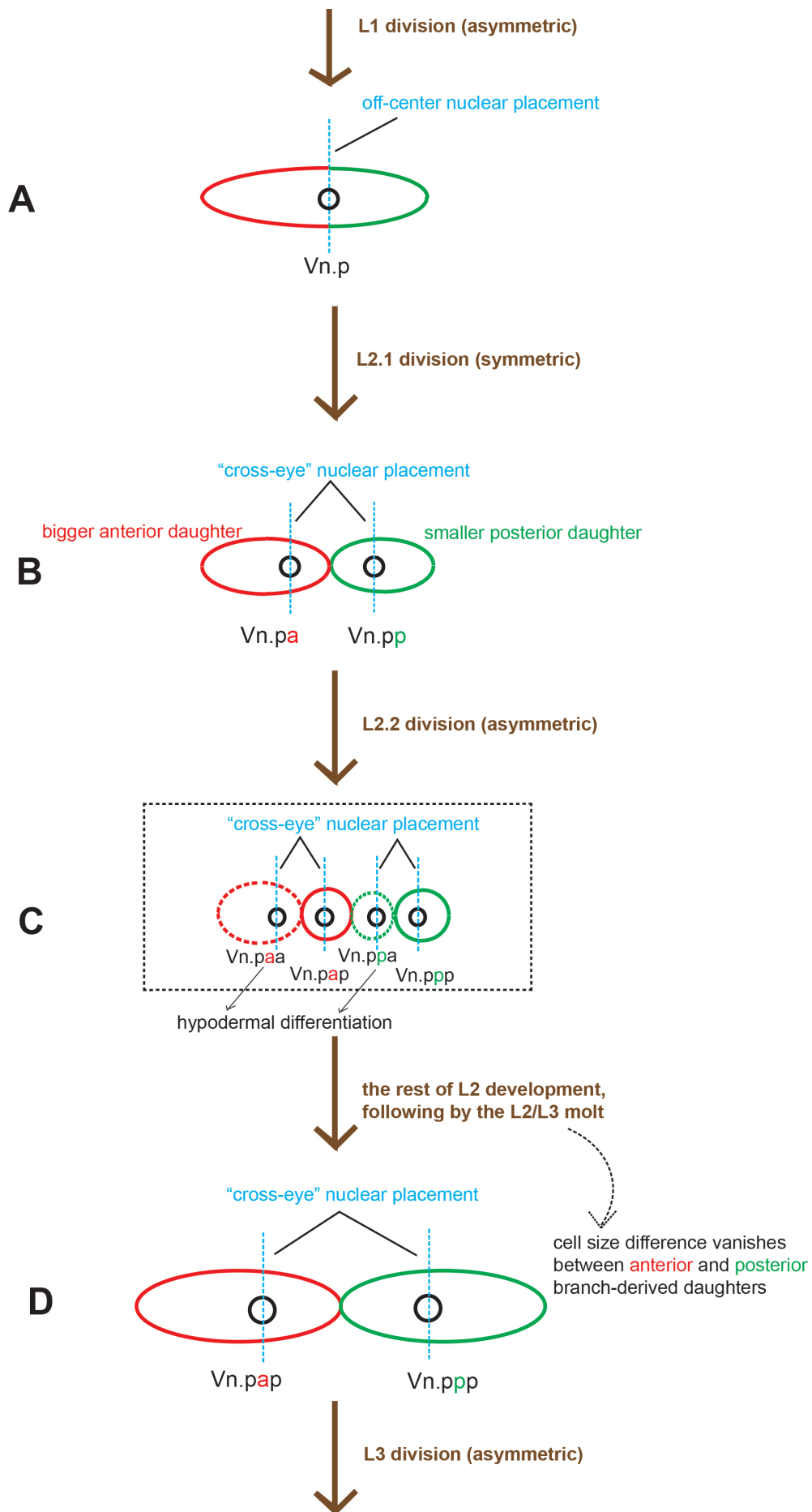


Figure 5.3: Schematic drawing showing nuclear positioning in the wild-type L2 and L3 divisions. Figures are derived from **Figure 5.2** but not drawn to scale, as size differences are exaggerated for demonstration purposes. **A)** Just before the L2.1 division. **B)** Between the L2.1 and L2.2 divisions. **C)** The speculated situation just after the L2.2 division. The dashed box denotes that the cell sizes and nuclear positioning have not actually been measured, but rather speculated based on the L2.2 anaphase data. **D)** After the L2/L3 molt, just before the L3 division.

In summary, nuclear positioning across L2.1 symmetric, L2.2 asymmetric, and L3 asymmetric divisions revealed two general trends. First, the initial placement of the seam nucleus along the A-P axis does not seem important. A symmetric division can initiate with a displaced seam nucleus and still generate two proliferative daughters; likewise an asymmetric division can start out with the seam nucleus closer to either end of the cell and still invariably produce a differentiative anterior daughter and a proliferative posterior daughter.

According to Hertwig's rule, the nucleus tends to take up a position at the center of a cell (Wilson, 1925). Even though the nucleus is often displaced in various polarized cells, the biological role of this off-center placement is mostly unknown. An example of a biologically relevant nuclear displacement is in the *C. elegans* EMS division, where the posterior but not anterior daughter nucleus is anchored to the posterior cell cortex by centrosomes. This nuclear anchoring is controlled by WNT and SRC signaling in the EMS and affects the asymmetric nuclear localization of POP-1/TCF (Sugioka and Sawa, 2010). The regulation and the biological relevance of seam cell nuclei off-center placement is unknown, however my data suggest that the displacement along the A-P axis does not appear to influence the fate outcome of SC divisions.

Secondly, seam daughters appear to retain their identity based on whether they are derived from the anterior branch (red) or the posterior branch (green)

of the L2.1 symmetric division. The anterior-branch daughters seem to pair with the posterior-branch daughters prior to the L2.2 and L3 divisions, suggesting that their branch identities persist through divisions. Even though at this point I cannot explain whether this identity is of any importance, it will be interesting to investigate whether the trend is retained for the L4 division too. Additionally, I would be curious to examine what happens in the L1 division to give rise to Vn.p cells, which all exhibit nuclear displacement towards the posterior.

5.2.3 WT spindle positioning analysis shows displaced cleavage furrows in the L2.2 asymmetric division

In addition to the nuclear positioning trends, spindle positioning analysis in WT worms also yielded an intriguing observation, whereby the cleavage furrow was displaced specifically in the L2.2 asymmetric division.

A closer look at the anaphase spindle data (actual data in Figure 5.2, cartoon drawing in Figure 5.4) demonstrated that while the cleavage furrow perfectly bisected the segregating chromosomes in the L2.1 and L3 divisions (Figure 5.4 A and C, blue dashed lines and text), during L2.2 anaphase it was displaced more towards the anterior set of chromosomes in both the Vn.pa ($p=10^{-4}$) and the Vn.pp ($p=10^{-6}$) cells (Figure 5.4B, blue dashed lines and text). This L2.2-specific furrow displacement could potentially be a mechanism to ensure that a differentiative anterior daughter was generated from this asymmetric division.

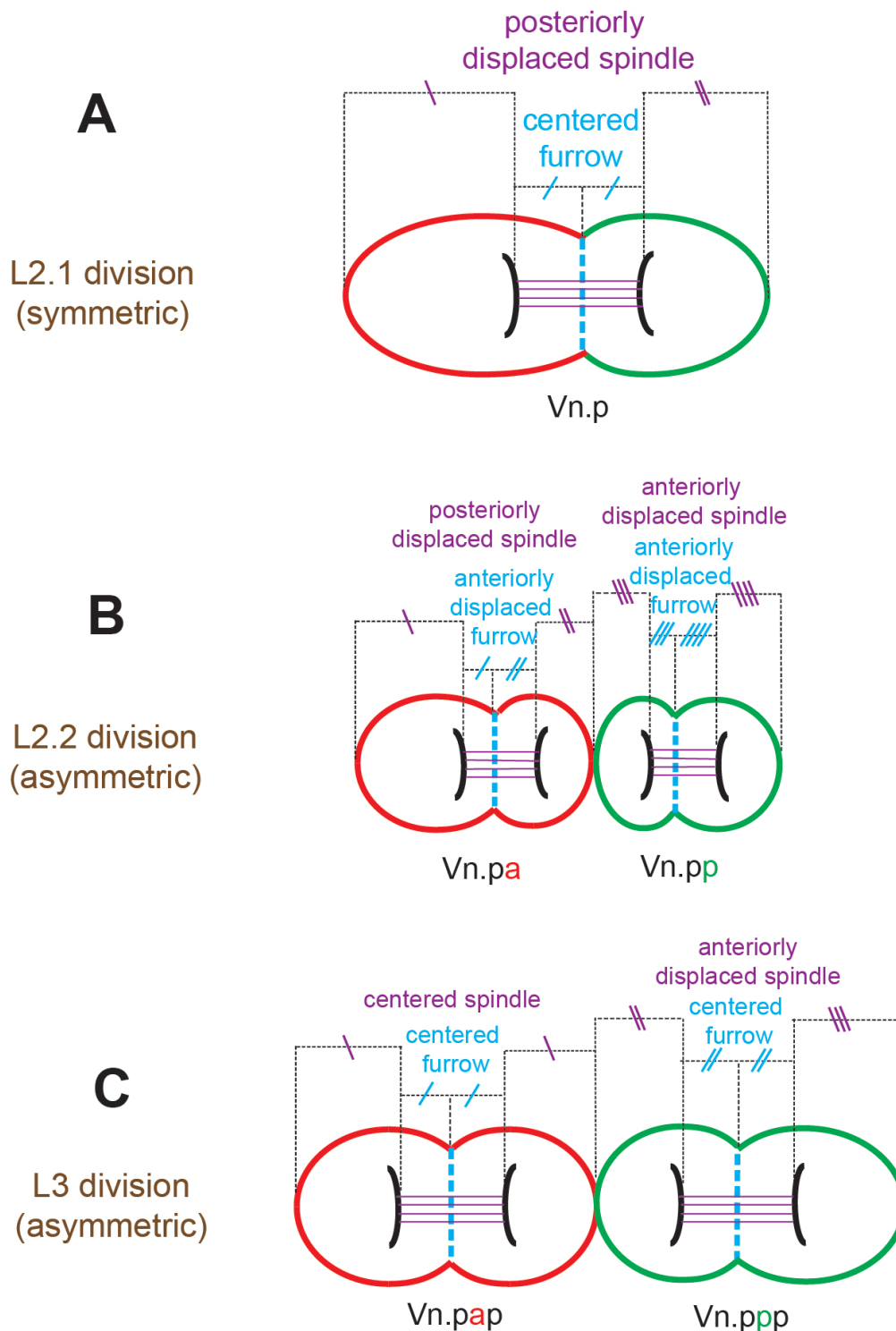


Figure 5.4: Schematic drawing showing anaphase spindles during the wild-type L2 and L3 divisions. Figures are derived from **Figure 5.2** but not drawn to scale, as size differences are exaggerated for demonstration purposes. Blue dashed lines represent the cleavage furrows and parallel purple lines represent the midzone spindles (neither structure was directly visualized, but their positions were inferred from other cellular markers). Different numbers and directions of hashes across the top represent distinct distance measurements within a particular division.

It is important to note the difference between spindle displacement and cleavage furrow displacement as mechanisms to generate unequal-sized daughters. The former concerns displacing the entire mitotic spindle complex, whilst both sets of segregating chromosomes remain equidistant to the cleavage furrow, such as in the case of the *C. elegans* zygote (see Figure 1.7A). The latter involves spindle arms of unequal lengths, with one set of the daughter chromosome closer to the cleavage furrow than the other set, i.e. the cleavage furrow is displaced with respect to the whole mitotic spindle complex. There are two precedents of a displaced cleavage furrow in asymmetric divisions, in *Drosophila* neural stem cells and in the *C. elegans* Q.a neuroblast (see Figure 1.7 B-C).

Spindle and furrow placements in SC divisions are indicated by purple and blue texts in Figure 5.4, respectively. The two mechanisms are not mutually exclusive for generating daughter size asymmetry. For example, during anaphase of the L2.2 division, both the spindle complex and the cleavage furrow were anteriorly displaced in the Vn.pp daughter (Figure 5.4B). A smaller, differentiative anterior daughter was generated as a result, however the relative contribution of each displacement to the final cell size is unclear. By contrast, the Vn.pa daughter also generated a differentiative anterior daughter, but from the combination of a posteriorly-displaced spindle complex (presumably as a result of the “cross-eyed” nuclear positioning phenomenon) and an anteriorly-displaced cleavage furrow at anaphase. Hence it is possible that the anteriorly-displaced cleavage furrow was particularly crucial for the Vn.pa cell division at L2.2. The anterior half of the Vn.pa cell was larger at

anaphase due to the posteriorly-displaced mitotic spindle complex. Hence in this particular situation, perhaps the anteriorly-displaced cleavage furrow provided a critical cue that the nascent anterior daughter is to differentiate despite its large size by “holding close” the anterior chromosomes at anaphase.

The size of the two Vn.pa division daughters was not measured after cytokinesis completion (the cell sizes drawn in Figure 5.3C were only a surmise), so it is unknown whether the anterior daughter became smaller between anaphase and abscission relative to the posterior daughter. Cell size is important for fate allocation in the second round of the *C. elegans* Q neuroblast divisions (Ou et al., 2010), but it may not necessarily correlate with cell fate in the seam. For example, the L2.1 SC division generates two unequal-sized daughters (Figure 5.3B), both of which have the proliferative seam fate. Conversely, the L3 division of the anterior branch-derived daughters (Vn.pap) generates a differentiative anterior daughter and a proliferative posterior daughter, despite having centered spindles and a centered furrow (Figure 5.4C). Regardless of whether anterior differentiation in the Vn.pa division is size-dependent, the anterior-directed cleavage furrow displacement may be an important mechanism to ensure that a differentiative anterior daughter is generated from this division (further discussed in Sections 5.3.1 and 5.3.2).

5.2.4 Spindle positioning appears unaffected by *nmy-2* knockdown

Having gained an understanding of WT spindle positioning in the L2 and L3 divisions, I proceeded to examine whether the positions would change following *nmy-2* knockdown.

The *nmy-2* knockdown spindle positioning dataset is largely similar to the WT situation (Figure 5.5). Divisions took place along the A-P axis, and consistent with my previous finding in Section 3.2.6, spindle rotation was so rarely observed in *nmy-2* knockdown that it was not taken into account. There was off-center placement of the nuclei in all three divisions; the characteristic “cross-eyed” nuclear placement in the anterior (red) and posterior (green) branch-derived daughters persisted through the L2.2 and L3 divisions; SC sizes equalized before the L3 division - all these trends previously seen with WT divisions also held true in the *nmy-2* knockdown worms. Thus spindle positioning in SC divisions appeared largely unaffected by *nmy-2* perturbation, and there was no evidence that spindles were altered to symmetrize L2.2 and L3 divisions. The displaced cleavage furrows observed in the WT L2.2 asymmetric division remained displaced in *nmy-2* knockdown.

The one notable exception was the cleavage furrow placement in the L2.1 symmetric division. In WT worms the cleavage furrow was displaced only in the L2.2 division and remained perfectly centered for the L2.1 and L3 divisions (Figure 5.2). In *nmy-2* knockdown worms, the situation held true for the L2.2 and L3 divisions, but the cleavage furrow in the L2.1 division was also displaced towards the anterior daughter chromosome ($p=0.001$), similar to the case in L2.2. This was particularly unexpected because L2.1 is a symmetric

division, and I previously hypothesized that the cleavage furrow displacement in the WT situation is a mechanism for ensuring an asymmetric division. There is no evidence that *nmy-2* knockdown turned the L2.1 division asymmetric, so it is difficult to explain why the cleavage furrow became displaced in this symmetric division. This result may potentially call the cleavage-furrow-displacement-generating-asymmetry hypothesis into question, or it may have no biological relevance. Further studies to test this hypothesis will be required (discussed in Section 5.3.2).

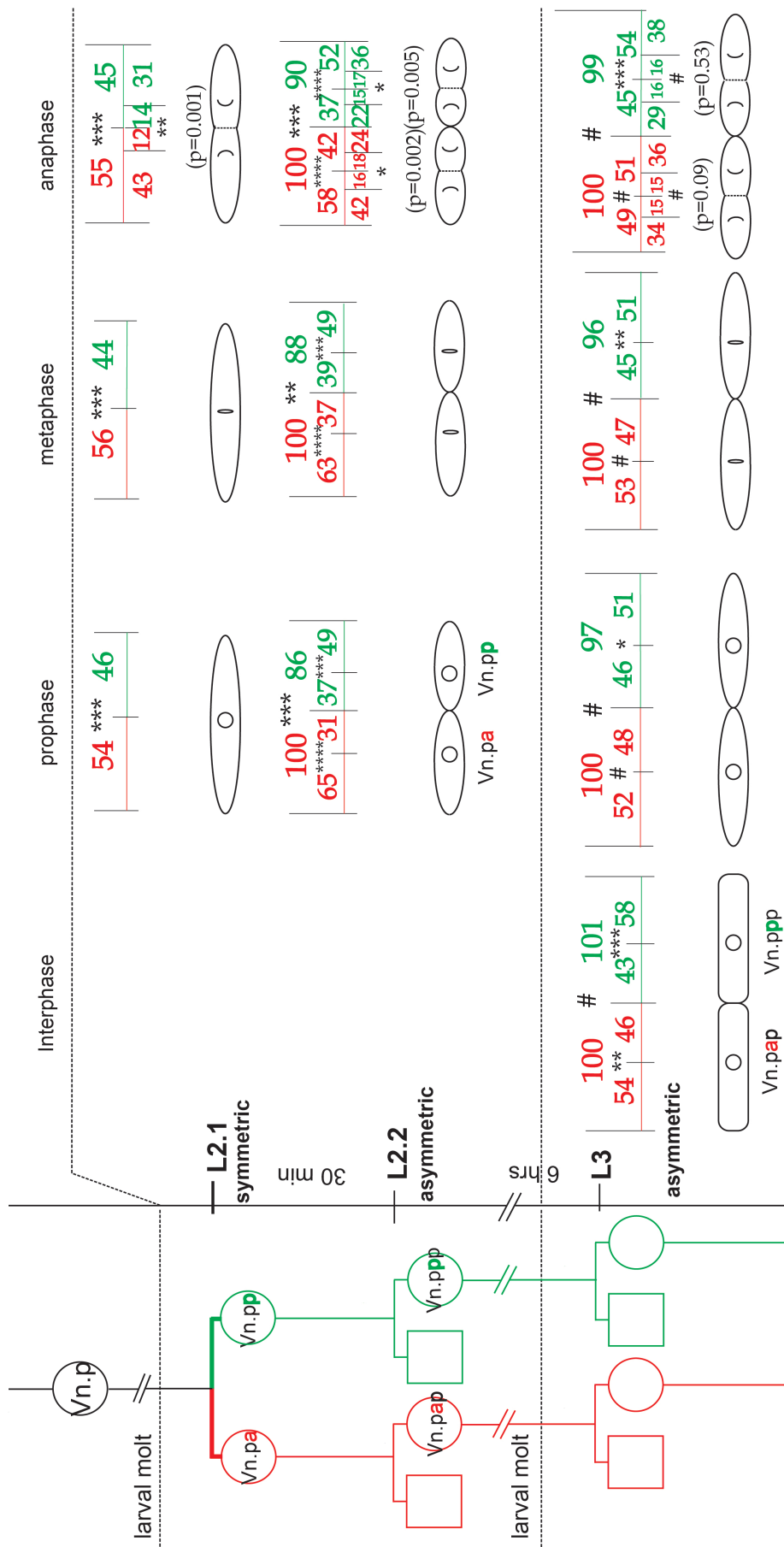


Figure 5.5: Spindle positioning during the L2.1 symmetric, L2.2 asymmetric, and L3 asymmetric divisions are mostly unaffected by *nmy-2* knockdown. Figures are drawn to scale along the A-P axis within each division. AW1116 worms (strain with *hels63[wrt-2p:gfp::PH;wrt-2p::gfp::H2B;lin-48p::mCherry]* and *ouls21[ajm-1p::mCherry]* in the *nmy-2* mutant background) were bleach synchronized to L1 and transferred to *nmy-2* RNAi plates at 25°C. Analysis was performed exactly as for **Figure 5.2**. $n > 50$ for each stage of each division. #: $p > 0.05$; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 10^{-5}$; ****: $p < 10^{-10}$.

It is worth noting that cell identity assignment in the *nmy-2* dataset was based on cell location in the worm at the time of image acquisition. Individual cells were not followed through for their exact lineages to be assigned, so it is always possible that the wrong identity was assigned based on position alone. This means that an anterior branch daughter could have been mis-assigned to the posterior branch, or that a V5-derived cell that would normally be excluded from the analysis could have been mis-assigned as a V4 cell and thus included. Location-based cell identification might also have led to a dataset that is skewed towards the WT situation, because SCs in the most perturbed areas of the worms, where cell identity could not be assigned with reasonable confidence, were excluded from the dataset presented in Figure 5.5. This was particularly relevant for the L3 division, because enough SCs could be lost following the L2.2 division to leave gaps in the seam (see Figure 3.5C), which rendered location-based cell identification more difficult and less reliable. Both L2 divisions were less affected because the worms tended to still have all of their SCs at those divisions.

5.3 Discussion

5.3.1 Cleavage furrow displacement in the L2.2 division may indicate a mechanism to switch between different modes of SC division

Anaphase spindle data from the WT worms (Figure 5.2, Figure 5.4) revealed that the cleavage furrow was displaced during L2.2 anaphase. This may provide an explanation for how worms rapidly switch between different modes of division going from L2.1 (symmetric) to L2.2 (asymmetric). The cleavage furrow was displaced significantly more towards the anterior set of chromosomes and may function to ensure that a differentiative (perhaps also smaller) anterior daughter is generated from this asymmetric division at L2.

As mentioned before, the two L2 divisions occur in rapid succession. Perhaps due to the time constraint, the L2.2 division has to employ such a mechanism to achieve an asymmetric division. Other SC asymmetric divisions are preceded by several hours of SC quiescence, which is an ample amount of time to implement the “usual” mechanisms, such as WNT signaling, to polarize the SCs and ensure the asymmetric mode of division. WNT signaling is still present in L2.1 (Wildwater et al., 2011) but is overridden to generate the symmetric division. The L2.2 asymmetric division is a unique case that immediately follows the symmetric division, so it may require an extra mechanism to antagonize or override the L2.1 instructions and/or the posteriorly-displaced spindle complex in the Vn.pa daughters. The cleavage furrow displacement at L2.2 anaphase is an enticing indication that this additional mechanism exists to facilitate a timely switch to the asymmetric mode of division.

5.3.2 Further experiments are necessary to determine whether cleavage furrow displacement is uniquely important to the L2.2 asymmetric division

Based on the WT division data, I proposed that the displaced SC cleavage furrow may be a unique case for the L2.2 division (particularly relevant for the Vn.pa daughters) to facilitate a rapid switch from the symmetric to the asymmetric mode of division. This notion faced a potential challenge when a similar displacement was observed in the L2.1 symmetric division upon *nmy-2* knockdown (Figure 5.5), with no suspected effect on the proliferative nature of that division. Furrow displacement at L2.1 anaphase was not observed in the WT situation, so it might just be an *nmy-2* knockdown phenomenon with no biological relevance.

One way to further address this potential contradiction is to examine the spindle positioning in male V5 and V6 lineages. At the beginning of the third larval stage, male V5.ppp, V6.pap and V6.ppp undergo an additional symmetric division to generate precursors to the male sensory ray structure. Both seam daughters derived from these symmetric divisions then rapidly perform an asymmetric division to generate an anterior hypodermal daughter and a posterior seam daughter (except for V6.pppp). It will be useful to examine the spindle positioning in these WT male-specific L3 symmetric and asymmetric divisions to see whether the pattern of cleavage furrow placement resembles that of the L2 divisions. Unfortunately this analysis cannot be

performed using the existing AW1095 strain due to the expression of *lin-48p::mCherry*, which obscures the V5-V6 region. While these new data of male V5 and V6 at the L3 divisions may be informative, it is crucial to keep in mind that these lineages are subjected to additional controls such as from the posterior Hox gene *mab-5*. After all, these male-specific L3 divisions occur at a different larval stage, with slightly longer time elapsed between the two divisions compared to the L2 situation, so their spindle positioning may be different to that in V1-4 at the L2 divisions anyways. An alternative is to use the *mir-48 mir-241;mir-84* triple mutant, which reiterates the L2 proliferative division at the L3 stage. However the number of SCs that undergo this reiteration varies (Abbott et al., 2005), so it may complicate the analysis.

It would also be useful to examine SC cleavage furrow placement in the WT hermaphrodite L1 and L4 divisions. If the cleavage furrow is placed exactly half way between the two sets of chromosomes in those two divisions, then it fits with my model, which proposes that a centered cleavage furrow is the norm for SC divisions and that a displaced furrow is a special case for L2.2 to overcome the time constraint and rapidly switch the division mode. The intriguing displacement observed in the *nmy-2* knockdown L2.1 division would then remain to be resolved. It is important to note that even if the L1 and L4 division data are not exactly as hypothesized here, they do not necessarily rule out the model. Each division can be unique despite the similarity in division outcomes, and each seam cell can be unique despite the similarity in cell lineages.

5.3.3 NMY II-dependent cortical polarity may play a role in displacing the cleavage furrow

In addition to the *C. elegans* V cells undergoing the L2.2 asymmetric division, displaced cleavage furrows were also reported in *Drosophila* NBs and in the *C. elegans* Q.a neuroblast. *Drosophila* NB divisions have basally-directed cleavage furrow displacement at anaphase in order to generate a larger apical NB daughter and a smaller basal GMC daughter. This furrow displacement is partially under the control of a cortical polarity pathway, whereby preferential displacement of myosin II from the apical cortex allows the apical NB to expand and pushes the cleavage furrow basally towards the nascent GMC (Cabernard et al., 2010; Connell et al., 2011; Roth et al., 2015). This myosin-based mechanism is conserved in the *C. elegans* Q neuroblast lineage, where the cleavage furrow is displaced towards the anterior to generate a smaller anterior daughter and a larger posterior daughter from the Q.a cell division (Ou et al., 2010). It has recently been shown that in *Drosophila* NBs, this cortical polarity cytokinesis pathway is spatially and temporally separate from the classic spindle-dependent cytokinesis pathway involving the centralspindlin complex, and that these two independent cytokinesis pathways are both necessary for the correct cleavage furrow positioning in NB asymmetric divisions (Roth et al., 2015).

Seeing as myosin II-based cortical polarity is conserved between both known cases of furrow displacement, it appears ever more important to closely re-examine NMY-2 localization in *C. elegans* seam cells, particularly during the

L2.2 asymmetric division where the cleavage furrows become displaced. By incorporating a DNA marker into the *nmy-2::gfp* reporter strain, perhaps more NMY-2 could be seen on the SC anterior cortex during L2.2 anaphase. This cortical NMY-2 may serve to displace the cleavage furrow, and/or to reduce the size of the future anterior daughters. If this is the case then once again we face the conundrum that *nmy-2* knockdown generated a loss of SC rather than a gain (see the previous discussion in Section 3.3.4). If NMY-2-based anterior cortical tension is important for specifying the anterior differentiative fate, then *nmy-2* perturbation would lead to fewer differentiated daughters and more seam daughters. Because we observed the opposite outcome in terms of SC numbers, this contradiction will need to be addressed if NMY-2 indeed preferentially localizes to the anterior cortex during L2.2 anaphase. Perhaps *nmy-2* knockdown produces complex pleiotropic effects on SC divisions that cannot be explained by the most simple and straightforward reasoning.

Of course it is also possible that the NMY-2-based cortical polarity cytokinesis pathway is a peculiarity of neuroblasts, and that in V1-V4 seam cells the cleavage furrow displacement comes from the classical spindle-dependent cytokinesis pathway and has nothing to do with cortical NMY-2 polarity. This would fit with my observation that the L2.2 cleavage furrow displacement was unaffected by *nmy-2* knockdown. In most metazoan cells, cytokinesis furrow positioning is dictated by cues from the mitotic spindle via the centralspindlin complex (Basant et al., 2015; White and Glotzer, 2012). If cortical polarity is found not be involved in SC furrow displacement, it would be interesting to

investigate exactly how the displaced furrows are specified by the spindle-based cytokinesis pathway in the L2.2 asymmetric division.

Chapter 6 *cdk-1* has possible cell cycle-independent roles in seam development

Recent work on *C. elegans* and *Drosophila* has generated a growing body of evidence suggesting crucial coupling between cell cycle progression and asymmetric divisions (Ishidate et al., 2014; Noatynska et al., 2013) (introduced in Section 1.2.3). CDK-1 is best known as the master cell cycle regulator in promoting entry to mitosis, but it also has at least two cell cycle-independent roles in *C. elegans* embryogenesis. It indirectly promotes OMA-1 degradation to allow oocyte-to-embryo transition (Shirayama et al., 2006), and it has been shown to cooperate with WNT to coordinate the cortical release of WRM-1 in the EMS division, thereby controlling the spindle rotation and orientation of that division (Kim et al., 2013). Hence I was interested in whether *cdk-1* may also have a cell cycle-independent role in post-embryonic development of the seam, possibly by interacting with *nmy-2* or *wrm-1*.

Forward genetics has generated useful resources that uncouple CDK-1's role in development from its role in cell cycle regulation. *cdk-1(ne2257)* is a temperature sensitive allele with a missense mutation in the T loop (or activation loop), however the mutation has no effect on cyclin B binding or cell cycle progression (Shirayama et al., 2006). Homozygous mutant worms have no obvious larval phenotypes; mutant embryos show normal cell divisions and well-differentiated cells and tissues (Shirayama et al., 2006). With no suspected cell cycle defects, this *cdk-1* allele was used for my analyses in this chapter.

6.1.1 Aim and hypotheses for this chapter

The aim of the work presented in this chapter was:

- To investigate whether *cdk-1* regulates SC asymmetric divisions independently of its role in cell cycle progression.

I hypothesized that:

- A major cell cycle regulator such as CDK-1 may play cell cycle-independent roles in SC development;
- In particular, CDK-1 may directly phosphorylate cortical WRM-1 in SCs as it does in the four-cell stage EMS.

6.2 Results

6.2.1 *cdk-1* mutation partially rescues *nmy-2* SC reduction phenotype across different larval stages

Using the *cdk-1(ne2257)* allele, I asked whether CDK-1 regulates post-embryonic SC development in a cell cycle-independent manner. While the *cdk-1* mutation alone had no effect on terminal SC numbers, it partially rescued the *nmy-2* loss of SC phenotype at 25°C (Figure 6.1A). In this dataset, *nmy-2* knockdown reduced terminal SC numbers from 16 to 9.5, and this reduction was partially rescued in the *nmy-2;cdk-1* double mutant, which had 14.5 SCs. Thus *cdk-1* and *nmy-2* exhibited antagonistic epistasis and *cdk-1* mutation

conferred a 77% rescue (5 out of 7.5 SCs) to the *nmy-2* loss of SC phenotype at 25°C. This finding was rather unexpected, and I further investigated whether the rescue occurred at a particular division or across different divisions.

Figure 6.1B shows the SC counts in each strain after each SC asymmetric division. There were fewer SCs in the *nmy-2* knockdown strain after each successive asymmetric division (red hashed bars), in accordance with my previous results. Overall SC numbers also decreased for the *nmy-2;cdk-1* double mutant after each division (green hashed bars) despite *cdk-1* rescue. *cdk-1* partially rescued *nmy-2* SC loss in each asymmetric division. Relative to overall *nmy-2* SC loss, *cdk-1* conferred a 80% rescue in L2.2, 70% in L3, and 42% in L4. The percentage of relative SC rescue was calculated using the terminal SC numbers from each of the following strains according to the formula:

$$\frac{(nmy-2;cdk-1) - (nmy-2)}{(WT) - (nmy-2)}$$

but it should only be taken as a qualitative guide to demonstrate rescue. For example the L4 dataset varied widely between Figure 6.1A and Figure 6.1B in terms of *cdk-1* rescue (77% in A versus 42% in B) due to the inherent variability of RNAi, but the overall message is the same – that *cdk-1* partially rescued the *nmy-2* loss of SC phenotype. In short, epistasis analysis suggests genetic interaction between *nmy-2* and *cdk-1* across different stages of SC development.

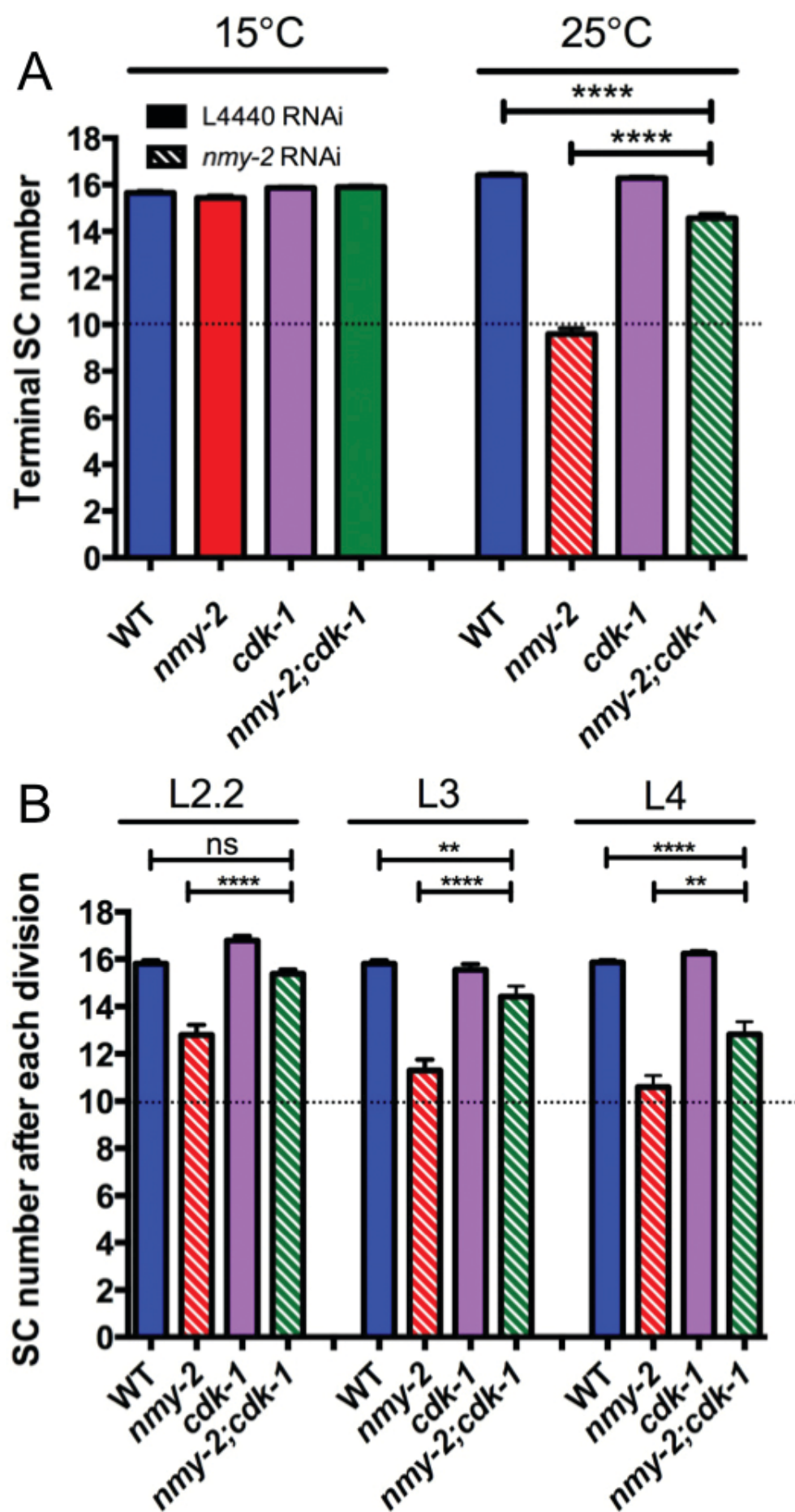


Figure 6.1: *cdk-1* mutation partially rescues *nmy-2* loss of SC phenotype. WT, *nmy-2*, *cdk-1*, and *nmy-2;cdk-1* strains with the *scmp::gfp* marker were bleach synchronized to L1 before being fed control or *nmy-2* RNAi at 15°C or 25°C. **A)** Terminal SC numbers were counted in young adults. **B)** SC numbers were counted after each SC asymmetric division. All counts of a particular strain in B) were performed on worms from the same plate at 25°C, except at different time points. The strains used were AW335, AW785, AW1103, and AW1107. n>50 for each sample. Dashed lines denote the 10 SCs that the worms hatch with. Error bars are the SEM. Unpaired T-test was used to determine significance (ns: p>0.05; **:p<0.01; ****:p<0.0001). At least three independent replicates of the experiments were performed for A and the experiment was performed just once for B.

6.2.2 *nmy-2* mutation partially rescues *cdk-1* embryonic lethality at 20°C

Having established genetic interaction between *nmy-2* and *cdk-1* in post-embryonic seam development, I next investigated whether the two genes may also interact during embryonic development. Both *nmy-2* and *cdk-1* alleles are temperature sensitive embryonic lethal. L4 animals of the *nmy-2* or *cdk-1* single mutant or the *nmy-2;cdk-1* double mutant were completely embryonic lethal at 25°C but produced viable progeny at 15°C (n>50). The incubation temperature was progressively lowered, but *nmy-2* and *cdk-1* embryonic lethality was not as straightforward as a yes/no binary choice at each temperature (which was the case for *nmy-2;wrm-1* double mutants in Figure 4.8B). Instead, brood size was counted to assess more subtle differences in embryonic lethality at different temperatures.

Figure 6.2 shows that at 22.5°C the single mutants and the double mutant were still mostly embryonic lethal. At 20°C, however, *nmy-2* mutant had a much higher brood size of 157, while *cdk-1* and *nmy-2;cdk-1* produced 14 and 40 viable progenies, respectively. Hence at 20°C, *nmy-2* mutation partially rescued *cdk-1* embryonic lethality, as the double mutant had a significantly

higher brood size than the *cdk-1* single mutant ($p < 0.01$). At 17.5°C, the *nmy-2* rescue of *cdk-1* was abolished, as both the *cdk-1* single mutant and the *nmy-2;cdk-1* double mutant increased their brood sizes to similar levels. This is probably because *nmy-2*'s effect on brood size was diminished at 17.5°C, seeing as *nmy-2* had a WT brood size at that temperature. At 15°C all four strains had similar brood sizes. Therefore, the 20°C dataset demonstrated antagonistic epistasis between *nmy-2* and *cdk-1*, by showing that *nmy-2* mutation partially rescued *cdk-1* embryonic lethality. Taken together, these experiments suggest genetic interactions between *nmy-2* and *cdk-1* during embryonic and post-embryonic development.

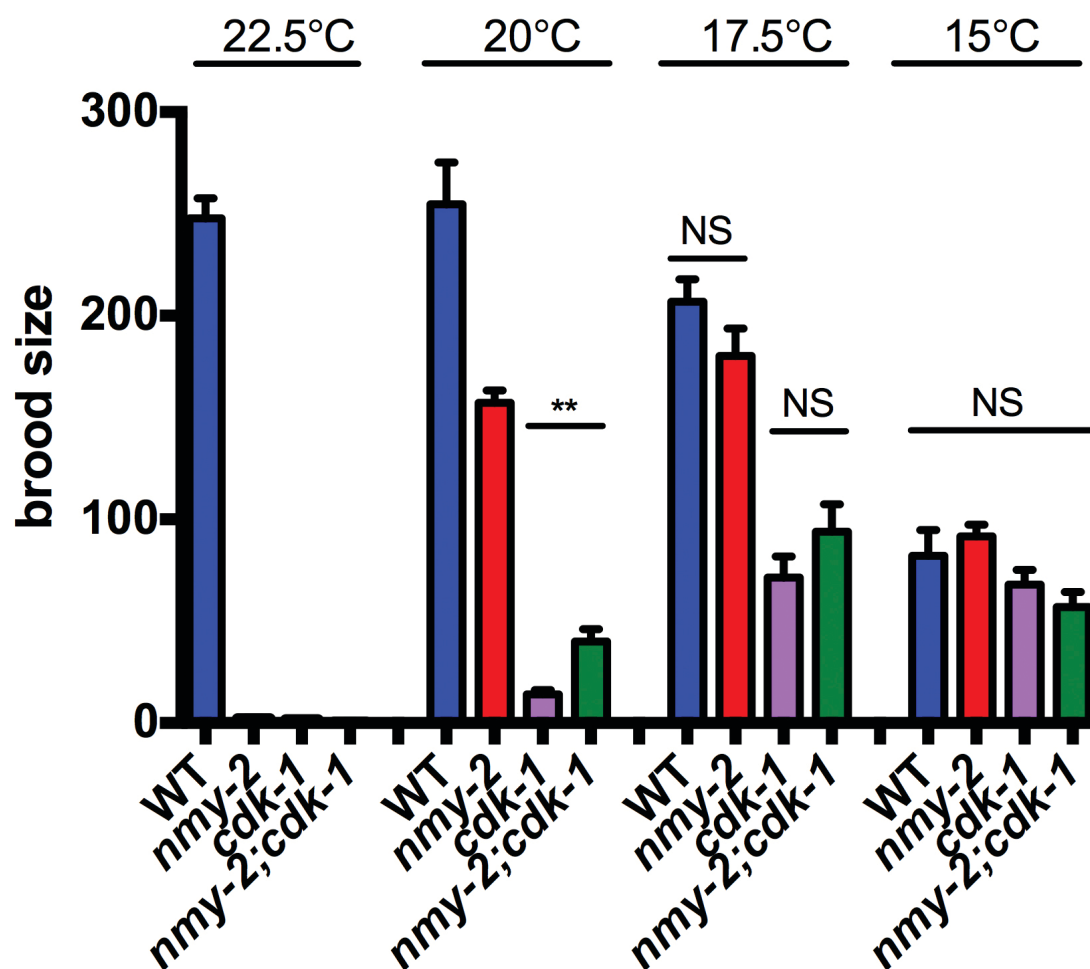


Figure 6.2: *cdk-1* embryonic lethality is partially rescued by *nmy-2* at 20°C.

Brood sizes were determined for each strain at the indicated temperatures. The strains used here were AW335, AW785, AW1103, and AW1107. $n > 10$ for each sample; error bars are the SEM; unpaired T-test was used to determine significance (ns: $p > 0.05$; **: $p < 0.01$). Two independent replicates of the experiment were performed.

6.2.3 *nmy-2* sensitizes *cdk-1*-related enlarged nucleus phenotype

A noticeable phenomenon in the *cdk-1* mutant was the enlarged SC nucleus (Figure 6.3A, arrows). It was seen in about 10% of the terminal SCs (Figure 6.3B, solid purple bar) in mutant worms that had undergone post-embryonic development at the restrictive temperature. SCs with enlarged nuclei were observed not only in young adults but also during earlier larval stages (data not shown). Despite the enlarged nuclei, SC numbers were WT at each larval stage in the *cdk-1* mutant (Figure 6.1, purple bars). These enlarged nuclei are reminiscent of the phenotypes seen in other *cdk-1* mutants, where normal DNA synthesis takes place despite failed mitotic entry (Boxem et al., 1999).

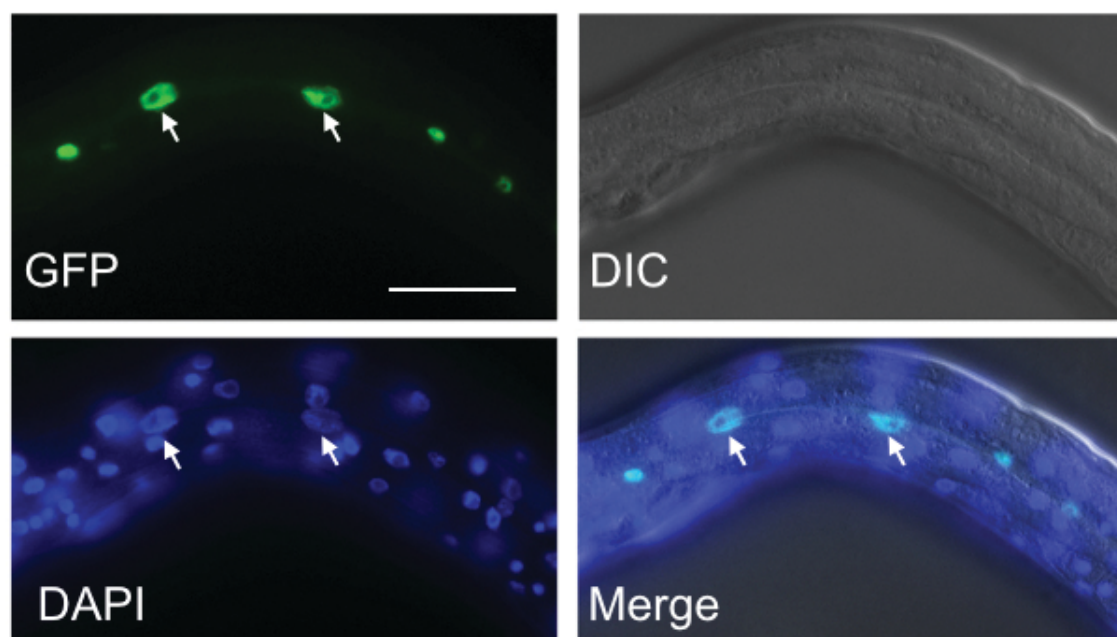
However, if mitotic entry was inhibited then the mutant would not have had WT SC numbers. Indeed SC with enlarged nuclei divided and differentiated as normal (presented in the next section) and there was no failed mitotic entry, rendering the appearance of these enlarged nuclei enigmatic.

The penetrance of enlarged SC nucleus was quantified, and *nmy-2* appeared to sensitize this *cdk-1* phenotype. Figure 6.3B shows that none of the strains had the enlarged nucleus phenotype at the permissive temperature of 15°C. At 25°C, 13% of the terminal SCs had an enlarged nucleus in the *cdk-1* mutant (purple solid bar). The addition of either *nmy-2* RNAi (purple hashed bar) or

nmy-2 ts mutation (green solid bar) to the *cdk-1* mutant roughly doubled the incidence of an enlarged nucleus to about 25% overall, even though *nmy-2* perturbation alone did not produce this phenotype. Applying both *nmy-2* RNAi and ts mutation to the *cdk-1* mutant led to an enlarged nucleus in 40% of all terminal SCs (green hashed bars), which is three times the incidence in the *cdk-1* mutant alone. Note that terminal SCs with enlarged nucleus were quantified as a percentage of total terminal SCs for each strain. Since the respective total SC numbers for the last four bars in Figure 6.3B were 16, 16, 16 and 15, the percentages roughly reflect overall penetrance in absolute numbers as well.

Therefore, *nmy-2* RNAi and mutation both sensitized *cdk-1*-related enlarged nucleus phenotype and the individual contributions from RNAi versus mutation were additive. In the absence of *cdk-1* mutation, neither *nmy-2* RNAi nor mutation alone produced enlarged SC nuclei. These results further suggest interactions between *nmy-2* and *cdk-1* in yet a different context, except this time *nmy-2* aggravates the *cdk-1* phenotype (negative epistasis) rather than conferring rescue (positive epistasis).

A



B

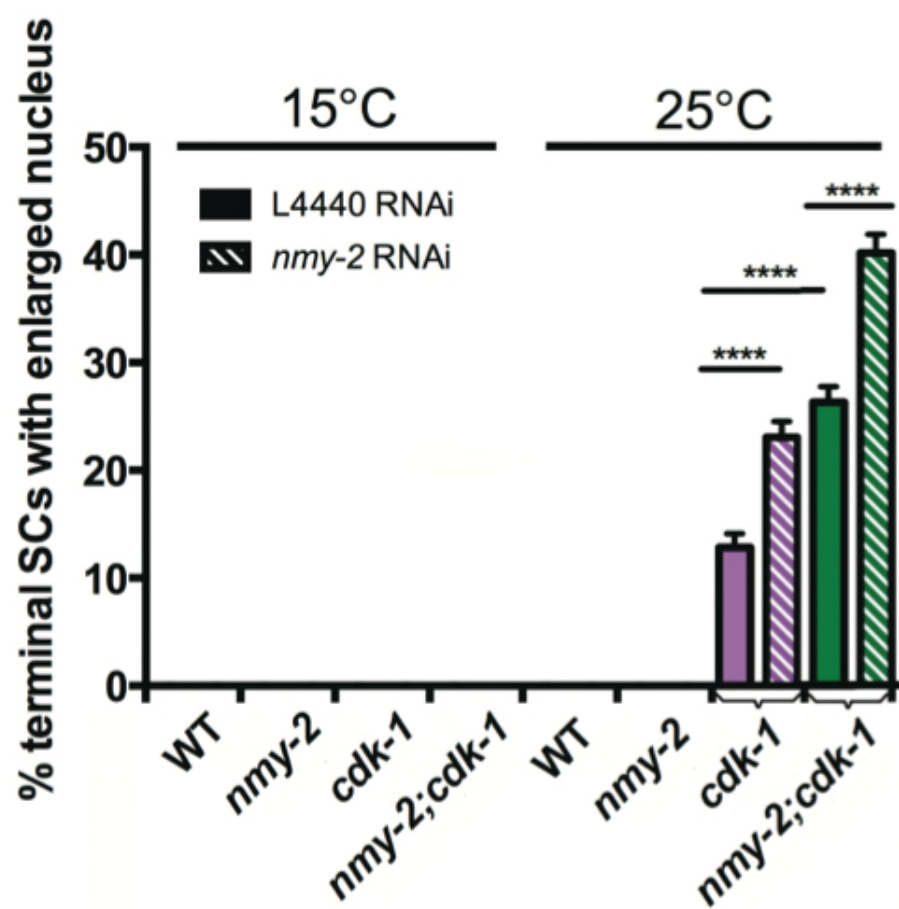


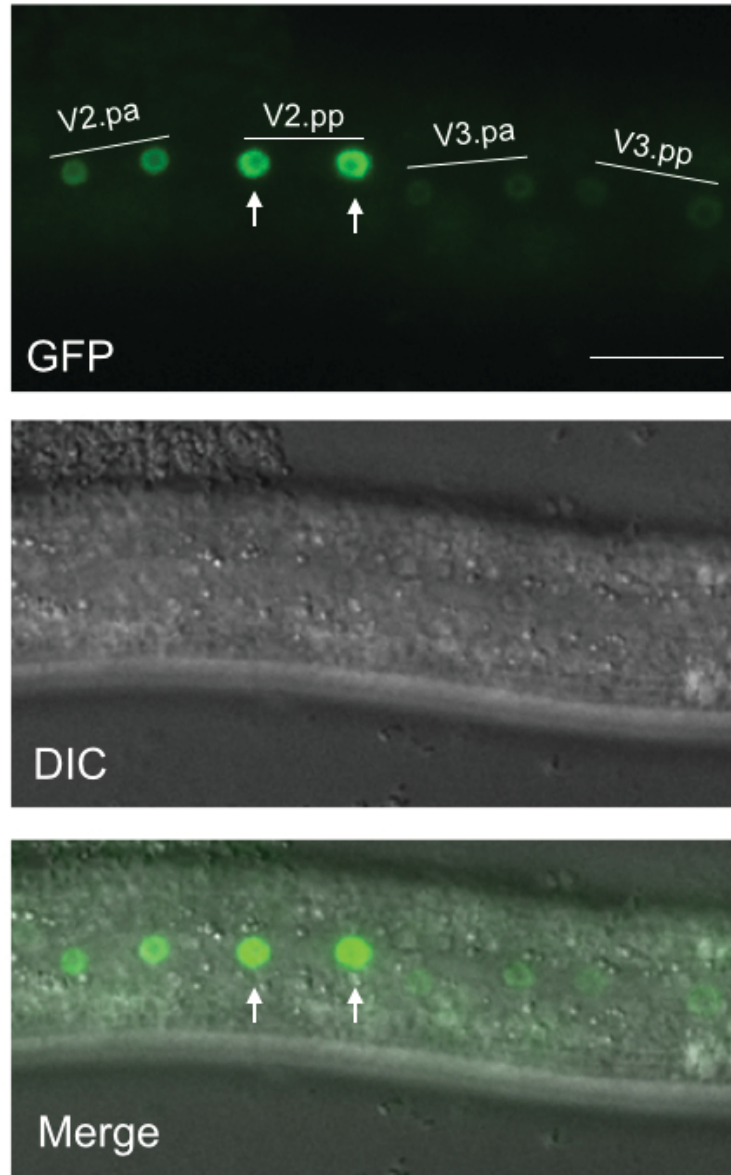
Figure 6.3: The *cdk-1* mutant has enlarged terminal SC nuclei and the phenotype is sensitized by *nmy-2*. **A)** White arrows: some terminal SC nuclei were enlarged in the *cdk-1* mutant at 25°C. Scale bar = 20 µm. **B)** Quantification of the enlarged nucleus phenotype. The four strains with the *scmp::gfp* marker were bleach synchronized to L1 and incubated at 15°C or 25°C on control or *nmy-2* RNAi. Terminal SCs with enlarged nucleus were quantified as a percentage of total terminal SCs for each strain. The strains used here were AW335, AW785, AW1103, and AW1107. n>50 for each sample. Error bars are the SEM. Unpaired T-test was used to determine significance (****:p<0.0001). At least three independent replicates of the experiments were performed.

6.2.4 SCs with enlarged nuclei divide and differentiate as normal

Curiously, SCs with enlarged nuclei appeared to execute divisions normally despite the extra genetic material. Figure 6.4A shows a worm just after the L2.2 asymmetric division. The V2.pp-derived daughters both had enlarged nuclei (arrows) compared to the neighboring V2.pa daughters, so they presumably arose from a V2.pp cell with an already-enlarged nucleus, which was capable of normal SC division. Additionally, terminal hypodermal cell numbers were counted using the *dpy-7p::2xNLS::yfp* marker in the *cdk-1* mutant (Figure 6.4B). Because SCs with enlarged nuclei were already observed in early larval divisions, if they divided normally to contribute hypodermal cells and self-renew, then the terminal hypodermal cell numbers would be normal. This was indeed the case. *cdk-1* mutant animals at 25°C had the same number of hypodermal cells as WT worms, suggesting that all SC divisions took place as normal despite the nuclear enlargement in a subset of these cells. The strain used here has both the *dpy-7p::2xNLS::yfp* and the *scmp::gfp* markers (plus *wrt-2p::gfp::PH* to outline the seam), so it was possible to simultaneously see enlarged SC nuclei and the normal number of hypodermal cells in the same *cdk-1* mutant animal. Terminal SCs with

enlarged nuclei also produced WT alae (data not shown) and were therefore capable of terminal differentiation.

A



B

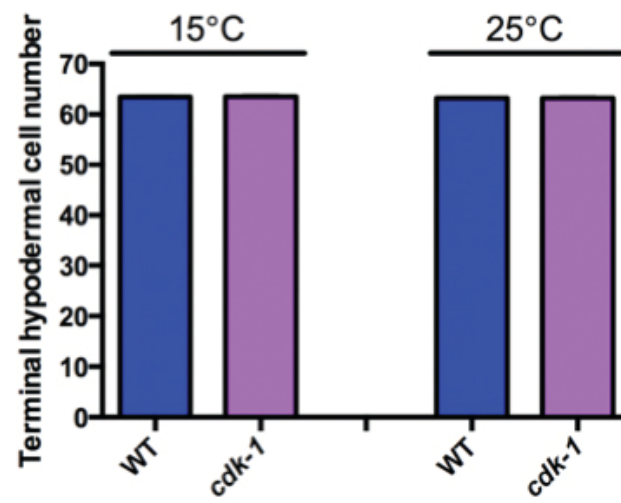


Figure 6.4: Seam cells with enlarged nuclei divide normally. **A)** A *cdk-1* mutant with *scmp::gfp* (AW1103) was placed at 25°C at the L1 stage. After the L2 asymmetric division the V2.pp daughters had enlarged nuclei (white arrows), presumably because they were derived from an enlarged V2.pp nucleus that divided normally. **B)** WT and the *cdk-1* mutant with *scmp::gfp* and *dpy-7p::2xNLS::yfp* markers were bleach synchronized to L1 stage and placed at appropriate temperatures on OP50 plates. Terminal hypodermal cell numbers were counted in the medial plane. The strains used in B were AW1015 and AW1150. n>30 for each sample. Error bars (too small to be seen) are the SEM. Unpaired T-test was used to determine significance and none of the four data groups is significantly different from any other group. Two independent replicates of the experiments were performed.

6.2.5 *wrm-1* and *cdk-1* show genetic interaction in the seam

In the four-cell stage *C. elegans* EMS blastomere, CDK-1 directly phosphorylates WRM-1 to preferentially displace it from the posterior cortex, thereby exposing an intrinsic polarity cue and allowing for the spindle rotation and division axis specification of that division (Kim et al., 2013). Cortical localization of WRM-1 is also important for SC divisions. WRM-1 localizes to the anterior cortex of SCs prior to an asymmetric division, so that the anterior daughter from the division inherits more cortical WRM-1 than the posterior daughter. Cortical WRM-1 asymmetry translates into the WRM-1 and POP-1 nuclear asymmetries, which ultimately specify different fates for the seam daughters. Therefore given the interaction between CDK-1 and cortical WRM-1 in the EMS, I sought to investigate their potential interactions in SC divisions.

A *wrm-1 cdk-1* double mutant strain was constructed (see Section 2.1.3.2 for the detailed crossing strategy) and the terminal SC numbers were counted in WT, *wrm-1* and *cdk-1* single mutants, and the *wrm-1 cdk-1* double mutant. The experiment took place at 25°C rather than at 26.5°C, because the *nmy-2;wrm-*

1 experiment previously demonstrated the masking effect of *wrm-1* mutation at 26.5°C in terms of SC number reduction (Figure 4.8A). At 25°C, while either *cdk-1* or *wrm-1* mutation alone had little effects on the terminal SC number, the double mutant only had ~13 SCs. This synergistic SC reduction suggests genetic interactions between *cdk-1* and *wrm-1* in seam cell development.

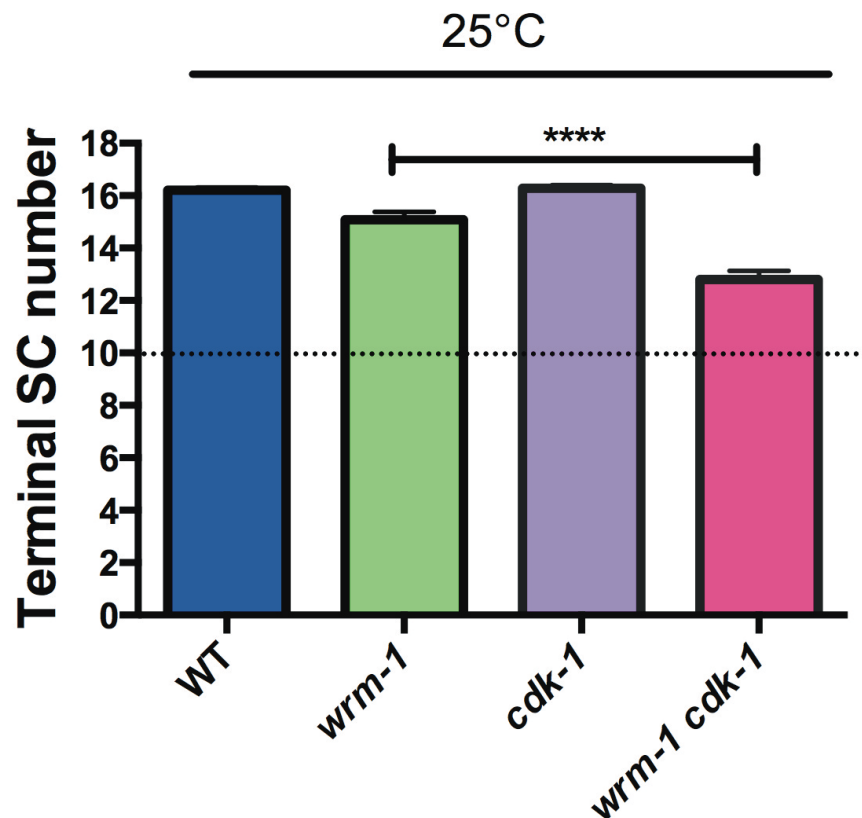


Figure 6.5: *wrm-1* and *cdk-1* show genetic interaction in the seam at 25°C. WT, *wrm-1* and *cdk-1* single mutants, and the *wrm-1 cdk-1* double mutant, all with the *scmp::gfp* marker, were synchronized to L1 and incubated at 25°C. Terminal SC numbers were counted in young adults. The strains used here were AW335, EW95, AW1103, and AW1160. $n > 50$ for each sample. Dashed line denotes the 10 SCs that the worms hatch with. Error bars are the SEM. Unpaired T-test was used to determine significance (****: $p < 0.0001$). Two independent replicates of the experiments were performed.

6.3 Discussion

6.3.1 *cdk-1* and *nmy-2* show genetic interaction in multiple contexts

In this chapter I used epistasis analysis to generate several lines of evidence for genetic interaction between *nmy-2* and *cdk-1* from embryogenesis to post-embryonic seam development. *nmy-2* partially rescued the *cdk-1* embryonic lethality (Figure 6.2), while *cdk-1* partially rescued the *nmy-2* loss of SC phenotype across multiple asymmetric divisions (Figure 6.1). The molecular bases of these rescues are completely unknown at the present and await further elucidation. The *cdk-1*-associated enlarged SC nucleus phenotype was enhanced by *nmy-2* (Figure 6.3). However, these SCs with nuclear enlargement divided as normal to generate normal terminal hypodermal numbers (Figure 6.4) and underwent proper terminal differentiation to produce WT alae. These data are very intriguing and beg an explanation for how *nmy-2* and *cdk-1* might interact during *C. elegans* development.

It is known that seam-derived hypodermal cells undergo endoreduplication and become tetraploid shortly after they are born, before they fuse with the hypodermal syncytium (Hedgecock and White, 1985). Perhaps the SCs in the *cdk-1* mutant inappropriately received instructions to endoreduplicate, possibly because they were mistaken for newborn hypodermal cells, without actually becoming hypodermal. Endoreduplication is thought to be the major mechanism for body size increase during post-embryonic development. It is under the control of the TGF- β growth signaling pathway, possibly via cyclin E regulation (Lozano et al., 2006). Intestinal nuclei, for example, undergo several

rounds of endoreduplication during larval development and increase the ploidy to 32C by adulthood (Hedgecock and White, 1985). It will be worthwhile to determine the ploidy of enlarged SC nuclei in the *cdk-1* mutant by DAPI staining and fluorescence intensity quantification (Lozano et al., 2006). Because the sizes of these enlarged nuclei were variable (data not shown), perhaps the ploidy of very large SC nuclei would exceed the tetraploidy that is associated with the seam-derived hypodermal nuclei. Subsequently, one may investigate how the *cdk-1* mutation, with no obvious defect in promoting mitotic entry, may lead to inappropriate endoreduplication in SCs, and how *nmy-2* perturbation may enhance this *cdk-1* phenotype.

6.3.2 CDK-1 is likely to work in parallel to WRM-1 in the seam

Genetic interaction between *cdk-1* and *wrm-1* was demonstrated in the seam, as the double mutant showed synergistically reduced SC numbers compared to the single mutants (Figure 6.5). This normally suggests that the gene products either work in parallel pathways or in the same pathway as a physical complex. It is known that WRM-1 forms a stable complex with LIT-1 kinase to phosphorylate POP-1 (Rocheleau et al., 1999), so it is unlikely that it also forms a complex with CDK-1 in the seam. Instead, CDK-1 likely functions in parallel to WNT signaling to regulate seam development.

Given that CDK-1 directly phosphorylates cortical WRM-1 in the EMS division (Kim et al., 2013), I originally hypothesized that this may also be the case in SC divisions to dissociate WRM-1 from the posterior cortex. Such relationship

between these two proteins may be demonstrated if one of the genes is epistatic to the other. Instead, my data showed synergistic results in the double mutant, suggesting that CDK-1 phosphorylation of WRM-1 is not the case in SCs. WRM-1 release from the EMS posterior cortex is key to unmasking the spatial cues for spindle rotation in that division. CDK-1 is thought to tightly couple this process to the cell cycle as the early embryo undergoes rapid cell divisions and patterning simultaneously (Kim et al., 2013). Spindle rotation and the necessity for cell cycle-coupling seen in embryogenesis is unlikely to be relevant for seam development, thus CDK-1 may not phosphorylate cortical WRM-1 in the SCs as it does in the EMS. Furthermore, Kim et al., (2013) noted the possibility that WRM-1 phosphorylation by CDK-1 in the EMS may occur in a signal-dependent manner. Perhaps P2/EMS signaling modifies WRM-1 at the P2/EMS junction (the posterior cortex of the EMS cell) to enable the subsequent recognition by CDK-1 at the appropriate timing. If this is the case, then the CDK-1 phosphorylation of WRM-1 is likely to be a unique scenario in the EMS division, without being applicable to the seam.

Embryonic lethality may be assessed, as a proof of concept, to demonstrate CDK-1 and WRM-1 interaction during embryogenesis. Brood sizes for *cdk-1* and *wrm-1* single mutants, but not the *wrm-1 cdk-1* double mutant, had already been analyzed at various temperatures (Figure 4.8, Figure 6.2). Given the known direct interaction between CDK-1 and WRM-1 in the four-cell stage embryo, one would expect that the double mutant has a brood size similar to either single mutant at an intermediate temperature between 15°C and 25°C, i.e. demonstrating that one gene is epistatic to the other.

In conclusion, under the recent notion that cell cycle progression and asymmetric divisions can be intricately coupled, I have demonstrated potential cell cycle-independent roles of *cdk-1* in regulating seam cell development via its interactions with both *nmy-2* and *wrm-1*. Little mechanistic exploration of how these interactions may occur has been pursued at this point, but the work should certainly be carried forth in the future given the enticing preliminary data.

Chapter 7 Final summary and remarks

7.1 Key findings

In this study I explored the role of NMY-2 in *C. elegans* seam cell divisions. I found that *nmy-2* was expressed strongly in the seam in late embryogenesis and the expression level decreased during larval development. NMY-2 cortical and cytoplasmic localization pattern was highly dynamic during post-embryonic SC divisions. Post-embryonic *nmy-2* knockdown using a combination of RNAi treatment and the *ts* alleles resulted in progressive SC loss after each asymmetric division. This led to a significantly reduced terminal SC count, indicating that *nmy-2* plays a role in seam development.

Three classes of cell fate transformations occurred in *nmy-2* knockdown and generated a net loss of SCs. At least two of these phenotypes were associated with cytokinesis failures and involved polyploid cells, indicating that proper cytoplasm segregation is important for ensuring the correct fate specification in SC divisions. However, cytokinesis failure alone did not fully explain the *nmy-2* knockdown phenotypes, because depleting other cytokinesis pathway components by RNAi resulted in the opposite phenotype, i.e. more seam cells, suggesting that NMY-2 has crucial cytokinesis-independent roles in the seam. Epistasis analysis revealed that *nmy-2* may function as a component of the RNT-1/BRO-1 pathway to regulate SC development in parallel to the WNT/ β -catenin asymmetry pathway.

Spindle orientation and positioning appeared unaffected by *nmy-2* perturbation. Analysis of the WT divisions revealed that the displacement of the nucleus along the A-P axis did not influence the cell fate outcome of divisions. By contrast, anterior displacement of the cleavage furrow in the L2.2 division might indicate a potential mechanism to ensure the asymmetry of this division. The furrow may potentially owe its displacement to cortical NMY-2 polarity.

Finally, genetic interactions between *nmy-2* and *cdk-1* and between *wrm-1* and *cdk-1* were established in various contexts including seam development. *cdk-1* thus represents a potential link between cell cycle regulation and fate specification in the seam.

7.2 The *C. elegans* embryo and the *Drosophila* neuroblast systems provide useful insights into *C. elegans* seam cell asymmetric divisions

We have gained invaluable insights into how asymmetric divisions are achieved and regulated from elegant studies of the *C. elegans* early embryo and the *Drosophila* neuroblasts. When studying asymmetric divisions, these two models are often referred to for formulating hypotheses and designing experimental approaches. Some molecular and physical mechanisms are remarkably conserved between the two systems, including the PAR-3/PAR-6/PKC complex and the heterotrimeric G protein and dynein-dependent force generation mechanism. These conserved elements may also be employed by

other systems, although neither example above has been studied in *C. elegans* seam cells yet.

Both the *C. elegans* embryo and the *Drosophila* NBs also relate to *C. elegans* seam cells in their own way. The *C. elegans* embryo and seam cells obviously concern the same organism, therefore certain molecular players can be seen to function in both embryogenesis and post-embryonic development. The WNT/ β -catenin asymmetry pathway, for instance, is crucial for both the EMS division at the four-cell stage and for SC asymmetric divisions in the developing larvae. Another example is GATA factors EGL-18 and ELT-6, which are required during embryogenesis to specify SCs and re-used during larval development to maintain their progenitor fate during SC divisions (Gorrepati et al., 2013). Thus when studying SCs, it is often useful to examine factors that are known to play a role in embryogenesis. One key player in the one-cell and four-cell stage embryonic divisions is NMY-2. The subsequent endeavor to understand the potential role of NMY-2 in SC development led to the conception of this thesis project.

The *Drosophila* NBs, on the other hand, provide an excellent parallel system to study stem-like divisions. While the *C. elegans* early embryonic divisions allowed us to learn a great deal about asymmetry divisions, those divisions are not stem-like and involve peculiarities such as the zygotic pseudocleavage, which is hardly relevant to any other system. By contrast, *Drosophila* NBs and *C. elegans* SCs both divide in a stem-like manner. Their reiterative asymmetric divisions always produce a daughter that acquires a distinct fate while the

progenitor self-renews. Therefore even though *Drosophila* NBs belong to a different organism, they offer a more appropriate comparison to *C. elegans* SCs in terms of stem-like divisions.

7.3 *nmy-2* knockdown has pleiotropic effects

A method was developed in this work to robust knock down *nmy-2* post-embryonically. Both RNAi and the ts mutation were required to achieve this knockdown, which was laborious and potentially limiting as discussed in Section 3.3.3. However, in hindsight, this combined regime does have the advantage of thoroughly perturbing *nmy-2* on both the mRNA and the protein level. My data suggested that *nmy-2* transcription decreases as larval development progresses, and myosin II is known to be extensively regulated post-translationally by various kinases and phosphatases. Thus using both the RNAi to destroy the mRNA and the ts mutation to interfere with the protein function enabled myosin II disruption on multiple levels, which each treatment alone would not be able to offer.

Myosin II is known to be involved in a wide range of biological processes downstream of multiple signaling pathways. Consistent with this knowledge, one of the themes that emerged from this work is that *nmy-2* knockdown produces pleiotropic phenotypes. A prevalent but undesired effect is cytokinesis failures, as discussed in Section 4.3.4. Although I had established that the cytokinesis involvement is not solely responsible for the *nmy-2* knockdown seam phenotypes, it presented a challenge to infer additional

NMY-2 functions from the gross phenotypes. The precise function of NMY-2 in the seam has not yet been fully determined, but this work supports that it may play multiple roles, including trafficking molecules (potentially RNT-1/BRO-1 pathway components), generating cortical contractility (potentially for reducing the size of the anterior daughter during and/or after an asymmetric division), and finally, executing cytokinesis. As a result, the protein localization pattern and the knockdown phenotypes were complex, and additional experiments were required to interpret these initial results by partially dissecting *nmy-2*'s multiple roles.

7.4 The power of lineage analysis and direct visualization

One major advantage of *C. elegans* as a model organism is its invariant cell lineage, which allows aberrant phenotypes to be traced back to single cells. Lineage analysis and direct visualization of cell fate changes are extremely powerful analytic tools, but they were under-used in this study. Quite a few speculations from my work may gain unequivocal support if supplemented by lineage analysis. For example, a main finding of my study was that *nmy-2* knockdown causes SC loss through inappropriate acquisition of the hypodermal fate. Although the interpretation of snapshot images seemed reasonable, it is worthwhile to directly follow through the cell lineage for confirmation.

Lineaging had not been performed in this study because it is not a trivial task, especially due to the penetrant and complex effects of *nmy-2* knockdown.

Because cell fate transformations occurred all along the length of the worm body and across different larval stages, a complete *nmy-2* knockdown lineage analysis would necessarily include all of the pertinent cells throughout larval development (at least from L2 onwards). Considering the pleiotropic effects of *nmy-2* knockdown and multiple classes of fate transformation phenotypes, the full *nmy-2* seam lineage will likely show large variations between individual animals, rendering the task even more difficult and potentially inconclusive. Nevertheless, even if the full lineage analysis is not performed, in the future it will still be beneficial to follow through some divisions on a smaller scale to verify different cases of *nmy-2*-related cell fate transformations.

7.5 Each seam cell at each division may represent a unique scenario

Another potential obstacle to this study is the sheer complexity of SC development. The ten SCs that the worm has at hatching could all potentially be different to one another. Even though V1-4 and V6 share the same division pattern, they may still have differences that do not contribute to a distinctive lineage outcome per se. For example, while genes like *mab-5* and *pal-1* (Hunter et al., 1999; Gilbert and Woollard, personal communication; Kenyon, 1986) control posterior SC development, perturbations of *ceh-20* or *unc-62* lead to seam hyperplasia primarily in the anterior (Hughes et al., 2013). Furthermore, it has been proposed that WNT signaling in the seam may employ a gradient of WNT ligands and/or receptors along the A-P axis (Gleason and Eisenmann, 2010), so the more anterior V cells may be

regulated differently than the posterior ones. Therefore grouping V1-4 together and treating them as a single lineage, as I did for the spindle positioning analysis in Chapter 5, may obliterate subtle differences amongst these individual SCs. For example, I originally did not distinguish between the anterior versus posterior branch-derived daughters in the WT spindle positioning dataset. This led to the inappropriate conclusion that the cell nuclei were more or less centered prior to the L2.2 and the L3 asymmetric divisions (data not shown). Had I not subsequently separated these cells based on actual observations, the “cross-eye” phenomenon and the maintenance of branch-identity across larval stages would have been completely overlooked. Again, a major merit of *C. elegans* is the feasibility of analyzing phenotypes at single cell resolution. While grouping some seemingly identical V lineage cells together reveals general trends about these cells, the finer resolution of cellular analysis may be lost.

In addition, the four asymmetric divisions that SCs undergo during larval development may all be different as well. The L1 division appears to be particularly robust. Mutagenesis screens in the past had generated a number of mutants with SC phenotypes, but few mutations perturb the L1 division (Alison Woollard, personal communication). The L2 asymmetric division is temporally and spatially unique in that it immediately follows the L2 symmetric division, with no molting in between for the worm to grow in size. Moreover, different sets of heterochronic genes govern stage-specific events at different larval stages, so the L3 and L4 divisions may also be subjected to different

regulations from each other. In short, one must be cautious to generalize about SC divisions, as each cell and each division may be unique.

Appendix

Table 3: List of *C. elegans* strains used in this study

(in the order in which they appeared in Chapters 3-6)

Strain	Genotype
N2	wild-type
AW1118	<i>unc-119(ed3)III;ouEx849[nmy-2p::pes-10p::gfp(pAW850)+unc-119+]</i>
AW1120	<i>unc-119(ed3)III;ouEx851[nmy-2p::pes-10p::gfp(pAW850)+unc-119+]</i>
JJ1473	<i>unc-119(ed3)III;zuls45[nmy-2p::nmy-2::gfp+unc-119+]V</i>
AW1049	<i>ouls15[bro-1CNE+pes-10p::nmy-2::gfp,ajm-1p::mCherry]</i>
AW876	<i>unc-119(ed3)III; ouEx750[bro-1CNE+pes-10p::nmy-2::gfp, ajm-1p::mCherry, unc-119+]</i>
LP162	<i>nmy-2(cp13[nmy-2::gfp + LoxP])I</i>
AW1092	<i>nmy-2(cp13[nmy-2::gfp + LoxP])I;ouls21[ajm-1p::mCherry]</i>
JR667	<i>unc-119(e2498::Tc1)III;wls51[scmp::gfp+unc-119+]V</i>
AW335	<i>wls51[scmp::gfp] him-5(e1490)V</i>
AW785	<i>nmy-2(ne3409)I; wls51[scmp::gfp], him-5(e1490)V</i>
AW786	<i>nmy-2(ne1490)I; wls51[scmp::gfp], him-5(e1490)V</i>
SV1009	<i>hels63[wrt-2p::gfp::PH,wrt-2p::gfp::H2B,lin-48p::mCherry]</i>
AW788	<i>nmy-2(ne3409)I;him-5(e1490)V;hels63[wrt-2p::gfp::PH,wrt-2p::gfp::H2B,lin-48p::mCherry]</i>
AW789	<i>nmy-2(ne1490)I;him-5(e1490)V;hels63[wrt-2p::gfp::PH,wrt-2p::gfp::H2B,lin-48p::mCherry]</i>
AW1050	<i>ouls18[bro-1CNE+pes-10p::nmy-2::gfp, ajm-1p::mCherry]</i>
AW1052	<i>nmy-2(ne3409)I;wls51[scmp::gfp] him-5(e1490)V;ouls18[bro-1CNE+pes-10p::nmy-2::gfp, ajm-1p::mCherry]</i>
AW1095	<i>hels63[wrt-2p::gfp::PH,wrt-2p::gfp::H2B,lin-48p::mCherry];ouls21[ajm-1p::mCherry]</i>
AW1116	<i>nmy-2(ne3409)I;hels63[wrt-2p::gfp::PH,wrt-2p::gfp::H2B,lin-48p::mCherry];ouls21[ajm-1p::mCherry]</i>
AW1015	<i>ouls10[scmp::NLS::tdTomato(pAW584)+wrt-2p::gfp::PH(pAW561)+dpy-7p::2xNLS::yfp(pAW516)]</i>
AW1018	<i>nmy-2(ne3409)I;ouls10[scmp::NLS::tdTomato+wrt-2p::gfp::PH+dpy-7p::2xNLS::yfp]</i>
AW1140	<i>ouls22[bro-1CNE+pes-10p::NLS::tdTomato(pAW582)+wrt-2p::gfp::PH(pAW561)+dpy-7p::2xNLS::yfp(pAW516)+ajm-1p::mCherry(pAW373)]</i>
AW1097	<i>ouls10[scmp::NLS::tdTomato+wrt-2p::gfp::PH+dpy-7p::2xNLS::yfp];ouls21[ajm-1p::mCherry]</i>
AW1106	<i>nmy-2(ne3409)I;ouls10[scmp::NLS::tdTomato+wrt-2p::gfp::PH+dpy-7p::2xNLS::yfp];him-5(e1490)V;ouls21[ajm-1p::mCherry]</i>
EW95	<i>wrm-1(ne1982)III;wls51[scmp::gfp]V</i>
AW861	<i>nmy-2(ne3409)I;wrm-1(ne1982)III;wls51[scmp::gfp] him-5(e1490)V</i>
AW862	<i>nmy-2(ne1490)I;wrm-1(ne1982)III;wls51[scmp::gfp] him-5(e1490)V</i>
AW943	<i>lit-1(or131)III;wls51[scmp::gfp] him-5(e1490)V</i>

AW983	<i>nmy-2(ne3409)I;lit-1(or131)III;wls51[scmp::gfp] him-5(e1490)V</i>
JK3437	<i>him-5(e1490)V;qIs74[sys-1p::pop-1::gfp,unc-119(+)]</i>
AW950	<i>nmy-2(ne3409)I;him-5(e1490)V;qIs74[sys-1p::pop-1::gfp]</i>
AW951	<i>unc-119(ed3)III;ouEx773[pCE-BiFC-nmy-2-VN173(pAW811)+pCE-BiFC-wrm-1-VC155(pAW812)+unc-119+]</i>
AW952	<i>unc-119(ed3)III;ouEx774[pCE-BiFC-nmy-2-VN173(pAW811)+pCE-BiFC-wrm-1-VC155(pAW812)+unc-119+]</i>
AW953	<i>unc-119(ed3)III;ouEx775[pCE-BiFC-nmy-2-VN173(pAW811)+pCE-BiFC-wrm-1-VC155(pAW812)+unc-119+]</i>
AW954	<i>unc-119(ed3)III;ouEx776[pCE-BiFC-nmy-2-VN173(pAW811)+pCE-BiFC-wrm-1-VC155(pAW812)+unc-119+]</i>
AW955	<i>unc-119(ed3)III;ouEx777[pCE-BiFC-nmy-2-VN173(pAW811)+pCE-BiFC-wrm-1-VC155(pAW812)+unc-119+]</i>
AW956	<i>unc-119(ed3)III;ouEx778[pCE-BiFC-nmy-2-VN173(pAW811)+pCE-BiFC-wrm-1-VC155(pAW812)+unc-119+]</i>
AW957	<i>unc-119(ed3)III;ouEx779[pCE-BiFC-nmy-2-VN173(pAW811)+pCE-BiFC-wrm-1-VC155(pAW812)+unc-119+]</i>
AW958	<i>unc-119(ed3)III;ouEx780[pCE-BiFC-nmy-2-VN173(pAW811)+pCE-BiFC-wrm-1-VC155(pAW812)+unc-119+]</i>
AW959	<i>unc-119(ed3)III;ouEx781[pCE-BiFC-nmy-2-VN173(pAW811)+pCE-BiFC-wrm-1-VC155(pAW812)+unc-119+]</i>
CB5253	<i>rnt-1(e1241) dpy-5(e61) unc-13(e51)I</i>
AW989	<i>dpy-5(e61) rnt-1(e1241) nmy-2(ne3409)I;wls51[scmp::gfp] him-5(e1490)V</i>
AW990	<i>dpy-5(e61) rnt-1(e1241)I;wls51[scmp::gfp] him-5(e1490)V</i>
AW991	<i>dpy-5(e61), nmy-2(ne3409)I; wls51[scmp::gfp] him-5(e1490)V</i>
AW992	<i>dpy-5(e61)I; wls51[scmp::gfp] him-5(e1490)V</i>
AW1103	<i>cdk-1(ne2257)III;wls51[scmp::gfp] him-5(e1490)V</i>
AW1107	<i>nmy-2(ne3409)I;cdk-1(ne2257)III;wls51[scmp::gfp] him-5(e1490)V</i>
AW1150	<i>cdk-1(ne2257)III;wls51[scmp::gfp]V;ouls10[scmp::NLS::tdTomato+wrt-2p::gfp::PH+dpy-7p::2xNLS::yfp]</i>
CB185	<i>lon-1(e185)III</i>
AW1154	<i>lon-1(e185),cdk-1(ne2257)III;wls51[scmp::gfp]V</i>
AW1160	<i>wrm-1(ne1982) cdk-1(ne2257)III;wls51[scmp::gfp] him-5(e1490)V</i>

* I have generated all except the shaded strains during the course of this study. Of the shaded strains, AW335 was previously generated in our lab, CB5253 was a gift from the Hodgkin Lab (University of Oxford), and the rest of were obtained from the *Caenorhabditis* Genetics Center.

Table 4: Primers used in this study

Purpose	Name	Sequence (5' - 3')
Genotyping	nmy-2-OF	GAAGGAATTCGTATATGCCGTCAAGG
	nmy-2-OR	AGATCTTCACAGCGTTCTTCCAAAGC
	ne3409-IF	GAAAGAAGCCGTCAGAAATTGTTTC
	ne3409-IR	TCAACCGAGATTCAATTGAATTTTTTTAGA
	ne1490-IF	ACAAGCAACAAATGGAAAAAATGGACGCGA
	ne1490-IR	AGTCTCGTCTTTAAAACCAGCCGATC
	lit-1-OF	CAAATGATTATCGATTTATTGGGAACCCCA
	lit-1-OR	CGCGAGGGGAAATTATCAATTACCTACCTT
	lit-1-IF	CGTCTGCGATTCCACTCGTGTATTTG
	lit-1-IR	ATTTGGTTTCGTATAGCAGCACGCGT
	wrm-1-OF	CCAAAGCCATACAGTATCTATGAGATCG
	wrm-1-OR	GACGTTCTGACATATCTTTGATAGTTGC
	wrm-1-IF	TAGTAATTGAATTTGCAATTGGAAATCG
	wrm-1-IR	CTTTGACTTCGTTTCCGGTCTTCCCA
	rnt-1-OF	TCCAATAAACTGATGATTCCACCTGAGTAGTCTG
	rnt-1-OR	TGTATTATTCATTTGCGAGTTGTAGCAGCC
	rnt-1-IF	CCGAAAATTCCATTTGACAATCGTCAT
	rnt-1-IR	CCACCATCATCGGCGCCGAATATT
	cdk-1-OF	GCTGTGCGAGAGATCAGCTTGCTCAAAG
	cdk-1-OR	AACTCAGTCGGGTGTGCCGAGAACTCTGA
	cdk-1-IF	CCGATTTTCGGAATTGCCAGAGCTATTGATT
	cdk-1-IR	TTCATGCGTGTAACGCGAATCGGAAT
Molecular cloning	GFP-F	GGAGAAGAAGCTTTTCACTGGAGTTGTCC
	GFP-R	CCATGCCATGTGTAATCCCAGCAGCTGT
	M13-F	TGTA AACGACGGCCAGT
	M13-R	CAGGAAACAGCTATGACC
	nmy-2CDS1-F	ATGACATCATCTCGACAAAAAGATG
	nmy-2CDS1-R	GATATTGAACAATACGATAAACGGCG
	nmy-2CDS2-F	TCAACGCTACGAGAACTTCTTGCC
	nmy-2CDS2-R	TTCTCCATCAACGACGTCTCGG
	nmy-2CDS3-F	CAGAAAATTTTCGGCTGACAAGGC
	nmy-2CDS3-R	TTAGTTGCGAACTGAGTCGCG
	nmy-2CDS.AgeI-F	ACCGGTATGACATCATCTCGACAAAAAGATG
	nmy-2CDS.AgeI-R	ACCGGTGTTGCGAACTGAGTCGCG
	wrm1CDS.NcoI-F	CCATGGCAATGGATGTGGATTGCGCAGAAAC
	wrm1CDS.XhoI-R	CTCGAGCCATTAGTTGTCGATGATGCTGC
	SDnmy-1-F	AAACCAGATTGGAGAAGTTGAGC
	SDnmy-1-R	TTCTGTCTTCTGATTGAGAGTTTCG
	SDnmy-2-F	GTCAAAGAAGTCAAATCTCTGTTGG
	SDnmy-2-R	CGTTTGTCTTTTCTAACTGATCC
	nmy2p-F	TATCTGAAATTTGAAAAAAGTGAGTTATTTTTTTCG
	nmy2p-R	TATTACCGCTGGAGCTGTTGTTGTAATCA

Table 5: Plasmids constructed for this study

Plasmid	Description
pAW742	<i>SDnmy-1</i> RNAi in pCRII-TOPO II rev
pAW743	<i>SDnmy-2</i> RNAi in pCRII-TOPO II rev
pAW744	<i>SDnmy-1</i> RNAi in L4440
pAW745	<i>SDnmy-2</i> RNAi in L4440
pAW746	<i>bro-1CNE+pes-10p</i> in pDONR P1-P4 (Gateway fragment 1 of 3)
pAW747	<i>nmy-2</i> genomic DNA in pDONR P4r-P3r Gateway fragment 2 of 3)
pAW748	GFP in pDONR P3-P2 (Gateway fragment 3 of 3)
pAW749	<i>bro-1CNE+pes-10p::nmy-2::gfp</i> in pcDNA6.2v5PL-DEST
pAW804	<i>nmy-2</i> cDNA fragment 1 in TOPO XL fwd
pAW805	<i>nmy-2</i> cDNA fragment 2 in TOPO XL fwd
pAW806	<i>nmy-2</i> cDNA fragment 3 in TOPO XL fwd
pAW807	<i>nmy-2</i> cDNA fragment 1+2 in TOPO XL fwd
pAW808	<i>nmy-2</i> cDNA fragment 1+2+3 (FL) in TOPO XL rev
pAW809	<i>nmy-2</i> BiFC cDNA in TOPO XL
pAW810	<i>wrm-1</i> BiFC cDNA in TOPO XL
pAW811	<i>nmy-2</i> in pCE-VN173
pAW812	<i>wrm-1</i> in pCE-VC155
pAW849	<i>nmy-2</i> full-length promoter in TOPO XL fwd
pAW850	<i>nmy-2</i> full-length promoter in pPD107.94

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