Investigating a role for Myt1 kinase in the *Drosophila* intestinal epithelium

by

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Abstract

Myt1 kinase performs several functions during *Drosophila* development. Myt1 has firstly been described as a regulator of the G2/M DNA damage checkpoint in the developing wing disc. In addition, Myt1 has been shown to regulate several aspects of male and female gametogenesis, while also coordinating cell cycle exit of germline associated somatic cells. Many unknowns remain, however, including the mechanism by which Myt1 functions in somatic cells, as well as the degree to which this mechanism is conserved in other *Drosophila* tissues. Furthermore, a role for Myt1 in adult flies beyond gametogenesis has not yet been described.

The *Drosophila* adult intestinal epithelium has been shown to possess a population of multipotent stem cells that give rise to differentiated epithelial cell types. Given that the majority of these intestinal stem cells proceed regularly through the mitotic cycle, I reasoned that Cdk1 inhibitory phosphorylation must be required to maintain intestinal stem cell homeostasis. Furthermore, these cells frequently produce transient daughter cells known as EBs that exit the mitotic cell cycle to produce absorptive enterocytes in a Notch dependent manner. This system, therefore, provides an excellent opportunity to investigate the formerly described regulatory roles of Myt1 in a previously unexplored setting. In this thesis, I examine the function of Myt1 in the adult fly intestine and provide evidence that Myt1 is an essential regulator of intestinal homeostasis. I demonstrate that Myt1 regulates cell division in the intestinal epithelium, and also show that it is required to promote mitotic cell cycle exit in EBs, a normally post-mitotic cell. Furthermore, I demonstrate that Myt1 activity in the *Drosophila* intestine is dependent on Cyclin A/Cdk1 and provide evidence that regulation by Myt1 occurs in G1 phase, a phase in which Myt1 activity has never before been described *in vivo*.

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The heavens declare the glory of God; the skies proclaim the work of his hands.
Day after day they pour forth speech; night after night they reveal knowledge.
They have no speech, they use no words; no sound is heard from them.
Yet their voice goes out into all the earth, their words to the ends of the world.

Psalm 19:1-4

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List of Abbreviations

AurA-T288p: phospho-specific antibody to residue T288 of Aurora A

APC/C: Anaphase promoting complex/ cyclosome

Bam: Bag of Marbles

BMP: Bone morphogenetic protein

Cdks: Cyclin-dependent kinases

DMSO: Dimethyl sulfoxide

EB: Enteroblast

EC: Enterocyte

EE: Enteroendocrine cell

Esg: Escargot

EGFR: Epidermal growth factor receptor

ER: Endoplasmic reticulum

Fzr: Fizzy-related

G1: Gap/ growth phase 1

G2: Gap/ growth phase 2

GFP: Green fluorescent protein

GSC: Germline stem cell

ISC: Intestinal stem cell

JAK/STAT: Janus kinase/ signal transducer and activator of transcription

JNK: Jun-N-terminal kinase

M phase: Mitotic/ meiotic phase

MARCM: Mosaic analysis with a repressible cell marker

NRE: Notch response element

PBS: Phosphate-buffered saline

PCM: Pericentriolar material

PCNA: Proliferating cell nuclear antigen

PH3: Phosphorylated histone H3

Plk1: Polo like kinase 1

Pros: Prospero

RNAi: RNA interference

siRNA: small interfering RNA

S phase: Synthesis phase

Su(H): Suppressor of Hairless

TOR: Target of Rapamycin

VFP: Venus fluorescent protein

1. Introduction

1.1 Summary

Myt1 is a member of the Wee-like kinase family and regulator of the master mitotic kinase Cdk1. Studies in *Drosophila melanogaster* and cell culture have demonstrated the role of Myt1 in cell cycle regulation and development to be highly dynamic. Herein, I discuss cell cycle regulation by the Wee-like kinases in order to highlight areas of progress in understanding their complex functioning, as well as research questions that have yet to be answered.

1.2. Overview of the cell cycle

The most fundamental characteristics of all organisms are their abilities to grow and proliferate. Indeed, every creature, both unicellular and multicellular, is programmed first to mature, and then to multiply. But how do these things transpire? As L.L. Larison Cudmore once said, “Every living thing is made of cells, and everything a living thing does is done by the cells that make it up (1977)”. Most basically, reproduction and organismal growth rely on the cell cycle, where a cell grows, replicates its DNA, and subsequently divides into two daughter cells, each receiving equal DNA content. The fidelity of this process is critical to facilitate organismal health and survival, with errors in division and DNA replication leading to genome instability and disease.

The prototypical cell cycle progresses through four phases including an initial gap (or growth) phase (G1), the DNA synthesis phase (S), a second gap phase (G2), and division of the cell in mitosis or meiosis (M). Variations upon this cell cycle exist in numerous organisms and tissues, a few of which are discussed at a later point.

1.3. Cell cycle regulation by cyclin dependent kinases

Though simple in its objective, cell division is a highly complex and dynamic process. Before proceeding through mitosis, somatic cells must grow significantly in size and accurately replicate their nuclear DNA. Additionally, cells can progress to the next phase of the cell cycle

only after passing stringent checkpoints that ensure certain conditions have been met (Figure 1-1). For example, the spindle assembly checkpoint in M phase ensures that cells do not segregate chromosomes until every chromosome is properly attached to the spindle. Such checkpoints are necessary regulatory measures that prevent the cell from failing mitosis or producing unfit progeny.

Studies in the 1970s revealed that there must be diffusible molecules responsible for regulating the state of the cell cycle. This was demonstrated through cultured mammalian cells in S phase, that when fused to a cell in G1, caused the G1 cell to replicate its DNA (Rao and Johnson, 1970). Similarly, a cell in M phase fused to a cell passing through interphase caused the cell in interphase to condense its chromosomes, a sign of M phase entry (Rao and Johnson, 1970).

Among these diffusible regulatory molecules that mediate cell cycle phase transitions are the essential group of proteins known as the cyclin dependent kinases, or Cdks. Cdks are serine/ threonine protein kinases whose activity is required to facilitate progression from one phase of the cell cycle to the next. As their name implies, Cdks acts in complex with cyclins, proteins whose expression waxes and wanes throughout the cell cycle, only allowing Cdks to exert an effect in a bipartite state. Entry into S phase is coordinated through the activity of Cyclin E/Cdk2 (Koff et al., 1991), after which inactive Cyclin A/Cdk1, Cyclin B/Cdk1, and Cyclin B3/Cdk1 complexes begin to form in preparation for mitosis (Figure 1-1). Additional Cdks function throughout the cell cycle to mediate sequential phase advancement once the cell has met certain checkpoint requirements (Figure 1-1) (Edgar and Lehner, 1996; Nasmyth, 1996; O'Farrell et al., 1989). My research focuses on Cdk1, the major mitotic kinase that catalyzes the transition from G2 phase into M phase.

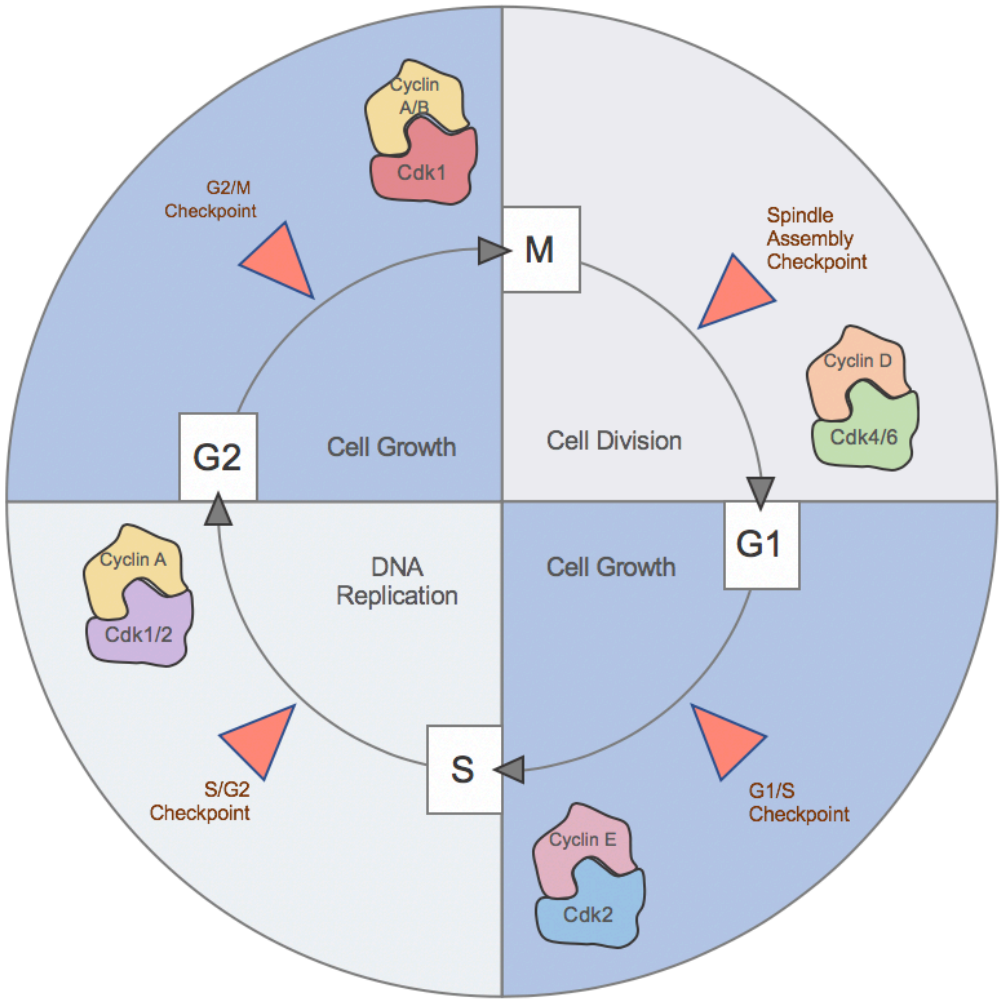

Figure 1-1. Phase advancement in the canonical cell cycle is driven by Cdk activity following checkpoint satisfaction. Both flies and vertebrates possess Cdk1, Cdk2, Cdk4 and Cdk6. Cyclin D can complex with Cdk4 or Cdk6 to promote G1 phase. Cdk2 in complex with Cyclin E promotes S phase entry only after the cell reaches an appropriate size and ensures no DNA damage is present in G1. The S/G2 phase checkpoint prevents Cyclin A/Cdk1 and Cyclin A/Cdk2 mediated progression into G2 phase until faithful completion of DNA replication. Cyclin A/Cdk1 and Cyclin B/Cdk1 promote M phase entry, but only if no DNA damage is present at the G2/M checkpoint. The spindle assembly checkpoint prevents anaphase entry until the mitotic spindle is correctly attached to the chromosomes.

1.4. Cyclin dependent kinase 1

Cdk1 was originally characterized in *Xenopus* egg extracts as the maturation-promoting factor (MPF) responsible for M phase entry. This was shown via microinjection experiments, where cytoplasmic extract from meiotic oocytes injected into G2-arrested eggs caused the G2-arrested eggs to enter meiosis (Masui and Markert, 1971). MPF was purified in the late 1980s by Manfred Lohka, who identified two components that were together capable of facilitating G2-arrested *Xenopus* egg entry into M phase (Lohka et al., 1988). Further work revealed that MPF was composed in part of a protein kinase, which was later determined to be the homologue of yeast *cdc2* (later named Cdk1). It was subsequently discovered that Cdk1 requires heterodimerization with Cyclin B to become active (Draetta et al., 1989; Dunphy et al., 1988; Gautier et al., 1988; Meijer et al., 1989).

In the decades since the identification of the Cyclin B/ Cdk1 complex, dozens of Cdk1 substrates have been validated (Enserink and Kolodner, 2010), with upwards of 300 putative substrates identified (Holt et al., 2009). These substrates are involved in a myriad of mitotic processes, many of which are reviewed elsewhere (Enserink and Kolodner, 2010; Ubersax et al., 2003). The extent of Cdk1 involvement in regulating mitotic and meiotic events only underscores the necessity of properly restricting Cdk1 activity throughout the cell cycle.

1.5. Discovering the Wee-like kinases

Cells that cannot degrade Cyclin B in mitosis are unable to exit M phase; similarly, cells that cannot inhibit Cdk1 in interphase enter M phase prematurely (Draetta et al., 1989). These conditional occurrences point to the necessity of regulating Cdk1 activity. There are a number of means by which Cdk1 is inhibited including physical associations with inhibitors, degradation of cyclins, and phosphorylation (Morgan, 1995). In our lab, we study Cdk1 inhibitory phosphorylation by the Wee-like kinases, regulatory proteins conserved in all

metazoans. To date, their only confirmed regulatory activity is to phosphorylate Cdk1 in an inhibitory manner.

To provide a basis for our current understanding of the Wee-like kinases, it is helpful to go all the way back to the beginning of their story. The first publication on Wee1 came out in 1975. At that time, Paul Nurse was screening fission yeast (*S. pombe*) for cell division cycle (cdc) mutants that were either elongated at the time of cell division or failed to divide at all (Nurse, 2004). During the course of his screen, Nurse stumbled upon fission yeast that were *smaller* than wild-type, indicating they divided before reaching their normal size (Nurse, 1975). This was a shocking discovery, as fission yeast had previously been known to divide only after reaching a critical size. In his original paper, Nurse describes *wee* mutants (indicating their smaller size) as having a normal length cell cycle but possessing a smaller volume at the time of cell division (Nurse, 1975). They are further described as having a shortened G2 phase (entering M phase prematurely) and a longer G1 phase such that the cell can reach a critical size before beginning DNA replication (Nurse, 1975). These observations not only provided the first clues towards Wee-like kinase involvement in regulating mitotic activity, but also gave evidence for the existence of cell cycle checkpoints, where certain conditions must be met before the next phase of the cell cycle can begin. Nurse's *wee* mutants demonstrated that there must be both G1/S and G2/M checkpoints.

Over the next decade, Paul Nurse and others began to describe numerous other cell cycle regulators. Among these were cdc2, cdc25, and cyclins. Mutant *S. pombe* referred to as *wee2* phenocopied the *wee1* mutation, with a double mutant showing no change in phenotype from either *wee1* or *wee2* independently (Thuriaux et al., 1978). Subsequent experiments revealed that the *wee2* mutation mapped to cdc2 and that the *wee1* phenotype must be caused by loss of the *wee* gene product (Nurse and Thuriaux, 1980). This suggested that wee1 might act as a negative regulator of mitosis through control of cdc2 (Nurse and Thuriaux, 1980). This interaction network was further expanded through the work of Peter Fantes, who found that

cdc25 mutations causing fission yeast to grow too long before mitosis were suppressed by mutations in both wee1 and cdc2 (Fantes, 1979). Based on these findings, it seemed likely that wee1 negatively regulated cdc2 while cdc25 positively regulated cdc2 through inhibition of wee1 (Fantes, 1979; Fantes, 1981). This ground-breaking research provided the foundation for our current understanding of Wee-like kinase functioning and cell cycle control.

Ensuing work in both mammalian cell culture and *Drosophila* has added more insight into the molecular mechanisms of Wee-like kinase activity. Wee1 is a conserved eukaryotic nuclear protein that phosphorylates tyrosine 15 (Y15) on Cdk1 (Figure 1-2) (Gould and Nurse, 1989). Another of the Wee-like kinases known as Myt1 (Membrane-bound tyrosine and threonine kinase) is found only in metazoans. Myt1 localizes to the endomembrane and targets both Y15 and the adjacent threonine residue (T14) on Cdk1 (Figure 1-2) (Kornbluth et al., 1994; Liu et al., 1997; Mueller et al., 1995). In all eukaryotes, Wee-like kinase activity is antagonized by Cdc25 phosphatases that facilitate Cyclin/ Cdk1 activity through the removal of inhibitory phosphates (Figure 1-2). Numerous unknowns still remain with respect to Wee-like kinases, however, including the extent of their redundancy throughout development, and how they function differentially in various cell types.

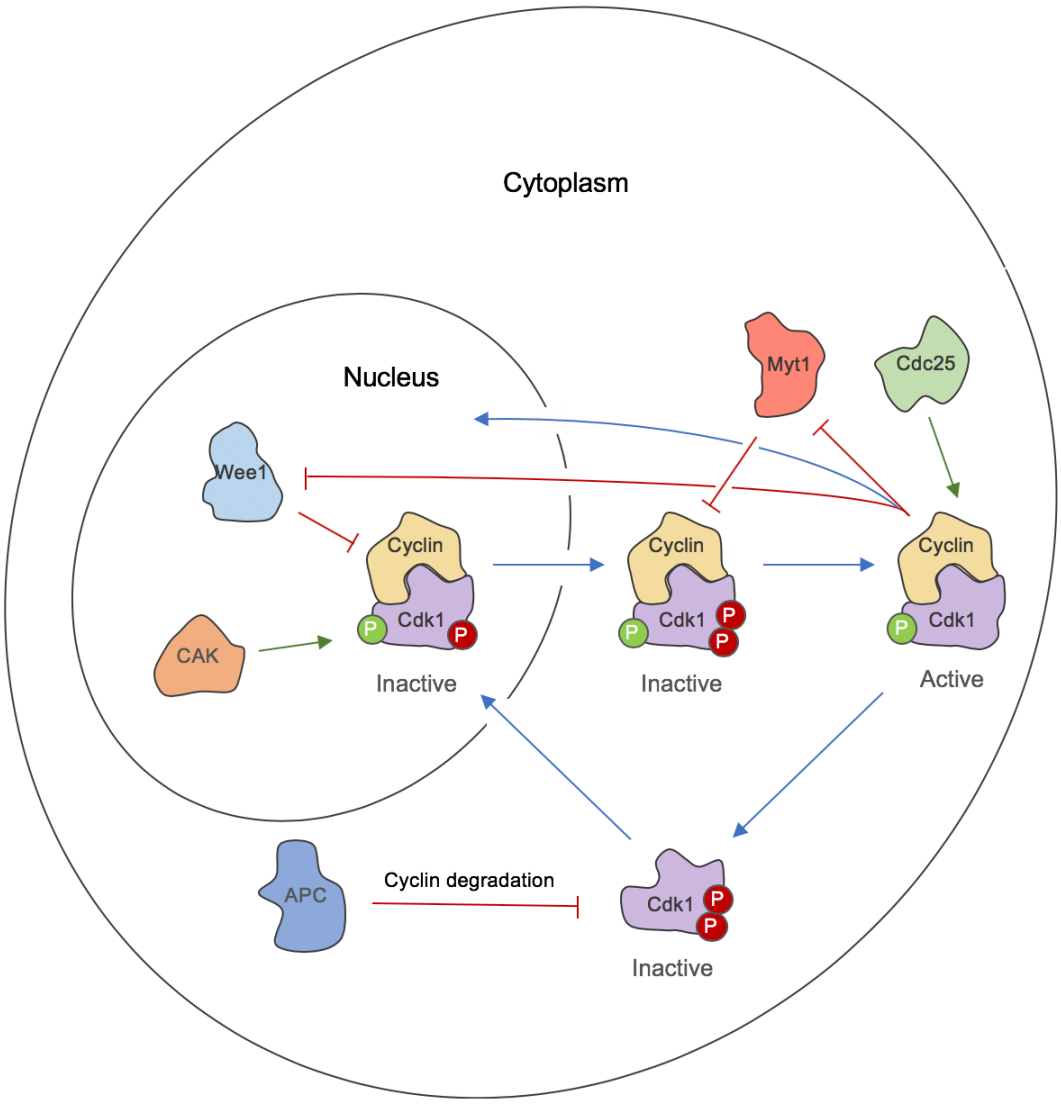

Figure 1-2. Mechanisms of Cdk1 activation and inhibition. Cdk1 must first form complexes with Cyclins to become active. Following this, nuclear Cyclin/ Cdk1 is inhibited by Wee1 phosphorylation of Y15, while cytoplasmic pools of Cyclin/ Cdk1 are targeted by Myt1 on both T14 and Y15. Cdk1 activating kinase (CAK) phosphorylates Cdk1 on T161, priming it for activity. Cdc25 activity removes inhibitory phosphates, activating Cdk1. Cdk1 initiates mitotic events in the cytoplasm and nucleus, also inhibiting Wee1 and Myt1. Subsequent activity by the anaphase promoting complex (APC) facilitates cyclin destruction and Cdk1 inhibition. Blue arrows indicate movement, green arrows indicate positive regulation, red lines indicate negative regulation.

1.6. Wee-like kinases during *Drosophila* development

As in all metazoans, Wee1 and Myt1 can both be found within *Drosophila* (Cornwell et al., 2002; Price et al., 2002). *Drosophila* Wee1 (dWee1) was originally identified in a screen for cDNA clones that rescued fission yeast *wee* mutants (Campbell et al., 1995), while dMyt1 was identified and cloned using previously described *Xenopus* and human amino acid sequences (Cornwell et al., 2002; Price et al., 2002). Characterization of these regulators has primarily been done within our laboratory, where mutant alleles of dWee1 and dMyt1 were generated through ethyl methanesulfonate (EMS) mutagenesis (Jin et al., 2005; Price et al., 2000).

1.6.1. Wee1 in the embryonic DNA replication checkpoint

Studies of the abovementioned *dwee1* mutants demonstrated that Wee1 is only essential during early embryonic development (Price et al., 2000). In *Drosophila*, this stage is characterized by rapid, synchronous cell cycles that proceed through S phase and nuclear division only (Orr-Weaver, 1994). These cycles occur largely without transcription and are thus regulated by maternally provided gene products (Orr-Weaver, 1994). Maternal Wee1 is essential during early embryogenesis, with embryos from mutant *wee1* females arresting in syncytial cycle 13 (Price et al., 2000). Normally, cycles 10-13 are distinguished by lengthening of interphase through increased activation of the DNA replication checkpoint, mediated by Chk1 and ATR (Crest et al., 2007; Sibon et al., 1997; Sibon et al., 1999). Embryos lacking maternal Wee1, however, show a highly similar phenotype to *grp* (Chk1) and *mei-41* (ATR) mutants, with no lengthening of interphase in the late syncytial cycles, and nuclei failing to separate during cycles 11 and 12 (Price et al., 2000). The similarity between the *wee1*, *grp*, and *mei-41* mutant phenotypes suggests that Wee1 might function in the DNA replication checkpoint. This hypothesis is supported by experiments where Wee1 overexpression in a *mei-41* background partially rescues the *mei-41* phenotype (Price et al., 2000). A role for Wee1 in

the DNA replication checkpoint in *Drosophila* has yet to be shown mechanistically however, and many questions still remain with respect to its functioning in embryogenesis. Wee1 is not essential for zygotic development due to Wee1 and Myt1 redundancy, with *wee1 myt1* double mutants resulting in synthetic lethality (Jin et al., 2008).

1.6.2. Several roles have been observed for dMyt1 in development

Characterization of *myt1* mutants, viable due to Wee1 redundancy, began with the observations that adult mutants possess thoracic bristle defects, and male mutants are sterile (Jin et al., 2005). Analysis of Myt1 in these developmental contexts, as well as others, reveals Myt1 function to be highly dynamic.

Work in the larval imaginal disc has demonstrated that Myt1 is required in response to DNA damage, at least in some contexts (Jin et al., 2008). Wing disc cells exposed to DNA-damaging radiation typically arrest before making the G2/M transition, thereby allowing time for DNA repair to occur. Accordingly, very few mitotic cells are observed under these conditions (Brodsky et al., 2000). In *myt1* mutant discs however, cells fail to activate the DNA damage checkpoint in G2 phase, and irradiated wing discs instead possess many mitotic cells (Jin et al., 2008). This is in contrast to *wee1* mutant discs that respond similarly to the wild-type control (Jin et al., 2008). Myt1, and not Wee1, therefore acts as a regulator of the DNA damage checkpoint under these circumstances.

Myt1 may also serve as a regulator of mitotic exit in differentiating cell types. During spermatogenesis, somatic cyst stem cells generate two large polyploid cyst cells that encapsulate the gonialblasts as they divide and differentiate to produce sperm (Jin et al., 2005). Cyst cells normally become quiescent after differentiation, however upon Myt1 loss they can proceed through ectopic divisions (Jin et al., 2005). A similar phenomenon is observed in follicle cells of the *Drosophila* ovary. In *myt1* mutants, the follicle cells, also derived from stem cell precursors, can undergo ectopic division rather than exiting the mitotic cell cycle (Jin et

al., 2005). These results both implicate Myt1 as a mediator of cell cycle exit during progenitor cell differentiation. Further evidence of this may be found in the sensory organ precursor (SOP) cells that divide and differentiate to give rise to the socket, shaft, neuron, and sheath composing the adult bristles. Expression of Cdk1 transgenes that cannot be phosphorylated on Y15 results in supernumerary SOPs with a shortened G2-phase arrest (Ayeni et al., 2016). This disrupts progenitor differentiation into secondary neuronal and non-neuronal cell types (Ayeni et al., 2016), resulting in bristle defects highly similar to that of *myt1* mutants. Though it has not been shown that the *myt1* bristle phenotype arises through failure to inhibit Cdk1 in G2-phase arrested SOPs, it may be that a similar mechanism is at play.

Experiments in the male and female germline have further shown that Myt1 is an essential meiotic regulator. During MI of *Drosophila* oogenesis, Myt1 is required to prevent defects in chromosome segregation (Jin et al., 2005). During a 4-day G2-phase arrest in spermatocytes, Myt1 is further necessary to inhibit Cyclin A/ Cdk1 complexes (Varadarajan et al., 2016). Loss of this inhibition causes disruption of the fusome, an intercellular bridge connecting germ cell cysts, as well as premature centriole disengagement that results in multipolar spindle formation during meiosis I (Varadarajan et al., 2016). Notably, Myt1 is not responsible for regulating the timing of MI entry as mediated by Cyclin B/ Cdk1, thus this demonstrates a novel role for Myt1 in regulating Cyclin A dependent functions of Cdk1 during male meiosis (Varadarajan et al., 2016).

In summary, several distinct roles have so far been described for Myt1 in *Drosophila* development. Firstly, Myt1 regulates the G2/M DNA damage checkpoint in some contexts; secondly, Myt1 may mediate cell cycle exit in several differentiating cell types; thirdly, Myt1 regulates distinct processes in both male and female meiosis. How Myt1 functions mechanistically in these contexts is unclear, and further work must be done to elucidate how differences in subcellular localization, physical associations, and phosphorylation by Myt1 kinase influences its tissue specific and cell specific functions. Additional research questions

remain, including how the aforementioned functions may be conserved throughout development and into adulthood.

1.7. What is the role of Myt1 kinase in other contexts?

Early work on Myt1 was conducted primarily in *Xenopus* and cell culture. Myt1 was originally described in *Xenopus* egg extracts as a membrane-associated protein kinase that targets two Cdk1 residues (Kornbluth et al., 1994). Later work demonstrated that Myt1 possesses a hydrophobic trans-membrane domain on its C-terminus, causing it to localize to the golgi and endoplasmic reticulum (ER) (Liu et al., 1997). How these differences in localization and Cdk1 phosphorylation affect the role of Myt1 relative to Wee1 is not entirely clear. Several investigations have been undertaken to answer these questions, but their results have been somewhat paradoxical.

Myt1 is predominantly thought to mediate G2 phase arrest, though the prevention of early M phase entry could be context dependent. Flow cytometric analysis of HeLa cells overexpressing Myt1 demonstrated that these cells were delayed in mitotic entry due to prolonged G2 phase arrest (Liu et al., 1999). In *Drosophila* S2 cells, overexpression of Myt1 similarly decreased rates of proliferation (Cornwell et al., 2002). Additional experimentation overexpressing kinase-inactive Myt1 in mammalian cells prolonged G2 phase, however overexpression of Myt1 truncated at the C-terminus, and therefore lacking a Cyclin B interaction motif, could not prolong G2 phase arrest (Liu et al., 1999; Wells et al., 1999). Taken together, these results suggest that Myt1 does mediate G2 phase arrest, perhaps through sequestration of Cyclin B/ Cdk1 to the golgi and ER.

Confounding conclusions have been reached through RNA interference studies, however. Depletion of Myt1 in S phase-synchronized HeLa cells via siRNA showed that these cells entered mitosis at a rate comparable to controls (Lewis et al., 2017; Nakajima et al., 2008). This was in contrast to Wee1 knockdown alone, which accelerated the G2/M transition,

indicating that Wee1 is primarily responsible for arresting mammalian cells in G2 phase (Lewis et al., 2017; Nakajima et al., 2008). Another group reported similar findings in HeLa cells, where Myt1 depletion did not affect normal cell cycle timing; the same study, however, demonstrated that upon ionization radiation to induce double-stranded DNA breaks, cells lacking Myt1 entered mitosis without undergoing DNA repair (Chow and Poon, 2013). This finding implicates Myt1 in the DNA damage checkpoint (Chow and Poon, 2013). Depletion studies in mammalian cells therefore suggest that Myt1 is not necessary for G2 phase arrest in healthy cells, and that only upon DNA damage is Myt1 activity required to facilitate cell arrest.

The disparate results reported here may reflect the limitations of overexpression studies, utilizing cell culture, or the complex nature of Wee1 and Myt1 redundant functioning. They also highlight a need for greater in-depth and *in vivo* analysis of Myt1 activity throughout the cell cycle. Of note, these depletion studies do corroborate evidence for the role of Myt1 in *Drosophila*. A requirement for Myt1 in mediating the DNA damage checkpoint is consistent with what has been described for Myt1 function in the *Drosophila* wing disc (Jin et al., 2008), and work in *Drosophila* spermatogenesis similarly shows that Myt1 does not coordinate the timing of G2 phase arrest (Varadarajan et al., 2016). Within somatic cells specifically, the evidence would therefore indicate that Myt1 is necessary for proper checkpoint functioning during periods of DNA damage. In part, my thesis will further investigate a role for Myt1 in somatic stem cells to clarify the nature of Myt1 function throughout the cell cycle.

1.8. Maintaining DNA integrity via Cdk1 regulation

An aforementioned study indicates that dMyt1 has a role in mediating the DNA damage checkpoint (Jin et al., 2008). Mechanistically, it is unclear how Myt1 might accomplish this. Complicating matters, dWee1 has been associated with the DNA replication checkpoint, albeit in a context where only S and M phases are occurring (Price et al., 2000). Do both Wee1 and

Myt1 have checkpoint functions in *Drosophila*? Precedent for redundant functioning between Wee-like kinases in the DNA replication checkpoint has been found in fission yeast, where *mik1* mutants (mik1 is another Wee-like kinase in fission yeast) have no phenotype on their own, while *wee1 mik1* double mutants fail to complete DNA replication before entering mitosis (Lundgren et al., 1991). It is clear that Cdk1 must be inhibited to allow time for DNA replication and repair to occur, but the nature of this inhibition with respect to Wee1 and Myt1 in *Drosophila* requires further investigation. In this section, I describe our current understanding of the S/M and G2/M checkpoints with respect to Cdk1 regulation in order to provide a framework from which to understand putative Myt1 involvement in these checkpoints.

1.8.1. The S-M checkpoint

Cdk1 activity is not limited to M phase. In fact, Cdk1 is required to promote G2 phase entry (Saldivar et al., 2018), and studies in mice have further revealed that Cdk1 is capable of compensating for loss of Cdk2 by forming heterodimers with Cyclin E (Aleem et al., 2005; Malumbres and Barbacid, 2009; Satyanarayana and Kaldis, 2009). Accordingly, it has been suggested that Cdk1 and Cdk2 may act synergistically to instigate DNA replication, but that low levels of Cdk1 activity are drowned out by much higher levels of Cdk2 activity (Bashir and Pagano, 2005). Whether Cdk1 functions as an S phase regulator under conditions where Cdk2 is present, however, is not entirely clear. In *Drosophila* embryogenesis, there is evidence that inhibitory phosphorylation of Cdk1 promotes the onset of late-replicating DNA, thereby increasing the duration of S phase in cycle 14 (Farrell et al., 2012). This suggests that Cdk1 does act as a regulator of origin firing (Duronio, 2012; Farrell et al., 2012).

Certainly, high levels of Cdk1 activity in S phase must be avoided to prevent mitotic entry and DNA lesions during DNA replication. The S-M checkpoint employs ATR and ATM to ensure that Cyclin B/Cdk1 does not become active until all DNA has been faithfully

replicated (Eykelenboom et al., 2013). Loss of ATR, even in a normal cell cycle, can result in cytokinesis failure and cell death that is dependent on Cdk1 activity (Eykelenboom et al., 2013). In mammalian cells specifically, ATR inhibits Cdk1 until DNA replication is completed, after which Cdk1 phosphorylates FOXM1, a transcription factor of the Forkhead Box family, that upregulates many mitotic genes (Saldivar et al., 2018). This inhibition occurs through ATR activating phosphorylation of Chk1, which stimulates Chk1 autophosphorylation and dissociation from the chromatin, such that it can inhibit Cdk1 through inhibitory phosphorylation of Cdc25 (Figure 1-3) (Smits et al., 2006; Sørensen and Syljuåsen, 2012). Evidence in *Xenopus* cell extracts suggests that Wee1 may also be the subject of activating phosphorylation by Chk1 (Figure 1-3) (Lee et al., 2001), however this has not been shown in flies or mammals. The majority opinion is therefore that Wee1 must inhibit Cdk1 independently of Chk1 to prevent DNA damage accumulation in S phase. This was evidenced through studies in several human cell lines that showed accumulation of DNA damage and single-stranded DNA in S phase upon Wee1 loss alone (Beck et al., 2010; Hauge et al., 2017). Taken together, these data suggest that ATR-mediated and Wee1-mediated Cdk1 inhibition are necessary for regulating Cdk1 activity in S phase.

Might there also be a role for Myt1 kinase in S phase? This has not been directly demonstrated, however depletion of Myt1 in mammalian cells can also result in DNA damage accumulation (Beck et al., 2010), suggesting a role for Myt1 in the S phase checkpoint. Furthermore, in mammalian cells where Cyclin B/Cdk1 complexes are present through G1 phase, Wee1 and Myt1 are both required to inactivate Cdk1 and prevent mitotic entry before DNA replication occurs (Pomerening et al., 2008; Potapova et al., 2009).

In *Drosophila* specifically, there is little evidence for Wee1 or Myt1 involvement in the S/G2 transition, beyond the previously described role for Wee1 in the embryonic DNA replication checkpoint. Within *Drosophila* larval neuroblasts, expression of Cdk1 that cannot be inhibited on Y15 or T14 results in severe genome instability, demonstrating that Myt1 dual

phosphorylation of Cdk1 could be important during periods of DNA replication in flies (Ayeni et al., 2014). Also consistent with this idea, *Drosophila myt1* mutants show a consistent phenotype wherein cellular DNA appears decondensed throughout the cell cycle (Campbell lab, unpublished observations; Jin et al., 2008). It is conceivable that this phenotype could be the result of DNA damage accumulation due to premature Cdk1 activity in S phase, and that Myt1 is therefore required to inhibit Cdk1 during DNA replication.

Collectively, the data described in this section shows that Cdk1 must be inhibited in S phase, and that this inhibition relies on Wee-like kinase activity in some contexts. An *in vivo* requirement for the Wee-like kinases in S phase beyond embryogenesis has yet to be shown, however.

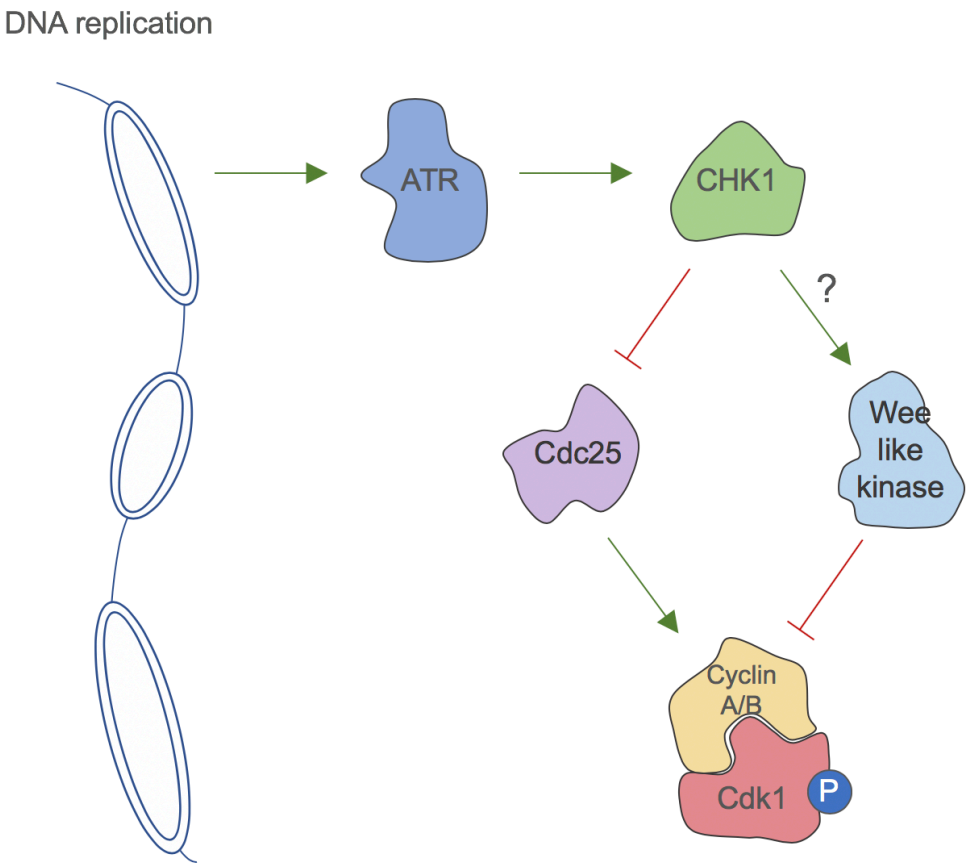

Figure 1-3. Inhibition of Cdk1 during S phase. During periods of DNA replication, ATR is activated and phosphorylates Chk1. Chk1 then inhibits Cdc25, preventing dephosphorylation of Cdk1. Concurrently, Wee-like kinases inhibit Cyclin/ Cdk1 complexes through phosphorylation, a process that may be promoted by Chk1 phosphorylation of Wee1. Question mark indicates an unknown interaction.

1.8.2. The G2/M checkpoint

DNA damage can be caused by many agents either intrinsic or extrinsic to the cell. No matter the source of the DNA damage, however, the cell responds through a common checkpoint mechanism to inhibit Cdk activity, and thereby facilitate G2 phase arrest. Evidence in both *Drosophila* and cell culture indicates that Myt1 may be involved in directly inhibiting Cdk1 in G2-phase cells that have accumulated DNA damage, however it is not clear how this might come about.

The major downstream effector kinase responsible for controlling cell arrest in response to DNA damage is Chk1. In similar fashion to the S phase checkpoint, Chk1 is phosphorylated in response to DNA damage (Sanchez et al., 1997; Walworth and Bernards, 1996). Chk1 then phosphorylates Cdc25 in an inhibitory manner (Zeng et al., 1998), preventing Cdk1 activation through the maintenance of Wee-like kinase mediated Y15 inhibitory phosphorylation. Evidence in *S. pombe* further suggests that Wee1 becomes hyperphosphorylated upon UV irradiation or Chk1 overexpression, indicating that Chk1 may also phosphorylate Wee1 to mediate the DNA damage response in G2 phase (O'Connell et al., 1997).

In *Drosophila*, it appears that Myt1, rather than Wee1, is the major G2/M checkpoint Cdk1 inhibitory kinase (Jin et al., 2008). How Myt1 might become activated under these circumstances remains to be established; it is plausible that Myt1 might be activated by Chk1 kinase activity, or that Myt1 could act independently of Chk1 (similar to Wee1 in the DNA replication checkpoint). Further research must be conducted to elucidate the mechanism by which Myt1 acts, as well as the extent to which this role for Myt1 is conserved in *Drosophila* tissues and other organisms.

1.9. Myt1 coordination of cell cycle exit in differentiating cells

In addition to the demonstrated requirements for inhibitory phosphorylation of Cdk1 in S and G2 phase, there is also evidence from *Drosophila* that Cdk1 must be phosphorylated during mitotic exit. This was observed in somatic cells of both the male and female gonads, where Myt1 loss resulted in a failure of certain differentiated cells to become quiescent (Jin et al., 2005). It is unclear how this occurs mechanistically, though there is precedent for a cellular requirement of Cdk1 inhibitory phosphorylation during mitotic exit. Whether or not this is true during scenarios wherein cells not only exit mitosis, but also exit the mitotic cell cycle, requires investigation.

1.9.1. Examples of inhibitory phosphorylation of Cdk1 during mitotic exit

Of necessity, Cdk1 activity must be restricted following metaphase to allow cells to exit mitosis. Cdk1 inhibition at metaphase is most often thought to occur through APC/C ubiquitination of cyclins that targets them for degradation (Morgan, 1999; Shirayama et al., 1999). A number of experiments have shown, however, that Cdk1 inactivation occurs prior to mitotic cyclin degradation, and that this is reliant on inhibitory phosphorylation of Cdk1 (D'Angiolella et al., 2007). This was demonstrated in *Xenopus* egg extracts, where Cdk1 inhibitory phosphorylation on Y15 was transiently observed in cells exiting M phase (D'Angiolella et al., 2007). Furthermore, expression of non-inhibitable Cdk1 caused cells to remain in M phase by preventing assembly of APC/Cdc20 complexes (D'Angiolella et al., 2007). Experiments in mammalian cells support these observations. Pharmaceutical inhibition of Wee1 in HeLa cells dramatically slows the metaphase to anaphase transition, with cells eventually arresting in mitosis (Lewis et al., 2017), while neural stem cells depleted of both Wee1 and Myt1 also have significantly prolonged mitosis (Toledo et al., 2015). Furthermore, cells lacking Myt1 inhibitory phosphorylation fail to properly assemble the golgi and ER during mitotic exit (Nakajima et al., 2008). Lastly, cells depleted of Cdc20 by RNAi were still

capable of exiting mitosis due to inhibitory phosphorylation of Cdk1 by Wee1 and Myt1 (Chow et al., 2011). It appears, therefore, that in addition to APC/C activity, inhibitory phosphorylation of Cdk1 plays a significant role in promoting anaphase. Further substantiation is required to determine whether or not inhibitory phosphorylation of Cdk1 is required during mitotic exit within any *Drosophila* tissues.

1.9.2. The mitotic to endocycle switch

Differentiating cells exit the mitotic cycle and become highly specialized to perform unique tasks. During differentiation, some cells enter a cell cycle variant known as the endocycle, which is characterized by oscillating growth and DNA synthesis phases without cytokinesis. Cells that enter the endocycle therefore achieve a copy number >2C and become known as polyploid. Some polyploid cells, such as nurse cells in the female germline, or cells of the salivary gland, can reach a copy number of >1000C (Hammond and Laird, 1985a; Hammond and Laird, 1985b). This is an effective and efficient means of regulating tissue size, initiating cell morphogenesis, or simply increasing gene number such that tissue specific gene products might be more readily manufactured (Zielke et al., 2013). Evidence in *Drosophila,* particularly in both the male and female gonads, indicates that there may also be a distinct role for Myt1 in regulating terminal differentiation of cells that undergo the mitotic to endocycle switch.

Cells face a unique challenge in entering the endocycle, and such a dramatic cell cycle shift requires a significant overhaul of cellular machinery. To begin endocycling, cells must first exit the canonical mitotic cycle following G2 phase (Zielke et al., 2013). This is a switch that is not entirely understood, though it is known that entrance into the endocycle requires Cdk1 downregulation. This is directed by Notch signaling that manipulates downstream cell cycle regulators (Zielke et al., 2013). In *Drosophila*, follicle cells of the ovary are produced by a population of stem cells that proceed through division and differentiation to give rise to the

final polyploid product (Wang et al., 2012). Here, oocyte Delta expression activates the Notch receptor in follicle cells, which represses Cdc25 activity via the Hindsight transcription factor, while activating Fzr to promote the APC (Figure 1-4) (Zielke et al., 2013). This inhibits Cdk1 (Figure 1-4) and allows Cyclin E/Cdk2 to initiate DNA replication without cytokinesis (Schaeffer et al., 2004; Shcherbata et al., 2004).

This Notch-mediated pathway of Cdk1 downregulation is conserved in other *Drosophila* tissues, as well as other organisms. In the mature *Drosophila* hindgut, Fzr (downstream in the Notch signaling pathway) mediates the switch between the mitotic and endocycle (Cohen et al., 2018), perhaps through mitotic cyclin degradation. In diploid cells of the *Drosophila* imaginal discs, salivary glands, and nervous system, Cdk1 depletion results in endocycling rather than continuance of the mitotic cycle (Hayashi, 1996; Weigmann et al., 1997). The same is even true in fission yeast, where Cdk1 inhibition causes re-replication to occur (Hayles et al., 1994). These findings demonstrate that Cdk1 must be inhibited prior to initiation of endoreplication, however the exact nature of this inhibition in many tissues remains an unanswered question. If Cdk1 fails to be initially inactivated by phosphorylation, does this prevent entrance into the endocycle? This is a question that is unclear, though the presence of mitotic polyploid cyst and follicle cells in the *Drosophila* testis and ovary respectively, due to Myt1 loss (Jin et al., 2005), would indicate that inhibitory phosphorylation of Cdk1 may contribute to proper execution of the mitotic to endocycle switch. Further evidence that Myt1 may be involved in this switch can be found in an overexpression study in the developing *Drosophila* eye, where ectopic Myt1 expression enhanced the phenotype caused by mutations in Delta, a Notch ligand (Price et al., 2002). This result suggests that Myt1 may be a downstream target of the Notch signaling pathway (Price et al., 2002). In part, my thesis will investigate the formation of endoreplicating cells in the *Drosophila* midgut, another context where the mitotic to endocycle switch occurs in a Notch dependent manner.

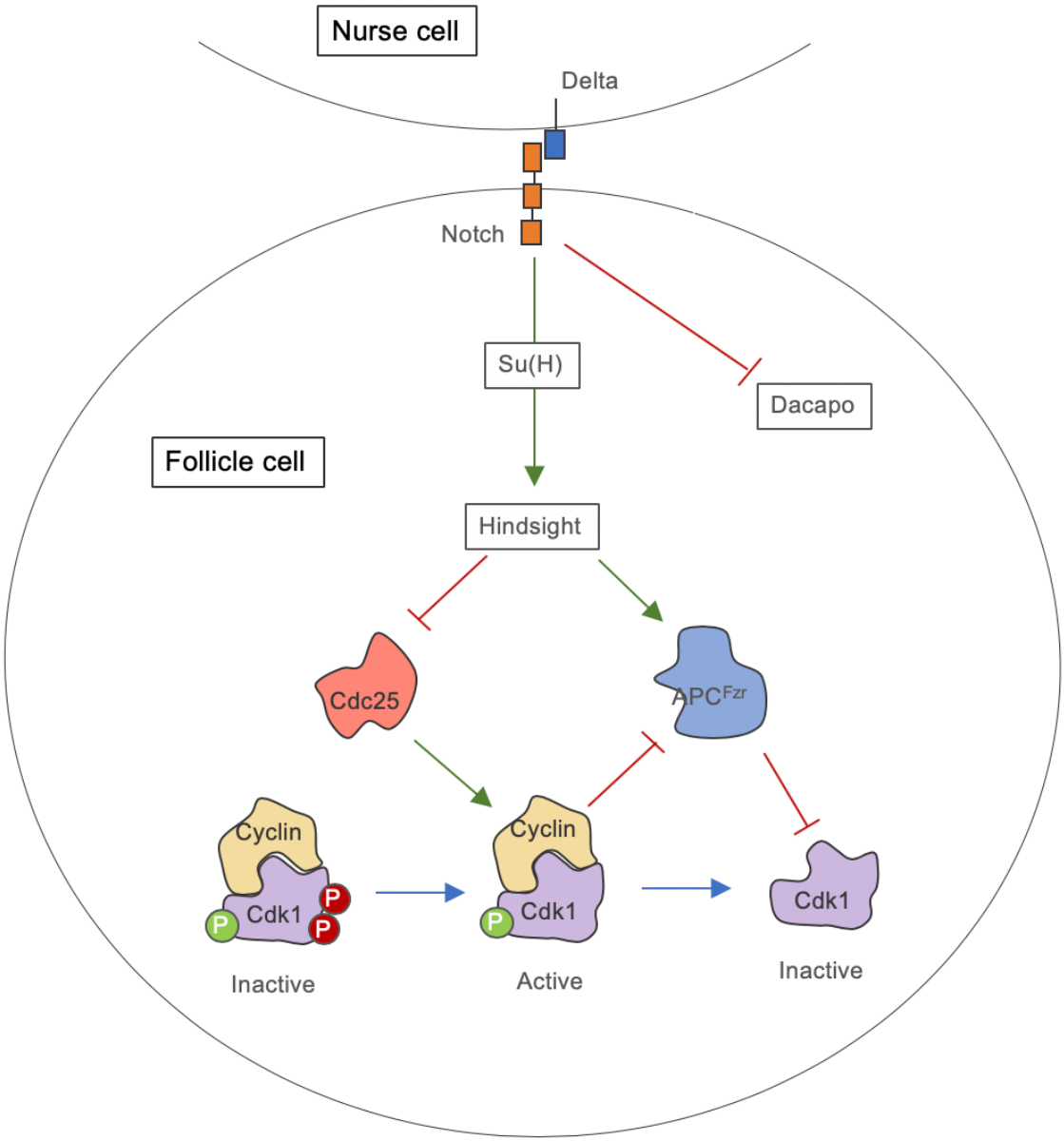

Figure 1-4. Notch signaling inactivates Cdk1 to initiate endoreplication. Nurse cells expressing Delta activate Notch in the follicle cell. This causes Su(H) mediated activation of Hindsight, which facilitates APC^{Fzr} degradation of mitotic cyclins, and inhibition of Cdc25 phosphatase, which prevents Cyclin/ Cdk1 complex activation. Notch also inhibits Dacapo to initiate Cyclin E/ Cdk2 activity. Red lines indicate negative regulation, green lines indicate positive regulation, and blue arrows indicate progressive regulatory steps.

1.10. Thesis Objective

This chapter has demonstrated that our understanding of Wee1 and Myt1 functioning is far from complete. Specifically, it appears that Myt1 is not universally required to inhibit Cdk1 in G2-arrested cells, and that there are contexts outside of healthy G2 phase arrest that necessitate Myt1 activity (upon DNA damage for example). The goal of my thesis is to utilize the well-established *Drosophila* model to better characterize Myt1 function relative to the cell cycle *in vivo*. Specifically, I employ Myt1 depletion studies to explore the cell-cycle timing of Myt1 activity in somatic cells of the adult intestinal epithelium. In so doing, I strive to comprehend how Myt1 functions in a context where somatic cells are undergoing both division and differentiation, thereby teasing apart the apparent pleiotropy observed in *Drosophila myt1* mutants.

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2. Myt1 regulation of Cdk1 in the *Drosophila* intestinal epithelium

2.1. Summary

Myt1 kinase serves several functions in *Drosophila* development. The mechanism by which Myt1 operates in somatic cells, as well as the degree to which such a mechanism is conserved in other *Drosophila* tissues, remains unclear. The *Drosophila* adult intestinal epithelium provides an excellent model system to investigate the function of Myt1 kinase in somatic cells. This tissue is highly similar to the vertebrate intestinal epithelium, possessing multipotent stem cells that can either self-amplify or divide asymmetrically to produce specialized cell types. Because intestinal stem cells (ISCs) complete a full cell cycle once per day, I hypothesized that Cdk1 inhibitory phosphorylation is required to maintain ISC homeostasis. Furthermore, ISCs produce transient daughter cells known as enteroblasts (EBs) that exit the mitotic cell cycle, undergoing the mitotic-to-endocycle switch to produce absorptive enterocytes (ECs) in a Notch dependent manner. This is therefore an ideal system in which to explore Myt1 function. Herein, I examine the function of Myt1 in the adult intestine and provide evidence that Myt1 is an essential regulator of intestinal homeostasis. I demonstrate that Myt1 is required to promote mitotic cell cycle exit in EBs, and further demonstrate that Myt1 activity in the *Drosophila* intestine is dependent on Cyclin A/Cdk1 activity.

2.2. Introduction

2.2.1. The *Drosophila* intestine as a model system

The *Drosophila* intestine is an excellent model system for studying cell cycle regulation in the context of differentiating cells. This is not only because of the incredible tractability of *Drosophila* as a model organism, but also because of the similarities between the fly and mammalian intestines. The fly intestine, in similar fashion to the human intestine, is composed of an epithelial monolayer surrounded by visceral muscle cells. The *Drosophila* intestine contains a foregut, midgut, and hindgut, which are functionally similar to the mammalian stomach, small intestine, and large intestine respectively (Singh et al., 2011). The midgut is the best characterized and most commonly studied of these regions, and can be further divided into the anterior midgut, middle midgut, and posterior midgut (Figure 2-1A). Additionally, the fly intestine possesses a large population of multipotent stem cells capable of replacing damaged and aging cells throughout the lifetime of the fly (Micchelli and Perrimon, 2006). This is comparable to the mammalian adult small intestine that also contains proliferative ISCs able to maintain epithelial homeostasis through tissue renewal (Barker et al., 2008). Loss of proper stem cell functioning can result in gut dysplasia or metaplasia, as well as chronic disorders such as inflammatory bowel disease (Li and Jasper, 2016).

Drosophila ISCs are derived from larval gut precursors and subsequently reside in the adult epithelial monolayer (Micchelli and Perrimon, 2006; Micchelli, 2012). ISCs can divide symmetrically to produce two identical daughter ISCs, or asymmetrically to produce both a daughter ISC and an intermediate enteroblast (EB) that further differentiates into an absorptive enterocyte (EC), or to a secretory enteroendocrine cell (EE) (Fig. 2-1B) (Biteau and Jasper, 2014; Micchelli and Perrimon, 2006; Micchelli, 2012). There is also evidence that under some circumstances ISCs can differentiate directly into pre-EEs (Zeng and Hou, 2015). These outcomes are controlled via bidirectional Notch signaling, where high Notch signaling results in an intermediate EB that subsequently differentiates into an EC, while low Notch signaling

in conjunction with *Delta* expression and activation of the transcription factor *Prospero* induces ISC differentiation into an EE (Guo and Ohlstein, 2015; Perdigoto et al., 2011; Zeng et al., 2015). Together, ISCs, EBs, EEs, and ECs form the intestinal epithelium, an essential stratum that contributes to fly health in numerous ways.

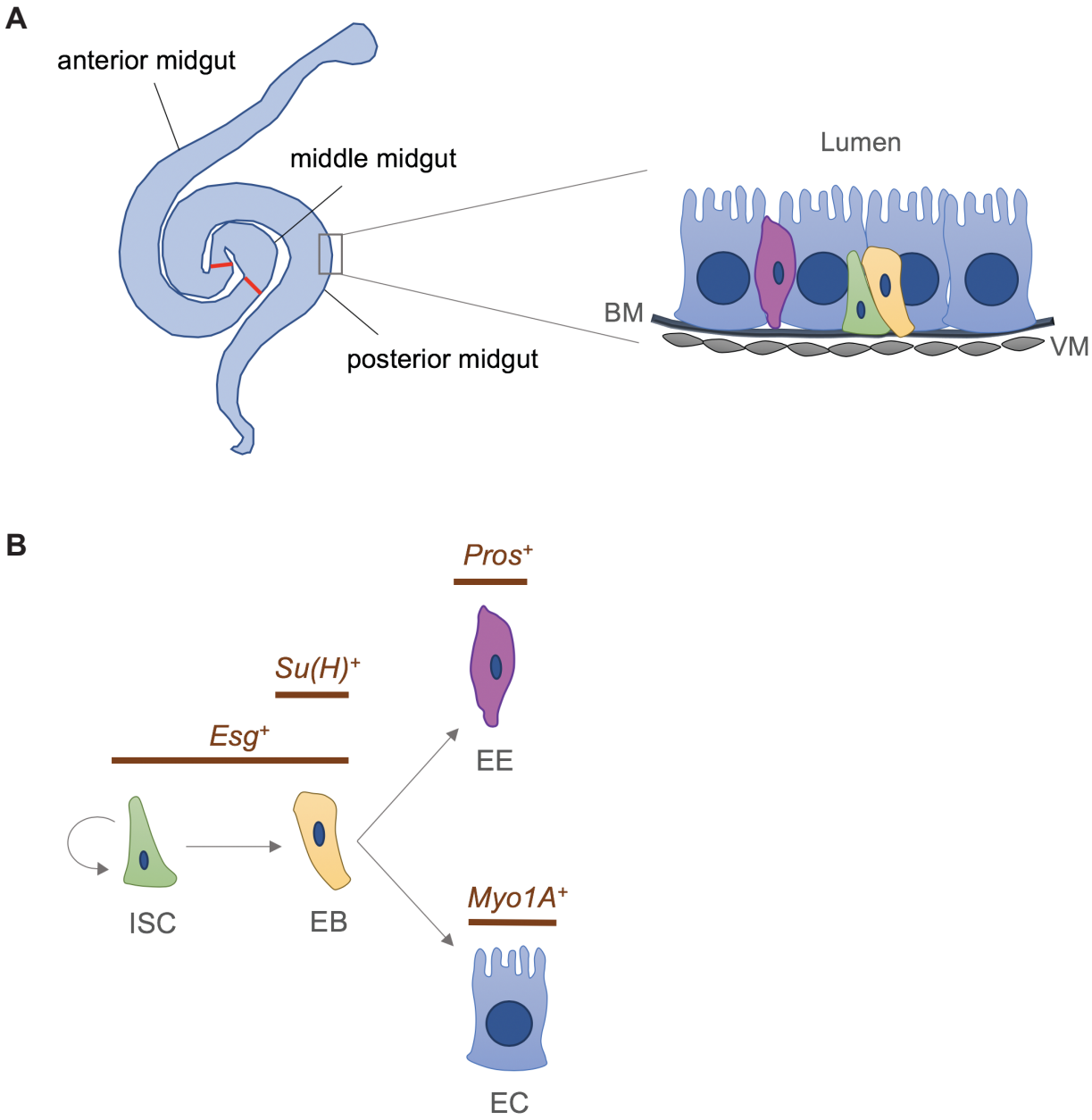


Figure 2-1. The *Drosophila* midgut. A) The midgut portion of the fly intestine can be subdivided into the anterior, middle, and posterior midgut. The epithelium is composed of a monolayer of cells attached to the basement membrane (BM) and surrounded by visceral muscle (VM). B) The intestinal epithelium is composed of self-renewing intestinal stem cells (ISCs) that divide to produce an enteroblast (EB) that differentiates into an enteroendocrine (EE) cell or an enterocyte (EC). Shown above each cell type (dark orange) are commonly employed markers specific to the cell type.

2.2.2. Epithelial regeneration depends on ISC maintenance and proliferation

The midgut epithelium turns over approximately once every 7-9 days in healthy adult flies, with rates of cell turnover increasing dramatically in response to enterocyte loss, enteric infection, or other intestinal stressors (Jiang et al., 2009; Xiang et al., 2017). This process depends on ISC divisions that produce appropriate ratios of differentiated cell types. Under homeostatic conditions, ISCs cycle once per day (Ohlstein and Spradling, 2006), with 20% and 80% of mitoses constituted of symmetric divisions to produce new ISCs, and asymmetric divisions to produce EBs respectively (de Navascués et al., 2012).

Much work has been done to uncover mechanisms regulating ISC maintenance, proliferation, and differentiation. ISC identity is maintained by niche signaling from the visceral muscle and the basement membrane (Jiang and Edgar, 2011), as well as by *escargot* (*esg*), a transcription factor of the Snail family (Korzelius et al., 2014). A number of signaling pathways are further involved in regulating ISC division, including JAK/STAT, necessary for stimulating ISC proliferation in response to damaged ECs (Jiang et al., 2009), EGFR, which promotes ISC division under both homeostatic and regenerative conditions (Biteau and Jasper, 2011; Buchon et al., 2010; Jiang et al., 2011; Jin et al., 2015; Xu et al., 2011), Insulin signaling (Choi et al., 2011; O'Brien et al., 2011), JNK (Biteau et al., 2008), Wingless (Lee et al., 2009; Lin et al., 2008), TOR (Amcheslavsky et al., 2011; Kapuria et al., 2012), BMP (Guo et al., 2013), and Hippo (Karpowicz et al., 2010; Ren et al., 2010; Staley and Irvine, 2010). These pathways work conjunctively to stringently regulate ISC proliferation in response to systemic and environmental cues. Many questions remain however, including how these signaling pathways manipulate basic cell cycle machinery to accomplish their goals, and how intrinsic cell cycle cues contribute to ISC maintenance and differentiation.

Studies in the gut related to cell cycle regulators are few, however regulation of ISC proliferation has been shown to occur on a transcriptional level by E2F1 (Jiang et al., 2009) as well as by Capicua, a transcriptional repressor that is negatively regulated by EGFR/Ras

signaling (Jin et al., 2015). Both overexpression of E2F and depletion of Capicua allow for augmented String and Cyclin E production causing increased ISC proliferation due to ectopic Cdk activity (Jiang et al., 2009; Jin et al., 2015). Little work has been done to understand the role other Cdk1 regulatory proteins play in controlling ISC division or differentiation.

In this study, I employ genetic manipulation of the *Drosophila* intestine to investigate a role for Myt1 in regulating cell proliferation and differentiation. I demonstrate that Myt1 is required in both ISCs and EBs, and that this activity prevents both DNA damage accumulation and overproduction of intestinal progenitors that fail to properly differentiate. Furthermore, I show that Myt1 mediates the mitotic-to-endocycle (ME) switch in EBs in a Cyclin A dependent manner. Hence, Myt1 inhibition of Cdk1 activity is required to prevent ectopic mitoses in EBs and to ensure proper differentiation of daughter cells arising from asymmetric ISC division.

2.3. Materials and Methods

2.3.1. Longevity assay

The lifespan study was conducted as in (Linford et al., 2013). Flies were collected within 8 hours post-eclosion and kept on standard cornmeal food at 25ºC for the duration of the experiment. Flies were allowed to mate for 3 days, after which female flies were separated out into a maximum of 30 flies per vial. Flies were transferred to fresh food every 2-4 days (no anaesthesia), and the number of dead flies in the older vial was recorded until no flies remained. Flies that escaped during tipping were recorded as 'censored,' and were considered to have exited the experiment the day of escape. Data was recorded in GraphPad Prism 7, after which survival curves and statistical analysis was performed in the same program. A log-rank (Mantel-Cox) test was used to determine significance between experimental groups.

2.3.2. Immunofluorescence

Flies of desired genotype and age were anaesthetized, scored, and put on ice. Flies were transferred to 200 µL phosphate-buffered saline (PBS) and the intestines were teased out with forceps. Once 5 guts had been collected, these were transferred to a microfuge tube containing 200 µL of fresh PBS on ice. After the desired number of guts was collected, the PBS was removed, and the tissue was incubated in 500 µL 8% formaldehyde fixative in PBS for 25 min. Following this, guts were washed 3 x 5 min in PBS + 0.2% Triton X-100 (PBT) then incubated in blocking solution of PBT + 3% BSA (PBTB) for 1 hour. The samples were then incubated overnight with 1º antisera diluted in blocking solution. Next, guts were washed 3 x 5 min and 1 x 15 min in PBT then incubated with 2º antibody in PBTB. Finally, guts were washed 5 x 10 min (once with Hoechst 33258, 1:1000 dilution, added to PBT) and then mounted onto a slide with coverslip. The antibody dilutions used for these experiments are recorded in Appendix C. Z-stack images were acquired using a Zeiss Axioplan microscope equipped with a CCD camera. Objective lenses used were Plan-Apochromat 20x/0.8 M27 or Plan-Apochromat 63x SF25. The Z-stack images were merged in Volocity 4, exported as TIFFs, and processed with Adobe Photoshop software. All experimental and control images were captured with identical camera settings and identically manipulated in Photoshop (brightness, contrast, and false colour manipulations). Quantifications of cell numbers were conducted by manual counting within the indicated region of the gut. Where experiments indicate that the 'gut' was analyzed, this refers to the whole midgut. Where experiments indicate that 1 frame was analyzed, this refers to all of the gut visible in the microscope field, looking just anterior of the midgut-hindgut transition zone (region R4c of the midgut). Guts were straightened out before mounting to control for variation in intestinal folding. Statistical analysis was performed in GraphPad Prism using an unpaired t-test with Welch's correction to control for differences in variance.

2.3.3. Temperature sensitive transgene expression

The UAS-GAL4 system was employed for cell type specific expression of transgenes. Here, the UAS enhancer promotes expression of downstream targets only when the GAL4 transcriptional activator is present. To allow cell-type specific expression, the GAL4 is coupled to a cell type specific enhancer. To allow for adult specific expression, this system is coupled to a temperature sensitive GAL80 ($GAL80^{ts}$), which restricts GAL4-mediated expression below 25ºC but releases GAL4 above 29ºC. Thus, flies are grown to adulthood <21ºC and are then transferred to 29ºC to induce transgene expression 3-5 days post-eclosion. RNAi experiments were conducted using flies with UAS-RNAi insertions. These flies were crossed to flies possessing a suitable GAL4 driver to generate progeny possessing both the GAL4 and UAS-RNAi constructs, such that RNAi can be expressed upon shifting flies to 29ºC. In all experiments, flies expressing $myt1^{RNAi}$ were analyzed after 7 days of transgene expression. A mutant phenotype was consistently observed at this point, though in a minority of flies a mutant phenotype could be seen as early as 4 days post-temperature shift. List of driver lines possessing GAL4 transcriptional activators, and the cell type(s) in which they induce transgene expression: esg^{ts} – ISC and EB; esg^{ts}*; Su(H)-GAL80* – ISC; GBE^{ts} – EB; $Pros^{ts}$ – EE; $Myo1A^{ts}$ – EC; How^{ts} – VM. See text or Appendix B for full genotypes.

2.3.4. Mosaic analysis with a repressible cell marker (MARCM)

MARCM is a technique used to generate clones of mutant cells (in an otherwise heterozygous background) in tissues wherein cell division occurs (Wu and Luo, 2006). First, this technique requires the generation of heterozygous flies with one chromosome possessing *tub-GAL80* distal to an FRT insertion site, and its homologue possessing a mutant allele distal to the same FRT site. Flies are also heterozygous for *Actin-GAL4* and *UAS-GFP*. Next, this technique employs the FLP-FRT system to generate cells homozygous for a mutant allele that are simultaneously labeled with GFP using the UAS-GAL4 system. Upon heat shock, heat shock-

activated FLP recombinase recombines aforementioned FRT sites on homologous chromosomes, resulting in paired daughter cells homozygous for *tub-GAL80* or the mutant allele respectively. GAL80 restrains Actin-GAL4 mediated UAS-GFP expression in heterozygous tissue or wild-type clones, while mutant clones no longer possess GAL80 and express GFP, allowing for unambiguous identification of cell genotypes. For my experiment, recombinant *FRT80B, myt1* flies were generated and crossed to a MARCM stock to create progeny with the genotype *hs-FLP, UAS-GFP, act-GAL4/ +; ; FRT80B, tub-GAL80/ FRT80B, myt1*. In this fly, all tissue is heterozygous until heat shock, which induces recombination in some G2-arrested cells that then divide to produce a cell homozygous for the mutant allele and expressing GFP (*hs-FLP, UAS-GFP, act-GAL4/ +; ; FRT80B, myt1/ FRT80B, myt1*), as well as a wild-type cell with no mutant allele but possessing two copies of the GAL80 such that it does not express GFP (*hs-FLP, UAS-GFP, act-GAL4/ +; ; FRT80B, tub-GAL80/ FRT80B, tub-GAL80*). These flies were compared to control flies without any mutant allele, and which therefore generated WT GFP positive clones of the genotype *hs-FLP, UAS-GFP, act-GAL4/ +; ; FRT80B/ FRT80B,* as well as WT GFP negative clones of the genotype *hs-FLP, UAS-GFP, act-GAL4/ +; ; FRT80B, tub-GAL80/ FRT80B, tub-GAL80*. Flies were maintained at RT for 4 days post-eclosion to allow for complete gut development, then heat shocked in a 38.5ºC water bath for 2 x 30 min separated by 5 min on ice to increase the number of recombination events (fewer events were observed without putting flies on ice). Flies were left at RT for 7 days post-heat shock and then analyzed. Clones were determined as adjacent GFP positive cells. Single GFP positive cells were not included in analysis to prevent inclusion of cells with spontaneous GFP expression.

2.3.5. Measuring nuclear fluorescent intensity of γ-H2Av

Control esg^{ts} or $esg^{ts}>myt1^{RNAi}$ flies were subjected to the immunofluorescent protocol above, with staining for GFP, DNA, and γ-H2Av. Following this, Z-stack images of the gut epithelium

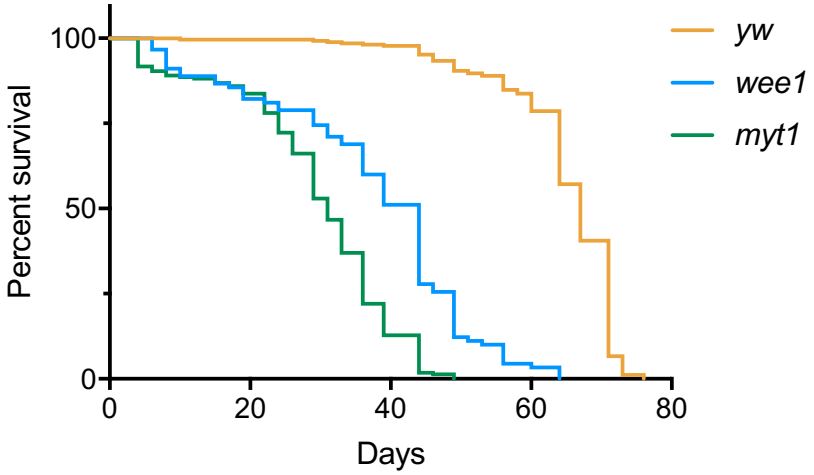
were acquired using identical camera settings for both the control and experimental group. Following this, projected Z-stack images were merged and exported as unprocessed TIFFs to ImageJ. The nuclear area was then identified by Hoechst staining, and this region was then selected for further analysis (region of interest tool on image J). The area of the nucleus and the integrated density (product of the area and Mean Gray Value) of γ-H2Av staining within the nucleus were measured. Following this, 5 background measurements adjacent to the progenitor cell were taken from each image. The corrected total cell fluorescence (nuclear signal intensity corrected for background noise) was then determined as: Integrated density – (area of nucleus x mean fluorescence of background readings).

2.4. Results

2.4.1. Loss of Myt1 activity decreases fly longevity

Flies mutant for *myt1* possess defects in certain mitotic and meiotic cells (Jin et al., 2005; Jin et al., 2008), however defects affecting adult fly health have not previously been described. *Drosophila* intestinal hyperproliferation defects are already known to result in increased mortality (Guo et al., 2014; Li and Jasper, 2016). This occurs as the result of a compromised epithelial barrier, which increases susceptibility to resident microbes that can trigger chronic inflammation and epithelial dysplasia (Guo et al., 2014; Li and Jasper, 2016). Accordingly, I assessed survivability of *wee1* and *myt1* null mutant flies as a potential indicator of requisite Wee1 or Myt1 functioning in the fly gut.

Relative to *yw* controls that had a median lifespan of 67 days, both *wee1* and *myt1* mutant flies had significantly decreased longevity, with median lifespans of 44 and 31 days respectively (Figure 2-2). Notably, *myt1* flies were significantly shorter-lived than *wee1* mutants (Figure 2-2). These results demonstrate that compromised Cdk1 inhibitory phosphorylation decreases fly longevity, and that Myt1 may be more important for maintaining the epithelial barrier of the intestine than Wee1. This is a reasonable assertion given that adult *wee1* and *myt1* mutant flies do not possess any other defects known to affect longevity.



	P Value	
	yw	*wee1*
myt1	<0.0001	<0.0001
wee1	<0.0001	

Figure 2-2. Loss of Wee1-like kinases significantly reduces survivability. Median survival is 67, 44, and 31 days for *yw* (n=277), *wee1* (n=90), and *myt1* (n=228) mutant flies respectively. p<0.0001 for all comparisons as determined by a log-rank (Mantel-Cox) test.

2.4.2. Myt1 loss promotes proliferation in the intestinal epithelium

The lifespan data suggested that both Wee1 and Myt1 may be important in maintaining the intestinal epithelium. Flies with a compromised intestinal epithelium often undergo increased ISC proliferation to replace damaged and dying cells (Jiang and Edgar, 2011). I therefore visualized mitotic activity in the intestines of 7-day old flies homozygous for null alleles of *wee1* (Price et al., 2000) and *myt1* (Jin et al., 2005) using the common mitosis marker phospho-histone H3 (Hendzel et al., 1997).

Control *yw* flies showed 4.2 +/- 2.8 mitoses per gut, however *wee1* mutant intestines showed 22.2 +/- 17.9 mitoses/ gut and *myt1* mutant flies showed 164.5 +/- 54.3 mitotic cells per gut (Figure 2-3). Thus, *wee1* and *myt1* mutant flies both exhibited significantly increased ISC proliferation indicative of compromised epithelial homeostasis. Consistent with the longevity data, these results showing that *myt1* mutant intestines were more affected than those of *wee1* flies led me to conclude that Myt1 is the major Wee1-related kinase operating in the *Drosophila* intestine. This data is further consistent with RNA sequencing from whole *Drosophila* guts showing that Myt1 is more highly expressed relative to Wee1 (Dutta et al., 2015). I next set out to address important unknowns such as the stage of development during which Myt1 activity is required and how Myt1 loss affects the distribution of differentiated epithelial cell types in the intestine.

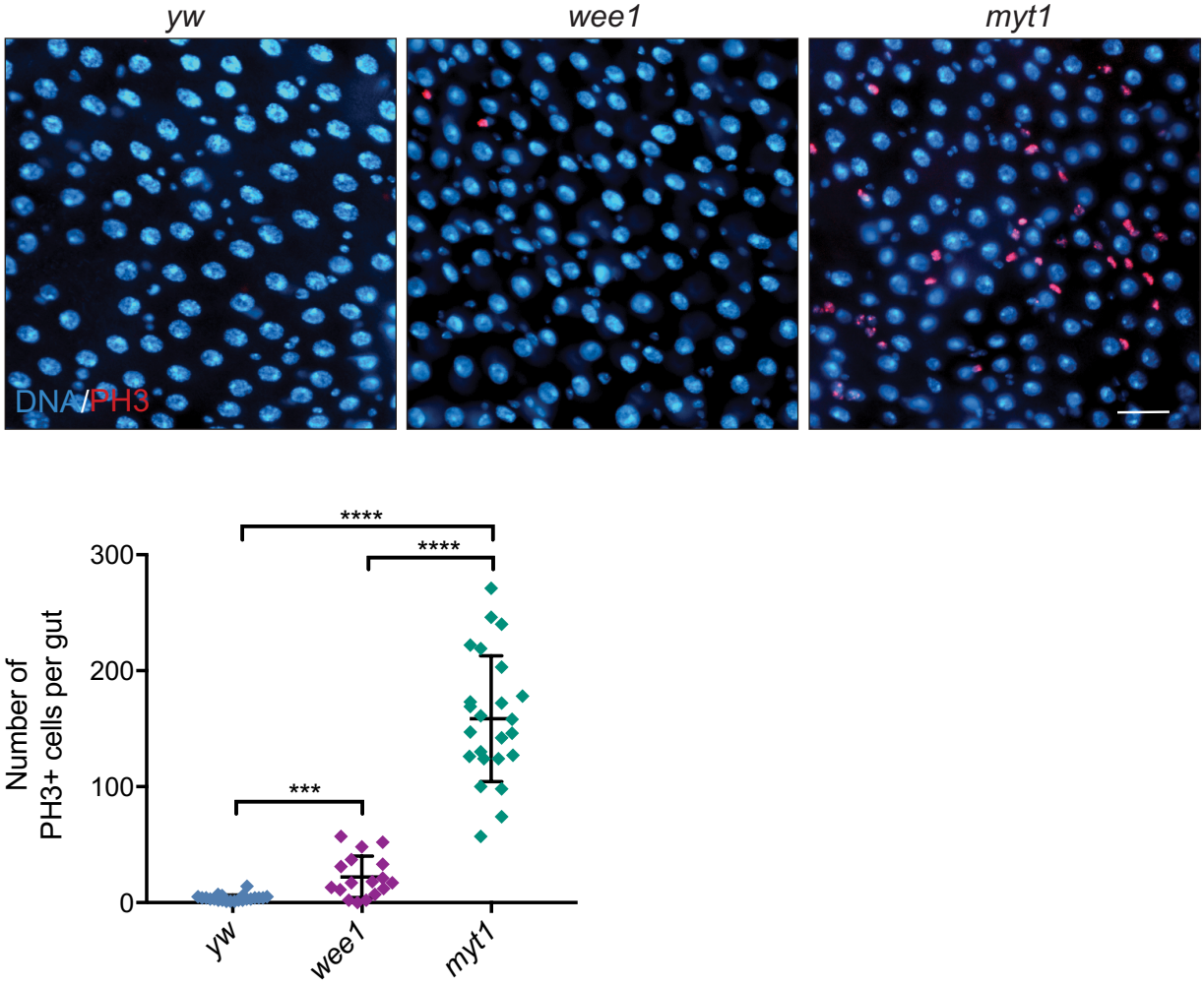


Figure 2-3. Hyper-proliferation in fly intestines devoid of Myt1. Intestines from 7d old female flies were dissected and stained for PH3 and DNA, and the number of PH3 positive cells per gut was quantified. *yw* control flies showed an average of 4.2 +/- 2.8 mitoses per gut (n=22), *wee1* mutants possessed an average of 22.2 +/- 17.9 mitoses per gut (n=17), and *myt1* mutant flies showed 164.5 +/- 54.3 PH3 positive cells per gut (n=24). Scale bar = 20 µm

2.4.3. Larval *myt1* mutants also show increased proliferation in the intestinal epithelium

ISCs are the only mitotic cells within the intestinal epithelium. These cells are derived from larval precursors (Takashima and Hartenstein, 2012), therefore I also analyzed the larval intestine to assess how the intestinal developmental program might be affected in *myt1* mutants. Guts from wandering 3rd instar larvae heterozygous for the *myt1* null allele showed 2.9 +/- 1.6 mitotic cells per frame (n=7) whereas *myt1* mutants displayed 83.1 +/- 38.0 PH3 positive cells per frame (n=10; Figure 2-4). These results suggest that the increased proliferation observed in the adult *myt1* mutants could be a consequence of earlier developmental defects. Given that only a small population of mitotically active intestinal cells normally survive the larval to pupal transition (Takashima and Hartenstein, 2012), it is possible that hyperproliferation during the larval stage of *myt1* mutants disrupts formation of the adult epithelium, resulting in gut dysplasia and compensatory ISC proliferation in adulthood. To determine if there was a requirement for Myt1 in the adult intestinal epithelium specifically, further analysis was conducted.

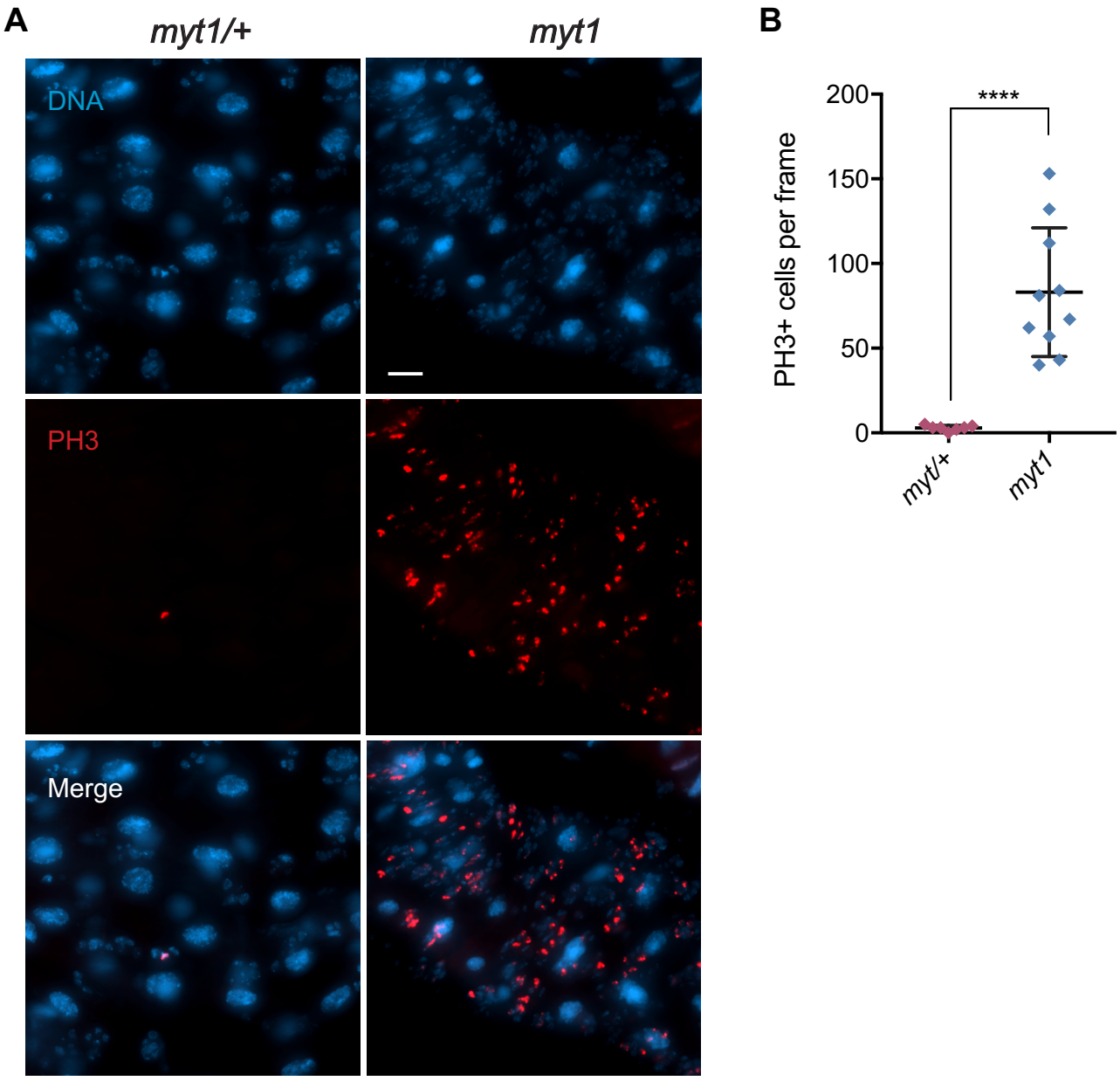


Figure 2-4. Larval *myt1* intestines also show increased proliferation. A) Intestines from 3rd instar larvae heterozygous and homozygous for a *myt1* null allele respectively were dissected and stained for DNA and PH3. Scale bar = 20 µm. B) Quantification of mitoses per frame in control heterozygotes (n=7) and *myt1* mutant (n=10) larval intestines.

2.4.4. Myt1 intrinsically regulates cell division in the adult intestinal epithelium

To test whether Myt1 activity is required specifically in the adult intestinal epithelium, I employed the MARCM system (Lee and Luo, 1999) to generate ISC clones possessing a *myt1* null allele. In this system, heat shock induced FLP-FRT mediated recombination during the mitotic cell cycle facilitates production of cells homozygous for a mutation in an otherwise heterozygous background. Since the mutant cells are GFP positive they can be easily distinguished from their heterozygous neighbours. Seven days following clone induction (heat shock), most *myt1* mutant clones possessed one to several mitotic cells as judged by PH3 labeling, with DNA also appearing dispersed and disorganized (Figure 2-5A-B). This was in contrast to WT clones in which DNA appeared normal and no mitotic cells were ever observed under these same experimental conditions (Figure 2-5A-B). Quantification of clone sizes showed that *myt1* mutant clones contained significantly more cells per clone than wild-type cells (Figure 2-5C), however this increase was not as substantial as expected given that some mutant clones are composed primarily of mitotic cells. One possible reason for this outcome is that loss of Myt1 results in either prolonged mitoses or arrests cells in mitosis. No increase in PH3 positive cells was observed in heterozygous or wild-type tissue surrounding *myt1* clones (Figure 2-5D). These data showing that loss of Myt1 activity causes excess mitoses specifically in *myt1* ISC clones indicates that Myt1 operates in a cell autonomous manner, intrinsically regulating cell division within the adult intestinal epithelium.

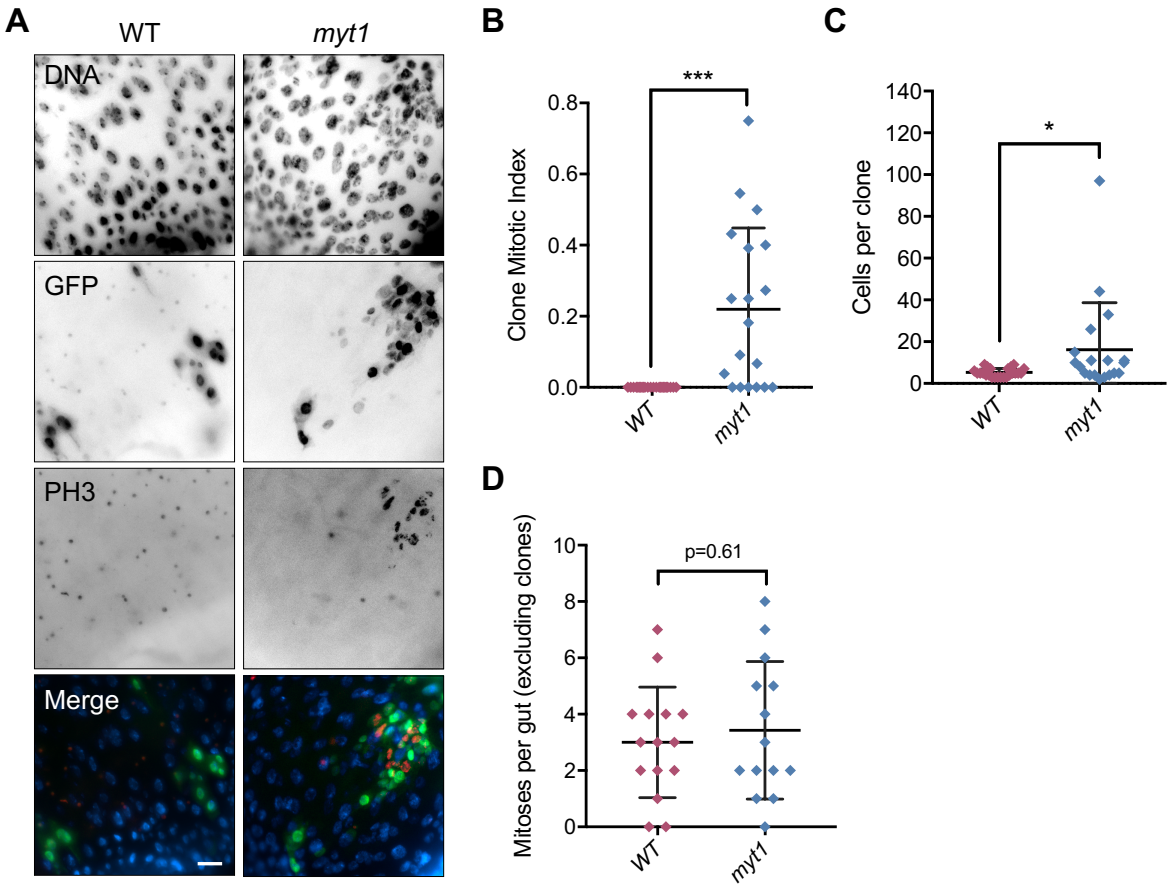


Figure 2-5. *myt1* MARCM clones show an increase in mitotic cell number. A) Visualization of WT and *myt1* mutant GFP positive mitotic clones in the posterior midgut. Scale bar = 20 µm. B) Quantification of PH3 positive cells reveals no clones with mitotic cells in the wild-type control (n=24 clones) whereas mutant clones exhibited a mitotic index of 0.22 +/- 0.23 (n=19 clones). C) The same WT and *myt1* clones possessed on average 5.3 +/- 2.0 cells and 16.2 +/- 22.5 cells per clone respectively. D) Quantification of PH3 positive cells in GFP negative tissue (in guts possessing clones) shows no difference between control (n=15) and *myt1*/+ (n=14) flies.

2.4.5. Validation of *myt1*^{RNAi}

Investigations utilizing *myt1* mutants can be difficult for a number of reasons. Firstly, expression of many constructs in a *myt1* mutant background causes increased lethality during development, for reasons unknown. Furthermore, studies employing this zygotic viable mutant do not allow for temporal control over loss of gene function. In addition to employing MARCM, I sought to address these issues using RNAi directed against Myt1. Previously, no one in our lab had successfully employed RNAi against *myt1* in several different *Drosophila* tissues (male germline, female germline, wing discs). This may be due to the stability of Myt1 protein in these tissues. Since RNAi against Myt1 had not been evaluated in the intestine, I tested five different RNAi lines against *myt1* and found 3 that recapitulated the mutant phenotype. I selected the fly line that gave the strongest intestinal phenotype (VDRC #105157) and utilized this for all subsequent experiments.

To test whether this line successfully depleted Myt1, I expressed EGFP-Myt1 with or without the RNAi construct. EGFP-Myt1 localized predominantly to the nuclear envelope of ISCs and EBs in control intestines, however upon 7 days co-expression with *myt1*^{RNAi}, the EGFP-Myt1 signal was almost completely absent (Figure 2-6). This data therefore supports genetic evidence that this RNAi line is capable of depleting endogenous Myt1. It is also the first report of Myt1 localizing to the nuclear envelope, the significance of which remains unclear.

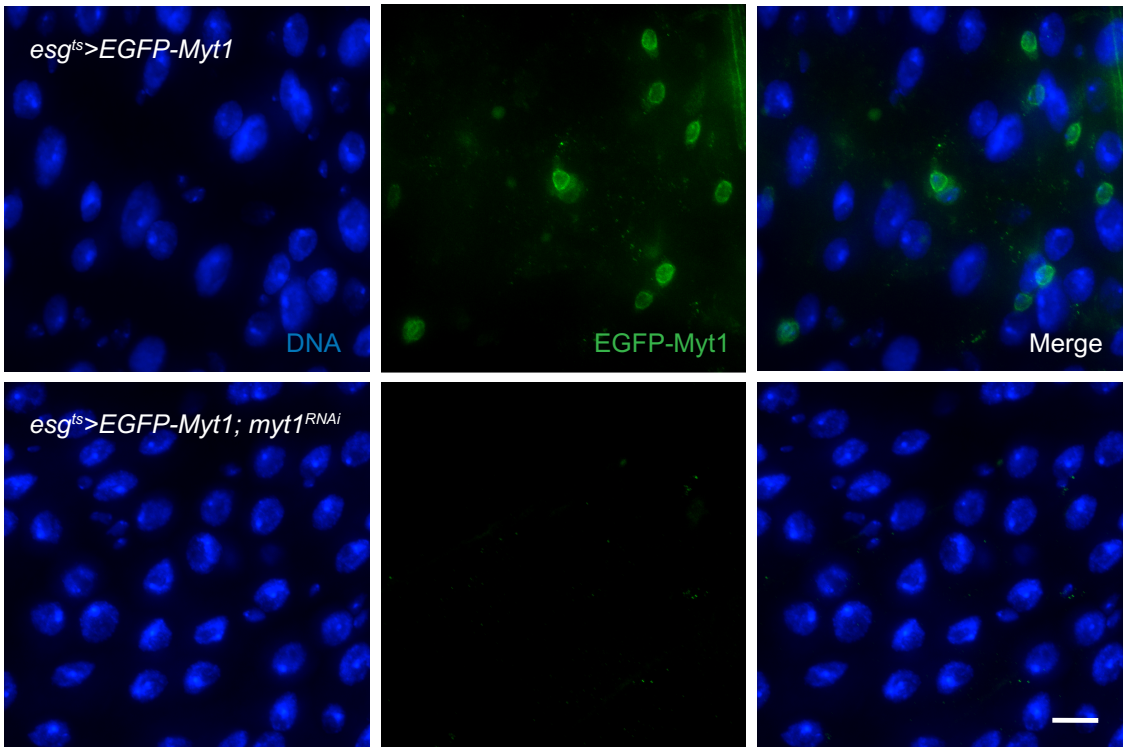


Figure 2-6. Validation of *myt1*^{RNAi}. Comparison of EGFP-Myt1 expressed alone or with *myt1*^{RNAi} in ISCs and EBs. Flies were grown up at 18ºC, with adults transferred to 29ºC 4d post-eclosion. Analysis was conducted 7 days after temperature shift. Scale bar = 10 μm

2.4.6. Myt1 maintains progenitor cell homeostasis

In the intestinal epithelium, multipotent ISCs divide asymmetrically to give rise to EBs that differentiate into secretory enteroendocrine cells or polyploid enterocytes. Previous work has demonstrated that these differentiated cells, as well as visceral muscle, can provide mitogenic cues to ISCs (Jiang et al., 2009; Lee et al., 2009; Lin et al., 2008). MARCM analysis demonstrated that Myt1 acts in a cell autonomous manner, however the cell type requiring Myt1 activity has yet to be determined. To elucidate this, I employed a temperature sensitive UAS-GAL4 system to express validated UAS-RNAi against *myt1* in each epithelial cell type independently, as well as in ISCs and EBs together. I observed that RNAi against *myt1* expressed in ISCs and EBs together with *esg-GAL4, UAS-GFP, tub-GAL80^{ts}* (hereafter referred to as *esg^{ts}*) increased the number of mitotic cells (Figure 2-7), a phenotype similar to that observed in the *myt1* mutant MARCM clones. This shows that Myt1 acts as a negative regulator of mitosis in ISCs and/ or EBs. I next analyzed depletion of Myt1 in ISCs alone using *esg-GAL4, UAS-2X-EYFP; Su(H)GBE-GAL80, tub-GAL80^{ts}* (hereafter referred to as *esg^{ts}; Su(H)-GAL80*) and in EBs alone using *Su(H)GBE-GAL4; tub-GAL80^{ts}, UAS-GFP* (hereafter referred to as *GBE^{ts}*). Depletion in both ISCs and EBs independently also resulted in significant increases in PH3 positive cells (Figure 2-7), indicating that Myt1 activity is required for normal proliferation in both of these cell types, rather than in ISCs alone as expected. Further analysis of *myt1^{RNAi}* expressed in EEs with *UAS-GFP, tub-GAL80^{ts}; pros-GAL4* (hereafter referred to as *Pros^{ts}*), ECs with *Myo1A-GAL4, tub-GAL80^{ts}, UAS-GFP* (hereafter referred to as *Myo1A^{ts}*) or visceral muscle with *tub-GAL80^{ts}; How-GAL4^{ts}* (hereafter referred to as *How-GAL4^{ts}*) showed no difference in the number of mitotic cells relative to controls driving UAS-GFP alone (Figure 2-7). This showed that *myt1^{RNAi}* expressed in these cell types does not affect ISC proliferation, and that Myt1 expression is required only within ISCs and EBs to prevent ectopic mitoses.

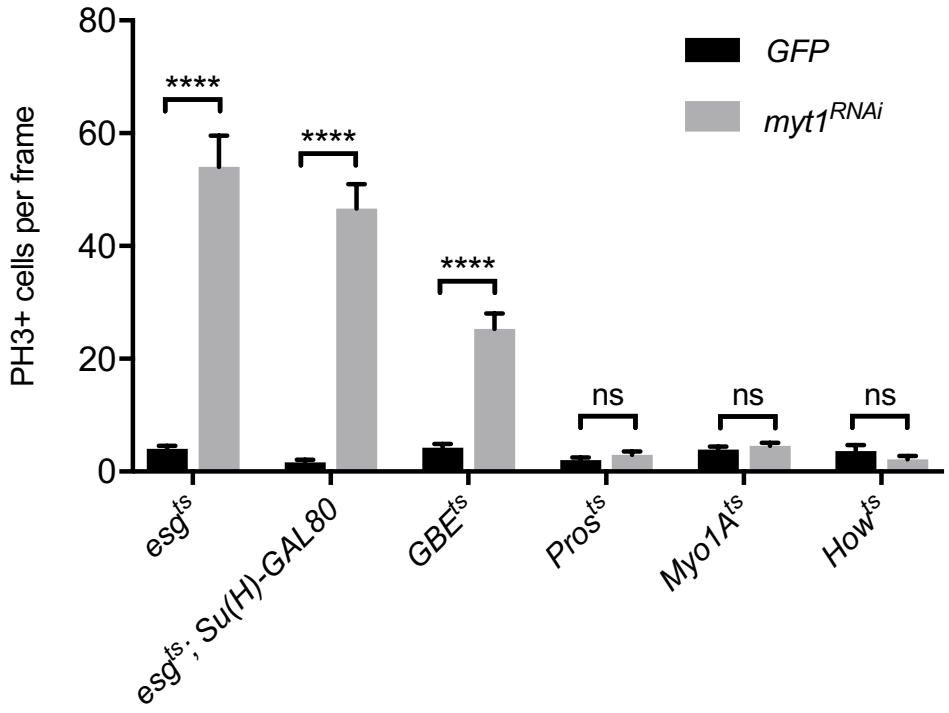


Figure 2-7. Myt1 is required in ISCs and EBs to prevent over-proliferation. Temperature sensitive GAL4 drivers specific to each epithelial cell type were used to analyze the requirement for Myt1 in the intestine. *esg*^{ts} (ISC and EB specific), *esg*^{ts}*; Su(H)-GAL80* (ISC specific), and *GBE*^{ts} (EB specific) driven depletion of Myt1 resulted in significant increases in mitoses (n=26, 21 and 24 respectively) relative to expression of UAS-GFP alone (n=25, 16, and 16 respectively). *Pros*^{ts}*, Myo1A*^{ts}*, and How*^{ts} driving *myt1*^{RNAi} (n=18, 25, and 19 respectively) showed no significant change in mitoses relative to expression of UAS-GFP alone (n=17, 19, and 16 respectively). Analysis was done on projected Z-stack images of guts taken 1 frame anterior from the midgut-hindgut transition zone. All flies were kept at 29ºC for 7 days before dissection. Error bars show S.E.M.

2.4.7. Myt1 promotes interphase in ISCs and EBs

To investigate how Myt1 loss in ISCs and EBs affects these same cell types, I next characterized flies in which Myt1 was depleted in both ISCs and EBs, as well as in ISCs and EBs independently. Schematics in each figure of this section identify cell types expressing *myt1*^{RNAi} in red, cell types expressing GFP (or a GFP-tagged construct) in green, and cell types expressing both *myt1*^{RNAi} and GFP in yellow.

Upon 7 days of *esg*^{ts}*>myt1*^{RNAi} expression (ISCs and EBs), posterior midguts were analyzed with PH3 labeling. The *myt1* knockdown showed an average of 54.1 +/- 24.6 mitoses per frame whereas the control was much lower with 4.0 +/- 2.9 mitoses per frame (Figure 2-8B). Furthermore, unlike the control where ISCs and EBs were uniformly distributed and rarely PH3 positive, the Myt1-depleted ISCs and EBs formed clusters of cells, many of which were mitotic and weakly GFP positive (Figure 2-8C). Stem cell identity is maintained by the transcription factor *escargot* (Korzelius et al., 2014), thus weak GFP expression due to lessened *esg-GAL4* activity could indicate a loss of stemness, perhaps as a result of rapid cell cycling or prolonged mitoses.

I subsequently analyzed the ISC-specific knockdown of Myt1 using *esg-GAL4* with *Su(H)*-driven expression of GAL80 to prevent RNAi expression in EBs. Quantification of the Myt1-depleted stem cells showed 46.6 +/- 19.8 mitoses, which was significantly higher than the controls with 1.6 +/- 1.8 mitoses per frame (Figure 2-9A). Closer analysis revealed that these *esg*^{ts}*; Su(H)-GAL80>myt1*^{RNAi} guts possessed mitotic cells that were either weakly GFP positive or not GFP positive at all, unlike controls in which all mitotic cells were clearly GFP positive (Figure 2-9B). These results could indicate that these ISCs have moved into a state of senescence, perhaps caused by an inability to exit mitosis, or that Myt1 produced in ISCs is necessary for inhibiting mitotic re-entry in newly formed EBs following asymmetric ISC division. It is possible that this demonstrates an EB-specific requirement for Myt1 given that

the majority of PH3 positive cells were not GFP positive, as would be expected of EBs but not ISCs.

RNAi against *myt1* expressed in EBs alone with GBE^{ts} also caused significantly increased cell proliferation, from 4.3 +/- 2.6 mitoses per frame in controls to 25.3 +/- 13.6 mitoses per frame (Figure 2-10A). To elucidate which cell types were being affected in this experiment, I analyzed these intestines where EBs were labeled with GFP. As expected, mitotic EBs were never observed in the control group, however GFP positive and PH3 positive cells were frequently seen in Myt1-depleted cells (Figure 2-10B). These results showed that loss of Myt1 specifically in EBs was sufficient to permit ectopic entry of EBs into mitosis. Because *GBE-GAL4* can be weakly expressed in ISCs (Edan Foley, pers. communication), I confirmed this result using the Notch response element *Suppressor of Hairless* tagged with GFP (Su(H)-GFP), an EB-specific reporter (Lucchetta and Ohlstein, 2017; Ohlstein and Spradling, 2007). Upon expressing $myt1^{RNAi}$ in ISCs and EBs, I observed Su(H) positive cells that were also PH3 positive (Figure 2-11), confirming that EBs do become mitotic upon Myt1 loss.

Collectively, these results can be interpreted in two different ways. One possibility is that Myt1 activity plays two roles within the intestinal epithelium: both in ISCs to maintain normal rates of proliferation by promoting interphase arrest, and in EBs independently to prevent inappropriate re-entry into a mitotic cell cycle. A second possible explanation is that Myt1 has no ISC-specific function, and that it is only required in EBs to promote mitotic exit following asymmetric ISC division and EB production.

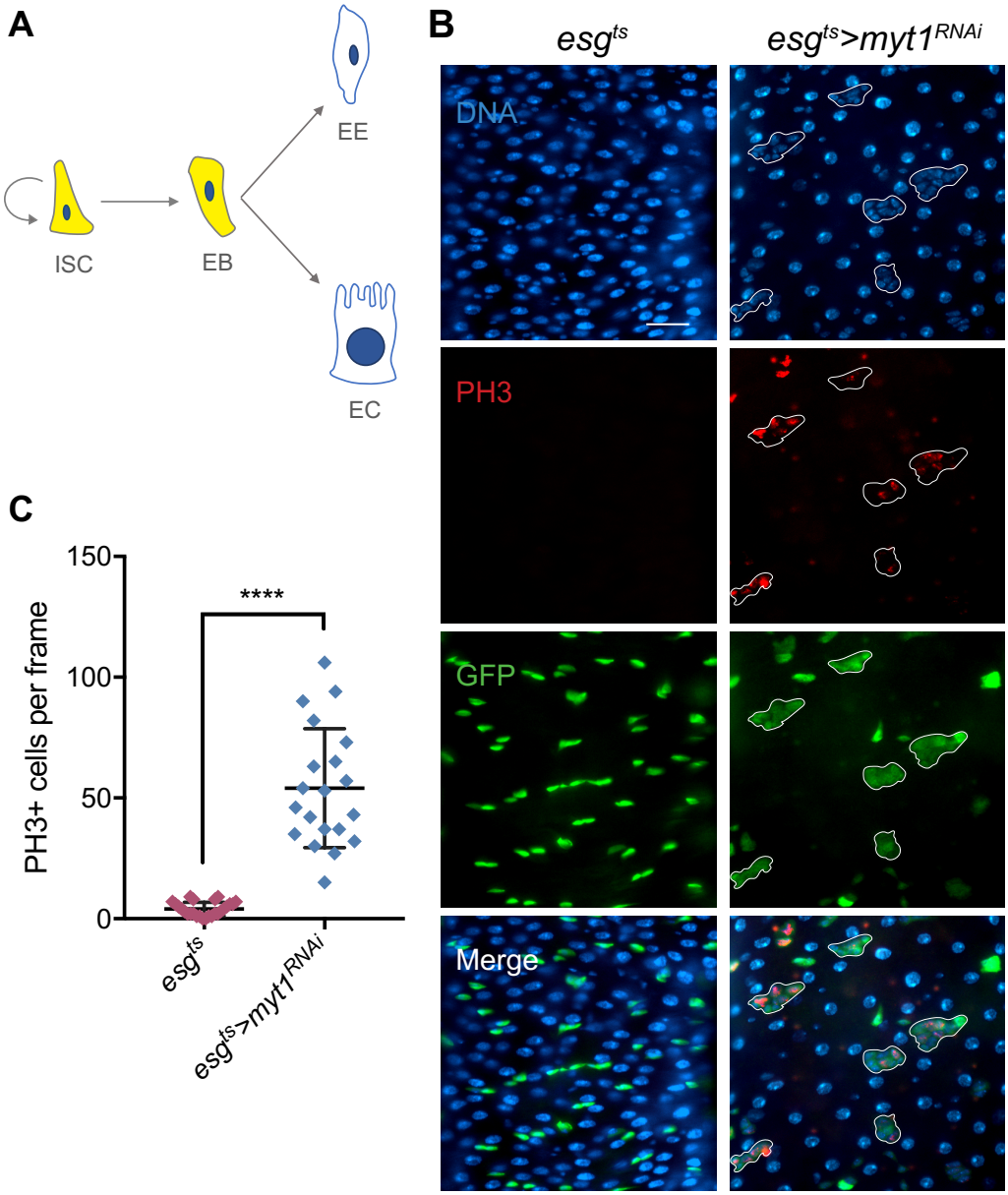


Figure 2-8. Myt1-depleted progenitors form clusters of mitotic cells. A) *myt1*^{RNAi} and GFP expressed in both ISCs and EBs with *esg*^{ts}. B) Visualization of *esg*^{ts}*>myt1*^{RNAi} (n=25) reveals that a significant proportion of progenitors become mitotic (PH3 positive) and group together when Myt1 is depleted (progenitors outlined in white). Few mitotic cells were observed in the control (n=26), and GFP positive progenitors were uniformly dispersed in the epithelium. Scale bar = 20 µm C) Quantification of mitotic cell number in progenitors depleted of Myt1. Flies were analyzed 7 days after temperature shift to 29ºC.

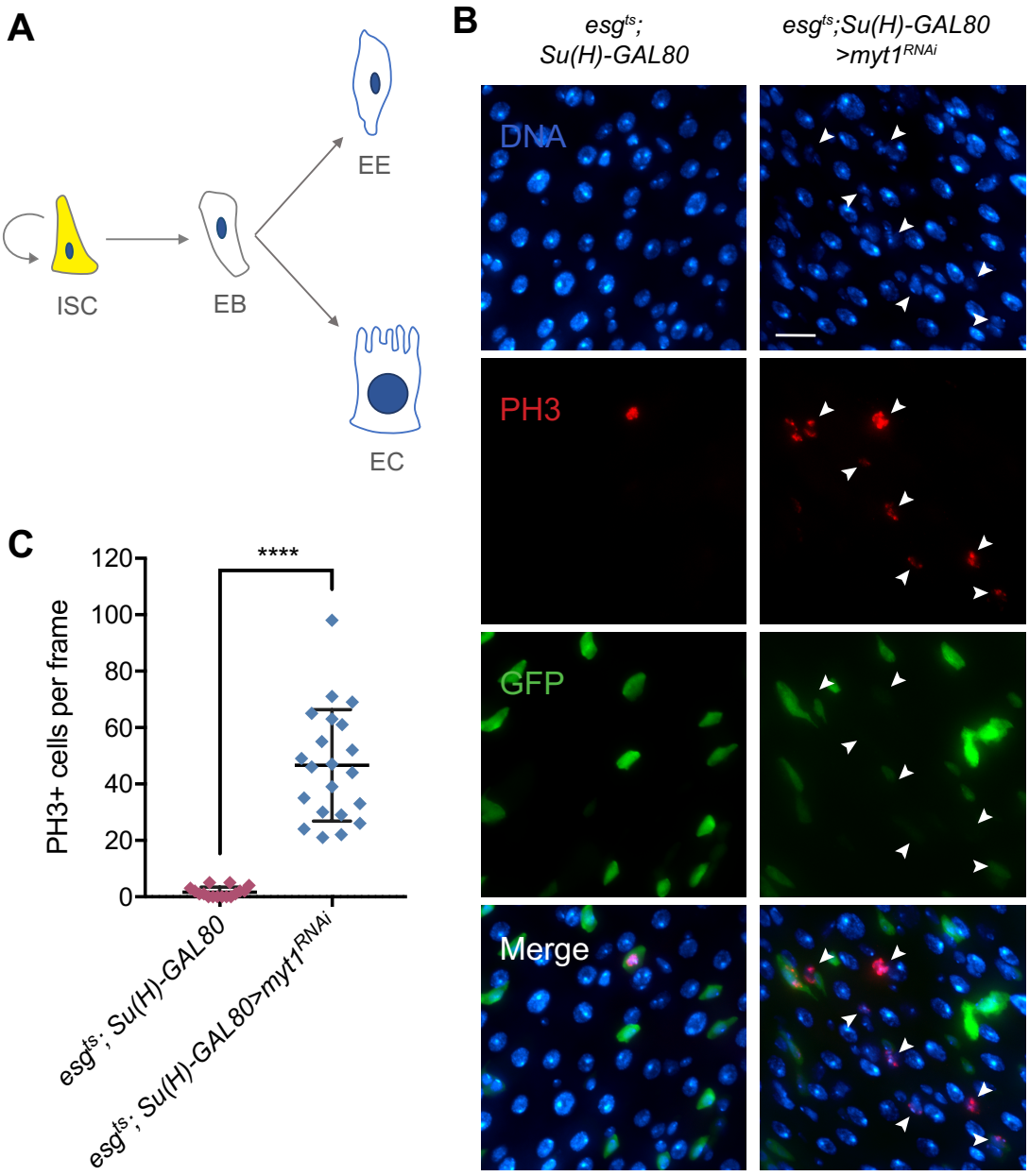


Figure 2-9. ISC-specific knockdown of Myt1 causes ectopic mitoses. A) Schematic showing ISCs expressing GFP and *myt1*^{RNAi} using *esg*^{ts}*; Su(H)-GAL80*. B) Visualization of intestines shows significant numbers of PH3 positive cells, many of which are not GFP positive (arrowheads). In control guts, PH3 positive staining was only observed in GFP positive cells. C) Myt1 depletion in ISCs (n=21) significantly increased the number of mitotic cells relative to the control (n=16). Flies were analyzed 7 days after temperature shift to 29ºC. Scale bar = 20 µm.

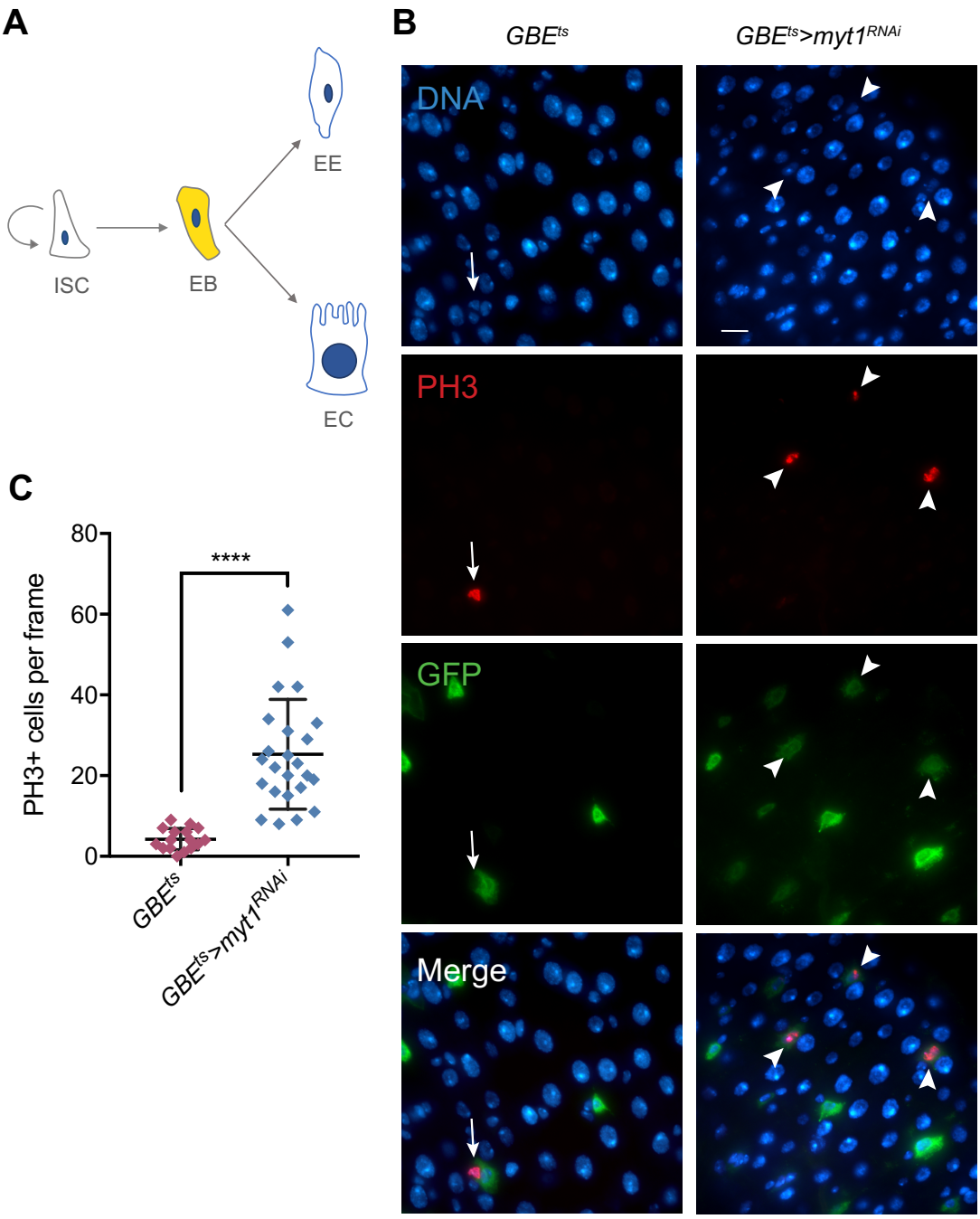


Figure 2-10. Mitosis in Myt1-depleted enteroblasts. A) *myt1*^{RNAi} and GFP expressed in EBs with *GBE*^{ts}. B) Z-stack projections of control expressing GFP alone or GFP and *myt1*^{RNAi} in EBs. Mitotic GFP positive cells (EBs) were never observed in the control. Arrow points to mitotic ISC next to an EB. In *GBE*^{ts}*>myt1*^{RNAi}, GFP and PH3 positive cells were commonly seen (arrowheads). C) Myt1 depletion in EBs significantly increased the number of mitotic cells (n=24) relative to the control (n=16). Scale bar = 20 µm.

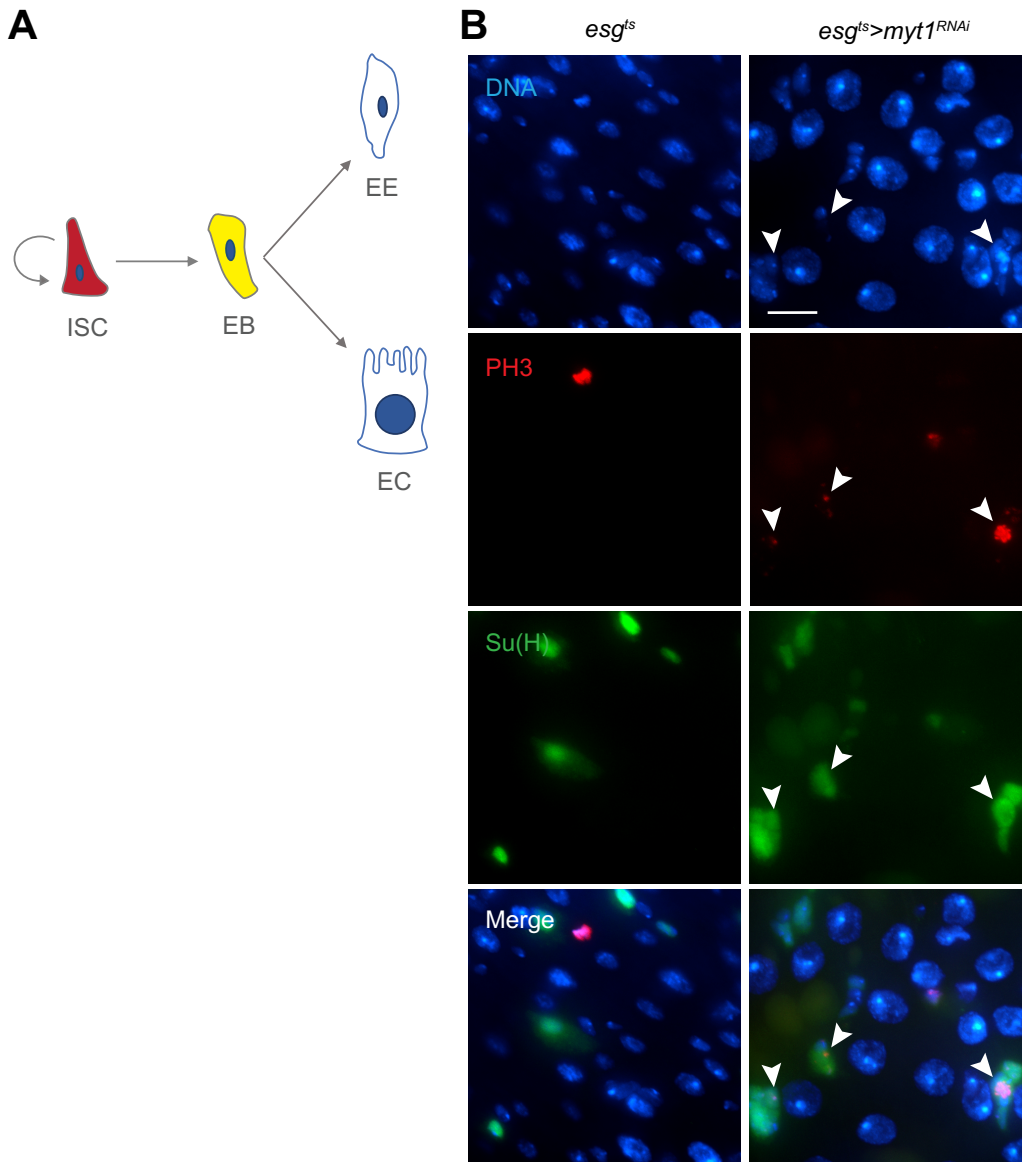


Figure 2-11. Enteroblasts become mitotic upon Myt1 depletion. A) ISCs and EBs express *myt1*^{RNAi} while EBs express Su(H)-nls-GFP reporter. Co-staining for GFP and PH3 shows no overlap in control, whereas guts with progenitors depleted of Myt1 shows consistent Su(H) and PH3 overlap (arrowheads). Scale bar = 10 μm.

To determine which of the abovementioned interpretations is correct, I conducted an experiment to elucidate if Myt1 loss in ISCs alone was sufficient to produce mitotic EBs. I generated a fly line expressing NRE-GFP (Notch response element tagged with GFP, (Lucchetta and Ohlstein, 2017), an EB-specific reporter, as well as *myt1*^{RNAi} in ISCs alone. While control guts never showed co-staining for PH3 and NRE-GFP, ISC-specific Myt1 depletion gave rise to PH3 positive cells that were also NRE positive ~63% of the time (Figure 2-12). This demonstrated that Myt1 loss in ISCs is sufficient to promote mitosis in EBs. It is unclear, however, if Myt1 is necessary for EB exit from mitosis, or if it prevents re-entry into mitosis following asymmetric ISC division. Given that EB-specific depletion of Myt1 with the *GBE*^{ts} driver also enables EBs to re-enter mitosis, it is possible that ISC-specific Myt1 also prevents mitotic re-entry within EBs. Additionally, it seems that Myt1 is required to promote interphase in ISCs since a significant proportion of mitotic cells in *esg*^{ts}*; Su(H)-GAL80>myt1*^{RNAi} flies (~37%, Figure 2-12C) were not GFP positive and were therefore likely to be ISCs. Overall, it is clear from my results that Myt1 loss in progenitors prevents exit from the mitotic cell cycle in EBs, a normally post-mitotic cell. EBs execute the mitotic-to-endocycle switch to produce differentiated ECs, thus further work is warranted to examine the effect of Myt1 loss on cell fate outcome.

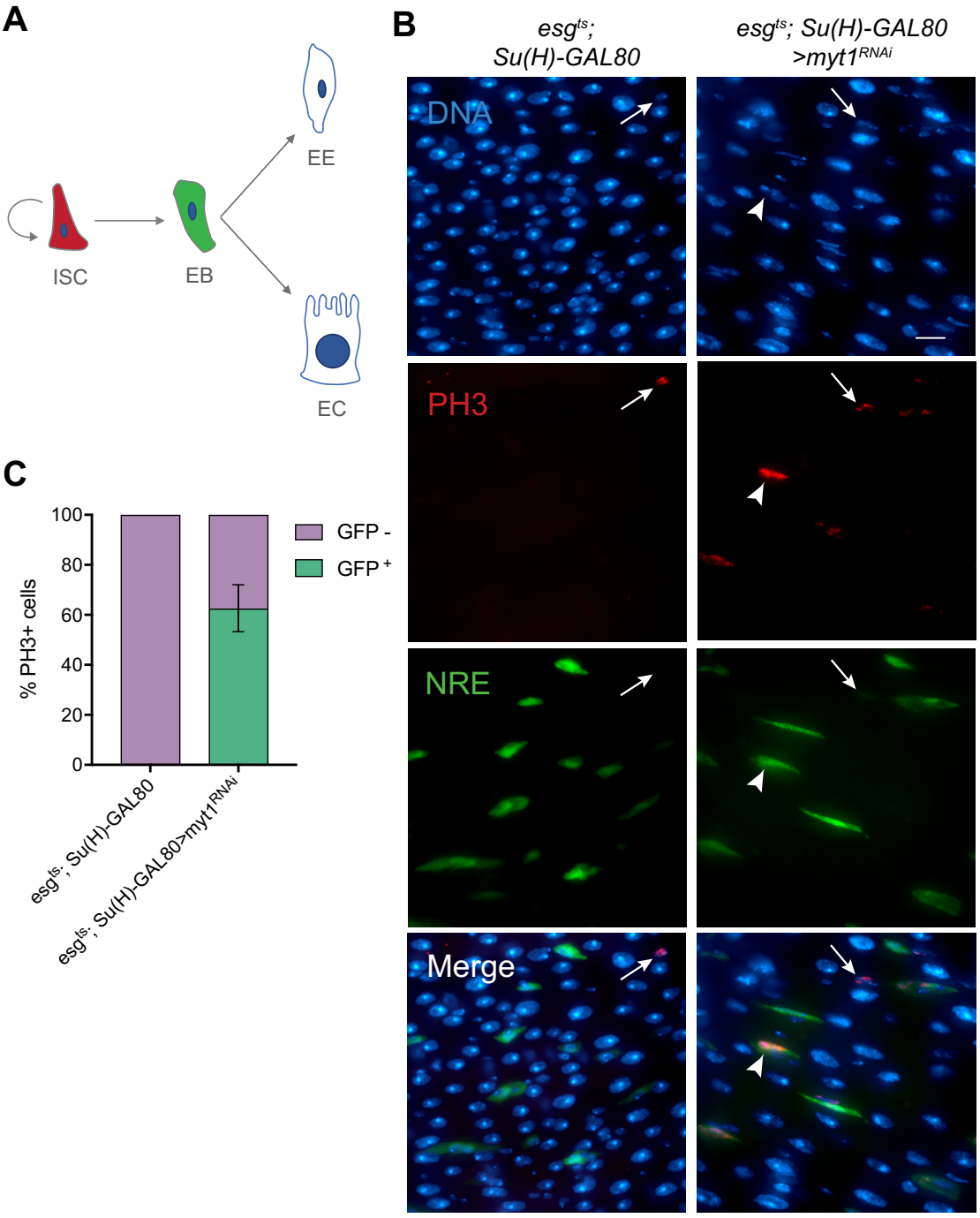


Figure 2-12. ISC-specific Myt1 depletion results in ectopic ISC and EB mitoses. A) Guts from flies expressing NRE-GFP in EBs and *myt1*^{RNAi} in ISCs. B) Z-stack projections of control and *myt1*^{RNAi} stained for PH3. Mitotic GFP positive cells (EBs) were never observed in the control, while NRE-GFP and PH3 positive cells were observed upon Myt1 loss (arrowhead). Arrows point to GFP negative, PH3 positive cells. C) Myt1 depletion in ISCs resulted in a fraction of PH3 positive cells that were NRE positive (n=12 guts), while PH3 and NRE positive cells were never observed in controls (n=10 guts). Scale bar = 10 μm.

2.4.8. Ectopic ISC and EB mitoses produce excess undifferentiated progenitors

Epithelial homeostasis is maintained by a careful balance of cell division, cell differentiation, and cell death. Upon perturbation of these processes, intestines quickly become dysplastic, developing anomalous growths that can lead to premature fly death. For example, Notch deficient flies are disrupted in their ability to form differentiated enterocytes, and instead produce tumors laden with excess progenitors and enteroendocrine cells (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006).

Flies deficient for Myt1 exhibit excessive cell proliferation. Accordingly, I next assessed how increased mitosis due to Myt1 depletion affects the balance of cell types in the intestinal epithelium. To do this, I utilized an esg^{ts} line similar to that previously employed, but with reporters as follows: *esg-GAL4, UAS-his-CFP, Su(H)-nls-GFP; tub-GAL80*^{ts}. In these flies, all ISC and EB nuclei are CFP positive while EB nuclei are also GFP positive. Thus, it is possible to quantify both ISC and EB numbers. In control esg^{ts} flies, I observed near-equal numbers of ISCs and EBs, however in $esg^{ts}>myt1^{RNAi}$ flies, the progenitor population in the intestinal epithelium contained approximately 60% EBs and 40% ISCs (Figure 2-13A). Further quantification of ISCs revealed that this was not due to diminished ISC numbers; rather, ISCs were more abundant upon Myt1 loss (Figure 2-13B) indicating that the number of EBs present was also proportionately increased.

Analysis of differentiated cell types was also conducted. I employed the esg^{ts} *Flp-Out* line (hereafter esg^{ts}*F/O*) with the genotype *esg-GAL4, tub-GAL80*^{ts}*, UAS-GFP; UAS-flp, Act>CD2>GAL4* (Jiang et al., 2009). Here, all progenitors and their clones were marked following a shift to 29ºC due to activation of Flp, which removes CD2 from the third chromosome to allow Actin-GAL4 expression. Thus, epithelial turnover (and production of differentiated cells) could be assessed. Under homeostatic conditions, approximately 10% of progenitors differentiate into EEs while 90% become ECs (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). Staining against Prospero (EE-specific) revealed a slight

decrease in the proportion of EEs within Myt1-depleted clones after 3 weeks RNAi expression, though no statistically significant difference was observed after only one week of *myt1*^{RNAi} expression (Figure 2-13C-D). Measurement of the internuclear distance between GFP positive ECs, however, demonstrated that there was a small but significant increase in the distance between EC nuclei after one week (Figure 2-13E). This showed that fewer ECs were present in *esg*^{ts}*F/O>myt1*^{RNAi} flies relative to the control. Overall, I therefore conclude that there was no impact on EB fate outcome in Myt1-depleted progenitors; rather, the defect with respect to EBs appeared to be in their ability to differentiate at all, since there was an accumulation of undifferentiated EBs in *esg*^{ts}*>myt1*^{RNAi} flies. Thus, phenotypic analysis demonstrated that ectopic mitotic activity in progenitors due to Myt1 loss inhibits progenitor differentiation into ECs, and, to a lesser extent, EEs. It is further possible that these superfluous undifferentiated cells may not be true EBs or ISCs but could instead be cells that are becoming senescent or entering a progenitor-like state.

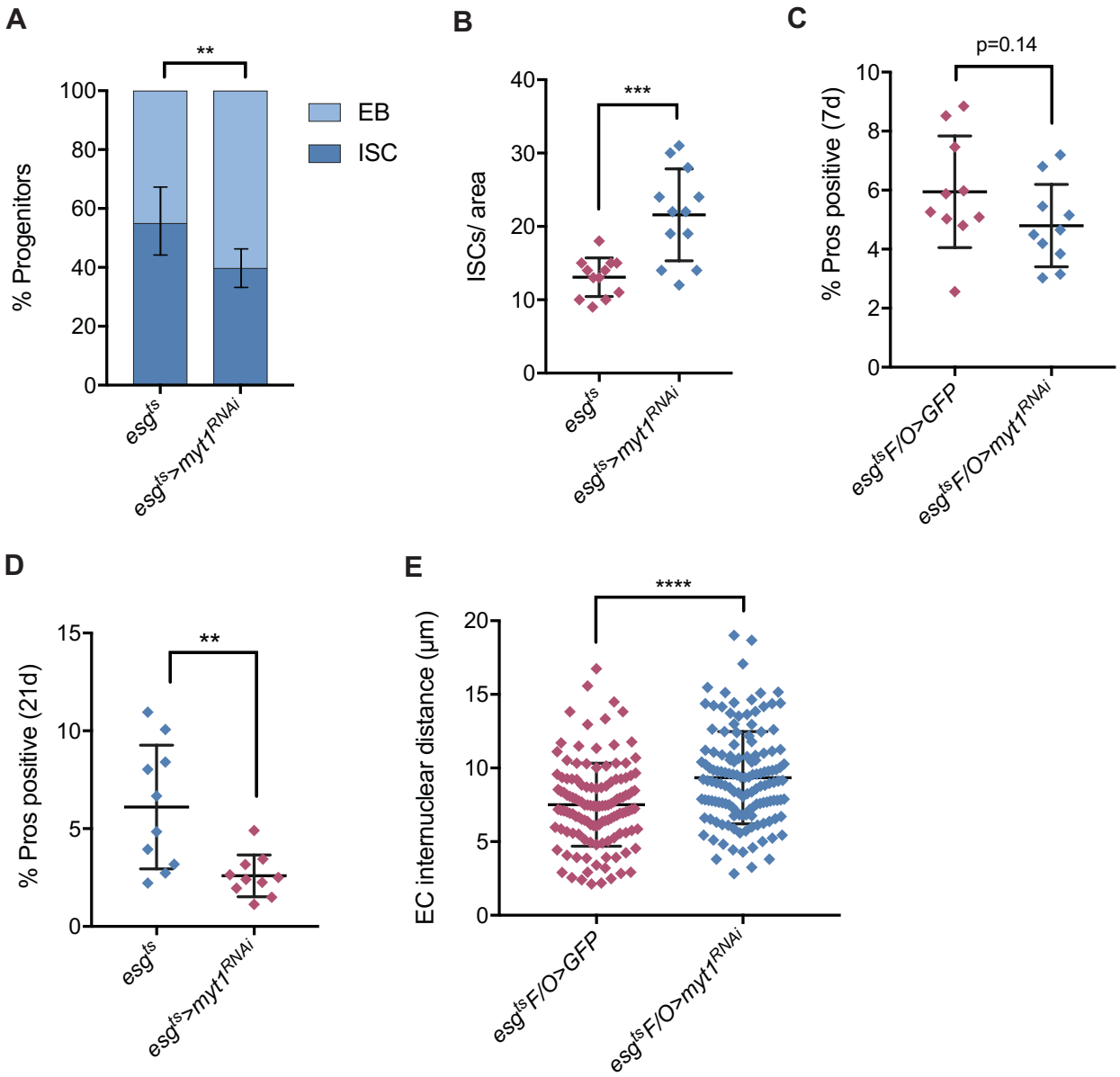


Figure 2-13. Overproduction of undifferentiated progenitors in Myt1 knockdown. A) Quantification of ISC and EB proportions using *esg-GAL4, UAS-his-CFP, Su(H)-nls-GFP; tub-GAL80^{ts}*. Control flies possess approximately 55% ISCs and 45% EBs (n=12). Myt1 loss causes a shift to 40% ISCs and 60% EBs (n=12). B) Quantification of ISC number in a 100 µm x 100 µm area 1 frame anterior of the midgut-hindgut transition zone. ISC numbers increase from an average of 13.1 +/- 2.6 in the control (n=12) to 21.6 +/- 6.3 ISCs upon Myt1 depletion (n=12). C-D) The percentage of EEs (Pros positive nuclei) in GFP positive clones does not change in *esg^{ts}>myt1^{RNAi}* flies (n=10) relative to the control (n=10) after 7d (C) but is significantly lower 21d after *myt1^{RNAi}* induction (n=10) relative to the control (n=10) (D). E) The average distance between ECs (identified by large polyploid nuclei) in GFP positive clones increases from 7.5 µm +/- 2.8 µm in controls to 9.3 µm +/- 3.1 µm in *esg^{ts}>myt1^{RNAi}* intestines. Twenty gut were analyzed, with at least 5 measurement taken from each gut. All analyses were conducted 7 days after shift to 29ºC, unless otherwise stated.

2.4.9. Cdk1 inhibitory phosphorylation maintains progenitor cell homeostasis

Though Myt1 activity is necessary to prevent ectopic mitoses in gut progenitors, it is not clear that this effect is due to Cdk1 inhibitory phosphorylation. Consequently, I examined how defects in Cdk1 inhibitory phosphorylation affected gut progenitor homeostasis by an alternative approach.

A previous study demonstrated that Cdk1 is present in all Delta positive ISCs and a subset of EBs, but not in differentiated ECs or EEs (Amcheslavsky et al., 2011). I corroborated this data by immunolabeling against the PSTAIR motif of Cdk1 within the intestinal epithelium. PSTAIR staining was observed in 100% of ISCs and 6.8% of EBs, but not in ECs or EEs (Figure 2-14). This supports previous work showing that Cdk1 is present in ISCs and a fraction of EBs.

I next assessed the requirements for Cdk1 inhibitory phosphorylation in the *Drosophila* midgut by expressing previously characterized transgenic Cdk1 inhibitory phosphorylation mutants (Ayeni et al., 2014) in progenitor cells. Expression of $Cdk1^{WT}$-VFP (Cdk1WT), $Cdk1^{T14A}$-VFP (Cdk1A), $Cdk1^{Y15F}$-VFP (Cdk1F) and $Cdk1^{T14A,Y15F}$-VFP (Cdk1AF) had variable effects on the midgut. Mitotic cell number relative to Cdk1WT was not significantly different with Cdk1A expression (unable to be phosphorylated on T14). In contrast, Cdk1F (unable to be phosphorylated on Y15) and Cdk1AF (unable to be phosphorylated on T14 or Y15) both resulted in significant increases in mitotic cell number, with Cdk1AF expression causing significantly more mitoses than Cdk1F expression (Figure 2-15). Dual inhibitory phosphorylation of Cdk1 is therefore more important for maintaining intestinal homeostasis than Y15 inhibitory phosphorylation alone, consistent with my previous results indicating that the dual Cdk1 inhibitory kinase Myt1 serves an essential role in this tissue.

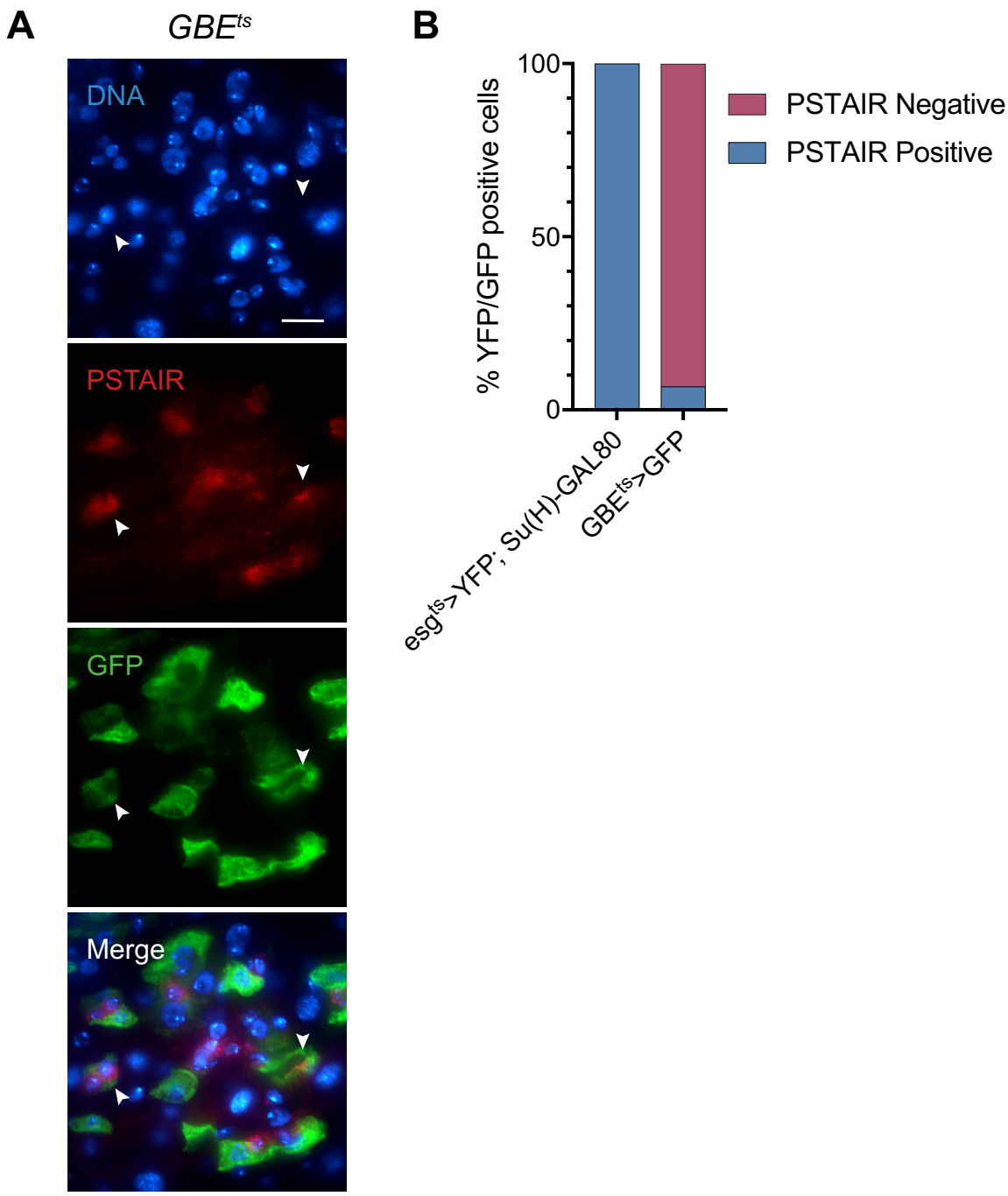


Figure 2-14. PSTAIR immunostaining is observed in all ISCs and a subset of EBs. A) Staining for the PSTAIR motif shows EBs (GFP positive) that are positive for the PSTAIR motif (arrowheads). Scale bar = 20 μm. B) Proportions of ISCs and EBs positive or negative for PSTAIR staining are shown. All ISCs are PSTAIR positive (n=8 guts), 6.8% of EBs are PSTAIR positive (n=10 guts).

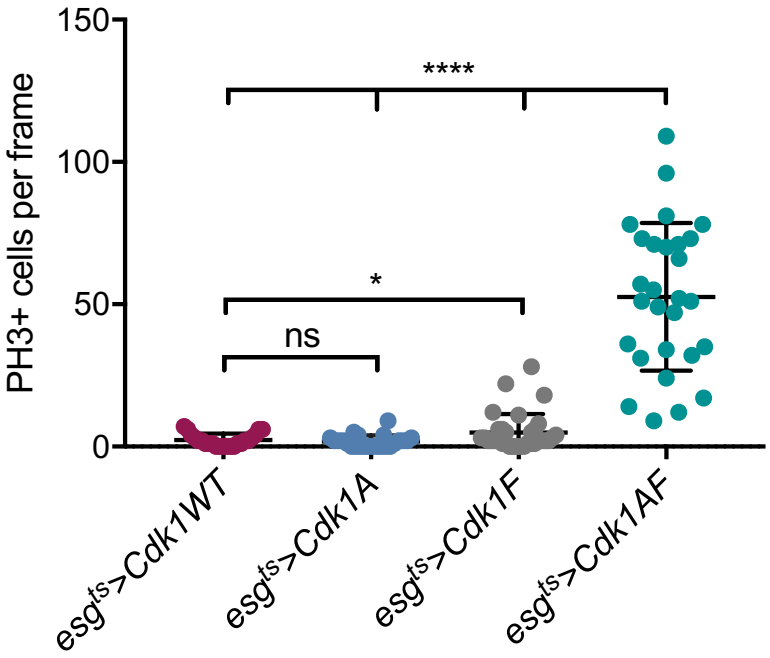


Figure 2-15. Expression of Cdk1 inhibitory phosphorylation mutants in the adult midgut. Numbers of PH3 positive cells were quantified one frame anterior from the midgut-hindgut transition zone. Cdk1WT (n=23), Cdk1A (n=24), Cdk1F (n=33), and Cdk1AF (n=28) were expressed in ISCs and EBs for 7d at 29ºC.

I also tested whether overexpression of the Cdc25 phosphatase String was sufficient to induce mitosis specifically within EBs. A previous study found that String overexpression alone with GBE^{ts} was not sufficient to induce cell division, and rather that String co-overexpression with Cyclin E was required to promote mitosis in EBs (Kohlmaier et al., 2015). At odds with this study, when I overexpressed String with GBE^{ts} I found that EBs indeed became mitotic, producing EB clusters (Figure 2-16). This is consistent with the idea that Cdk1 inhibitory phosphorylation is required to promote EB exit from the mitotic cell cycle. Taken together with the phospho-acceptor mutant data, this result also supports the hypothesis that loss of Myt1 dual inhibitory phosphorylation of Cdk1 is the molecular mechanism underlying the *myt1* mutant intestinal phenotype.

After establishing that inhibitory phosphorylation of Cdk1 is necessary to maintain homeostasis within the intestinal epithelium, I wondered if Myt1 might inhibit specific cyclin-Cdk1 complexes. During *Drosophila* spermatogenesis, Myt1 inhibits Cdk1 in a Cyclin A specific manner (Varadarajan et al., 2016), though the extent to which this mechanism is conserved remains unclear. Accordingly, I depleted Cyclin A in progenitors of the intestine to determine if Myt1 activity is Cyclin A specific in this context. In contrast to control intestines showing 1.7 +/- 0.5 mitoses/ frame (n=13), no mitoses were observed in guts with ISC-specific Cyclin A depletion (n=15, Figure 2-17C). Phenotypic analysis further revealed that Cyclin A depletion in ISCs produced large stem cells with polyploid nuclei (Figure 2-17A), showing that Cyclin A is required for mitosis but not DNA replication. Co-expression of $CycA^{RNAi}$ and $myt1^{RNAi}$ in ISCs (n=10) rescued the increase in mitosis observed upon $myt1^{RNAi}$ expression alone (n=15), with large polyploid stem cells also observed under these conditions (Figure 2-17B-C). Thus, $CycA^{RNAi}$ is epistatic to $myt1^{RNAi}$, though it is not clear from this result if Myt1 inhibits Cdk1 in a Cyclin A specific manner, or if Cyclin A depletion merely prevents mitosis in ISCs expressing $myt1^{RNAi}$.

Next, I examined if Cyclin A knockdown could prevent mitosis in EBs depleted of Myt1. Because flies carrying both *GBE^{ts}* and *CycA^{RNAi}* transgenes were lethal, I employed the novel EB-specific driver *28E03-GAL4* (Lucchetta and Ohlstein, 2017). After making a temperature sensitive version of this driver, I expressed *myt1^{RNAi}* alone or with *CycA^{RNAi}* in EBs. Expression of *myt1^{RNAi}* with *28E03^{ts}* resulted in EBs that possessed weak PH3 staining (Figure 2-18A). Upon *CycA^{RNAi}* co-expression, however, almost no PH3 positive EBs were observed, and the number of PH3+ cells/ frame decreased from 9.3 +/- 1.5 in Myt1-depleted EBs (n=11) to 3.0 +/- 0.6 (n=16, Figure 2-18A-C). Because Cyclin A has no known function in EBs, this result suggests that mitotic entry in EBs lacking Myt1 is dependent on Cyclin A and could also indicate that Myt1 is a selective regulator of Cyclin A/Cdk1 complexes.

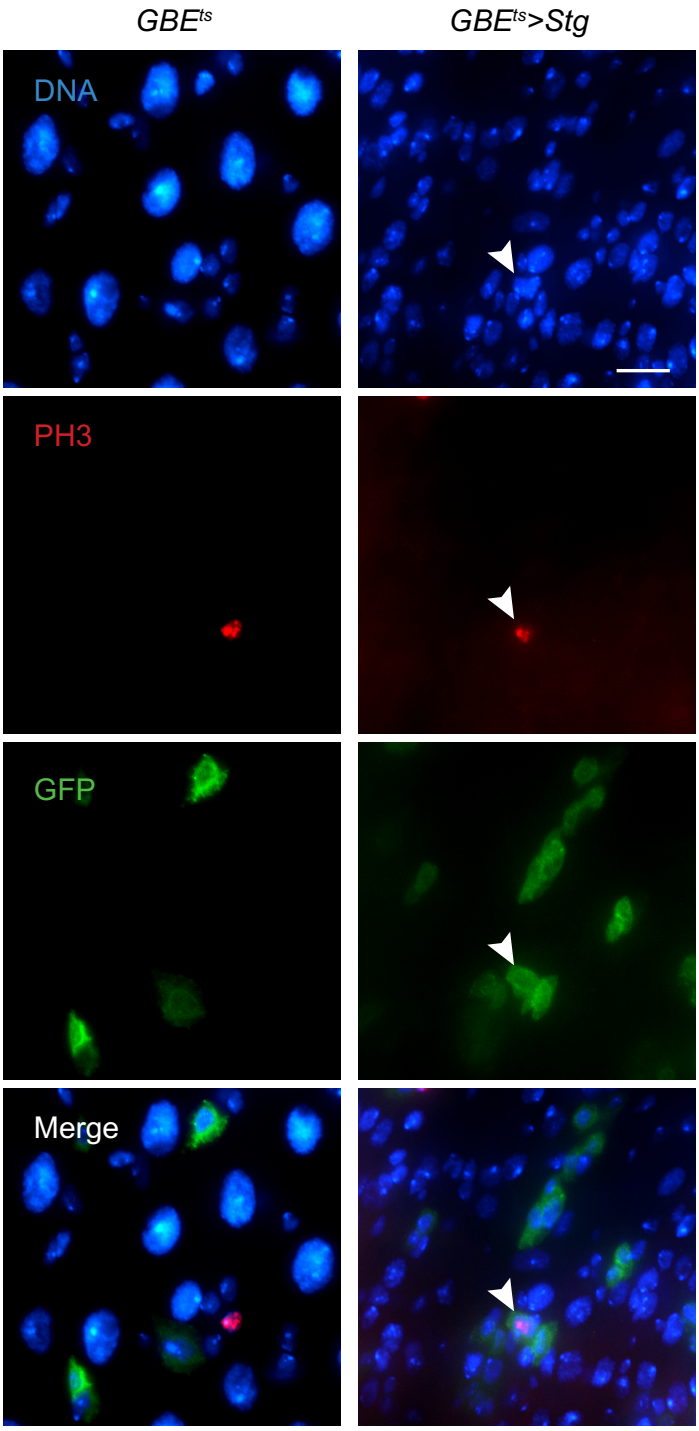


Figure 2-16. String overexpression in EBs induces mitosis. Immunolabeling for PH3 shows no overlap between GFP positive EBs in control *GBE^{ts}* guts, while guts overexpressing String phosphatase in EBs possess GFP positive EBs that are also PH3 positive (arrowhead). Intestines were analyzed 7 days after temperature shift to 29ºC. Scale bar = 10 µm.

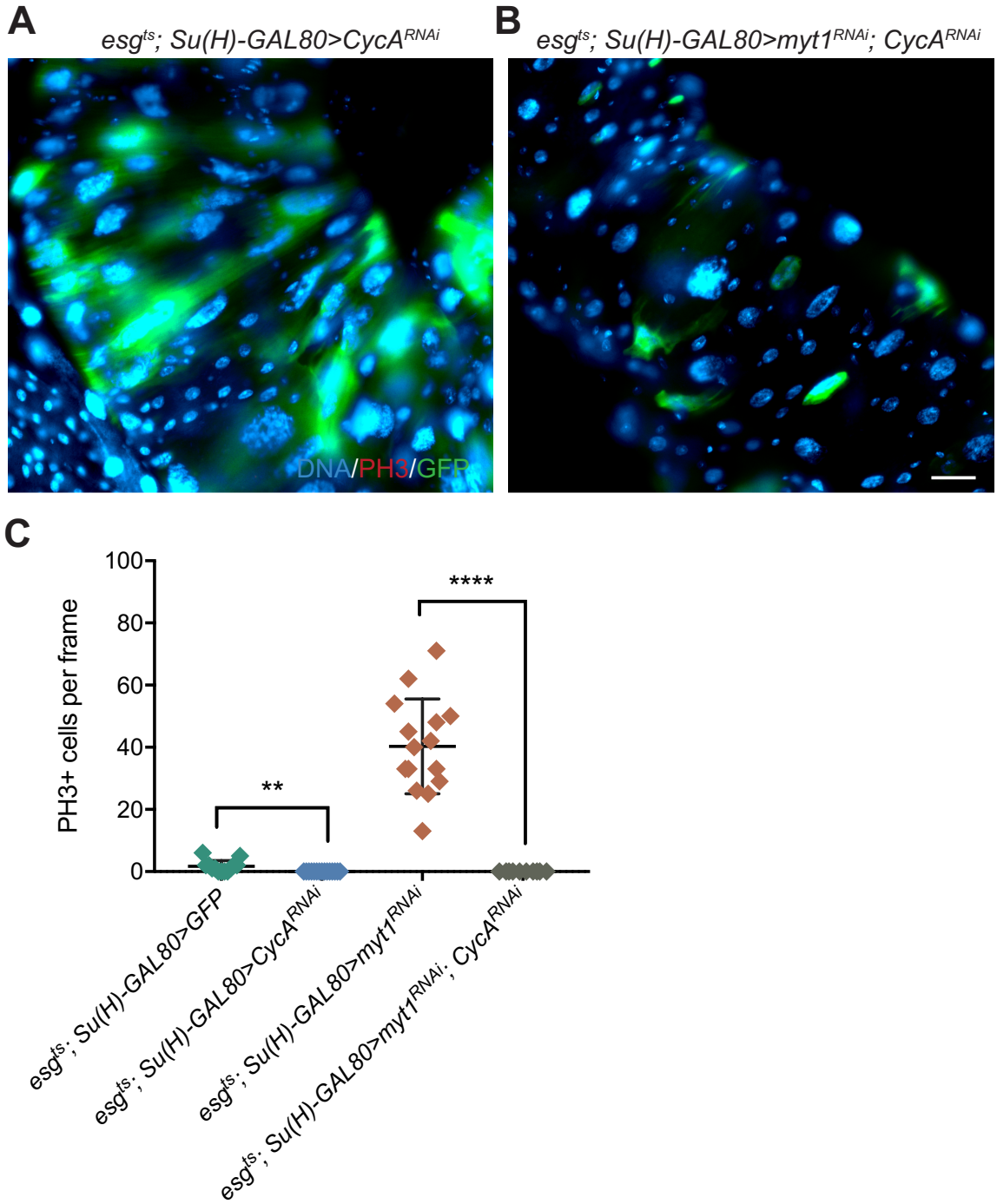


Figure 2-17. Cyclin A is required for mitosis in ISCs. A) ISC specific Cyclin A depletion produces large polyploid cells (stem cells are GFP positive), with no mitoses observed. B) Co-expression of *myt1*^{RNAi} and *CycA*^{RNAi} also results in large polyploid cells with no mitoses observed. Scale bar – 32 µm. C) Quantification of PH3+ cells in posterior midguts 7 days after transgene expression.

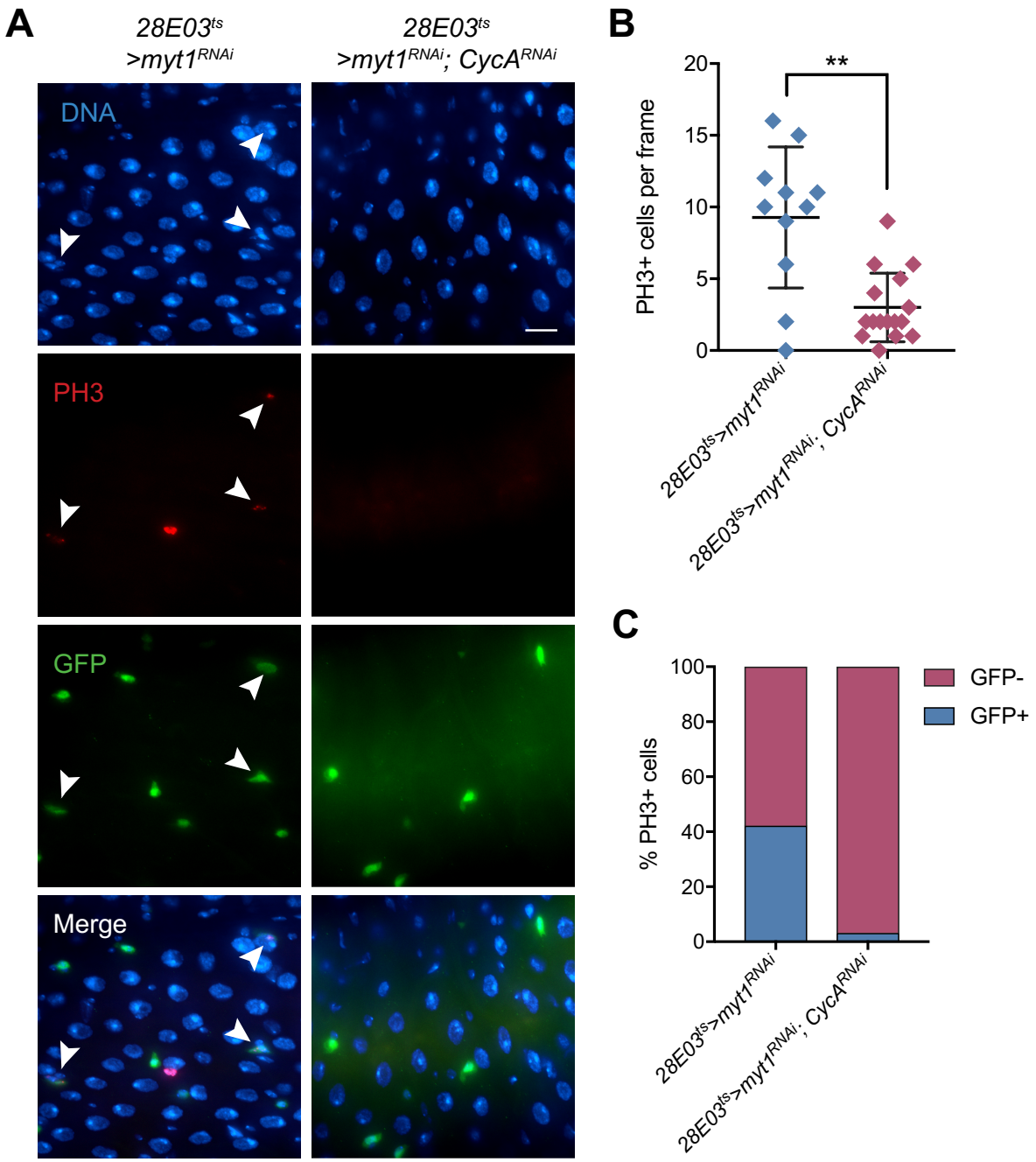


Figure 2-18. Loss of Cyclin A prevents mitosis in Myt1-depleted EBs. A) *myt1*^{RNAi} was expressed alone in EBs (GFP positive), or with RNAi against *Cyclin A*. Arrows point to PH3+ EBs. Scale bar = 10 µm. B) Quantification of PH3+ cells per frame. C) Graph showing proportions of PH3+ cells that are GFP+ (EBs) or GFP- (non-EBs).

2.4.10. Membranes appear uncompromised in *myt1* depleted progenitors

During *Drosophila* spermatogenesis Myt1 both localizes to and helps to maintain the structural integrity of the fusome (an ER-derived organelle) in a Cyclin A-dependent manner (Varadarajan et al., 2016). Similar results were reported for Myt1 localization to the Golgi apparatus in mammalian cell culture (Nakajima et al., 2008). Given that Myt1 function in EBs also appears to be Cyclin A dependent, and that EGFP-Myt1 localizes to the nuclear envelope, I wondered if intestinal progenitors might possess compromised nuclear envelopes upon Myt1 depletion. Immunofluorescent analysis of the nuclear envelope protein lamin revealed stronger staining upon $myt1^{RNAi}$ expression, but the nuclear envelopes of progenitors (as judged by their smaller nuclei) otherwise appeared comparable to controls (Figure 2-19A). In contrast to other contexts where Myt1 promotes ER integrity, this result suggested that Myt1 is not responsible for maintaining the structural integrity of ER-derived organelles in ISCs and EBs.

I also investigated whether cells were able to successfully undergo cytokinesis upon Myt1 loss, given that $myt1^{RNAi}$ often resulted in tight clustering of nuclei. Anillin localizes to the cleavage furrow and is necessary for cytokinesis (Zhao and Fang, 2005), thus I employed this as a marker for cytokinesis in gut progenitors. Anillin showed similar localization in both the control and $myt1^{RNAi}$, though staining was also noticeably stronger in the Myt1 knockdown (Figure 2-19A). Since anillin is degraded by the APC/C at the end of mitosis (Zhao and Fang, 2005), these results could reflect lower APC/C activity upon Myt1 loss. I also analyzed a membrane marker, mCherry-CD8, expressed in the *myt1* mutant. Membrane formation appeared similar in both the mutant and the heterozygous control (Figure 2-19B), though analysis in the mutant was more difficult due to the smaller size and clustering of progenitors. These results showed that though abnormal amounts of lamin and anillin may be present after Myt1 depletion, both the nuclear membranes and cell membranes appear otherwise uncompromised.

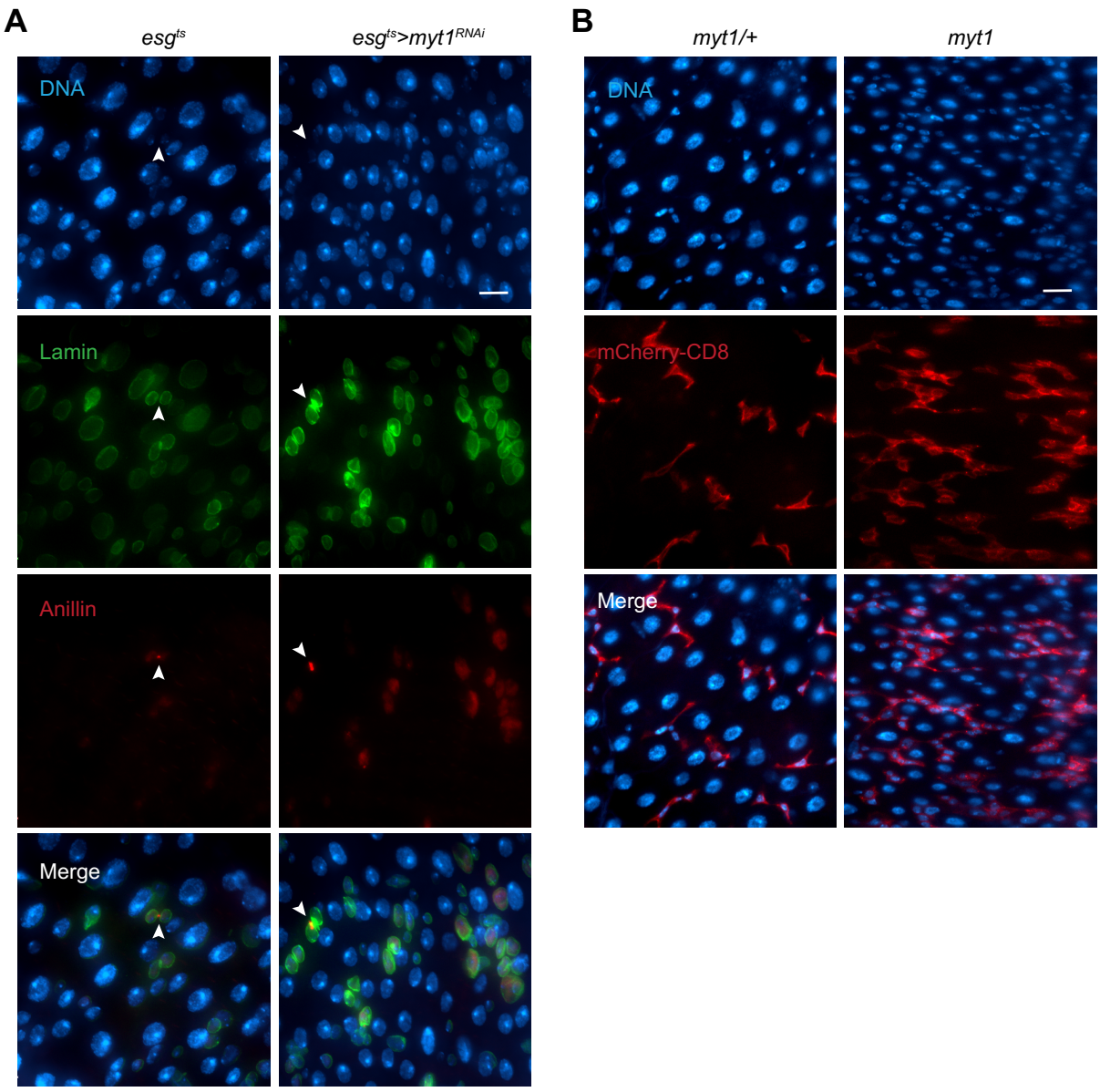


Figure 2-19. Membranes remain intact upon Myt1 loss. A) Microscopy images of lamin and anillin in *esg*^{ts} control and *esg*^{ts}>*myt1*^{RNAi}. Arrowheads point to anillin accumulation at the cleavage furrow. Scale bar = 20 µm. B) UAS-mCherry-CD8 expressed by *esg*^{ts} in *myt1* mutants and a heterozygous control. Scale bar = 30 µm.

2.4.11. Cell cycle analysis of intestinal progenitors

To better understand the timing of Myt1 activity during the cell cycles of ISCs and EBs respectively, I next assessed how proportions of ISCs and EBs in each phase of the cell cycle were affected by Myt1 loss. I employed a previously described fluorescent ubiquitination-based cell cycle indicator (FUCCI) (Zielke et al., 2014) to assess phase dynamics in the fly intestine. This transgenic fly line utilizes tagged degrons from E2F1 and Cyclin B, which are degraded in S phase by $CRL4^{Cdt2}$ and M phase by the APC/C respectively (Zielke et al., 2014). E2F1 is tagged with GFP while Cyclin B is tagged with mRFP such that cells in G1 phase are GFP positive, S phase cells are RFP positive, and cells in G2/M are both GFP and RFP positive (yellow). Cells that exhibit a very weak fluorescent signal remained unclassified, as it is not clear if they are cells that are dying, becoming senescent, or otherwise in an aberrant cell state.

The majority of ISCs in the *Drosophila* intestine are arrested in G2 phase (Zielke et al., 2014). Myt1 is a known regulator of G2 phase arrested cells so I hypothesized that Myt1 loss would result in a decreased proportion of progenitors in G2 phase. Control analysis with the fly-FUCCI line expressed in ISCs alone indicated that approximately 36%, 4%, and 50% of ISCs were in G1, S, and G2/M phases respectively, with 10% of cells remaining unclassified (Figure 2-20A, C, n=9). In contrast, *myt1* knockdown flies possessed almost no detectable S or G2/M phase cells, reduced numbers of G1 phase cells (21%), and a majority of cells for which a cell cycle phase could not be determined (Figure 2-20B-C, n=12). The near absence of S and G2 phase cells suggests that ISCs do not properly initiate DNA replication or G2 phase arrest when Myt1 is lost. Incomplete S phase has previously been shown to cause genomic instability (Beck et al., 2010; Malumbres and Barbacid, 2009; Sørensen and Syljuåsen, 2012), therefore the large increase in cells which were not classified, and which reside in an aberrant cell state when Myt1 is depleted, could also indicate that Myt1 activity is necessary for proper initiation or coordination of S phase in ISCs. Furthermore, the observation that fewer ISCs are in G1

phase after Myt1 loss suggests that Myt1 activity might be required as early as G1 phase to prevent premature mitosis.

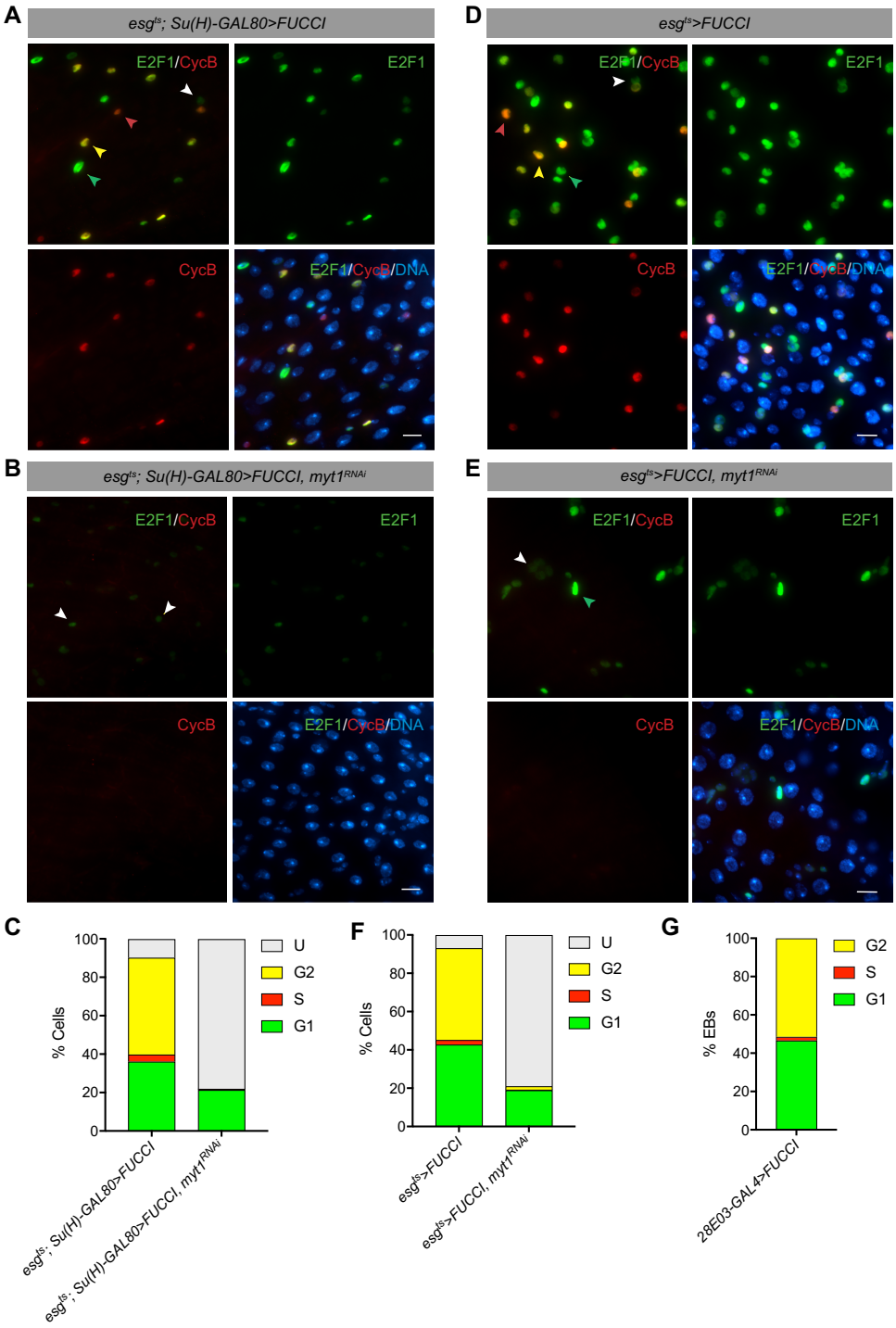


Figure 2-20. Cell cycle shift upon Myt1 loss. Fly-FUCCI cell cycle distribution in control (A) and Myt1-depleted (B) ISCs, or in control (D) and Myt1-depleted (E) ISCs and EBs together. Colored arrowheads point to cells representative of each cell cycle phase: green – G1; red – S; yellow – G2; white – unclassified (U). Scale bars = 10 μm. (C, F, G) Quantification indicated cell types in each of the indicated cell cycle phases.

In addition to examining ISCs alone, I analyzed FUCCI expression in ISCs and EBs together. Previous analysis demonstrated that *esg^{ts}>myt1^{RNAi}* resulted in a progenitor population possessing approximately 60% EBs and 40% ISCs (see Figure 2-13). I therefore reasoned that I should be able to approximate the EB-specific response to Myt1 depletion after determining the normal cell cycle distribution of EBs (since the ISC-specific response was already known). Cell cycle analysis of EBs alone using *28E03-GAL4*, an EB-specific GAL4 (Lucchetta and Ohlstein, 2017), showed that approximately 2% of EBs were in S phase while 47% and 51% were in G1 and G2/M phase respectively (Figure 2-20G, n=13). Similar to what was observed in ISCs alone, *esg^{ts}>myt1^{RNAi}* resulted in a significant change to the proportion of S and G2/M phase ISCs and EBs (Figure 2-20D-F). In contrast to controls that showed 43%, 2%, and 48%, of progenitors in G1, S, and G2/M phases respectively, with 7% unclassified (U; n=15), *myt1* knockdown flies exhibited 19%, 2%, and 79% of cells that were in G1, G2/M, or unclassified respectively, with nearly no S phase cells observed (n=14, Figure 2-20F). Given that a proportion of S and G2/M phase EBs were observed in control guts and that *myt1^{RNAi}* resulted in fewer G1, S and G2/M phase cells, it is reasonable to infer that Myt1 is also required for a normal length G1 phase, as well as entry into S and G2 phase in EBs.

The observed loss of G1 and S phase cells in *myt1* knockdown flies, along with a simultaneous increase in mitotic cell number (as judged by PH3 labeling), led me to hypothesize that Myt1 is required to prevent precocious Cdk1 activity in late G1 phase. If this were true, I would expect to observe aberrant S phase entry, with *myt1* mutant cells expressing both S and M phase markers. Accordingly, I employed *esg^{ts}* in a *myt1* mutant background to express GFP tagged PCNA, an essential S phase protein that acts as a co-factor for DNA polymerase (Moldovan et al., 2007). Nuclear PCNA could be detected only in PH3-negative cells of the heterozygous control, confirming that these markers distinguish S and M phase cells as expected (Figure 2-15). This contrasted with *myt1* mutant midguts in which progenitors possessing both nuclear PCNA and PH3 were observed (Figure 2-21). These results are

consistent with my hypothesis that Myt1 activity prevents precocious Cdk1 activity during interphase, resulting in aberrant mitotic activity that interferes with cell differentiation and instead promotes cell senescence.

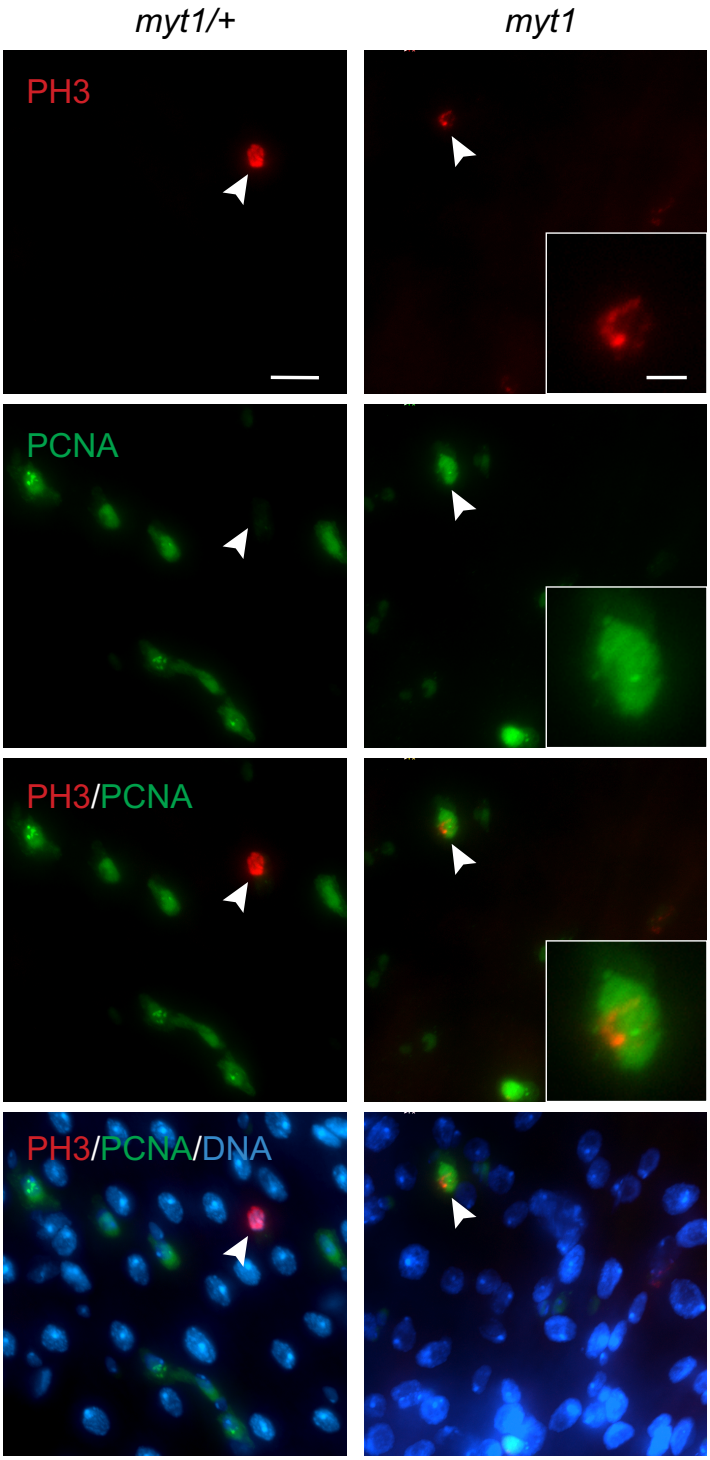


Figure 2-21. Mitotic activity during S phase in *myt1* mutant intestine. Midguts from 7 day old control *myt1/+* and *myt1* mutant flies expressing *esg^{ts}>PCNA::GFP* and stained for PH3. Arrowheads point to PH3 positive cells. Scale bar = 10 µm. Inset scale bar = 2.5 µm.

2.4.12. Myt1 prevents DNA damage accumulation

Ectopic Cdk1 activity in S phase disrupts DNA replication, causing an accumulation of DNA double stranded breaks as well as single stranded DNA near replication forks (Sørensen and Syljuåsen, 2012). As just discussed, cell cycle analysis of Myt1-depleted intestines indicates that some progenitor cells proceed through an S phase that is disrupted by mitotic activity. Additionally, the DNA of progenitors in Myt1-depleted guts appears characteristically dispersed and disorganized (see Figure 2-5), as previously reported in other tissues (Jin et al., 2005). If lack of Myt1 inhibitory phosphorylation allows precocious Cdk1 activity at the G1/S phase transition, intestinal progenitors deficient for Myt1 would likely experience DNA damage. ATM and ATR kinases phosphorylate H2Av in response to double-stranded DNA breaks (Joyce et al., 2011; Shiloh, 2006), thus this chromatin modification serves as a marker of DNA damage. Accordingly, I analyzed *esg^{ts}>myt1^{RNAi}* flies for phosphorylated histone H2Av (γ-H2Av).

The nuclear γ-H2Av signal was significantly increased in the *esg^{ts}>myt1^{RNAi}* guts relative to the controls, with a higher intensity of diffuse nuclear staining observed in addition to an increase in the relative number of progenitor specific γ-H2Av foci (Figure 2-22). These results showing that DNA damage accumulates when Myt1 is lost are therefore consistent with my hypothesis that Myt1 promotes interphase from late G1 phase onward. Notably, the strongest γ-H2Av signals were observed in cells with the weakest GFP signal, indicating that these cells may become dying or losing their cell identity as a direct result of DNA damage accumulation. Collectively, my results therefore indicate that Myt1 loss results in Cyclin A/Cdk1 activity during G1 and S phase, thereby promoting premature mitotic activity and inhibiting cellular differentiation.

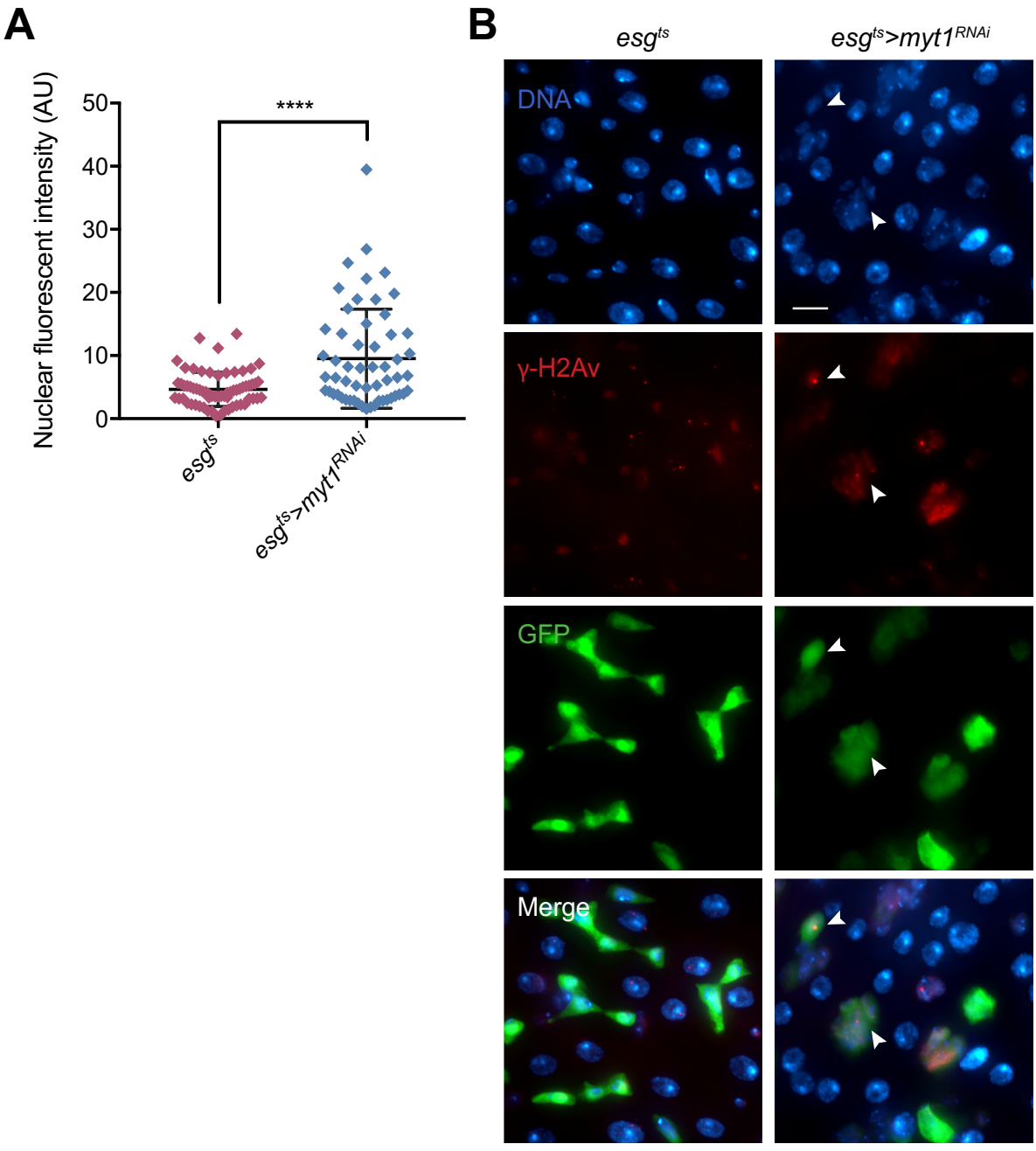


Figure 2-22. Myt1 prevents DNA damage accumulation in progenitors. A) The mean nuclear fluorescent intensity of γ-H2Av in GFP positive cells was approximately 2-fold greater in Myt1-depleted progenitors (n=8 guts) relative to controls (n=8 guts). B) Visualization of DNA damage in progenitors shows a stronger diffuse signal and more foci (arrowheads) in *esg*^{ts}>*myt1*^{RNAi} guts relative to the *esg*^{ts} control. Scale bar = 20 μm.

2.5. Discussion

Previous work has demonstrated the roles of Myt1 in *Drosophila* to be diverse. During development, Myt1 inhibition of Cyclin A/Cdk1 during G2 phase is essential for normal male meiosis (Varadarajan et al., 2016). It is also required for faithful mitotic exit in both male and female germline-associated somatic cells before they transition into endocycles, although the molecular mechanism is not yet known (Jin et al., 2005). Furthermore, loss of Myt1 causes elevated DNA damage and apoptosis in diploid imaginal wing disc cells, again by an unknown mechanism (Jin et al., 2008). Here I show that Myt1 activity is essential for normal epithelial homeostasis in the adult intestine, a tissue well suited for dissecting underlying mechanisms linking these diverse developmental defects. Loss of Myt1 resulted in aberrant progenitor cell proliferation as well as defects in EB differentiation into specialized polyploid enterocytes. I further demonstrate that Myt1 regulation of mitotic exit in EBs is Cyclin A dependent, showing that Myt1 likely acts through inhibitory phosphorylation of Cyclin A/Cdk1.

2.5.1. Myt1 regulation of epithelial homeostasis

The balance of epithelial cell types is strictly enforced within the *Drosophila* intestine. For example, dying ECs use epidermal growth factors to cue ISC division, thereby facilitating their own replacement in the intestinal epithelium (Liang et al., 2017). How intrinsic cues regulate progenitor homeostasis is less understood. Herein, I showed that Myt1 can act autonomously to regulate progenitor cell proliferation. Analysis of *myt1* mutant clones revealed increased cell division and larger clones relative to controls. This demonstrated that Myt1 loss disrupts the coordination of cell division in the intestinal epithelium. Further examination of Myt1 function in the fly intestine using cell-type specific RNAi revealed that Myt1 loss in each progenitor cell type caused ectopic mitoses within normally quiescent EBs. Higher numbers of mitotic ISCs were also observed upon ISC-specific depletion of Myt1. It was unclear from my results whether Myt1 has ISC-specific functions, however. For example, it is possible that

mitotic induction in EBs elicited increased ISC proliferation, which is a normal response to epithelial stress (Jiang and Edgar, 2011). Cell cycle analysis of Myt1-depleted ISCs showed a clear decrease in the fraction of ISCs in interphase, however, consistent with an ISC-specific role for Myt1. Thus, I conclude that Myt1 functions in both ISCs and EBs to promote interphase, though further work will need to be done to determine the consequences of Myt1 loss on ISCs and their progeny.

How did increased cell division in Myt1-depleted guts affect the balance of cell types in the intestinal epithelium? Within one week, Myt1 loss resulted in significant increases in the populations of ISCs and EBs, with a contemporaneous decrease in the number of absorptive enterocytes. Furthermore, upon prolonged RNAi expression against Myt1, a significant reduction in enteroendocrine cells was also observed. These data demonstrated that Myt1 loss causes excess progenitor production while impeding progenitor differentiation. Furthermore, a large proportion of progenitors entered an aberrant cell state upon Myt1 loss, as indicated by weak cell cycle reporter signal intensity. The rise in the number of cells with indeterminate cell cycle identity suggests that apoptosis may increase in *myt1* mutant guts. Immunostaining against the common cell death marker cleaved Caspase-3 was unsuccessful, thus further work needs to be done to determine if apoptosis increases in *myt1* mutant guts. Overall, it is clear that Myt1 promotes cell differentiation by allowing progenitors to exit the mitotic cell cycle, thereby maintaining epithelial homeostasis.

2.5.2. Myt1 promotes the mitotic to endocycle switch

Differentiating EBs undergo the mitotic to endocycle (ME) switch to become polyploid absorptive enterocytes, a process that requires permanent down-regulation of Cdk1 (Zielke et al., 2013). Though it is not entirely clear how Cdk1 is downregulated in EBs, previous work demonstrated that co-overexpression of Cyclin E and the Cdc25 phosphatase String allowed for ectopic mitoses in EBs, suggesting that String inhibition prevents Cdk1 activation

(Kohlmaier et al., 2015). The full nature of Cdk1 inhibition during the ME switch requires further exploration.

Consistent with previous results suggesting that Myt1 promotes exit from the mitotic cell cycle in differentiating cells (Jin et al., 2005), I find that Myt1 is necessary for allowing normally post-mitotic EBs to execute the ME switch. This was shown through Myt1 depletion in ISCs or EBs, both of which resulted in mitotic EBs. Furthermore, I demonstrated that re-entry into mitosis in Myt1-depleted EBs is dependent on Cyclin A, suggesting that Myt1 promotes the ME switch through inhibitory phosphorylation of Cyclin A/Cdk1.

How does unscheduled Cyclin A/Cdk1 activity enable EBs to enter mitosis? My own Fly-FUCCI data, as well as preceding work (Kohlmaier et al., 2015), has shown that EBs proceed through a period of high APC/C activity in G1 phase, followed by lower APC/C activity in S and G2 phase. It has also been previously demonstrated that Cyclin A represses the APC/C activator Fzr/Cdh1 at the G1/S transition, thereby allowing accumulation of mitotic cyclins and String phosphatase (Dienemann and Sprenger, 2004; Erhardt et al., 2008; Jaspersen et al., 1999; Lukas et al., 1999; Reber et al., 2006; Sigrist and Lehner, 1997; Zachariae et al., 1998). Accordingly, I propose that Cyclin A is responsible for inhibiting Fzr/Cdh1 following the G1/S transition in EBs, such that mitotic cyclins can accumulate. I further propose that Myt1 kinase functions to inhibit Cyclin A/Cdk1 during G1 phase, allowing Fzr/Cdh1 to facilitate degradation of mitotic cyclins (Figure 2-23). Thus, when Myt1 is absent from EBs, Cyclin A/Cdk1 inhibits Fzr/Cdh1 activity prematurely in G1 phase and permits Cyclin B/Cdk1 to initiate mitosis. In support of this hypothesis, lower APC/C activity was shown in Myt1-depleted guts, as represented by increased levels of the APC/C substrate anillin (see Figure 2-19A) (Zhao and Fang, 2005).

Exactly what Cyclin A is doing in differentiating EBs will be an important area for future research. An intriguing possibility is that Cyclin A/Cdk is also necessary for chromatin re-modeling during cell differentiation, since Cyclin A has an established role in this context

(Erhardt et al., 2008; García Del Arco et al., 2018). Furthermore, studies in other experimental systems indicate that Cyclin A begins to accumulate in G1 phase and is required to initiate DNA replication (Coverley et al., 2002; Fung and Poon, 2005; Pagano et al., 1992; Rape and Kirschner, 2004; Yam et al., 2002). This is also true in the *Drosophila* bristle lineage, where Cyclin A is required to coordinate DNA replication in endocycling cells (Sallé et al., 2012). In addition to mediating Fzr/Cdh1 inhibition, Cyclin A may therefore regulate aspects of DNA replication in differentiating EBs.

Given that Cyclin A has established functions in S phase, it is unclear how Myt1 inhibition might operate spatially and temporally to restrict Cyclin A/Cdk complexes. It is possible that Myt1 inhibits Cyclin A/Cdk1 in G1 phase, after which Notch signaling downregulates Myt1 to facilitate Cyclin A activity in S phase. Previous work from our lab showed that ectopic Myt1 expression in the developing eye enhanced the mutant phenotype of the Notch ligand *Delta*, suggesting that Notch signaling indeed regulates Myt1 activity (Price et al., 2002). It is also possible that Myt1 might restrict EB-specific Cyclin A/Cdk1 activity in a dosage-sensitive manner, as has been suggested for *rca*-1, another Cyclin A regulator (Dong et al., 1997). Alternatively, Myt1 may only be required to inhibit cytoplasmic pools of Cyclin A/Cdk1 from initiating mitosis, thus allowing nuclear Cyclin A to promote S phase. This would make sense in light of localization data from S2 cells showing that Cyclin A localizes throughout the cell during interphase, with strong localization also observed at kinetochores (Erhardt et al., 2008). In summary, I believe Myt1 promotes the mitotic to endocycle switch in EBs by selective inhibition of Cyclin A/Cdk1 complexes, though further work needs to be done to understand the full nature of this interaction.

Another interesting question is how Myt1 depletion in ISCs promotes mitosis in EBs. One possibility is that *myt1* transcripts persist through asymmetric ISC divisions, allowing for expression in EBs. Alternatively, Myt1 might also inhibit Cyclin A/Cdk1 in ISCs. Within the context of differentiating cells in the *Drosophila* embryo, it has been shown that Cyclin A is

required to inhibit Fzr/Cdh1 in the final cell cycle before terminal mitosis, allowing Cyclin B and String to initiate cell division (Reber et al., 2006). If this mechanism were conserved in ISCs undergoing asymmetric division, it is possible that unregulated Cyclin A/Cdk1 could therefore facilitate excess String and Cyclin B/Cdk1 activity such that newly-produced EBs never exit mitosis. In support of this hypothesis, my data indicates that ISC-specific Cyclin A is absolutely required for mitosis, with Cyclin A depletion producing large polyploid stem cells. Further work needs to be done, however, to understand ISC- and EB-specific mechanisms of Myt1 regulatory activity.

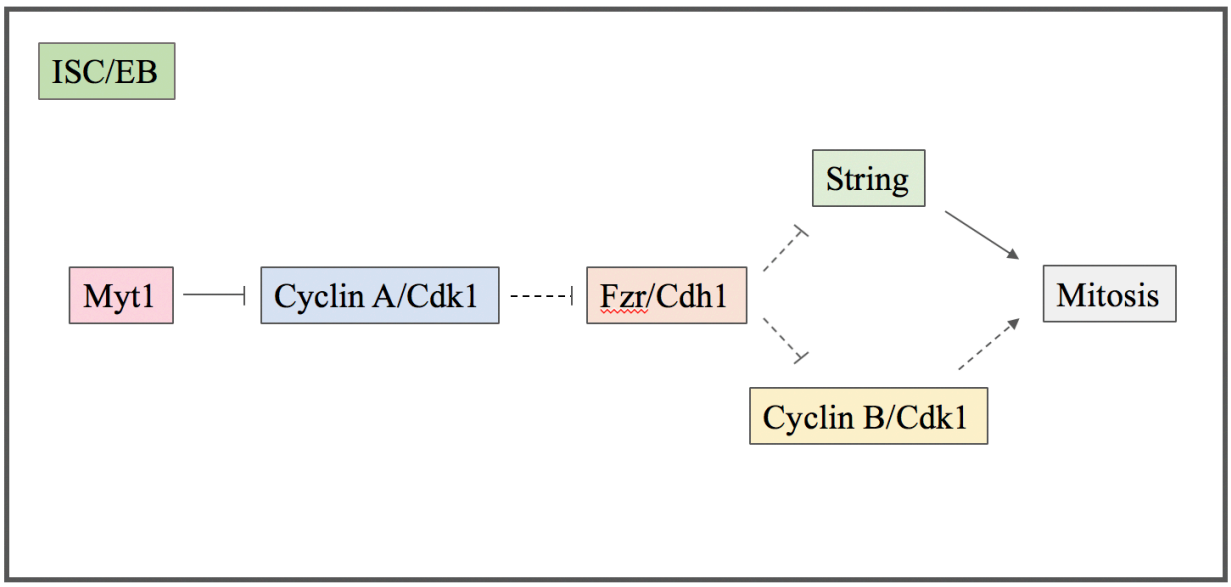


Figure 2-23. Mechanisms of intestinal progenitor regulation by Myt1. I propose that Myt1 inhibits Cyclin A/Cdk1 complexes in ISCs and EBs, enabling Fzr/Cdh1 to inhibit String and Cyclin B/Cdk1. This allows cells to complete mitotic exit and continue through interphase. Dashed lines indicate regulatory activity that is confirmed in other contexts, but which has not yet been established in *Drosophila* intestinal progenitors.

2.5.3. Reconciling Myt1 as a regulator of G1 phase

Previous work in other experimental systems has shown that Myt1 inhibition of Cyclin B/Cdk1 is important during pre-meiotic G2 phase arrest and coordination of endomembrane remodeling during mitotic exit (Nakajima et al., 2008). More recently, work from our laboratory showed that Myt1 inhibition of Cyclin A/Cdk1 is essential during a developmentally programed G2 phase arrest in *Drosophila* male meiosis (Varadarajan et al., 2016). I have now shown that Myt1 activity is also required to maintain intestinal epithelium homeostasis, both for normal ISC proliferation and the mitotic to endocycle switch in EBs. The latter defect can be suppressed by simultaneous elimination of Cyclin A, suggesting that Myt1 inhibition of Cyclin A/Cdk1 activity allows intestinal progenitor cells to exit the mitotic cell cycle and undergo terminal differentiation.

At what point in the cell cycle does Myt1 act? Myt1 loss resulted in a decreased fraction of G1 phase intestinal progenitors, a near-absence of S and G2 phase progenitors, and a contemporaneous increase in mitotic cell number. This increase in mitotic cells at the expense of interphase cells demonstrated that progenitors successfully enter G1 phase but fail to complete G1 and either fail to execute the G1/S phase transition without Myt1 or have hurried S and G2 phases. This was unexpected given that Myt1 has previously been described only as a regulator of G2 phase in *Drosophila* (Jin et al., 2008; Varadarajan et al., 2016). The fact that the Myt1 EB phenotype is Cyclin A dependent also supports a role for Myt1 in G1 phase since Cyclin A is synthesized in G1 phase and becomes active in S phase of cells in other contexts (Coverley et al., 2002; Fung and Poon, 2005; Pagano et al., 1992; Rape and Kirschner, 2004; Reber et al., 2006; Yam et al., 2002). Furthermore, Myt1-depleted guts exhibited increased DNA damage and possessed some cells expressing both S and M phase markers. This supports the idea that Myt1 loss results in compromised S phase coordination. Altogether, this data suggests that Myt1 prevents mitotic activity before the G1/S phase transition in cells executing the ME switch.

A role for Myt1 in early interphase is not altogether unexpected. Work in mammalian cells, *Xenopus* egg extracts, and *Drosophila* embryogenesis demonstrates a precedent for Wee-like kinase inhibition of Cdk1 in S phase (Beck et al., 2010; Hauge et al., 2017; Lee et al., 2001; Price et al., 2000). Furthermore, RNAi depletion of Myt1 in mammalian cells leads to DNA damage, suggesting that Myt1 might coordinate S phase in this context (Beck et al., 2010). Interestingly, another study in mammalian cells showed that both Wee1 and Myt1 were required to inhibit Cyclin B/Cdk1 complexes when they were present in G1 phase (Pomerening et al., 2008; Potapova et al., 2009), supporting the idea that Myt1 can act at this phase of the cell cycle. Within *Drosophila* specifically, expression of Cdk1AF in larval neuroblasts results in severe genome instability, again supporting the idea that dual inhibitory phosphorylation of Cdk1 may be required in this tissue during DNA replication, or to arrest the cell cycle in response to DNA damage. Furthermore, several tissues within *myt1* mutants exhibit disorganized and decondensed nuclei in addition to increased mitotic cell numbers (Campbell lab, unpublished observations; Jin et al., 2008), which could be attributed to aberrant Cdk1 activity at the G1/S phase transition. Further work must be done to confirm Myt1 involvement in G1 phase of fly gut progenitors, and to determine the extent to which this mechanism may be conserved in other *Drosophila* tissues. It is possible that Myt1 activity at different points in the cell cycle is context-dependent, and that this mechanism is active only in differentiating cells. This could make sense given the tremendous variability in reports of Myt1 activity relative to the cell cycle.

In summary, I find that Myt1 is necessary to maintain epithelial homeostasis within the *Drosophila* intestine. Without Myt1, progenitors in the intestinal epithelium exhibit increased mitosis, producing excess progenitors that fail to differentiate. This leads to a loss of epithelial integrity and increased mortality. I show that the increase in mitotic EBs arises from a failure to inhibit Cyclin A/Cdk1, but whether this is also true in ISCs remains unclear. Loss of Myt1

results in decreased numbers of progenitors in interphase, with an increase in cells in an aberrant state, as well as cells exhibiting DNA damage. Furthermore, cells positive for S phase and M phase markers could be observed. This supports a role for Myt1 in mediating G1 phase completion and proper S phase entry, though further work needs to be done to verify that Myt1 inhibits Cyclin A/Cdk1 at this point of the cell cycle. Such a mechanism, if validated, could not only tie together the aforementioned *myt1* mutant phenotypes but could further clarify how Myt1 performs a specialized role distinct from Wee1 within several developmental contexts.

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Appendix A: Investigation into mechanisms of Cyclin A/Cdk1mediated centriole disengagement during *Drosophila* spermatogenesis

A.1. Summary

Cdk1 in complex with Cyclin B is most commonly thought to comprise the maturation promoting factor driving cells into M phase; the role of other cyclins in M phase is less understood. Recent work has demonstrated that Cyclin A bound Cdk1 may have a distinct role in promoting some M phase events. Specifically, a study from our lab has shown that in *Drosophila*, regulation of Cyclin A/Cdk1 is responsible for maintaining fusome and centrosome integrity during spermatogenesis (Varadarajan et al., 2016). This was demonstrated through male *myt1* null mutants that are unable to inactivate Cyclin A/Cdk1 in G2 phase. In these flies, I propose that the Cyclin A/Cdk1 complex prematurely targets substrates at the fusome and centrosome in G2 phase, resulting in abnormal fusome and centrosome behaviour. This portion of my thesis was devoted to testing this hypothesis by searching for putative Cyclin A/Cdk1 substrates at the centrosome. I also further characterized the abnormal centrosomes of *myt1* spermatocytes in an effort to search for centrosome proteins prematurely targeted by Cyclin A/Cdk1.

A.2. Introduction

A.2.1. *Drosophila* spermatogenesis

Animal development requires precise spatial and temporal coordination of cell cycle progression and differentiation to produce specialized cell types. Mitosis is a process that produces two genetically identical daughter cells and is necessary for organismal development and growth, while meiotic divisions result in four genetically unique daughter cells called gametes. Gametes from male and female parents fuse together, giving rise to genetically diverse offspring.

During gametogenesis, germ-line stem cells (GSCs) undergo asymmetric division and differentiation to produce gametes. In male *Drosophila melanogaster*, GSC derived cells proceed through both mitotic and meiotic divisions before differentiating into sperm (Figure

A-1) (Cenci et al., 1994; Davies and Fuller, 2008). This process begins at the apical tip of the testis in the germinal proliferation center, where the GSCs are encapsulated by cyst progenitor cells attached to the hub (Fuller, 1993). First, GSCs undergo an asymmetric division to produce both primary spermatogonia as well as another stem cell (Hardy et al., 1979). The primary spermatogonia then proceed through four synchronous mitotic divisions with incomplete cytokinesis, giving rise to 16 cell cysts of primary spermatocytes interconnected by ER-derived cytosplasmic bridges called fusomes (Varadarajan et al., 2016; Fuller, 1993). The 16-cell cyst stage begins with a short G1 phase followed by S phase and a prolonged G2 phase arrest (Fuller, 1993). Spermatocytes remain arrested in G2 phase for 93 hours during which the cells undergo a period of enhanced gene transcription and rapid cell growth that culminates in meiosis I and II, spermatid differentiation and sperm elongation (Varadarajan et al., 2016; Fuller, 1993).

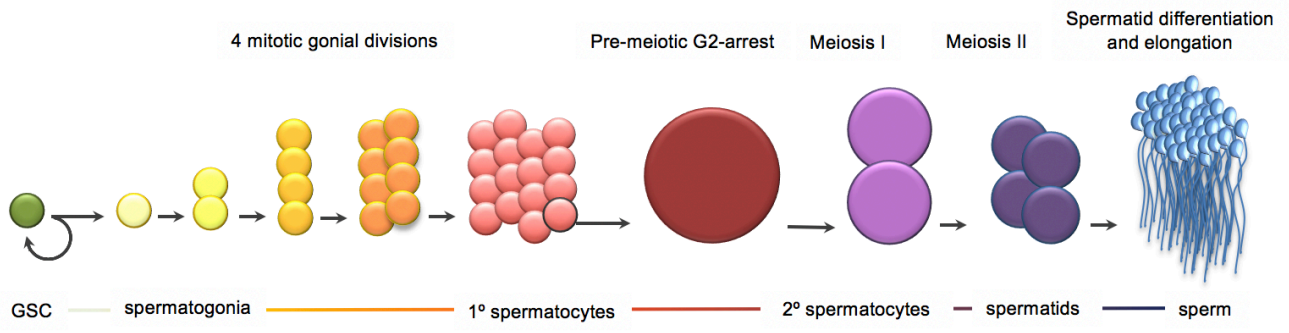


Figure A-1. *Drosophila* spermatogenesis. GSCs attached to the hub of the testis divide asymmetrically to produce one stem cell and one daughter that differentiates into a spermatogonial cell. Spermatogonia proceed through 4 mitotic divisions, giving rise to 16-cell cysts of primary spermatocytes. These spermatocytes arrest in a 93-hour G2-phase arrest, after which they proceed through meiosis I to produce secondary spermatocytes. Secondary spermatocytes then proceed through meiosis II, giving rise to spermatids that then elongate and differentiate to produce functional sperm.

A.2.2. The role of Myt1 in pre-meiotic G2 phase arrest

Myt1 regulation of G2 phase arrest during meiosis is common to many organisms. For example, immature oocytes in *Xenopus lavis, Asterina pectinifera,* and *C. elegans* exhibit a 'G2-like' prophase-I arrest that is dependent on Myt1 regulation of Cyclin B/ Cdk1 (Burrows et al., 2006; Furuno et al., 2003; Gaffré et al., 2011; Inoue and Sagata, 2005; Karaiskou et al., 2004; Kishimoto, 2011). This is in contrast to Wee1, which (at least in *Xenopus* oocytes) is undetectable until oocyte maturation or fertilization (Murakami and Vande Woude, 1998; Nakajo et al., 2000; Walter et al., 2000). In *C. elegans,* Myt1 activity is also required for regulation of G2 phase arrest in spermatogenesis, as demonstrated by dominant negative mutations that prevent MI entry (Lamitina and L'Hernault, 2002; Burrows et al., 2006, #28992}.

Myt1 activity in *Drosophila* meiosis has been characterized predominantly through the use of a *myt1* null mutant made in our laboratory (Jin et al., 2005). Zygotic *myt1* mutant *Drosophila* appear largely healthy into adulthood, presumably due to functional redundancy with Wee1, however the females are semi-fertile, and males are fully sterile (Jin et al., 2005). Original work by Zhigang Jin showed that during spermatogenesis, both mutant gonial cells and somatic cyst cells proceed through ectopic mitotic divisions. Furthermore, though meiosis I and II both still occurred, DNA was unequally divided into spermatids, and the seminal vesicles of *myt1* mutants were empty (Jin et al., 2005; Varadarajan et al., 2016). Subsequent analysis by Ramya Varadarajan further characterized the role for Myt1 in regulating meiotic divisions. She showed that Myt1 is not required to prevent precocious division of spermatocytes, rather Myt1 inhibits Cyclin A/Cdk1 complexes from initiating fusome degradation and centriole disengagement during G2 phase (Varadarajan et al., 2016). Thus, Myt1 serves multiple functions during *Drosophila* spermatogenesis. One major unknown remaining from this aspect of our research is the identity of the protein(s) aberrantly targeted by Cyclin A/Cdk1 in the absence of Myt1. Using *myt1* mutants as a model, I attempted to

answer this question by identifying centrosome protein(s) targeted by Cyclin A/Cdk1 during pre-meiotic G2 phase arrest.

A.2.3. Centrosomes and the centrosome cycle

The *myt1* phenotype is characterized by premature centriole disengagement during G2 phase of spermatogenesis (Varadarajan et al., 2016). To understand centriole disengagement, we must look at it in light of the centrosome cycle. Mature centrosomes function as the microtubule organizing centers that facilitate segregation of nuclear DNA during cell division. Centrosomes also play a critical role during animal development in mediating cell polarity and motility in some cell types (Mottier-Pavie and Megraw, 2009; Tang and Marshall, 2012). For example, centrosomes undergo drastic changes during maturation that enable them to form the bipolar spindle that segregates DNA. In many stem cell lineages, the orientation of the spindle determines how cell fate determinants are asymmetrically segregated (Chen et al., 2018), thereby linking cell fate and proliferation with centrosome behavior.

Cells typically begin G1 phase possessing two centrosomes connected by a flexible linker (Conduit et al., 2015). At this stage, centrosomes are composed of barrel-shaped centrioles that incorporate minimal pericentriolar material (PCM). Upon entering S phase, centrioles, like DNA, replicate themselves such that each centriole is attached or 'engaged' orthogonally to a newly formed pro-centriole. In *Drosophila*, this process is dependent on the kinases Cdk2 and Polo-like kinase 4 (PLK4) (Habedanck et al., 2005; Wong et al., 2015). Throughout G2 phase, mother and daughter centrioles grow in size, however only at the G2/M transition do centrioles mature and begin incorporating significant PCM such that they become capable of organizing microtubules (Conduit et al., 2015). During meiosis, centriole pairs composing the centrosome remain attached until anaphase-I, after which they disengage such that each centriole becomes a microtubule organizing center contributing to bipolar spindle formation in meiosis II.

In contrast to the canonical meiotic centrosome cycle, the *myt1* mutant phenotype is characterized by centriole disengagement occurring early in G2 phase (Varadarajan et al., 2016). This untethering of the centrosome cycle from the rest of the cell cycle eventually results in the formation of a multi-polar spindle during MI and production of aneuploid spermatids that cannot develop into functional sperm following MII.

A.2.4. Understanding centriole engagement and disengagement

Centriole disengagement mechanisms are not well understood in either mitotic or meiotic cell cycles. During meiosis, both the tether keeping centrosomes in close proximity and the linker holding mother and daughter centriole pairs together are disassembled allowing for centrosome separation and centriole disengagement respectively (Agircan et al., 2014; Fry, 2015). Several proteins involved in centrosome tethering have been characterized in humans, including C-Nap1, rootletin, Cep68 and Cep215 (Graser et al., 2007), however components involved in centriole-centriole cohesion have remained more elusive. Evidence from mammalian cell culture indicates that pericentrin (PCNT) and Cep215 together facilitate centriole cohesion, while Plk1 activity followed by separase cleavage of PCNT results in disengagement (Hatano and Sluder, 2012; Pagan et al., 2015). Work in *Drosophila* has not identified a role for separase in centriole-centriole cohesion however, so it remains unclear which proteins at the centrosome might act as a cohesion scaffold or a tether holding mother-daughter centriole pairs together. Consequently, my search for a candidate protein targeted by Cyclin A/Cdk1 at the centrosome was necessarily broad.

A.3. Materials and methods

A.3.1. Fly Stocks

Flies used in the following experiments were raised at 21°C or 25°C dependent on experimental requirements and as noted in Figures. See Appendix B for a full list of fly stocks used in these experiments.

A.3.2. Determining putative Cdk1 and Polo centrosome substrates

Group-based prediction systems (GPS) for predicting kinase-specific phosphorylation sites were used to determine whether the *Drosophila* homologs of known centrosome proteins possessed putative Cdk1 phosphorylation site(s) or Polo binding and phosphorylation site(s). GPS 2.0 (Xue et al., 2008) and GPS Polo 1.0 (Liu et al., 2013) were used to predict Cdk1 phosphorylation sites and Polo binding and phosphorylation sites respectively. A medium threshold was used for both programs. Protein sequences were first put into GPS 2.0 after which proteins with predicted Cdk1 phosphorylation sites were analyzed using GPS Polo 1.0. Proteins with predicted Polo binding and phosphorylation sites were then analyzed further with an RNAi screen and sterility assay.

A.3.3. Sterility assay

A sterility assay was employed to determine the effectiveness of RNAi employed against centrosome proteins during spermatogenesis. Virgin female flies expressing *bam-GAL4*, a UAS 'driver' transgenic strain expressed in late spermatogonia/ early spermatocytes, or *UAS-Dicer; bam-GAL4* (to enhance RNA interference) were collected and crossed to transgenic males carrying the relevant GAL4-responsive RNAi construct. Depending on the experiment, 5-30 individual F1 male progeny expressing the siRNA against a specific gene were then crossed to 3 *yw* virgin females (standard 'wild-type' control). After 7 days, these parents were tipped over or discarded and the number of F2 adult progeny were counted to assess male

fertility. F1 males that produced no progeny, <60 progeny, or >60 progeny were considered sterile, semi-fertile, and fertile respectively. This sterility assay was employed as described in Varadarajan et al., 2016, with modified values assigned to each fertility category (sterile, semi-sterile, fertile).

A.3.4. Immunofluorescence

Adult males 0-2 days old were anaesthetized and put on ice. 10-12 testes were teased out in 30µL PBS using forceps, then transferred to a drop of PBS on a coverslip. The tips of the testes were cut with a dissecting needle, after which the testes were gently squashed with a poly-lysine coated glass slide. The slide and coverslip were then snap frozen in liquid nitrogen and the coverslip removed with a razor blade. Two subsequent fixation protocols were utilized: 1) Methanol-acetone fix. Slides were immersed in 100% methanol at -20°C for 15 minutes, followed by 30 seconds in acetone at -20°C, and 10 minutes in 0.3% Triton X-100, 0.1% acetic acid in PBS. This was followed by washing with 0.3% Triton X-100 in PBS (PBST). 2) Formaldehyde fix. Slides were immersed in 95% ethanol at -20°C for at least 10 minutes. Slides were subsequently dried around the samples and a hydrophobic pen was used to encircle the testes. A drop of 3.7% formaldehyde was added for 10 minutes, then slides were briefly washed in PBS followed by washing with PBST. After fixation, slides were blocked with PBST + 3% BSA (PBSTB) for 1 hour followed by incubation with primary antibodies in PBSTB overnight at 4°C. Slides were then washed in PBT followed by incubation with secondary antibodies diluted 1:1000 in PBSTB for 1 hour. Slides were then washed in PBST and counter-stained with Hoechst 33258. Slides were mounted and imaged within 1-2 days. See Appendix B for a list of antibodies and concentrations used.

A.3.5. Live imaging of testes

To analyze fluorescent reporters in live spermatocytes, testes were dissected directly into PBS. They were subsequently moved into PBS on a coverslip upon which a slide was inverted for immediate viewing. Imaging was completed within 30 minutes of dissection.

A.3.6. Inhibitor treatments

Fly food was microwaved in a vial for 20 seconds to achieve a uniform liquid state. Following this, 550µL of food was pipetted into a fresh empty vial and was mixed with 50µL DMSO containing nocodazole or taxol at the appropriate initial concentration to achieve the desired final concentration. The food was allowed to solidify and cool, after which adult male flies 0-2 days old were put in the vial and allowed to feed on the food/ drug mixture for up to 3 days. Initial control treatments were performed at concentrations ranging from 50µM to 500µM to determine the optimal concentration for affecting microtubule dynamics. The testes of flies expressing *tubulin-GFP* and fed with food containing inhibitors were analyzed after 3 days to determine the optimal inhibitor concentration by looking for arrested spermatocytes. 200µM was found to be the optimal concentration at which both the taxol and nocodazole treated flies had a majority of spermatocytes arrested in metaphase I and at the G2/M transition respectively.

A.4. Results and Discussion

A.4.1. RNAi screen of centrosome proteins putatively targeted by Cyclin A/ Cdk1

Previous work indicated that the *myt1* centriole disengagement phenotype could be rescued by Polo depletion (Varadarajan et al., 2016). Since Polo commonly binds to and acts upon substrates pre-phosphorylated by Cdk1 (Elia et al., 2003), I reasoned that centriole disengagement might be mediated by Polo and Cyclin A/Cdk1 phosphorylation of proteins that

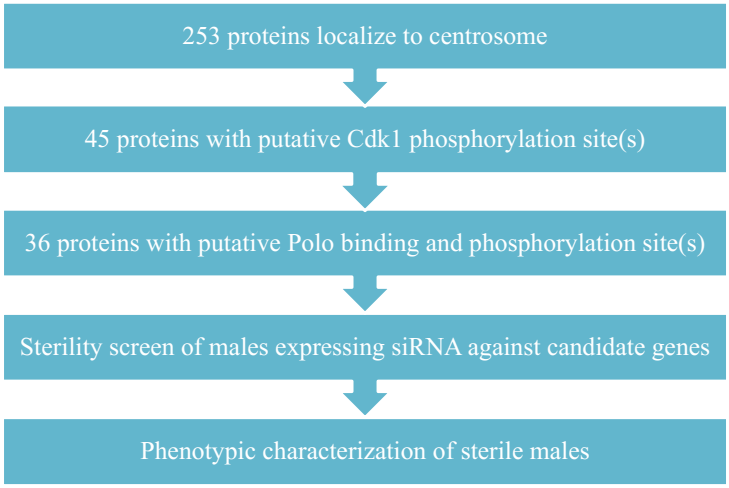
localize to the centrosome. To test this hypothesis, I first analyzed known centrosome proteins for putative Cdk1 phosphorylation sites, followed by a search for Polo binding and phosphorylation sites. Of the 253 *Drosophila* proteins known to localize to the centrosome that I analyzed, 45 contained at least one putative Cdk1 phosphorylation site, with 36 of the 45 also possessing at least one putative Polo binding and phosphorylation site (Figure A-2). These proteins served as candidates for subsequent analysis.

For these 36 candidate proteins, I first conducted an RNAi sterility screen. Male *Drosophila* with centrosome defects are typically sterile (Conduit et al., 2015), thus inducible double stranded RNA that affects centrosome dynamics in the male germ-line should result in male sterility. The expected outcomes for depletion of proteins involved in centriole-centriole cohesion or centriole disengagement were either premature centriole disengagement or failure of centriole pairs to disengage in anaphase I, respectively. A number of caveats must be mentioned, however. With respect to the RNAi screen, it is firstly possible that males classified as fertile may have been so as a result of an ineffective siRNA construct, or that the timing of siRNA expression may not have been conducive to candidate protein depletion. Additionally, there may be redundancy in this process such that several proteins work in tandem to maintain centriole-centriole cohesion, and single protein depletion is not sufficient to disrupt centriole cohesion or disengagement. Furthermore, it bears mentioning that though Cdk1 and Polo have known involvement in centriole disengagement, they may not directly target a protein at the centrosome and this may instead be accomplished by a downstream effector of these kinases.

Expressing siRNA against each candidate gene independently resulted in 6 experimental groups with sterile males, 3 groups with semi-sterile males and the remaining 27 with fertile males (Figure A-2B). Further analysis of siRNA-expressing spermatocytes in sterile and semi-sterile males via immunofluorescent labeling of centrosomes and spindle failed to show premature centriole disengagement or absence of centriole disengagement. For example, depletion of the kinesins Klp61F and Pavorotti resulted in failure to assemble a proper

spindle or complete MI but did not show premature disengagement at the G2/M transition, or engaged centrioles following MI (Figure A-3). This indicates that RNAi against these candidates was effective, however these proteins may not be involved in centriole cohesion or centriole disengagement. Thus, the RNAi screen did not provide Cyclin A/Cdk1 target candidates for further analysis.

A



B

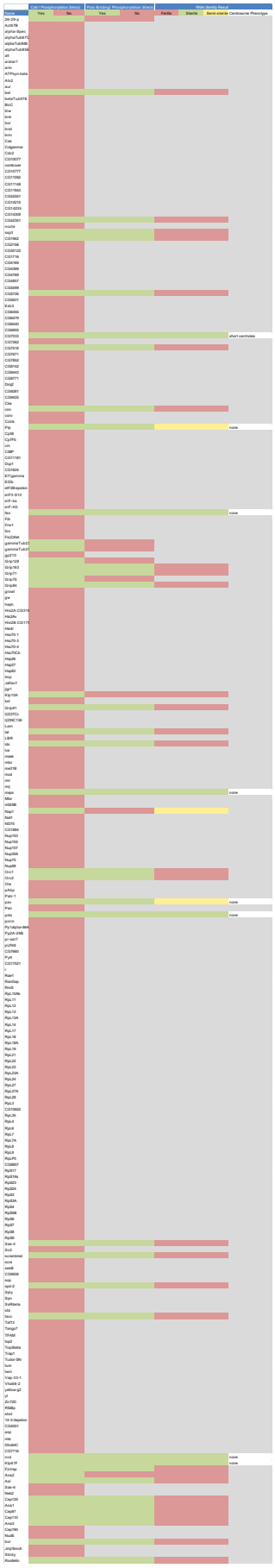


Figure A-2. RNAi screen of candidate genes involved in centriole-centriole cohesion and centriole disengagement. A) Experimental workflow for determining and analyzing candidate proteins for RNAi screen. B) List of proteins localized to the centrosome followed by outcomes of analysis of Cdk1 phosphorylation sites, analysis of Polo binding and phosphorylation sites, sterility assay, and phenotypic analysis. Columns are in order (left to right) by which analysis was conducted for each gene.

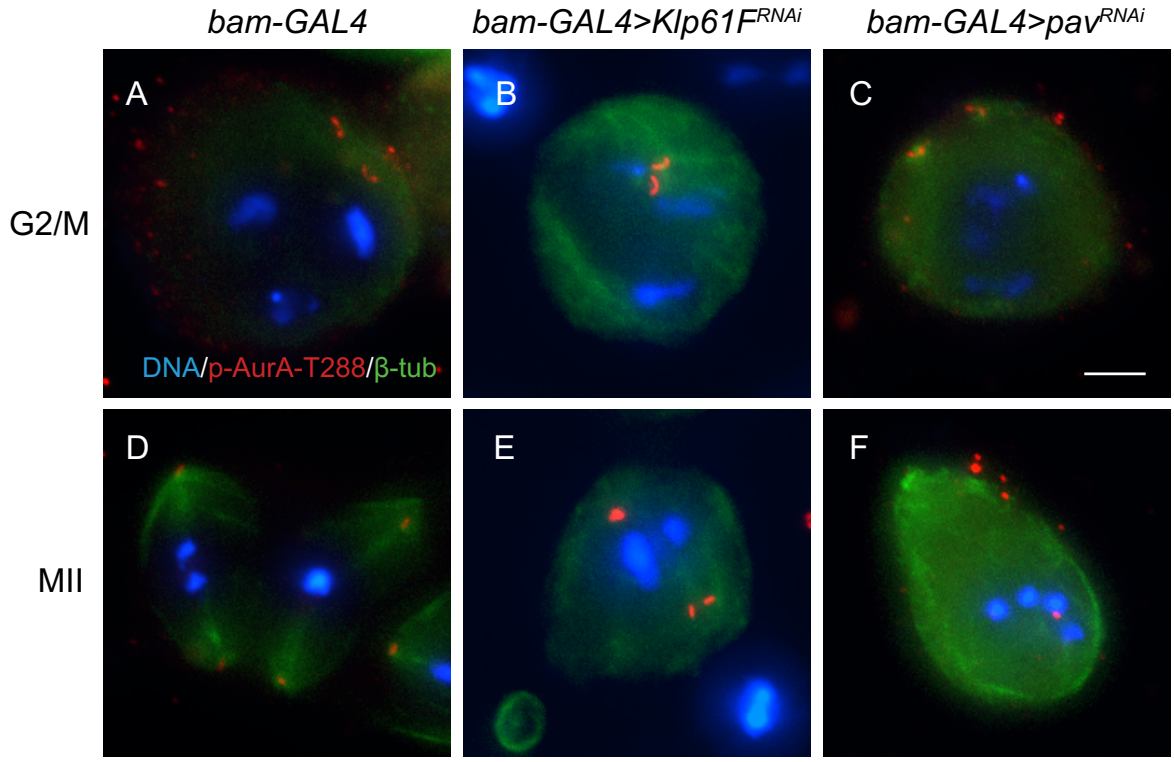


Figure A-3. RNAi against candidate genes results in normal centrosomes and abnormal meiosis. Phenotypic analysis of spermatocytes proceeding through either the G2/M transition or MII. At the G2/M transition, two pairs of engaged centrioles (red) are apparent in bam-GAL4 control, as well as in spermatocytes depleted of *Klp61F* or *Pav* (A-C). Normal anaphase II showing one centriole forming each spindle pole (D), and abnormal MII upon Klp61F or Pav depletion (E-F). Centrosomes appear disengaged but unable to form a spindle (E) or absent (F) in MII. Scale bar = 10 μm.

A.4.2. Centrosome composition appears unaffected in *myt1* spermatocytes

Though there are hundreds of proteins that localize to the centrosome throughout the cell cycle, the primary centrosome structure is maintained by approximately 20 proteins (Fu et al., 2015). These proteins are arranged into three zones; the core centriole and inner PCM, which are present throughout the cell cycle, and the outer PCM that is present in late G2 and M phase (Fu et al., 2015). Though the RNA interference experiments gave me no indication of which proteins were required for centrosome engagement during pre-meiotic G2 phase, I reasoned that visual analysis using reporters might provide another perspective on how loss of Myt1 affected centrosome structure. For these experiments, I analyzed the localization of several key centrosome proteins using a combination of fluorescent reporters and antibodies as indicated (Figure A-4). Of the proteins analyzed in late G2 spermatocytes, none appeared to be mis-localized in *myt1* mutants relative to the heterozygous control (Figure A-4), indicating that the overall construction of the *myt1* mutant centrosome may not be compromised. Further analysis of the remaining key centrosome components, as well as analysis at greater resolution, may be useful for identifying the Cdk1 substrate.

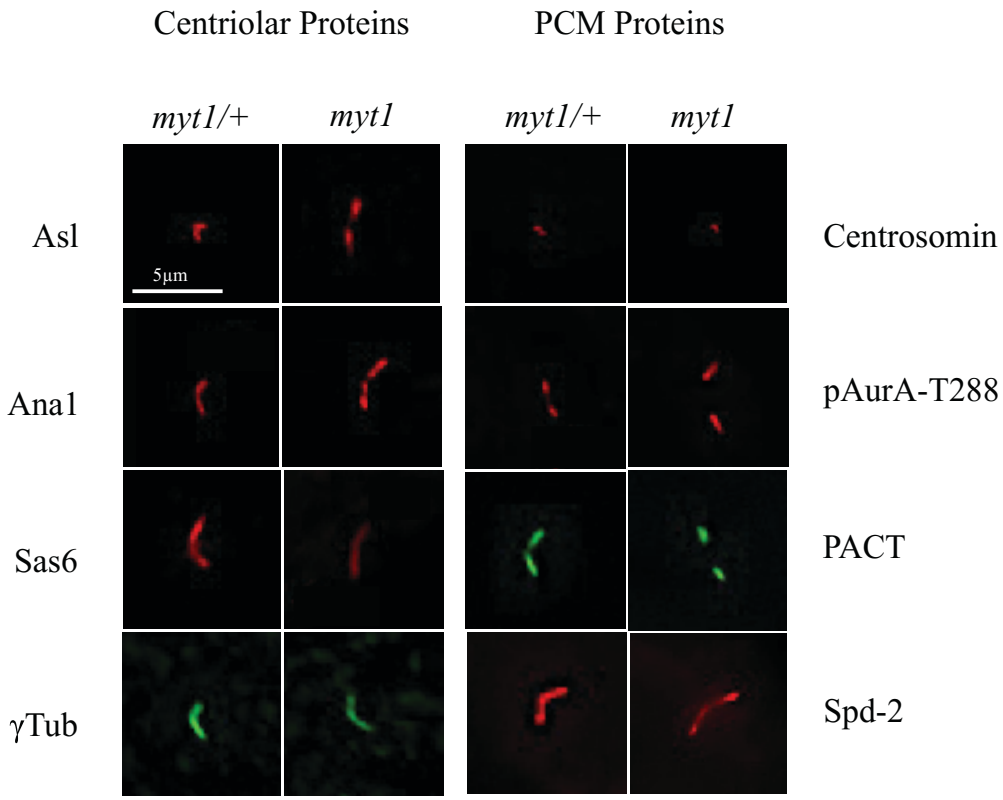


Figure A-4. Centrosome composition appeared to be unaltered in *myt1* mutant spermatocytes. Conserved centrosome components localized to either the centriole or PCM in wild-type centrosomes were analyzed in late G2 phase spermatocytes, staged as in Varadarajan et al., 2016. Asl-mCherry, Ana1-mCherry, anti-Sas-6, and anti-γ-tubulin were used to assess centriole components while anti-Cnn, anti-pAurA-T288, PACT-GFP, and anti-Spd-2 were used to analyze PCM proteins.

A.4.3. Assessing the role of microtubule dynamics on centriole disengagement

To determine if forces generated by microtubule assembly and disassembly might play a role on centriole disengagement in *myt1* mutant spermatocytes, I subjected these cells to nocodazole, which inhibits microtubule polymerization and arrests cells at the G2/M transition, as well as taxol, which prevents microtubule depolymerization, effectively arresting cells in metaphase. Upon 3 days of oral treatment with 200 μM nocodazole, nearly all spermatocytes were arrested at the G2/M transition, while treatment with 200 μM taxol resulted in a majority of spermatocytes arrested in metaphase (Figure A-5A). Treatment of *myt1* mutants with the same inhibitor dosages failed to rescue the centriole disengagement defect, as measured by number of centrosome foci (Figure A-5B). The proportion of spermatocytes with 4 centrosome foci (disengaged) was 90% and 88% for nocodazole and taxol treated flies respectively, which was very similar to the DMSO treated control group (Figure A-5B). Thus, microtubule forces do not appear to affect centriole-centriole cohesion within pre-meiotic G2 phase-arrested *myt1* mutant spermatocytes.

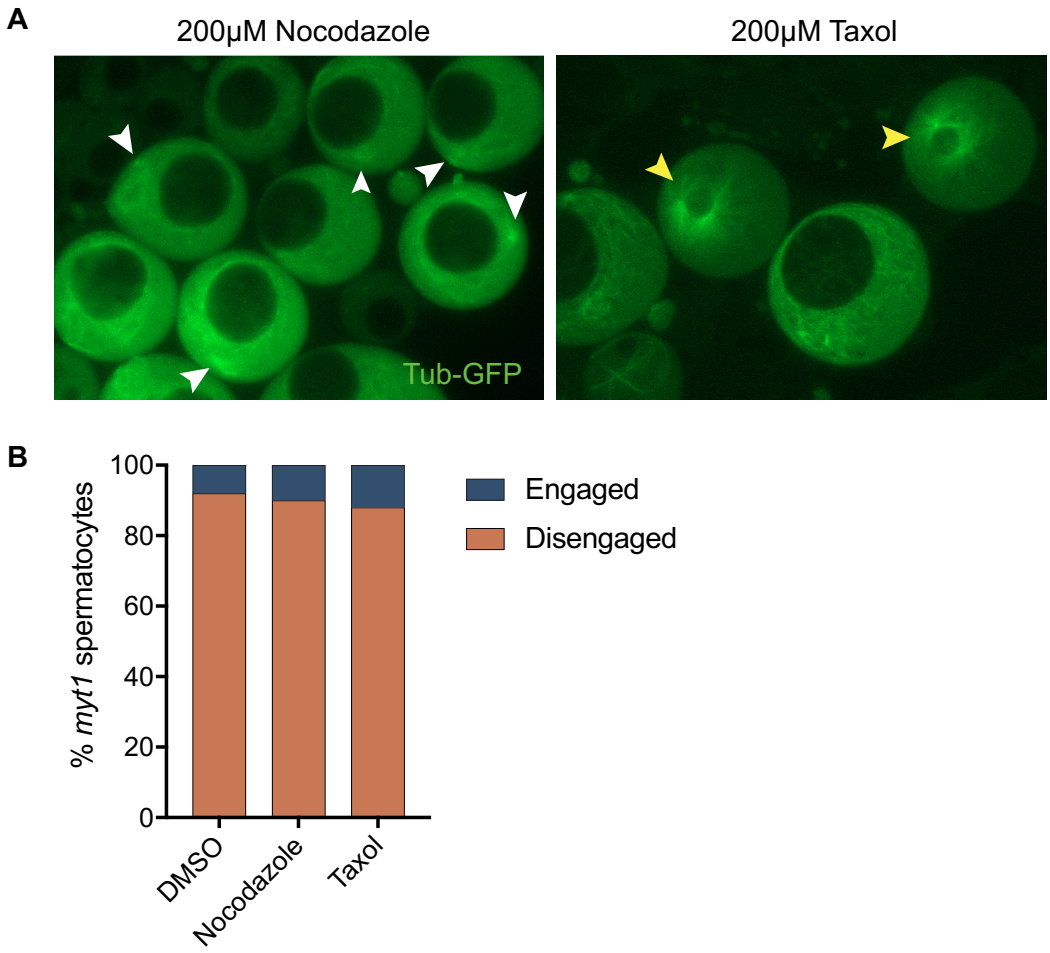


Figure A-5. Inhibition of microtubule polymerization or depolymerization does not rescue *myt1* centriole disengagement. A) Late G2 spermatocytes expressing Tubulin-GFP from flies fed 200 μM nocodazole or 200 μM taxol. Nocodazole fed flies possessed spermatocytes arrested predominantly in prophase as indicated by the appearance of tubulin foci (white arrowheads) while the majority of taxol fed fly spermatocytes were arrested in metaphase (yellow arrowheads). B) Quantification of *myt1* spermatocytes with engaged (2 centrosome foci) or disengaged (4 centrosome foci) centioles from flies fed regular food containing DMSO, nocodazole, or taxol. n>100 spermatocytes for each group.

A.4.4. *myt1* mutant spermatocytes possess supernumerary centrioles

Characterization of the timing of centriole disengagement demonstrated that centriole disengagement in *myt1* mutant spermatocytes occurs during mid-G2 phase (stage S3-S4) (Varadarajan et al., 2016). I undertook additional analysis with ubiquitously expressed PACT-GFP (centrosome localization motif from Plp) in a *myt1* mutant background. Approximately 30% of *myt1* spermatocytes possessed more than 4 centrioles (Figure A-6A) with some possessing as many as 8 centrioles, showing that mutant centrioles are capable of undergoing extra rounds of replication in addition to prematurely disengaging. These results also suggest that the centrosome defect in *myt1* mutant spermatocytes might occur during or immediately following S phase. This makes sense in light of the fact that fusome disruption due to ectoptic Cdk1 activity in *myt1* mutants also occurs either very early in G2 phase (Varadarajan et al., 2016), or perhaps as early as S phase. Future work will need to be done to elucidate the exact timing of the defect in *myt1* mutants as well as protein(s) targeted by Cyclin A/Cdk1 that facilitate centriole disengagement.

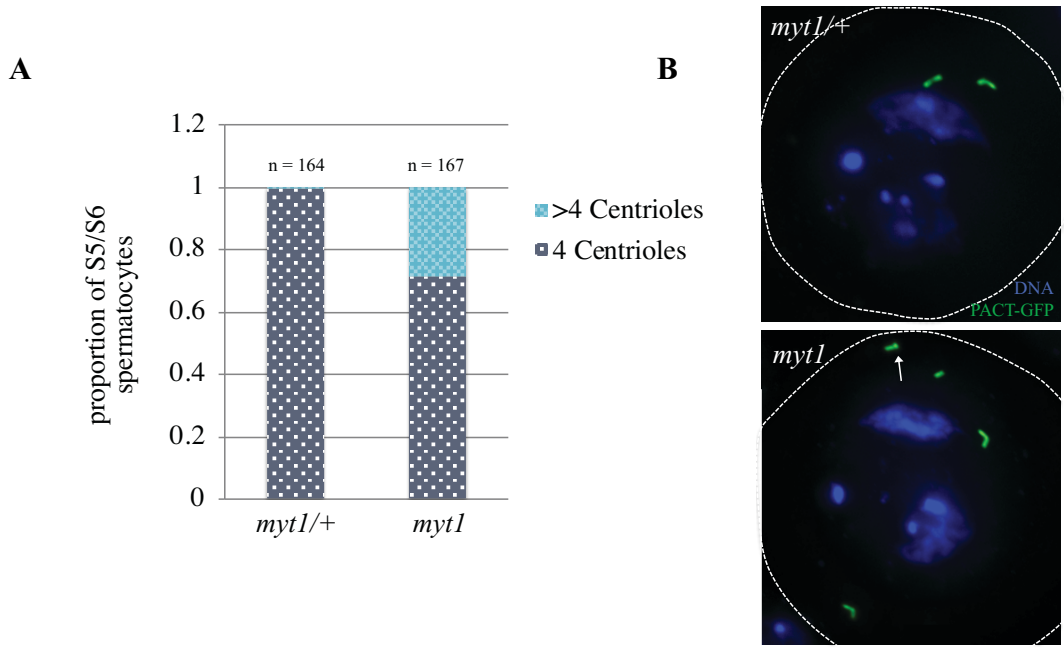


Figure A-6. *myt1* mutant spermatocytes can reduplicate centrioles. A) Quantification of centriole number in late G2 phase spermatocytes (S5/S6). Nearly all heterozygous control spermatocytes possess 4 centrioles while 30% of *myt1* spermatocytes possess more than 4 centrioles. B) Centrioles were marked with the PACT-GFP reporter. *myt1* spermatocytes possessed anywhere from 4-8 centrioles with centrioles in either the engaged or disengaged conformation. The arrow indicates a possible newly formed centriole.

A5. References

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Appendix B: *Drosophila* Stocks & Antibodies

B.1. *Drosophila* Stocks

Fly Line	Genotype	Source	ID/ Ref
bam-GAL4	*w; bam-GAL4 (VP16)*	Campbell Lab	N/A
UAS-Dicer	*P{w[+mC]=UAS-Dcr-2.D}1*	BDSC	24648
yw	y^1 *w*	Campbell Lab	N/A
myt1	$myt1^{R6}$	Campbell Lab	N/A
bel^{RNAi}	y^1 v^1; *P{TRiP.JF02884}attP2*	BDSC	28049
$CG1962^{RNAi}$	y^1 v^1; *P{TRiP.GL01480}attP2*	BDSC	43139
$CG7033^{RNAi}$	y^1 sc^* v^1; *P{TRiP.HMS01190}attP2*	BDSC	34711
$CG7518^{RNAi}$	y^1 sc^* v^1; *P{TRiP.HMC06523}attP40*	BDSC	77392
cnn^{RNAi}	y^1 sc^* v^1; *P{TRiP.GLC01863}attP40*	BDSC	57149
Plp^{RNAi}	y^1 sc^* v^1; *P{TRiP.HMC05936}attP40*	BDSC	65231
feo^{RNAi}	y^1 sc^* v^1; *P{TRiP.GL00393}attP2*	BDSC	35467
$gammaTub23C^{RNAi}$	y^1 v^1; *P{TRiP.JF01722}attP2*	BDSC	31204
$gammaTub37C^{RNAi}$	y^1 sc^* v^1; *P{TRiP.HMS00517}attP2*	BDSC	32513
$Grip163^{RNAi}$	y^1 v^1; *P{TRiP.HMJ21829}attP40*	BDSC	57821
$Grip84^{RNAi}$	y^1 sc^* v^1; *P{TRiP.HMS00627}attP2*	BDSC	33548
$Klp10A^{RNAi}$	y^1 sc^* v^1; *P{TRiP.HMS00920}attP2*	BDSC	33963
$Grip91^{RNAi}$	y^1 v^1; *P{TRiP.JF01719}attP2*	BDSC	31201
lat^{RNAi}	y^1 sc^* v^1; *P{TRiP.HMC06377}attP40*	BDSC	67274
lds^{RNAi}	y^1 sc^* v^1; *P{TRiP.HMS01389}attP2*	BDSC	34980
$msps^{RNAi}$	y^1 sc^* v^1; *P{TRiP.HMS01906}attP40/CyO*	BDSC	38990
$Nap1^{RNAi}$	y^1 sc^* v^1; *P{TRiP.HMC06207}attP2*	BDSC	65936
$Orc1^{RNAi}$	w^{1118}; *P{GD4477}v46521*	VDRC	46521
$Orc2^{RNAi}$	y^1 sc^* v^1; *P{TRiP.GL01560}attP40*	BDSC	43215
pav^{RNAi}	y^1 v^1; *P{TRiP.HMJ02232}attP40*	BDSC	42573
sas-4^{RNAi}	*P{KK100878}VIE-260B*	VDRC	106051
$scrambled^{RNAi}$	y^1 sc^* v^1; *P{TRiP.GL00517}attP2*	BDSC	36098
Spd-2^{RNAi}	*P{KK110116}VIE-260B*	VDRC	101882
$tacc^{RNAi}$	y^1 sc^* v^1; *P{TRiP.HMC06268}attP2*	BDSC	65982
ncd^{RNAi}	y^1 v^1; *P{TRiP.HMJ22094}attP40*	BDSC	58144
Klp61F^{RNAi}	*P{KK100504}VIE-260B*	VDRC	109280
Asl^{RNAi}	y^1 v^1; *P{TRiP.GL00661}attP40*	BDSC	38220
Cep135^{RNAi}	y^1 sc^* v^1; *P{TRiP.HMC06109}attP40*	BDSC	65357
Ana1^{RNAi}	y^1 v^1; *P{TRiP.HMJ23356}attP40*	BDSC	61867
bor^{RNAi}	y^1 sc^* v^1; *P{TRiP.HMC06013}attP40*	BDSC	65057
Rootletin^{RNAi}	y^1 v^1; *P{TRiP.HMJ22888}attP40*	BDSC	60494
PACT-GFP	*w; Ubi-PACT-GFP,* myt^{R6}*/Tm6*	Tim Megraw	*Martinez-Campos et al., 2004*
Asl-mCherry	*w; Ubi-Asl-mCherry/Cyo;* myt^{R6}*/Tm6*	Tim Megraw	*Novak et al., 2014*
Ana1-dTom	*w; Ubi-Ana1-dTom/Cyo;* myt^{R6}*/Tm6*	Tim Megraw	*Blachon et al., 2008*
Tub-GFP	*UASp-GFP-alpha-Tubulin/TM3, Sb*	David Glover	N/A
wee1^{ES1}	*w;* $wee1^{ES1}$*/cyo*	Campbell Lab	N/A
MARCM80B	*yw hsFLP UAS-GFP tubGAL4; FRT80B tubGAL80*	Bruce Edgar	N/A
FRT80B	*w; neoFRT80B*	BDSC	1988
FRT80B, myt1^{R6}	*w; neoFRT80B,* $myt1^{R6}$	This study	N/A
esg^{ts}	*esg-GAL4;* $tubGAL80^{ts}$ *UAS-GFP*	Edan Foley	N/A
	esg-GAL4; tub-$GAL80^{ts}$*; MKRS, Sb/ TM6B*	This study	N/A

	esg-GAL4, UAS-his::CFP, GBE-Su(H)-GFP:nls; tub-GAL80^{ts}	Lucy O'Brien	N/A
esg^{ts}; Su(H)-GAL80	*esg-GAL4, UAS-2X-EYFP/Cyo; Su(H)GBE-GAL80, tub-GAL80^{ts}/TM6B*	Bruce Edgar	N/A
esg^{ts}F/O	*esg-GAL4, tub-GAL80^{ts}, UAS-GFP; UAS-flp, Act>CD2>GAL4*		
Su(H)^{ts}	*Su(H)GBE-GAL4; tub-GAL80^{ts}, UAS-GFP*	Edan Foley	N/A
28E03^{ts}	*tub-GAL80^{ts}, UAS-GFP; 28E03-GAL4*	This study	N/A
Pros^{ts}	*tub-GAL80^{ts}, UAS-GFP; ProsV1-GAL4*	Bruce Edgar	N/A
Myo1A^{ts}	*Myo1A-GAL4; tub-GAL80^{ts} UAS-GFP*	Edan Foley	N/A
How^{ts}	*tub-GAL80^{ts} UAS-GFP; 24B-GAL4*	Bruce Edgar	N/A
UAS-myt1^{RNAi}	*P{KK102559}VIE-260B*	VDRC	105157
UAS-EGFP-Myt1	*P{UASp-EGFP-Myt1}1, y^{1} w*	Campbell Lab	N/A
UAS-FUCCI	*Kr/Cyo; UAS-GFP-E2F1, UAS-mRFP-nls-CycB/TM6B*	BDSC	55122
UAS-PCNA::GFP	*UAS-PCNA::GFP, neur-GAL4/TM6*	N/A	N/A
Su(H)-GFP	*Su(H)-nls-GFP/TM6*	Lucie O'Brien	N/A
NRE-GFP	*NRE-GFP*	Ben Ohlstein	N/A

B.2. Antibodies

Antibody	Source	Cat #/ Reference	Dilution
Mouse monoclonal anti-GTU-88	Sigma	T6557	1:100
Rabbit polyclonal anti-Sas-6	Jordan Raff	*Habedanck et al., 2005*	1:500
Rabbit polyclonal anti-Cnn	Tim Megraw	*Zhang and Megraw, 2007*	1:500
Rabbit polyclonal anti-phospho-AurA-T288	Abcam	N/A	1:500
Rabbit polyclonal anti-Spd-2	Maurizio Gatti	*Giansanti et al., 2008*	1:3500
Rabbit polyclonal anti-phospho-Histone H3 (Ser10)	Millipore	06-570	1:2000
Chicken polyclonal anti-GFP	ThermoFisher Scientific	PA-19533	1:2000
Rabbit polyclonal anti-RFP	ThermoFisher Scientific	R10367	1:1000
Mouse monoclonal anti-Delta	DSHB	C594.9B	1:100
Mouse monoclonal anti-Prospero	DSHB	MR1A	1:100
Mouse monoclonal anti-lamin DmO	DSHB	ADL84.12	1:200
Rabbit polyclonal anti-Cdk1/Cdc2 (PSTAIR)	Millipore	06-923	1:8000
Rabbit polyclonal anti-Anillin	C. Field	*Field and Alberts, 1995*	1:300
Mouse monoclonal anti-γ-H2Av	DSHB	UNC93-5.2.1	1:50
Goat anti-rabbit IgG (H+L) secondary antibody, Alexa Fluor 568	ThermoFisher Scientific	A11011	1:2000
Goat anti-mouse IgG (H+L) secondary antibody, Alexa Fluor 568	ThermoFisher Scientific	A11004	1:2000
Goat anti-mouse IgG (H+L) secondary antibody, Alexa Fluor 488	ThermoFisher Scientific	A32723	1:2000
Goat anti-Chicken IgY (H+L) secondary antibody, Alexa Fluor 488	ThermoFisher Scientific	A11039	1:2000

B.3. References

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