Investigating the Regulation of Separase during Meiosis I in *Caenorhabditis elegans*

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Dedication

To my family and extended family

To my partner

To my closest friends

To all of my teachers and mentors

To Dr. Chad Tillberg, Dr. Anne Jenkins Kruchten, Dr. Alberto Bressan, Dr. Alla Grishok, Dr. Germano Cecere, Dr. Ainhoa Ceballos, and Dr. Joshua Bembenek, without whom I would not have had this amazing experience

To the millions of worms who sacrificed their lives, so that I could study a couple proteins

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Abstract

Meiosis is a tightly regulated series of events leading to gamete formation. A key player in this process is the protease Separase (SEP-1). Known for its role in chromosome segregation, we have defined a role for SEP-1 in vesicular trafficking during cell division. Securin (IFY-1) is an inhibitory chaperone of SEP-1 that is degraded at anaphase onset after ubiquitination by the Anaphase Promoting Complex/Cyclosome (APC/C). After IFY-1 degradation, activated SEP-1 cleaves a subunit of cohesion to allow chromosome segregation. SEP-1 also displays dynamic localization during the cell cycle. In prometaphase I, IFY-1 localizes to kinetochores and mysterious cortical filaments. During anaphase I, SEP-1 transfers to the midbivalent, the region between homologous chromosomes where Cohesin localizes, and concurrently appears on vesicles, called cortical granules, in the cortex. How the dynamic localization of SEP-1 is regulated is currently unknown. Here, I investigated how the APC/C and IFY-1 regulate Separase during meiosis I. First, I observed that IFY-1 is degraded by anaphase I and is not present on vesicles. Second, APC/C activity is required for SEP-1 to localize to vesicles. *apc/c* RNAi results in arrested embryos with SEP-1 and IFY-1 remaining colocalized on cortical filaments. Third, partial *ify-1* RNAi causes precocious SEP-1 vesicle localization. These observations suggest that IFY-1 degradation is required for SEP-1 to localize to vesicles during anaphase I. To further test this, Non-Degradable Securin fused to GFP (GFP::IFY-1^{DM}) was generated. GFP::IFY-1^{DM} is stably expressed during anaphase I and causes embryonic lethality. GFP::IFY-1^{DM} causes chromosome

segregation defects, polar body extrusion failure and inhibits cortical granule exocytosis. Importantly, expression of GFP::IFY-1^{DM} causes a reduced and delayed localization of SEP-1 to vesicles during anaphase I. I conclude that degradation of IFY-1 regulates the localization of SEP-1 to vesicles to coordinate exocytosis with chromosome segregation. My findings suggest a novel role for the APC/C and IFY-1 in controlling the localization of SEP-1, in addition to regulating its protease activity for chromosome segregation and cortical granule exocytosis during anaphase I.

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Chapter 1

Introduction

The Cell Cycle

A fundamental aspect of cellular life is the ability of a cell to passage its genome to future generations. For eukaryotes, we describe this phenomenon in the context of the eukaryotic cell cycle (Fig. 1). Broadly, this phenomenon is captured by a single parental cell dividing to produce two daughter cells. It is necessary for the cell to faithfully navigate its way through the cell cycle, as failure to do so can be generally disastrous: In the case of unicellular organisms' senescence is one outcome, whereas in multicellular organisms there are myriad, and include aneuploidies, infertility, and cancers. To circumvent disaster, a conserved regulatory module operates, along with a series of checkpoints, to ensure a one-way progression from the beginning to end of the cell cycle. The first factors governing the regulation of cell cycle progression were discovered using yeast genetics in the 1970's (Hartwell et al., 1970). Since the discovery of these regulators, the knowledge of how the progression of the cell cycle is regulated has greatly expanded (Morgan, 2007). The linear progression through each stage of the cell cycle is governed by interactions between cyclin-dependent kinases (CDKs) and their activating Cyclins (Lim & Kaldis, 2013). The rapid destruction of Cyclins at specific time points ensures the one-way trajectory of the cell cycle (Reed et al., 2003). For simplicity, the cell cycle is often dichotomized between "interphase" and "M-Phase." Within each step of the cell cycle, specialized mechanisms are in place to ensure the fidelity of cell cycle progression.

The Cell Cycle: Interphase

Interphase is a composite of, generally, three independent phases: Gap 1 (G1), Synthesis (S), and Gap 2 (G2). Each of these phases functions in space and time and are under tight regulatory control.

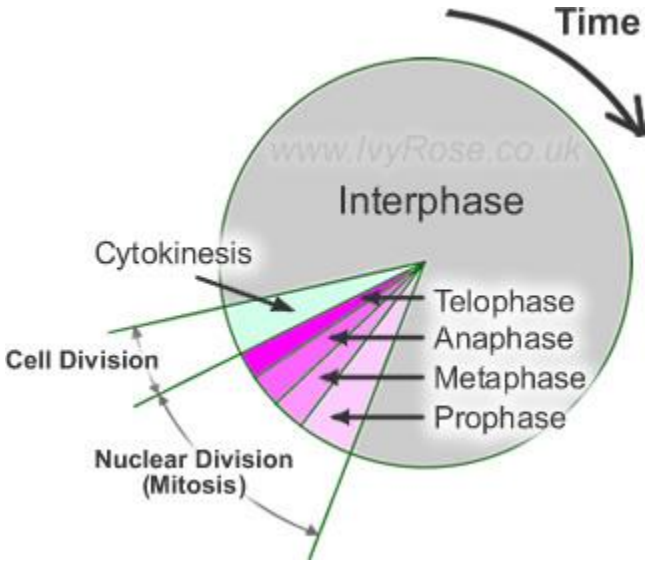


Figure 1. Cartoon generalization of the different phases of the cell cycle. The bulk of a cell's live is spent in interphase (grey). During M-phase, in this case, Mitosis (shades of purple) entails a series of steps to partition the duplicated genomes into two separate nuclei. Ultimately, the cells divide during a process called cytokinesis to produce two genetically identical daughter cells. Image courtesy of Dodd Waldwick.

Interphase: Gap 1 (G1)

The cell cycle typically consists of two phases of growth, which we term the Gap phases. The first Gap phase to occur is G1, which follows the exit of the previous cell division and prior to DNA duplication in S phase. Progression through G1 is tightly regulated. This regulation in part depends upon the regulation of G1 specific transcription factors, for instance, E2Fs, which are known to interact through E2F activators with the tumor suppressor Retinoblastoma protein (pRb; Hurford *et al.,* 1997; Giacinti & Giordano, 2006). Together, E2Fs and pRb ensure proper progression of the cell cycle in G1 (Henley & Dick, 2012). Misregulation of this pathway can result in tumorigenesis and oncogenesis through over proliferation and defects in apoptosis (Du *et al.,* 2009). To avoid these issues, there is one important checkpoint in G1, which serves to control proper cell cycle entry: The "commitment point" (also called 'restriction point' in metazoan and 'start' in yeast), which is conserved from yeast to mammals, instructs whether the cell is to reenter the cell cycle (Bertoli *et al.,* 2015). This checkpoint prevents premature destruction of G1-specific cyclins and thus ablation of G1-specific CDK1 activity. The switch to G1-to-S phase appears to be a field of active research with many open questions and controversy, but it appears established that the inactivation of APC/C-CDH1 and pRb via satisfaction of the commitment point, enabling entry into S-phase (Liu *et al.,* 2020).

Interphase: Synthesis (S)

During S-phase of the cell cycle the chromosomes are duplicated. Within S Phase there are three major checkpoints ensuring that DNA replication initiates once and that any damage to DNA is repaired (Bartek *et al.,* 2004). The first checkpoint is the "replication checkpoint," where the

DNA structure is monitored to ensure that replication has gone properly by maintaining the integrity of the replication fork as well as preventing the initiation of replication elsewhere in the genome until repairs to the DNA are corrected (Barnum & O'Connell, 2016). The two other key points of regulation that both function to delay cell cycle progression, into G2 phase and M-phase, by preventing activation of CDK1 by Cyclin B (Bartek *et al.,* 2004). These are the *S-M checkpoint* and the *intra-S phase checkpoint*. The S-M checkpoint delays cell cycle progression until the entire genome has been duplicated, while the intra-S phase checkpoint monitors for double-strand breaks and issues related to erroneous repair.

Additionally, and important for a successful cell cycle, it is well established that the factor required to hold duplicated chromosomes together are added only at this point in the cell cycle and is tied to DNA replication (Uhlmann & Nasmyth, 1998). This factor is called Cohesin. For the purpose of this research, it is only important to know that Cohesin is a tripartite ring complex that is conserved across eukaryotes and ensures that the duplicated genetic information will be appropriately paired to allow for proper sorting in metaphase and segregation during anaphase (Onn *et al.,* 2008). Without Cohesins, chromosomes do not position themselves properly during M phase and result in untimely, premature segregation (Michaelis *et al.,* 1997). The exact nature of how Cohesin associates with DNA is controversial with recent studies suggesting upwards of three modes of association (Brooke & Berkowitz; Srinivasan *et al.,* 2020). Emerging evidence suggests independently evolved mechanisms or deviations from the canonical replication-dependent association of Cohesin to DNA (Zuilkoski *et al.,* 2022). It is noteworthy that depending on the cell type, one subunit of Cohesin varies, and this is the Kleisin subunit. These Kleisin subunits are quite diverse and reflect a surprisingly large number of functions outside of merely holding chromosomes together during the cell cycle, so in addition to chromosome

segregation, this involves DNA repair, transcriptional control, and genomic integrity (Brooke & Berkowitz, 2014). Evidence is emerging of Cohesin not merely playing a passive role in DNA cohesion and interesting questions pertaining to the histone code and the "*cohesion code*," for instance, in the context of DNA repair are emerging avenues of research (Rudra & Skibbens, 2013; Zuilkoski *et al.,* 2022). Much interest is now dedicated to understanding how Cohesin, once thought to be merely a static entity waiting for its inevitable doom in anaphase, now appears dynamic, with structural variation throughout the cell cycle. How Cohesin performs various cellular roles and how its structure modulates throughout the cell cycle and how that is connected to various events is an active and undeveloped area of research (Rudra & Skibbens, 2013).

Interphase: Gap 2 (G2)

G2 is not necessarily required for cell division, though mechanisms are in place to repair breaks in duplicated chromosomes (Burgoyne *et al.,* 2007). Nevertheless, it is a crucial time to acquire and synthesize resources necessary for progression into M phase.

Members of a well-conserved family of kinases are especially important in preventing M phase entry at this time. These are the WEE1 serine/threonine kinases, which phosphorylate conserved inhibitory residues on Cdk1, blocking its activity, and are thus negative regulators of M-phase entry (Ghelli Luserna di Rorà *et al.,* 2020). Silencing of WEE1 kinase signaling is critical for CDK1 activation as phosphatases may remove the inhibitory phosphates deposited by WEE1from conserved CDK1 residues (Lundgren *et al.,* 1991). Hence, the WEE kinases are important for maintenance of G2 arrest. There is functional divergence in G2 arrest amongst

WEE1 kinases, where WEE1 arrest is related to insufficient cell size and another WEE kinase, MIK1 maintains G2 arrest via signals associated with the DNA damage checkpoint (Rhind & Russell, 2001).

It is also noteworthy to mention that members of the WEEf1 kinase family and members of the spindle assembly checkpoint associate with the Golgi apparatus and endoplasmic reticulum (Liu *et al.,* 2007; Hirose *et al.,* 2004). In humans, MYT1 kinase associates with these structures and is required to mediate Golgi and endoplasmic reticulum reassembly during M phase exit (Nakajima *et al.,* 2008). In flies, the RZZ complex mediates ER-Golgi vesicle trafficking in interphase cells (Hirose *et al.,* 2004). The significance and purpose of these connections to vesicle trafficking and checkpoint signaling is elusive and an undeveloped area of research.

M Phase: An Overview

Following the completion of interphase, the cell cycle transitions to M-phase. M-phase can be broadly categorized as consisting of two phenomena (Fig. 1). The first is the nuclear division (mitosis) where the duplicated genomes present in the mother cell's nucleus are partitioned into their own daughter nuclei. The second step being cell division (cytokinesis), where the mother cell is partitioned into two daughters via ingression of the cleavage furrow by the actomyosin contractile ring (Green *et al.,* 2012). Ultimately, these daughter cells are genetically identical to each other. Depending on the type of organism or cell type, M-phase includes mitosis or meiosis. In contrast to mitotic M-phase, during meiotic M-phase, dividing cells undergo two rounds of chromosome segregation and cytokinesis without an intervening period of genome duplication, resulting in haploid daughter cells. Meiosis is critical in sexually reproducing organisms for

gamete formation and generating genetic diversity. Proper execution of cell division is therefore critical for cellular and organismal viability. Improper progress of mitosis can lead to aneuploidy, which can be detrimental to cellular function (Kops *et al.*, 2005). Thus, it is important that cell division is tightly regulated both during chromosome segregation and cytokinesis. Key points of regulation exist during M phase in both mitotic and meiotic cell division; the best characterized to date being the metaphase-to-anaphase transition, which will be discussed in the context of mitosis. Throughout eukaryotes, the metaphase-to-anaphase transition constitutes a critical point of regulation that ensures that the sister chromatids of the mother cell are properly segregated to the daughter cells. The key molecular regulators of this process are well conserved throughout Eukaryotes and function to coordinate the timely activation of the conserved cysteine protease, Separase. This regulatory network is known as the mitotic checkpoint, which safeguards premature entry into anaphase from metaphase (Fig. 2). Once activated an irreversible process is initiated whereby Separase proteolyzes the Kleisin of Cohesin, a multisubunit complex holding replicated chromosomes together, liberating the chromosomes for segregation, the hallmark feature of anaphase (Zhang & Pati, 2017).

The Mitotic Checkpoint

The Spindle Assembly Checkpoint (SAC)

The transition from metaphase to anaphase is an irreversible cellular decision. The mechanism in place to prevent precocious entry into anaphase is the known as spindle assembly checkpoint (SAC). Early genetic studies using budding yeast identified many of the conserved components of the SAC (Hoyt & Roberts, 1991; Li & Murray, 1991). The SAC itself functions as a molecular

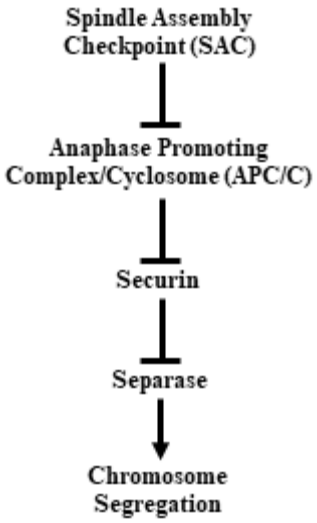


Figure 2. The mitotic checkpoint. This is the canonical regulatory network responsible for the transition for metaphase to anaphase. Satisfaction of the spindle assembly checkpoint (SAC) unleashes irreversible activation of Separase and chromosome segregation in anaphase.

surveillance system which monitors the attachment of microtubules emanating from the spindle poles to large proteinaceous structures on the chromosome known as kinetochores (Lara-Gonzalez *et a.,* 2021). Kinetochores assemble on a specialized locus (or loci) on the chromosome called the centromere. The size and number of kinetochores associated with chromosomes vary across metazoa; however, they generally require a specialized chromatin signature at the centromere for formation and share a common architecture, which is assembled in a hierarchical manner (Gascoigne & Cheeseman, 2011; Musacchio, 2015; Musacchio & Desai, 2017; Talbert& Henikoff, 2020*;* Lara-Gonzalez *et al.,* 2021). Kinetochores allow for the physical linkage of chromosomes to the spindle. The establishment of a proper spindle microtubule-kinetochore interface is critical for the transition from metaphase to anaphase. Microtubules attached to the kinetochore are known as k-fibers, and this attachment ultimately allows the chromosomes to move towards the poles during anaphase. Additionally, kinetochores function as signaling and sensory hubs, delaying anaphase entry by monitoring proper microtubule attachment to the spindle. The mere presence of a single unattached kinetochore is sufficient to elicit the SAC (Rieder *et al.,* 1995), safeguarding the proper entry into anaphase (Sacristan & Kops, 2015). While this suggests that the SAC is a robust hurdle for the cell to bypass for cell cycle progression, recent work as established that its fidelity exists on a spectrum, rather than merely existing in the cell as a bimodal "on/off switch" (Collin *et al.,* 2013). The initial formation of the SAC inhibitory signal is a rate limiting step (Simonetta *et al.,* 2009; Dick & Gerlich, 2013), and the strength of this SAC is directly proportional to the number of unattached kinetochores, with diminishing SAC inhibitory effectiveness as the number of unattached kinetochores decreases. It is critical for the generation of proper kinetochore attachments as defects can give rise to cell

division defects, like aneuploidy (Grengan *et al.,* 2011). While important work is being done to understand the nature of factors upstream of the canonical SAC signal, the "diffusible signal" is well established (Lara-Gonzalez *et al.,* 2021).

To promote entry into anaphase, the kinetochore elicits the SAC through the formation of a diffusible signal called the mitotic checkpoint complex (MCC). The MCC prevents APC/C from interacting with CDC20, a coactivator important for recruiting key substrates for ubiquitination by the APC/C (Musacchio & Salmon, 2007). The MCC protein complex is composed of SAC proteins BUBR1/MAD3, BUB3, and MAD2 bound directly to CDC20 (Musacchio, 2015) (Izawa & Pines, 2015; Faesen et al., 2017). In metazoans, a conserved adaptor called the RZZ complex is recruited to kinetochores to aid in both the elicitation and silencing of SAC signaling (Karess *et al.,* 2005). The RZZ complex is composed of Rod (*rough deal*), Zwilch, and ZW10 (Zeste-white 10), and is in part responsible for recruiting additional SAC proteins such as Mad1:Mad2 to the kinetochore to stabilize SAC signaling (Buffin *et al.,* 2005; Silió *et al.,* 2015). Furthermore, the RZZ complex recruits cytoplasmic dynein, a minus-end directed microtubule motor, to the kinetochore (Starr *et al.,* 1998). Proper localization of cytoplasmic dynein to the kinetochore by RZZ is essential for silencing of the SAC; dynein mediates the transport of SAC signaling factors towards the spindle poles (Hoffman *et al*, 2001). The molecular underpinnings of the link between RZZ to dynein has recently established whereby farnesylated Rod acts as a receptor the dynein assessor protein, Spindly (Mosalaganti *et al.*, 2017). Spindly is necessary for SAC silencing as mutating conserved domains prevent dynein recruitment to kinetochores, thus enhancing SAC signaling at the kinetochore (Gassmann *et al.,* 2010). Thus, the connection between SAC function and molecular motors is well established, though many mysteries remain.

Interestingly, and of particular relevance, is that the APC/C has been implicated in the control of these molecular motors during M phase in worms (Ellefson & McNally, 2011).

Anaphase Promoting Complex/Cyclosome: Activation

The correct bioriented attachment of kinetochores to the bipolar spindle satisfies the SAC. Once the SAC is satisfied, the entry into anaphase occurs via the activation of the large E3 ubiquitin ligase APC/C by allowing its association with its coactivator CDC20. APC/C-CDC20 recognizes specialized motifs on its substrates, called degrons, and polyubiquitinates these substrates to mark them for degradation by the 26S proteasome (Davey & Morgan, 2016). Two key substrates of the APC/C during mitosis are Cyclin B and Separase. Destruction of Cyclin B promotes exit from M-phase. Destruction of Securin, the inhibitory chaperone of Separase, promotes chromosome segregation, the hallmark of anaphase. Destruction of Securin liberates the proteolytic activity of Separase, allowing it to cleave the Kleisin subunit of Cohesin, which in turn liberates sister chromatids to move towards opposite spindle poles in anaphase (Holland & Taylor, 2006; Zhang & Pati, 2017).

Cytokinesis

The last step of cell division is intimately linked to the chromosome segregation of anaphase onset. This final step is called cytokinesis, which partitions the mother cell into two daughters. This process involves drastic physical changes to the sell size and shape and must be coordinated with membrane trafficking and chromosome segregation. The current understanding of cytokinesis, in the context of cell cycle progression, is that it always follows anaphase onset. While the mechanism behind anaphase onset is well established (Fig. 2), the precise mechanism

of establishing where and when the cell will ultimately partition into two is less certain (Pollard, 2017). However, this current understanding has recently been challenged whereby cytokinesis can occur without nuclei in flies (Bakshi *et al.,* 2022). There are still questions about the regulation of cytokinesis, especially because of documented variation in the process found through eukaryotes with some cytokinetic events being left incomplete (Chaigne & Brunet, 2022). Along these lines, some "essential" factors required to complete cytokinesis in somatic tissues, are not required for the completion of cytokinesis in the germline, as is the case for the microtubule motor protein ZEN-4 in the *C. elegans* hermaphroditic germline (Lee *et al,* 2018).

An extreme example illustrating this point that cytokinesis only appears to ever follow chromosome segregation in anaphase is the case of the *Drosophila melanogaster* syncytial embryo. In this example multiple, synchronous rounds of nuclear divisions and DNA duplication occur without intervening cytokinesis events (Calzone *et al.,* 2007). A well-established, conserved network ensures that cytokinesis eventually follows the onset of anaphase onset (Cundell *et al.,* 2013). The nature of slow synthesis and rapid destruction of Cyclins likely also buttresses this phenomenon (Reed *et al.,* 2003).

Despite some overall variations, cytokinesis generally follows a conserved series of steps and checkpoints in eukaryotes, especially animals (Fededa & Gerlich, 2012). During anaphase of mitosis, and after chromosomes have moved apart sufficiently by the bipolar anaphase spindle, the initiation of a series of five steps begin, collectively referred to as cytokinesis. These steps are as follows: 1) Central spindle assembly, 2) Division plane specificity, 3) Contractile ring assembly, 4) Furrow Ingression, and 5) Abscission.

To ensure proper chromosome segregation, cells establish division planes perpendicular to the spindle. How the division plane is specified is an active area of research, and there appear to be

multiple mechanisms involved (Pollard, 2017). In animal cells, the mitotic spindle is important for establishing the division plane (White & Glotzer, 2012). After chromosome segregation as initiated in anaphase, the metaphase spindle is reorganized into the anaphase spindle, in part through contribution of the microtubules to the spindle midzone, which serves as the basis for what will become the central spindle, which serves as an important regulatory hub for generating the plane of cell division in animal cells (Glotzer, 2009). In order to accomplish the formation of the central spindle, CDK1 must become inactive, leading to the removal of inhibitory phosphorylation of a number of key motor proteins, microtubule nucleators and stabilizers and other critical regulatory proteins (Green *et al.,* 2017). There are at least three pathways in which this is accomplished involving a centrosome-dependent, centrosome independent, and chromosome-based route (Goshima *et al.,* 2008; Goshima *et al.,* 2013). Importantly, inactivation of CDK1 in early anaphase allows a microtubule antiparallel bundling protein, PRC1, the chromosome passenger complex (CPC) and Centralspindlin to localize to the spindle midzone (Green *et al.,* 2012; Nishiyama *et al.,* 2020). The CPC is composed of multiple subunits, including Aurora B, a kinase that is a key regulator of cleavage furrow ingression and abscission timing (Carmena et al., 2012). Removal of CDK1 inhibitory phosphates from subunits of CPC allows this complex to relocalize to the spindle midzone (Mierzwa & Gerlich, 2014). Centralspindlin, a heterotetrameric complex consisting of two kinesin-6 motor proteins (MLPK1) and two RhoGAPs, which is important for stabilization of the central spindle, is then phosphorylated by Aurora B at conserved residues on MLPK1, thereby blocking Centralspindlin from associating with its inhibitor (Douglas *et al.,* 2012). Through inactivation of CDK1 the central spindle becomes stabilized and in turn serves as a hub for establishing division plane specificity in part due to the RhoGAPs associated with Centralspindlin. At this time as well,

PRC1 recruits a kinesin which limits microtubule growth to a narrow region between the spindle poles; a RhoGEF with a plasma membrane-interacting pleckstrin homology domain then allows Centralspindlin to associate with the equatorial plasma membrane in a confined area between the spindle poles, which will the serve as the site of furrow ingression (Green *et al.,* 2012). As the anaphase spindle moves the poles in opposite directions, the association of Centralspindlin to the plasma membrane stimulates the actomyosin contractile ring. After the furrow constricts near completion, the central spindle compacts and matures into the midbody, which serves as the site where abscission will take place (Green *et al.,* 2012). In what is known as the abscission checkpoint (also known as NotCut), Aurora B monitors for lagging chromosomes to ensure the genome is equally partition and thus delays abscission (Steigemann *et al.,* 2009). In this role, Aurora B phosphorylates a key component of the abscission machinery known as CHMP4, at conserved residues which keep it in a conformation incapable of associating with the ESCRT-III complex, preventing abscission (Capalbo *et al.,* 2012; Agromayor *et al.,* 2013). Once chromosomes have cleared the cleavage furrow area, the abscission signal is satisfied by a mechanism in which Aurora B substrate specificity changes, enabling removal of inhibitory phosphorylation of CHMP4 and CHMP4 association with the ESCRT-III complex to facilitate the completion of cytokinesis (Pike *et al.,* 2016). In this way, inactivation of CDK1-Cyclin B allows the CPC to regulate two key processes: 1) the assembly of the metaphase spindle into the central spindle, and 2) the NoCut pathway responsible for governing faithful abscission (Norden *et al.,* 2006; Ibarlucea-Benitez *et al.,* 2018). Through the careful regulation of these processes, the exit of M phase has now commenced, and the cell cycle will work its way through the process over again.

Meiosis

In many sexually reproducing organisms, cell division is modified for the purpose of gamete formation. The key difference between mitotic and meiotic cell division is the presence of two successive cell divisions without an intervening S-phase. During the first meiotic M phase (meiosis I), homologous chromosomes recombine, which aids in generating genetic diversity. After the APC/C is activated in anaphase I, homologous chromosomes segregate and the first cytokinesis occurs, resulting in two daughter cells of reduced ploidy. This is also called the reductive division. Next, during meiosis II, sister chromatids are segregated. Thus, anaphase II is analogous to mitotic anaphase. Ultimately, the logical framework of M phase entry, the spindle assembly checkpoint, the metaphase-to-anaphase transition, the abscission checkpoint, and meiotic M phase exit are conserved. Consequently, failure to properly control meiosis can result in aneuploidy, and pathology arising from improper execution of meiosis include infertility, intellectual disability and, in some cases, cancer (Nagaoka *et al.*, 2012).

The Metaphase-to-Anaphase Transition Regulators: Historical Contexts & Emerging Roles

While the regulators of Separase are known for their role in cell division, specifically during chromosome segregation, recent studies have illuminated additional functions, not all of which are canonically associated with cell cycle regulation. Several pieces of evidence point to a wider role for this pathway in orchestrating numerous cellular events to ensure faithful cell cycle progression (Kishimoto, 2015).

The Spindle Assembly Checkpoint (SAC)

The RZZ complex is primarily known for its role in removing checkpoint signals following satisfaction of the SAC. The Zeste-white 10 (ZW10) subunit of the RZZ complex has been shown to play a role in vesicular trafficking in interphase cells. ZW10 was found to localize to the endoplasmic reticulum (ER), where it interacts with the S/G_2 checkpoint RINT1 (Rad50-interacting protein) and the v-SNARE, Syntaxin 18, to mediate trafficking between the ER and Golgi apparatus (Hirose *et al.*, 2004). Additionally, ZW10 has been found to play a role in cytokinesis during meiosis in male flies, in addition to ER-Golgi apparatus trafficking (Wainman *et al.,* 2012). Work from our group suggests that ZW10 plays a role in vesical trafficking during meiosis in *C. elegans* oocytes, likely during cortical granule biogenesis (Bembenek *et al.*, 2007). Collectively, these studies suggest a conserved role for ZW10, and by extension the entire SAC, during vesicular trafficking, possibly throughout the cell cycle.

It is now appreciated that the SAC proteins function during interphase to preserve genome integrity. It has been known that key SAC components like MAD1, MAD2, and MPS1 localized to the nuclear envelope-cytoplasmic interface since the turn of the millennium (Campbell *et al.*, 2001). For instance, nucleopore proteins have been shown to promote assembly of the MCC, canonically known to delay anaphase onset (Rodriguez-Bravo *et al.*, 2014). Astonishingly, the tumor suppressor PTEN has been shown to recruit and catalyze the dimerization of one MCC component at the nuclear pore in interphase, Mad1 (Liu *et al.*, 2017). They demonstrated that PTEN was required for MAD1 localization to the nuclear envelope, but not kinetochores during interphase. Whether the rest of the MCC was able to associate with PTEN and MAD1 in the nucleoplasm was not determined but it is intriguing to ask whether PTEN could enable assembly of the MCC at the nuclear pore. This would insinuate that non-kinetochore-based mechanisms

are capable of generating the MCC. Indeed, a recent study using human HeLa cells established kinetochore-independent catalysis of the MCC though these cells were not in interphase (Li *et al.,* 2017). Nevertheless, SAC signaling is gaining appreciation for roles outside of its typically thought of function spindle region (Heasley *et al.,* 2017), and the community appears open to the possibility of non-kinetochore-based structures like the nucleopore in eliciting the MCC and be extension the SAC outside of its canonical role at kinetochores (Lara-Gonzalez *et al.,* 2021). Nevertheless, a wide range of implications arise if the nucleopore complex is shown to directly facilitate the assembly of the MCC in interphase. A general understanding of how other aspects of cell biology are affected by such signaling, like general homeostasis remain to be explored, or even a delay in nuclear envelope breakdown NEBD, and perhaps a connection to the ribosome-checkpoint discussed earlier which could ensure that the proper amount of translatable material is present in the cell. And on a final related note, non-canonical kinetochore formation has been documented in protists (Akiyoshi & Gull, 2014), and rather surprisingly a GPI-anchored protein is capable of eliciting the SAC in yeast.

Collectively, these observations implicate noncanonical activation of checkpoint processes like the SAC, long thought to be elicited by kinetochores, may be prevalent in different cellular contexts outside of the spindle region. Thus, the idea of such a phenomenon, and the insinuation of a shared regulatory hub governing many facets of the cell is of great interests and suggests a more direct, expansive signaling hub, possibly upstream of the master regulators of cell cycle progression, the CDK kinases.

The Anaphase Promoting Complex/Cyclosome (APC/C)

A universal feature of the cell cycle is controlled proteolysis of various proteins, in space and time, to ensure proper cell cycle progression. Regulated proteolysis is found in both Eukaryotes and Prokaryotes via post translational-based protein modifications (Amon, 1998). Prokaryotes are not known to possess the master regulator of cell cycle progression, CDK1, and the cyclins found in Eukaryotes; nevertheless, proteolytic systems exist to control cell cycle progress that are functionally analogous to the eukaryotic model, complete with proteasomes and ubiquitin-like proteins (Gottesman 1996; Hershko, 1998; Mogk *et al.*, 2007; Gur *et al.*, 2011; Varshavsky, 2011). Indeed, proteins with cyclin-like function (periodicity in this case) have been identified in bacteria (Domian *et al.*, 1997; Lori *et al.*, 2015).

While this analogous method of cell cycle progression control is fascinating with many open questions pertaining to substrate recognition and relevance to pathogenicity (Gur *et al.*, 2011), and with regulatory principles likely translatable across kingdoms (Mogk *et al.*, 2007; Mahmoud & Chien, 2018), arguably one of the best studied sources of regulated proteolysis stems from Eukaryotic models (King *et al.*, 1996). One of the best conserved pathways, which is especially apropos to cell cycle progression is the ubiquitin-proteosome system (Varshavsky, 2012). This system is largely a Eukaryotic innovation (Eme *et al.*, 2011), and relies on the attachment of one of the most conserved, ubiquitous proteins found in the cell, ubiquitin, to target proteins for degradation (Özkaynak *et al.*, 1984; Pickart 2001). Broadly speaking, failure to control the ubiquitin-proteosome system can prove fatal to Eukaryotic cells, for example, by reducing the available pool of amino acids necessary for translation (Suraweera *et al.*, 2012). Compounding the importance of this process, one estimate suggests approximately 5% of the human genome encodes factors responsible for coupling and decoupling ubiquitin and ubiquitin-like proteins

from those post-translationally labeled with them (Strieter & Korasick, 2012). This suggests a figuratively infinite and diverse number of ways for regulated protein proteolysis. Underscoring this idea is the diverse manifestation of maladies originating from errors in the ubiquitin-proteasome system. Under properly functioning ubiquitin signaling cells will maintain normal homeostasis whereby cellular adaption to environment, the robustness of said adaptation, and proteostasis are maintained. In contrast, disruption of ubiquitin signaling results in a large variety of phenomena that can affect aging, immunity, congenial genetic maladies, neurodevelopment and neurodegenerative diseases, cardiovascular disease, and cancers (Jentsch & Haendler, 2009; Powell *et al.,* 2012; Hu & Sun, 2016; Amer-Sarsour *et al.,* 2021; Basar *et al,* 2021; Le Geuerroué & Youle, 2021).

The cell is able to modulate ubiquitin signaling capabilities by implementation of a code, on par with the "*histone code*," called of the "*ubiquitin code*." This code allows for a diversity of functions beyond merely targeting proteins for destruction, enabling change in the protein's cellular behavior and localization via a combination of mono-, di-, and polymeric additions of ubiquitin moieties to lysine residues (Komander & Rape, 2012); however, intriguingly, emerging evidence has expanded the role of ubiquitination to non-lysine residues (McClellan *et al.,* 2019). Importantly, the E3 ubiquitin ligases are responsible for target specificity and dwarf the number of E1 and E2 ligases. In humans it is estimated that there are over 600 E3 ligases, compared to approximately 42 E2 ligases (Deshaies & Joazeiro, 2009; Morreale & Walden, 2016). Due to the diversity of E3 ligases, much effort is put into studying them. And it turns out that, arguably, the most well documented model for the proteasome-ubiquitination system involves centers around the APC/C E3 ubiquitin ligase.

The APC/C has been associated to numerous cellular processes outside of triggering the metaphase-to-anaphase transition. This is due to the APC/C associating with different activators: In M phase, its primary activator is CDC20, while in interphase it is CDH1 (Wäsch *et al.,* 2010). The APC/C was found to localize to centrosomes (Tugendreich *et al.,* 1995), and later found to play a role in ciliary polarity in the *Xenopus* epidermis (Ganner *et al.,* 2009). Additionally, the APC/C has been implicated in regulating axonal growth via its coactivator CDH1 in post-mitotic neurons, in addition to maintenance of asymmetrical distribution of proteins during neuronal division (Gieffers *et al.,* 1999; Konishi *et al.,* 2004; Slack *et al.,* 2007). In this context, it is likely serving a neuroprotective function by preventing ectopic cell death (Almeida, 2012; Veas-Pérez de Tudela *et al.,* 2015).

Interestingly, early studies from budding yeast cell division provided clues connecting cell cycle progression, proteolysis, and vesicle trafficking When components of what we now know to be the APC/C are mutated they result in cytokinesis defects (Hirano *et al.,* 1986). Cells were able to segregate their sister chromatids; however, the daughter cells failed to complete cytokinesis (Yamano *et al,* 1996). This observation is termed the *"Cut"* and is a classic phenotype indicative of cytokinesis failure. Additional studies from the 1990s from fission yeast defined additional cell cycle regulators with this hallmark feature of cytokinesis failure (Yanagida, 1998). The involvement of the APC/C in regulating cytokinesis is no longer controversial (Lindon, 2008), though the precise mechanisms remain an active area of research.

Work from our group has implicated a more direct role for the APC/C in vesicle trafficking that occurs during meiosis I of the worm *C. elegans* oocyte. While it is uncertain what the exact role of the APC/C is in this context, inactivation of the APC/C by RNAi appears to result in an abnormal distribution of vesicles at the cell cortex (Bembenek *et al.,* 2007). A recent *in silico*

study identified multiple putative APC/C substrates, which employed a successful proof of concept experiment (Franks *et al.,* 2020). These substrates reflect diverse processes with open areas of questions concerning how all these events are regulated in space and time with cell cycle progressions. Buttressing a link of the APC/C to vesicle trafficking, one of these proteins, the RhoGap, ARHGAP13, has been implicated in cytokinesis, in addition to chromosome segregation, in human T lymphocyte cell culture (David *et al.,* 2014).

These recent observations are intriguing in part because little is known how many of these processes are spatially and temporally connected to one another. The evidence that the SAC-APC/C pathway, canonically responsible for making sure chromosomes are properly segregated at the right place and at the right time, also have vesicle trafficking defects. Cell division is thus a potentially interesting to model chromosome segregation and vesicle trafficking temporally, given that genomes need to be segregated and membrane needs to be delivered to the ingressing cleavage furrow to successfully partition the mother cell into two daughter cells. Failure to coordinate these events can prove detrimental to cell function and viability. For instance, in mammalian oocytes, precocious initiation of cell cycle progression events during M phase can result in chromosome segregation defects and global issues with egg activation (Levasseur *et al.,* 2019; Thomas *et al.,* 2021). On the other end of the spectrum, when cell progression is slowed, it has been reported in human tissue culture that prolonged M phase also results in chromosome segregation defects (Shindo *et al.,* 2021).

Emerging research has implicated the APC/C as being functional outside of M phase. In interphase, it is generally regarded as inactive; however, APC/C-CDH1 does exhibit activity towards S phase and mitotic Cyclins, and absent its CDH1 coactivator DNA replication occurs precociously (Pines, 2011). In the cases where APC/C is activated, it can auto-ubiquitylate its

coactivator CDH1 to target it for degradation and cell cycle re-entry; how this would be achieved in this context is currently shrouded in mystery given that the APC/C is inhibited by Emi and how CDC20 could become associated with the APC/C is equally as uncertain as Emi is known to inhibit APC/C-CDC20 association at least *in vitro* (Pines, 2011; Sivakumar & Gorbsky, 2015). From a direct, spatiotemporal standpoint, little work has been done to directly monitor APC/C localization *in vivo* using live cell imaging. The APC/C can anchor to the centrosome toward the end of mitosis (Ban *et al.,* 2007), but the mechanism and high temporally resolved insight is lacking. Furthermore, the APC/C has been shown to localize to chromosomes via interactions with the Ska complex, but the exact molecular details of this movement are unknown (Sivakumar *et al.,* 2014). Directly monitoring the APC/C in real time could provide a lot of insight into how its cellular roles are performed. Taken together, could issues associated with timing of cell cycle progression events like chromosome segregation and membrane trafficking, considering the interesting roles of the SAC-APC/C pathway in both chromosome segregation be used vesicle trafficking, provide one model to understand how cell cycle progression events are coordinating in space and time? Could the two phenomena of the vesicle trafficking required for cytokinesis and chromosome segregation be coupled by a shared regulatory network?

Cell Cycle Progression via SAC-APC/C

Ultimately, the SAC-APC/C pathway is known to work together to properly activate a key protein during an important stage of a cell's life: Activation of the protease Separase to promote chromosome segregation in anaphase. Classic work done in yeast during mid-1990s and early-2000s, established the function and regulation of Separase during chromosome segregation. Recent technological innovations and studies of many cellular processes have implicated

Separase in a wide variety of functions. This implicates the SAC-APC/C pathway beyond its canonical function during the metaphase-to-anaphase transition.

Separase & Securin: Background & Emerging Roles

The understanding of the primary function of Separase is well established and known for its role in liberating chromosome segregation in anaphase by activation of its enzymatic, proteolytic activity. The general regulatory pathway controlling Separase protease activity is well established from classic *in vitro* and *in vivo* work from multiple models. Originally characterized in yeast as a protein required for proper cell cycles (Baum *et al.*, 1988), Separase was soon thereafter found implicated in and necessary for chromosome segregation during anaphase of mitosis (McGrew *et al.,* 1992; Funabiki *et al.,* 1996). The role of Separase at chromosomes during meiotic anaphase I and anaphase II is conserved with mitotic anaphase, as previously mentioned. Proper activation of Separase involves the proteolysis of the Kleisin subunit the Cohesin complex.

Another way to put this canonical role of Separase following satisfaction of the M-phase checkpoint signaling is that it destroys Cohesin. It is important to emphasize that there are two pathways for Cohesin removal during M-phase, and this was first demonstrated in human mitotic cells (Waizenegger *et al.,* 2000). The first pathway is the non-proteolytic, prophase pathway, which function in early M-phase, prior to anaphase onset. The non-proteolytic pathway is itself important for the maturation of the chromosome as the cell cycle progresses towards anaphase, specifically in removing bulk Cohesin from the chromosome arms (Kueng *et al.,* 2006).

The second pathway of Cohesin removal is the proteolytic pathway, which relies on the activation of the protease activity of Separase which cleaves centromeric Cohesin (Uhlmann *et*

al., 1999; Uhlmann *et al.,* 2000). This second pathway is what defines the canonical role of Separase during anaphase. Failure to properly regulate the protease activity of Separase can be catastrophic if anaphase initiates prematurely. It turns out that Separase protease activity is tightly regulated. And in order to understand how this is achieved, it is important to understand the structure of this protein. Separase is an enzyme related to the CD clan of cysteine proteases, which cleave the Kleisin subunit found in proteins such as REC8, SCC1 and RAD21 (Uhlmann, *et al.,* 2000). With the exception of *Drosophila melanogaster* in which Separase is encoded by two separate genes (Jäger *et al.,* 2001). There are three key structural features of Separase. The first feature is in the N-terminal region. In this region there are multiple ARM repeats which is important for protein-protein interactions, including with Securin. The sequence of the N-terminus is divergent among members of metazoa; however, the N-terminals folding pattern is conserved. The second and third features are in the C-terminus, and these are the active protease domain and the pseudo protease domain (Viadiu *et al.,* 2005). (Melesse *et al.,* 2018). The inactive proteae domains helps stabilize the active protease domain and the active protease domain is what is responsible for enzymatically cleaving the substrates of Separase (Lin *et a.,* 2016), Separase has been found to cleave Cohesin for chromosome segregation in many established model systems including yeast, plants, worms, flies, and humans (Luo & Tong, 2021).

The first documentation on the structure of Separase was conducted via NMR, and while the resolution was low, a general idea was painted about how Securin binds along the length of Separase, where it serves a dual purpose in protein folding and pseudosubstrate inhibition (Viadiu *et al.,* 2005). Fortunately, we now have excellent, near atomically resolved crystal structures of Separase in (Bachmann *et al.,* 2016), along with the Separase-Securin complex

spanning yeast, worms, and humans (Lin *et al.,* 2016; Boland *et al.,* 2017, Fig. 3; Tong *et al.,* 2017; Yu *et al.,* 2021). However, we are still limited on the knowledge of the entire structure of Securin across phyla owing to the difficult nature of resolving intrinsically disordered regions. For instance, the C-terminal region of metazoan and worm and human Securin is unknown due to it not achieving a relatively ordered structure by binding to Separase, or even another unknown factor (Boland *et al.,* 2017; Tong *et al.,* 2017). Despite this limitation, we now possess the ability to theoretically determine the effects of protein-protein-interactions on structural change of Separase that makes it more conductive to interact with one partner rather than another.

To avoid premature activity of Separase protease activity, it must be enzymatically restrained in this context. This control is directly achieved via an association with a class of proteins called Securins, which have been found in all Eukaryotic models to date (Funabiki *et al.,* 1996; Kitagawa *et al.,* 2002; Cohen-Fix *et al.,* 1996; Zou *et al.,* 1999; Leismann *et al.,* 2006; Kromer *et al.,* 2019; Yu *et al.,* 2021). Securins are intrinsically disordered proteins that serve as pseudosubstrate inhibitors of Separase (Sánchez-Puig *et al.,* 2005; Csismok *et al.,* 2008). As the *separase* transcript is translated, Securin co-translationally binds to the emerging Separase protein This association fulfills a second role of Securin as the chaperone of Separase. Waizenegger *et al.,* 2002; Nagao & Yanagida, 2006). In some systems like budding yeast and mammals, Securin is dispensable, though there is an increase in chromosome segregation defects and compromised genomic integrity (Jallepalli *et al.,* 2001; Mei *et al.,* 2001; Yamamoto *et al.,* 2006). This suggests complex regulation of Separase outside of Securin. The role of phosphoregulation was implicated in the regulation of Separase soon after discovery of its

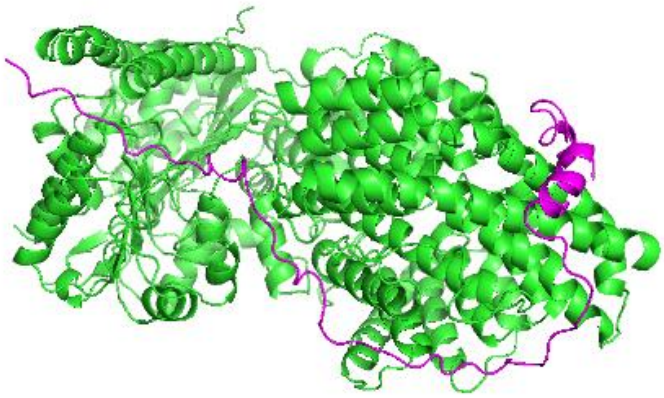


Figure 3. Structure of Separase and its inhibitor chaperone Securin. Crystal structure of *C. elegans* Separase (green) bound to Securin (magenta). Separase orientation N to C-terminus (left to right). Securin orientation C to N- terminus (left to right). Image modified from Boland *et al.* (2017).

regulation by Securin during chromosome segregation (Stemmann *et al.,* 2001; Huang *et al.,* 2005). Indeed, it was found that Cyclin-B can also bind and inhibit Separase (Herbert *et al.,* 2003; Chang *et al.,* 2003; Gorr *et al.,* 2005; Hellmuth *et al.,* 2015). Interestingly, it was found that while CDK1-Cyclin B can bind and inhibit Separase, Separase serves a role in negatively regulating CDK1 in absence of Securin (Gorr *et al.,* 2006). Indeed it was found that Separase functions as an additial layer of CDK- regulation by serving as an inhibitor, independent of its proteolytic activity (Stemman *et al.,* 2006; Shindo *et al.,* 2012).

Separase is also known to engage in autocleavage of itself in anaphase and this is kept in check by by protein phosphatase PP2A (Zou *et al.,* 2002; Holland *et al.,* 2007). Together, this suggests that while the proteolytic function of Separase for chromosome segregaton is well established, the regulatory control of Separase localizatoin remains elusive and is an open area of research. In addition to how its non-proteolytic function is regulated in this emerging web of regulation. Indeed, work form our lab has documented a conserved N-terminal motif of uncertain function, though it is likely involved in protein-protein interactions (Melesse *et al.,* 2018). Evidence from *C. elegans* suggests that the folding landscape around the motif targeted for cleavage by Separase is important in which (Monen *et al.,* 2015). Strikingly, a novel motif associated with its Cohesin substrate, SCC1 possesses a domain important for robust proteolysis, independent of the cleavage site (Rosen *et al.,* 2019). In light of the emerging roles of Separase during the cell cycle, regulatory and functional insights being gained need to be addressed. Consequenctly, the field of Separase biology has experienced an explosure of unanswered question as to how this protease is regulated in space and time to carry out chromsome segregation, in additon to its non-proteolytic activities (Zhang & Pati, 2017; Kamenz & Hauf; Luo & Tong, 2021).

The non-proteolytic activity of Separase has been most heavily studied in the context of M phase. Two examples of its non-proteolytic function are in polar body extrusion and M phase exit in mouse oocytes and budding yeast, respectively (Sullivan & Uhlmann, 2003; Kudo *et al.*, 2006). Additional proteolytic functions have also been uncovered related to M phase function including, telomere capping (flies), microtubule polymerization (plants), and anaphase spindle stabilization (Cipressa et al., 2016; Moschou et al., 2016). Separase has also been documented to be involved in centriole duplication and disengagement, and Separase was found to have a new substrate, Kendrin (Lee & Rhee, 2012; Matsuo *et al.*, 2012). Separase has also been known to cleave itself which in turn is important for exit from M phase (Zou *et al.*, 2002; Papi *et al.*, 2005). Importantly, regarding this last point of exit from M phase, of which chromosome segregation and vesicle trafficking are especially important, work from our group has implicated Separase in vesicle trafficking required in other meiosis I and first mitotic division in the *C. elegans* early embryo (Bembenek et al., 2007; Bembenek et al., 2010; Richie et al., 2011; Bai & Bembenek, 2017). Intriguingly, Separase has also been implicated in vesicle trafficking in other systems, such as plants and mice, suggesting this key role may be conserved throughout metazoa (Bacac *et al.*, 2011; Moschou *et al.*, 2013). In flies, Separase was identified as important for protein secretion, which is known to be important for cytokinesis (LeBrasseur, 2005; Bard *et al.*, 2006).

Interestingly, like the previous cell cycle regulators discussed, Separase has been documented to be activated during interphase for DNA damage responses, and strikingly even is S phase by controlling replication fork speed (Cucco *et al.*, 2018). The emerging roles of Separase outside of chromosome segregation call into question how it is regulated in these contexts. Whether the

regulators known control its activating during chromosome segregation also control Separase in these new roles remains to be determined.

There is ample opportunity to clarify fundamental aspects of cell biology, especially in the emergence of SAC-APC/C components having roles throughout the cell cycle. Whether these regulate Separase during its functions throughout the cell cycle is an open question. From our work in worms, we have documented an additional role for the protease activity of Separase during vesicle trafficking that takes place in M phase. In anaphase of meiosis I, Separase is responsible for promoting the exocytosis of vesicles called cortical granules, and it also localizes to the bases of the polar body which might be important for a function in meiotic cytokinesis (Bembenek *et al.*, 2007; Bai & Bembenek, 2017). During the first mitotic division, Separase is also required for cytokinesis, specifically by incorporating RAB-11-positive vesicles into the cleavage furrow and site of abscission, the midbody (Bembenek *et al.*, 2010; Bai & Bembenek, 2017). How Separase is regulated during vesicle trafficking events in M phase is unknown, and whether the SAC-APC/C control Separase during vesicle trafficking remains to be determined.

Chapter 2

Open Questions, A Model System, and Recent Findings

An Open Question in the Field of Cell Division: The Master Regulator of M-phase Entry & Exit

In the beginning the question was posed: How does a cell coordinate its multiple events in space and time as it progresses through the cell cycle? The kinase CDK1 together with its coactivator, Cyclin B, is regarded as the master regulator of cell cycle progression in Eukaryotes. Classically defined, CDK1-Cyclin B activation drives entrance into M phase while exit depends on inactivation so that the cycle can start anew (Skoufias *et al.,* 2007; Alberts *et al.,* 2014). The metaphase-to-anaphase transition marks this transition, and thus the APC/C is ultimately responsible for governing CDK1-Cyclin B activity here. Current evidence now suggests that a mere on/off switch in CDK1 activity dictates this transition is likely a simplistic model. Kishimoto (2015) pointed out early evidence from the 1980s calling into question whether CDK1-Cyclin B activity was necessary for M phase. In the early 2000s, it was found that CDK1 activity alone was not sufficient to maintain M phase in human and yeast culture, indicating an additional layer of phosphoregulation (Stegmeier & Amon, 2004; Skoufias *et al.,* 2017). In human cells there are multiple CDKs but CDK1 was shown to be the sole CDK required for the cell cycle, suggesting CDK1 has functional roles outside of M phase (Santamaría *et al.,* 2007; Satyanarayana & Kaldis, 2009), Further studies demonstrated that CDK1-Cyclin B activity does not merely go away during interphase (Lindqvist *et al.,* 2007; Potapova *et al.,* 2009; Gavet & Pines, 2010). Indeed, CDK1/Cyclin B activity is important throughout the cell cycle. For instance, CDK1-Cyclin B couples translation to proliferation and pronuclear union in miotic M phase and the first mitotic interphase following fertilization, respectively (Tachibana *et al.,* 2008; Shuda *et al.,* 2015). These observations call into question the threshold of CDK1-Cyclin B activity required for different events during the cell cycle, including cell cycle progression

(Kishimoto, 2015; Crncec & Hochegger, 2019). Interestingly in human cancer cells, in contrast to a previous report from a non-cancerous cell line in which CDK1 absence causes G$_2$/M arrest (Itzhaki *et al.,* 1997), CDK1 depletion was capable of being rescued by increased expression of CDK2 (Lau *et al.*, 2021). This novel finding is reminiscent of those found from previous studies implicating the importance of means to govern the phosphorylation state of Cdk1 substrates (Stegmeier & Amon, 2004; Skoufias *et al.*, 2017). Indeed, an open question about cell cycle progression in the context how much CDK1 is necessary and sufficient, is the robustness of the canonical understanding of M-phase entry, exit, and general cell cycle progression (Kishimoto, 2015). Nevertheless, while assigning the exact role for CDK1-Cyclin B, and other CDK1 in cell cycle progression is difficult and have been attributed to promiscuity of CDKs towards substrates, there is no question that they are collectively master regulators of cell cycle progression (Malumbres, 2014). Recently Kishimoto (2015) suggested that rather than CDK1/Cyclin B as the sole regulator mitotic entry and exit, to see the signaling responsible for these cell cycle changes as part of a larger signaling hub. To this latter point, the establishment of a stable M phase state, or a stable state of any phase of the cell cycle remains an open area of inquiry, especially as the minimum necessary level of CDK1-Cyclin B for cell cycle progression (Crncec & Hochegger, 2019). How the cell cycle transitions through each phase of the cell cycle is probably in part dependent on cellular context of a more complex kinase-phosphatase network, of which CDK1-Cyclin B is a part of, and it could be insinuated what Kishimoto (2015) meant that this would then depend upon different cellular processes within said stage. Indeed, the maturation promoting factor (MFP), which has been historically regarded as CDK1-Cyclin B, now appears to be a more complex combination of factors, of which the Greatwall kinase and CDK1-Cyclin B are constituents of (Hara *et al.,* 2012). The nature of this network governing

entry and exit of M phase, and progression through the cell cycle is not as simple as the activation and deactivation of CDK1-Cyclin B (Kishimoto, 2015; Crncec & Hochegger, 2019).

The Female Oocyte: A Historic Model of Cell Division

A powerful model system that that has been responsible for discoveries of key cell cycle regulators, like the Cyclins (Evans *et al.*, 1983), is the metazoan female oocyte. The oocyte is the end result of female gamete formation during meiotic M phase, during which a series of highly asymmetric divisions generate a single, cytoplasm rich cell containing many of the necessary components to ensure a robust early development (Larose *et al.*, 2019). The oocyte is colloquially known as the female egg. The eggs in animals are generated early in development and are set aside for later usage as the animal reaches sexual maturity. Importantly, depending on the species, the eggs are arrested in either meiotic prophase I (called the primary arrest, and the oocyte is referred to as the primary oocyte) or metaphase II (called the secondary arrest, and the oocyte is referred to as the secondary oocyte), and in both cases this arrest is maintained by high CDK1-Cyclin B activity, the SAC, and generally inactivation of the APC/C (Madgwick & Jones, 2007; Von Stetina & Orr-Weaver, 2011). Oocytes in some species, like humans can spend decades in the cell cycle and as a consequence to issues like Cohesin fatigue, are prone to chromosome segregation defects when fertilized, thus there is a lot of interest in understanding the control of the cell cycle in these cells as related to human aneuploidy, cancers, and genetic conditions (Nagaoka *et al.*, 2012). The egg is suspended in the cell cycle until a cue from the male gamete, the sperm, signals the oocyte to resume the cell cycle, in what is also known as "*egg activation"* (Fig. 4; Von Stetina & Orr-Weaver, 2011).

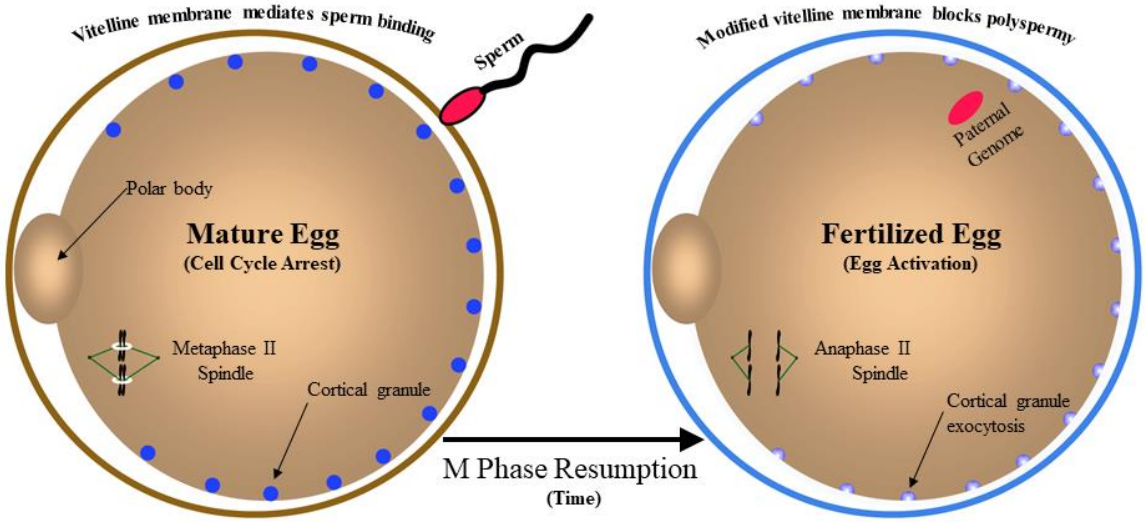


Figure 4. Egg activation in metazoa. In metazoans the female egg is arrested in meiosis I. In the case of mice, as exemplified in this image, the egg is referred to as the secondary oocyte and is arrested in metaphase II. In this egg, the polar body refers to the product of the first meiotic cell division reducing the ploidy of the cell. Specialized vesicles, cortical granules are labeled in blue. The extracellular matrix of the egg, called the vitelline membrane (brown), mediates sperm binding. Following fertilization, the egg is activated resulting in a number of events including the completion of sister chromatid segregation in anaphase and the exocytosis of specialized vesicles called cortical granules. Cortical granule exocytosis delivers material to the extracellular matrix, modifying it to block polyspermy/additional fertilization events. (Image provided by Dr. Josh Bembenek and adapted for this dissertation).

For example, mouse oocytes are suspended in metaphase II. Following fertilization, a wave of calcium signaling is responsible for the events of egg activation which include the completion of anaphase (anaphase II in this case) and the specialized exocytosis of vesicles present throughout metazoa, called cortical granules. Whether there is a direct coupling between the calcium wave and cortical granule exocytosis remains to be determined. Nevertheless, they are necessary for modifying the extracellular matrix surrounding the oocyte (Wessel et al., 2001). Cortical granules are specialized secretory vesicles derived from the Golgi apparatus. They remain near the endoplasmic reticulum (ER), both of which are moved to the cell cortex prior to anaphase I or II, depending on the organism (Sardet *et al.,* 2002). The exocytosis of these vesicles modifies the extracellular matrix so that additional fertilization events are inhibited.

The Regulation of Separase during Vesicular Trafficking in Meiosis: The beginning of a unified model of cell cycle progression?

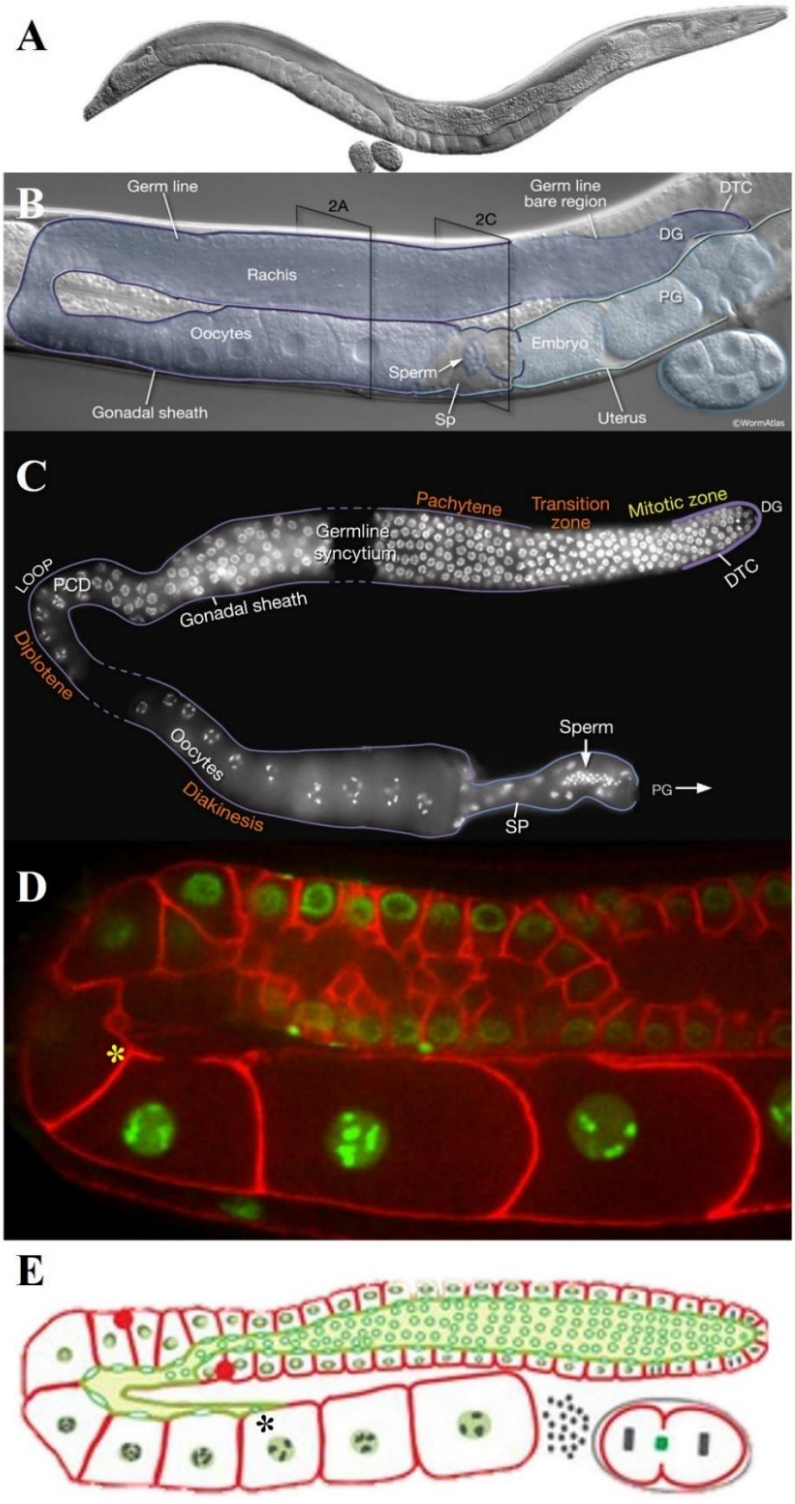
In the mid-2000s, our group has established a novel role for Separase during anaphase. Specifically, we uncovered a role for Separase in specialized vesicle trafficking events that take place in anaphase I during female meiosis in the worm, *Caenorhabditis elegans*. The oocytes of *C. elegans* are arrested in prophase I of meiosis and following signals from the sperm, the cell cycle resumes, and cortical granules are eventually exocytosed during anaphase I (Bembenek *et al.,* 2007). Intriguingly, we observed that Separase localized to cortical granules and is required for their exocytosis during anaphase I (Bembenek *et al.,* 2007). Exocytosis of cortical granules is responsible for eggshell formation. We refer to this extracellular matrix as the eggshell in worms. Separase has been implicated in vesicle trafficking in other systems (Bacac *et al.,* 2011). Further scrutinization of Separase during vesicle trafficking using the *C. elegans* embryo established it as

a regulator of RAB-11-positive vesicles required for the first cytokinesis (Bembenek *et al.* 2010). Interestingly, cortical granules are also labeled by RAB-11. More recently, in *C. elegans*, we found that Separase protease activity is required for the exocytosis of cortical granules during anaphase I, as well as for aspects of trafficking involving other RAB-11 positives vesicles near the final step of cytokinesis, abscission (Bai & Bembenek, 2017).

Collectively, our observations of Separase during specialized trafficking events during anaphase I touches upon an important question brought up at the beginning of this chapter: How is cell cycle progression coordinated in space and time with cellular events, broadly speaking? The events that take place following egg activation of the *C. elegans* oocyte occur rapidly compared to many other systems like mice, such as chromosome segregation, polar body extrusion and the construction of the eggshell, making it an excellent system for studying how processes can be coordinated with one another. How are chromosome segregation, of which the functional regulation of Separase is well established, and specialized vesicle trafficking events that we know take place during anaphase I regulated? The observation that Separase is a regulator of specialized vesicle trafficking suggests a connection between cell cycle progression and membrane trafficking.

Caenorhabditis elegans an established and powerful model used to address fundamental questions related to early development (Fig. 5). This is owed in part to its optical transparency, quick development, and ease of genetic manipulation (Corsi et al., 2015). The gonad is a rapid assembly line of early development, which makes the worm excellent source for gaining insight

Figure 5. *Caenorhabditis elegans* As a Model for Cell Division. **A**) Image of the adult nematode, *Caenorhabditis elegans*. **B)** DIC cross section of the anterior gonad arm of the adult hermaphrodite with important cytological landmarks labeled. The *C. elegans* germline is a long tube generated from a a single mitotic cell in the distal gonad (DG), the distal tip cell (DG), which functions as a bonafid germline stemcell. The germline is a syncytium and the shared cytoplasm is refered to as the Rachis. Cellulararization of the oocyte precursors is accomplished near the bend of germline. At the most proximal end of the gonad sheet the most terminal oocyte is fertilized by sperm in the spermatheca (SP) and enters the uterus, refered to as the proximal gonad (PG), where it is subsequently refered to as the fertilized one-cell meiotic embryo upon entry into the uterus. **C)** Icolated adult worm germline staned with DNA marker DAPI. Mitotic and subsequent meiotic cell cycle progression follow a distal germline to (DG) to proximal germline (PG). The stages of meiotic I prophase are labeled in orange. Programmed cell death (PCD) near the germline indicate at the cell death aids in formation of the single female meoitic gamete (oocyte). **D.** Loop region of the mid-gonad to emphasize shared cytoplasm of the germline. Note the cytoplasmic bridge (yellow asterisk) at the loop, after which the oocyte is now cellularized. Chromosmes are labeled with GFP and plasma membrane is red. **E**. Cartoon of the adult germline: Note the shared cytoplasm (greenish hue) and the numerous cytoplasmic bridges (see black asterisk for reference). Images (**A**-**C**) were adapted from WormAtlas (Lints & Hall, 2009) while (**D**-**E**) were addapted from Lee et al. (2018).


A
B
Germ line
2A
2C
Germ line
bare region
DTC
DG
Rachis
PG
Oocytes
Embryo
Sperm
Gonadal sheath
Sp
Uterus
©WormAtlas
C
Pachytene
Transition
zone
Mitotic zone
DG
Germline
syncytium
LOOP
PCD
DTC
Gonadal sheath
Diplotene
Sperm
Oocytes
PG
Diakinesis
SP
D
E

into fundamental aspects of cell division. In each gonad arm, oocytes are ovulated and subsequently fertilized in the spermatheca once every 23 minutes (McCarter et al., 1999). The first meiotic divisions occur soon thereafter, with the first mitotic division beginning an hour after fertilization. Coupled with the conserved nature of regulators fundamental to the cell cycle, *C. elegans* offers itself as an in utero window into early events of development inaccessible or technically arduous to assess in other established models of development. Significantly, *C. elegans* is the currently the only established model organism whereby we can monitor the entirety of meiosis through early embryonic development *in utero*. Lastly, to my knowledge, *C. elegans* is currently the only model system in which we can address the spatiotemporal regulation of Separase in real time at high resolution. This offers, for the first time, a completely novel approach to understanding Separase biology without having to utilize indirect indicators of localization like biosensors, so we can now assess how activity and function are coupled spatiotemporally, at high resolution.

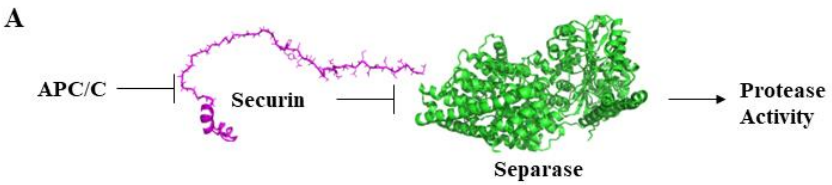
Central Hypothesis (Fig. 6)

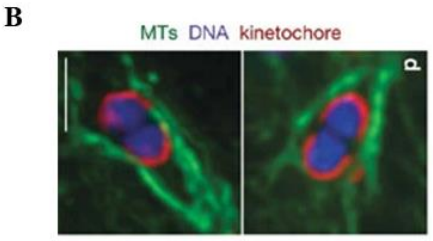
The connection of Separase to two important events of egg activation, chromosome segregation and vesicle trafficking, suggests these are coordinated in space and time. Importantly, this implies that the regulation of Separase during meiosis I remains elusive on two fronts:

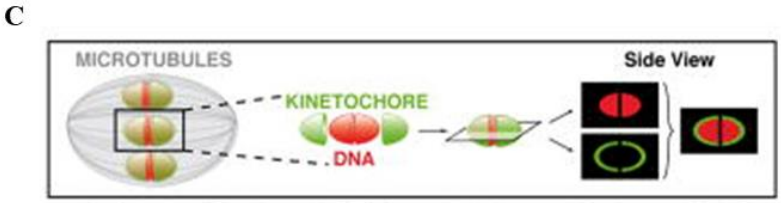
1) How is Separase protease activity regulated during the exocytosis of cortical granules that takes place during anaphase I?

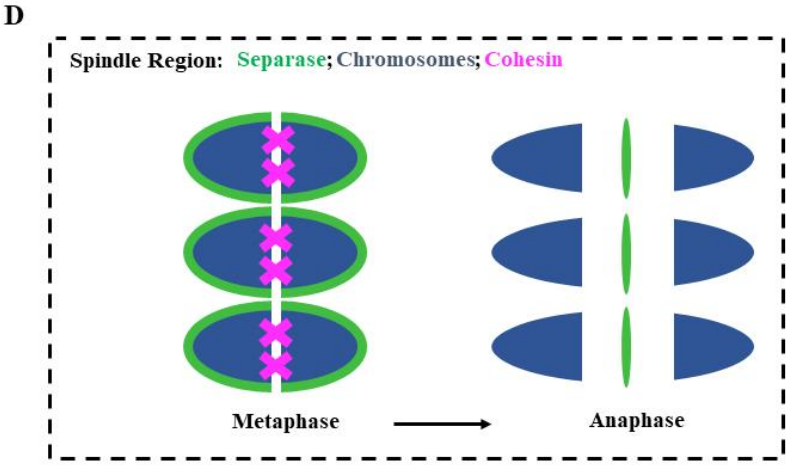
2) How is Separase spatially regulated: How does Separase localize from KFs in metaphase to vesicles in anaphase I?

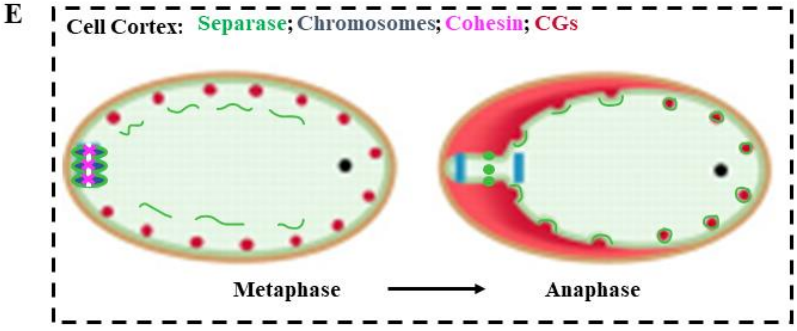
Figure 6. Central hypothesis: Cell cycle regulators known to control Separase protease activity also control its localization to and subsequent action at substrates during anaphase I. A) Subset of regulators of the metaphase-to-anaphase transition controlling Separase protease activity. **B**) Structure of *C. elegans* metaphase I chromosomes. Note the cup-shape of the kinetochore (red) surrounding the homologous chromosomes (DAPI). Area between the homologous pair is referred to as the midbivalent (MT = microtubules, green). **C**) Cartoon of the metaphase I spindle anatomy as viewed by confocal microscopy. The cross section represents the central plane of the chromosomes in the spindle. **D**-**E**) Cartoon depicting localization of Separase (green) during the metaphase-to-anaphase transition at the spindle **(D)** and cortex (E). Note: Separase localizes from kinetochore-based structures on the chromosomes and the cortex in metaphase, close to its substrates. In anaphase Separase is relocalized to sites of action at the spindle and at the cortex where it is needed to promote exocytosis of cortical granules (GCs). Note: Further, **D**) Cohesin (pink) is destroyed in anaphase at the spindle, **B)** where nothing is known about is substrate at the cortex. Images in (**A)** were adapted from Boland *et al.,* 2017; Images in (**B**) were modified from Wignal & Villeneuve, 2009; Images in **(C**) were adapted from Dumont *et al.,* 2010; Image (**E**) was modified form an image provided by Dr. Josh Bembenek.


A
APC/C
Securin
Protease
Activity
Separase


B
MTs DNA kinetochore


C
MICROTUBULES
Side View
KINETOCHORE
DNA


D
Spindle Region: Separase; Chromosomes; Cohesin
Metaphase
Anaphase


E
Cell Cortex: Separase; Chromosomes; Cohesin; CGs
Metaphase
Anaphase

To address these questions, my central hypothesis is that the canonical regulators of Separase protease activity required for chromosome segregation (Fig. 2), also control its proteolytic function during vesicle trafficking as well as is localization to sites of function at the spindle (Cohesin) and the cortex (cortical granules, unknown substrate; *see discussion*).

Chapter 3

Results

Securin is Degraded before Separase Localizes to Vesicles

Previously, in the worm *C. elegans*, we have documented that Separase localizes to specialized vesicles called cortical granules during meiotic during anaphase I (Bembenek *et al.*, 2007), and its protease activity is required for their exocytosis after anaphase I onset (Bembenek *et al.*, 2010; Mitchell *et al.*, 2014, Bai & Bembenek, 2017). The protease activity of Separase is required to cleave a subunit of Cohesin to promote chromosome segregation in anaphase, and this is canonically achieved by degradation of Securin, its inhibitory chaperone. Securin is also required for Separase localization to the spindle in yeast. Whether Securin regulates Separase localization from kinetochore cups (KCs) to the midbivalent region (MR) at the spindle and from kinetochore filaments (KFs) to cortical granules (CGs) at the cortex during anaphase is not known. The MR is documented to contain the substrate of Separase, Cohesin, in worms (Severson & Meyer, 2014). I suspect that Separase localizing to CGs may be necessary to promote their exocytosis (Bai & Bembenek, 2017).

Biochemical studies have established that Separase becomes proteolytically active when Securin is targeted for degraded by the anaphase promoting complex/cyclosome (APC/C) in anaphase (Kamenz & Hauf, 2017). Given that Securin inhibits Separase protease activity prior to anaphase is well established, I suspected that its inhibitory role extends to blocking Separase localization to its documented anaphase I localizations: MR and CGs (Bembenek *et al.*, 2007; Muscat *et al.*, 2015). Separase and Securin are known to form a complex both *in vivo* and *in vitro* studies, which enable Securin to conduct its canonical dual function as the main pseudosubstrate inhibitory chaperone of Separase (Kamenz & Hauf, 2017; Boland *et al.*, 2017). A previous *in utero* study on Securin during worm meiosis did not indicate whether Securin localizes to KFs in

meiosis I (Wang *et al.,* 2013), as might be expected if Securin and Separase form a complex in meiosis I. An alternative possibility is that Separase forms a complex with one of three Cyclin B orthologs, which is also known to complex with and inhibit the protease activity of Separase (Kamenz & Hauf, 2017; Boland *et al.,* 2017). To address these scenarios, and since Securin is arguably the best described regulatory chaperone of Separase, I decided to revisit Securin localization dynamics during meiosis I, as I suspected Securin could localize to KFs, as we previously reported for Separase (Bembenek *et al.,* 2007; Bai & Bembenek, 2017). I hypothesize that Securin degradation is required to promote Separase localization form KCs and KFs to the MR and CGs in anaphase I. To address this hypothesis, I conducted *in utero* live cell imaging of the of the -1 oocyte expressing the chromosome marker H2B::mCherry with Separase::GFP or GFP::Securin beginning from the prophase I arrest through anaphase I onset (Fig. 7).

Following egg activation, I noticed that Securin accumulates on structures resembling KFs in the cytoplasm, like Separase, suggesting aspects of Securin localization dynamics was previously overlooked (Fig. 7H,B; Wang *et al.,* 2013). To verify that Separase and Securin colocalize to these structures, I used CRISPR/Cas9 to insert fluorophores in frame of the genetic loci of both Separase and Securin, following established protocol (Paix *et al.,* 2015). I then co-expressed endogenously tagged Securin::GFP together with endogenously tagged Separase::mScarlet and monitored their dynamics during meiosis I (Fig. 8). I observed that both proteins colocalize to the same cytoplasmic structures following egg activation (Fig. 8). I conclude that Securin colocalizes to KFs with Separase following meiotic resumption.

Figure 7. Separase and Securin localization during meiosis I in the *C. elegans* oocyte. Representative images of meiosis in embryos expressing Separase::GFP (green, **A**-**F**) and GFP::Securin (green, **G**-**L**) together with chromosome marker H2B::mCherry (magenta). During prophase I arrest, in the -1 oocyte, Separase is confined to the cytoplasm and excluded from the nucleus (**A**), whereas Securin is present in both locations (**G**). In what we suspect corresponds to egg activation, Separase (**B**) and Securin (**H**) begin accumulating on linear elements (orange arrow) and cortical filaments (white arrow). Before ovulation and around NEBD, Separase (**C**) and Securin (**I**) display identical localization patterns, with both populating the nucleus with distinct kinetochore signal detected (white asterisk). Localization patterns remain largely identical through prometaphase I, with the linear elements (orange arrow) now closely associated with the spindle near the kinetochores (white asterisk) and an observed reduction in the number of cortical filaments (white arrow). Cytoplasmic Securin signal decreases as anaphase I approaches (Fig. 7B). Coincident with chromosome segregation in early anaphase I, Separase and Securin begin to show overlapping and distinct localization patterns at both the spindle and the cortical regions (**E,K**). In the spindle region, Separase and Securin both localize to the midbivalent region between homologous chromosomes (yellow arrowhead) and linear element signal is absent. At the cortex, Separase signal begins to localize to cortical granules in the anterior of the cell near the spindle (blue arrowhead) but is still present on cortical filaments posterior to the vesicles (white arrow). In contrast, Securin is not detected on vesicles, but filament signal is detected in the posterior region like Separase (white arrow). During mid-anaphase I (**F,L**), before cortical granules are exoctyosed, Separase is largely localized to the midbivalent region between the homologs while Securin is absent (yellow arrowhead). Separase is localized to cortical granules through the cortex (blue arrowheads), while Securin signal continues to be diminished. Distinct Separase and Securin signal at kinetochore cups, linear elements and cortical filaments is not detected. Images were acquired from both *in utero* and *ex utero* specimens and are variably shown as single plane or z-projections of various depths to emphasize localization dynamics.

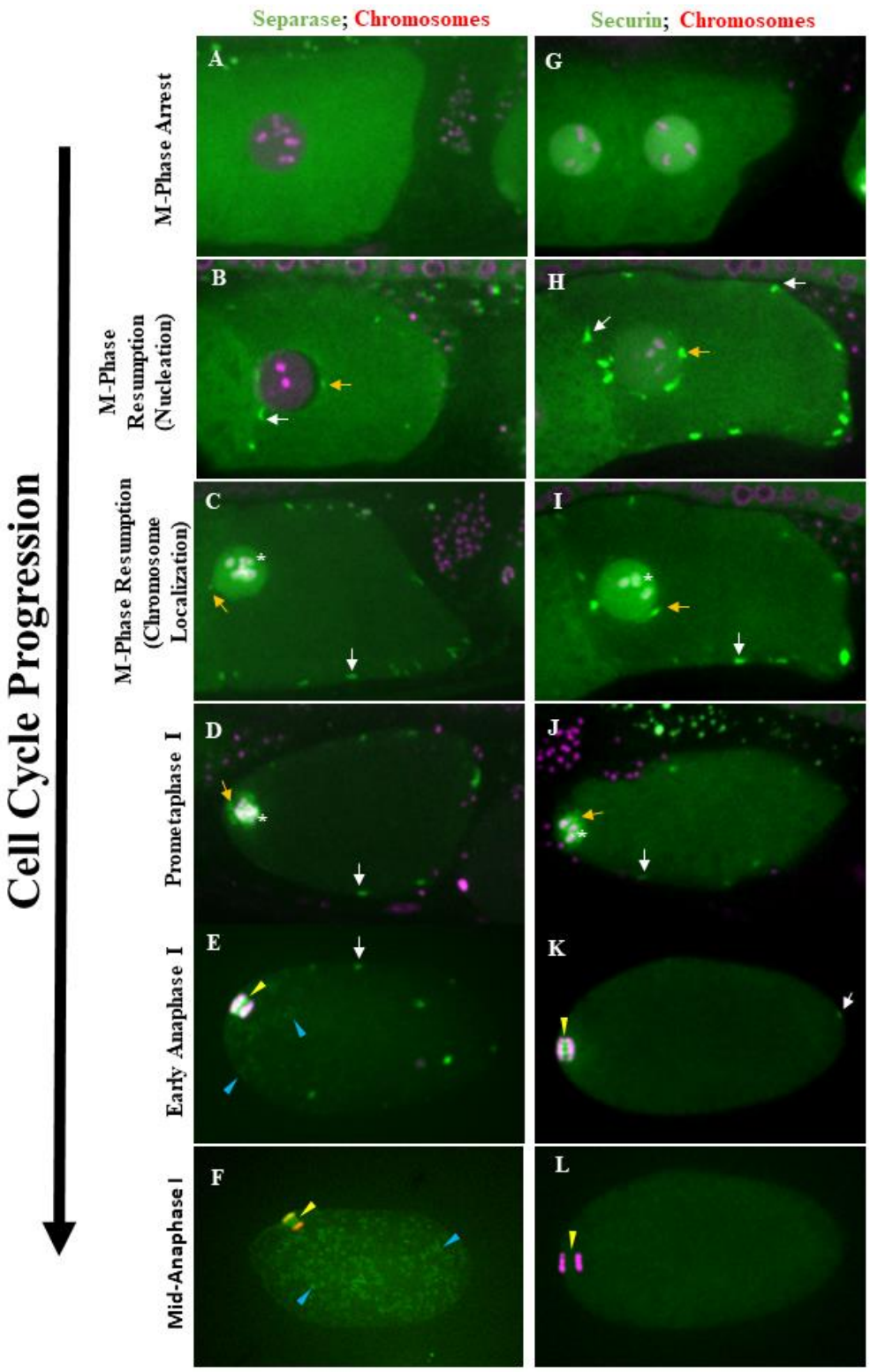
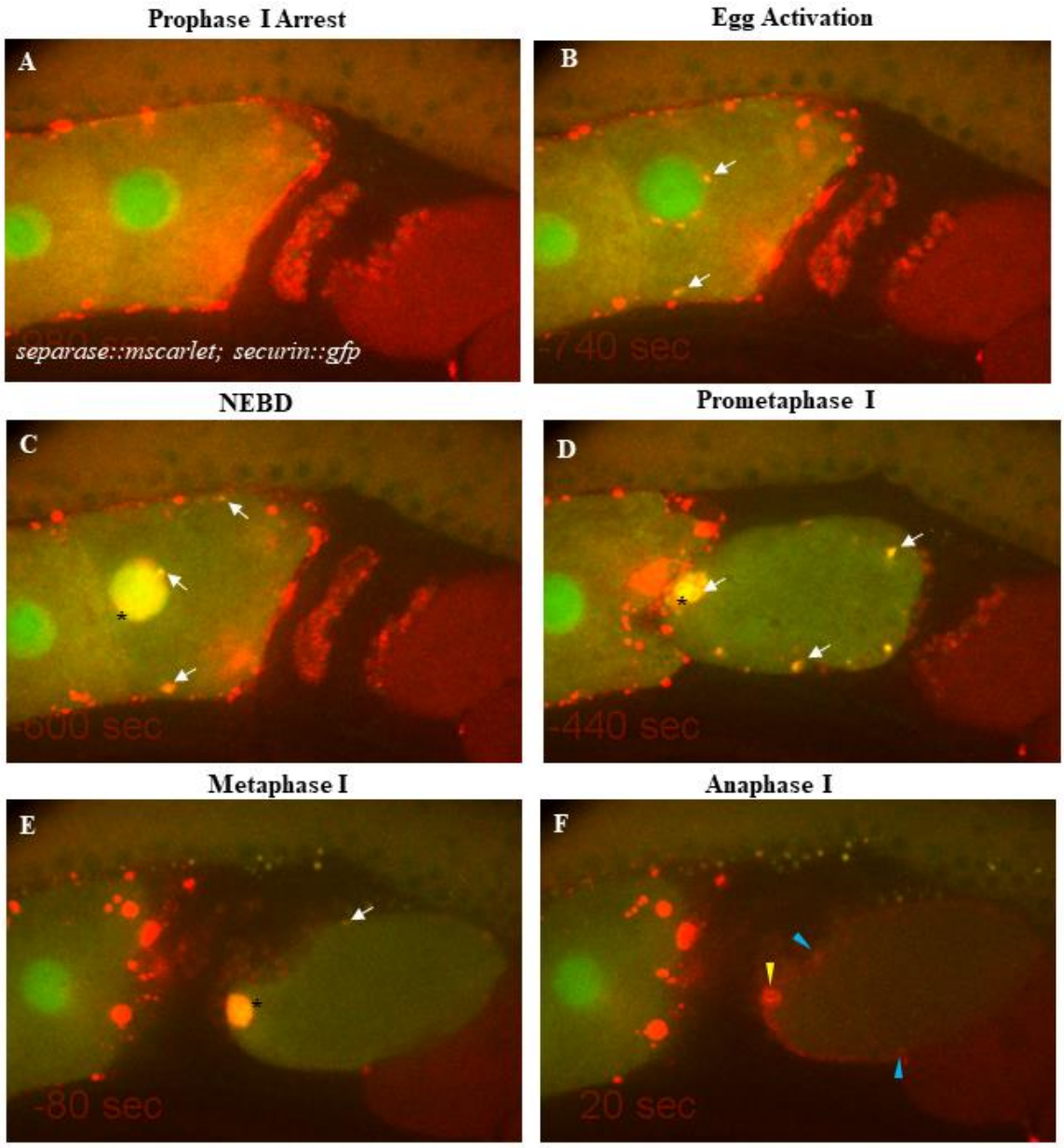

Separase; Chromosomes
Securin; Chromosomes
Cell Cycle Progression
M-Phase Arrest
M-Phase Resumption (Nucleation)
M-Phase Resumption (Chromosome Localization)
Prometaphase I
Early Anaphase I
Mid-Anaphase I
A
B
C
D
E
F
G
H
I
J
K
L

Figure 8. Separase and Securin colocalization dynamics during meiosis I. Representative images from an *in utero* time series of meiosis in embryos expressing endogenously tagged Separase::mScarlet (red) and endogenously tagged Securin::GFP (green). Localization dynamics are identical to Figure 1. During prophase I arrest Securin exists in two populations, one in the cytoplasm and on the nucleus, while Separase is confined to the nucleus (**A**). During early egg activation Separase and Securin appear together on linear elements and cortical filaments (white arrows; **B**). Next, after NEBD has commenced and prior to ovulation, Separase and Securin are both enriched within the soon-to-be deteriorated nucleus and are colocalize to kinetochores (black asterisks), in addition to the linear elements and KFs (white arrow) (**C**). Shortly after ovulation in early prometaphase I, the NEBD has largely completed and Separase and Securin remain colocalized to KFs and KCs (black asterisks; **D**), and this colocalization is largely maintained up until metaphase I when linear elements are not detected as labeled by either protein (**E**). Noted is the appreciable diminishment of Securin signal from the cytoplasm at this time. By mid-anaphase I, Separase is localized to its sites of action at the spindle (yellow arrowhead) and cortical granules (blue arrowheads). Images are z-max projections of 6µm (4 steps of 2µm) acquired through the spindle-plane.


Prophase I Arrest
A
separase::mscarlet; securin::gfp
Egg Activation
B
40 sec
NEBD
C
-600 sec
Prometaphase I
D
-440 sec
Metaphase I
E
-80 sec
Anaphase I
F
20 sec

Prior to ovulation, around the time of nuclear envelope breakdown (NEBD), Separase and Securin display identical localization patterns at the KCs and KFs (Fig. 7C,I; Fig. 8). As the cell cycle progresses towards anaphase, I noticed that the number of cortical filaments, to which Separase and Securin colocalize, decrease and this appears to be associated with degradation of cytoplasmic Securin, which has been previously described in worm and mouse oocytes (Fig. 7D,J; Fig. 8; Wang *et al.,* 2013; Thomas *et al.,* 2021). At anaphase I onset, which can be defined classical by poleward movement of homologous chromosomes or by Separase localization to the MR (Bembenek *et al.,* 2007; Muscat *et al.,* 2015), I noticed novel, previously undescribed localization dynamics of Separase and Securin (Bembenek *et al.,* 2007; Wang *et al.,* 2013; Bai & Bembenek, 2017). In early anaphase I, in the anterior of the cell, Separase localizes the to the MR and CGs, while it is retained on KFs and absent from CGs in the posterior of the cell. At this point in time, microtubules, kinetochore and spindle assembly checkpoint proteins are reorganized to form the anaphase I spindle, with key regulators of cytokinesis, such as the chromosome passenger complex (CPC), briefly localizing to the MR (Dumont *et al,* 2010). Unexpectedly and in contrast to what I initially predicted at anaphase I onset, Securin signal is detected at the midbivalent and appears absent from cortical granules (Fig. 7L; Fig. 8F). I am not aware of any studies that document the relationship between the tagging of Securin with ubiquitin by the APC/C and subsequently how long it takes for GFP-associated Securin signal to be degraded by the 26S proteosome. Furthermore, no studies to date have documented the relationship between the removal of Securin from Separase with respect to its protease activity. It is possible that the GFP::Securin signal I observe at the spindle could reflect multiple populations of Securin at different steps in its removal from Separase. Our unique system for monitoring Separase and Securin dynamics, coupled with known biosensors of Separase capable

of detecting its activity at the spindle following anaphase onset could be used to address these fascinating questions (Monen *et al.,* 2015)., which are beyond the scope of my studies. Nevertheless, by the time Separase has largely localized to the MR and CGs and is no longer present on obvious KC or KF in anaphase , Securin signal has been noticeably reduced compared to its levels in prophase I arrest (Fig. 7A,L; Fig. 8A,F).

These data suggest that that Securin may differentially regulate Separase localization during anaphase I at the spindle and at the cortex: At the spindle, in contrast to my prediction, Securin degradation does not appear to be required for the transfer of Separase from the KC to MR; in contrast, at the cortex, and consistent with the hypothesis that Securin has an inhibitory role on Separase localization, Securin degradation appears to be required for Separase to localize to CGs (Fig. 7F,L; Fig. 8F).

Based on my careful scrutiny and novel insight into Separase and Securin localization dynamics during meiosis I, I will classify anaphase into three distinct categories: 1) Early anaphase I: In the anterior of the cell in the spindle region, Separase and Securin colocalization to the MR and their localization at KCs is not clearly detected, and Separase localizes to CGs and is absent from the KFs. In the posterior of the cell, Separase and Securin remain localized to KFs and are absent from CGs.; 2) Mid-anaphase I: Securin is degraded largely from the cell and is no longer present at the MR and KFs, while Separase remains present at the MR and is no longer on KFs, having been transferred to CGs throughout the cortex but before their exocytosis; 3) Late anaphase I: The initiation of cortical granule exocytosis (CGE), after chromosome segregation has

progressed for some time, near the spindle pole as we have previously reported (Bembenek *et al.*, 2007; Bai *et al.*, 2017).

Activation of APC/C Activity is Required for Separase Localization

The regulation network governing Separase protease activity for chromosome segregation during anaphase is well established and is conserved throughout Eukarya for both mitotic and meiotic M phase, the M phase checkpoint (Fig. 2; Lara-Gonzalez *et al.*, 2021). Whether this pathway controls the protease activity of Separase required for CGE and its localization in meiosis I remains unexplored (Bembenek *et al.*, 2007; Bai & Bembenek, 2017). Our previous work indicates the protease activity of Separase is required for RAB-11 positive vesicle trafficking, which includes cortical granules (Bai & Bembenek, 2017). Our previous work, and my observations suggest a role for the SAC-APC/C pathway through Securin degradation in anaphase as an important for both proper activation of the protease activity of Separase and its localization to vesicles (Bembenek *et al.*, 2007; Bai & Bembenek *et al.*, 2017; Figs. 7,8). There is a dearth of studies regarding the regulation of Separase localization. In yeast, which undergo a closed mitosis where the nuclear envelope does not break down (Sazer *et al.*, 2014), Securin is documented to be important for Separase getting into the nucleus and for associating with the spindle (Kumada *et al.*, 1998; Hornig *et al.*, 2002), though the role of the SAC-APC/C pathway is unexplored in this context. With respect to cell divisions where the nuclear envelope breaks down (open cell division), in organisms such as in *C. elegans*, flies, and vertebrates, there are even fewer stringent studies. In *C. elegans*, the APC/C activity has been shown to be important for Separase to localize to CGs, though in an APC/C-null background, localization of Separase to KCs and KFs was not commented on (Kimura & Kimura, 2012). How Separase localizes to

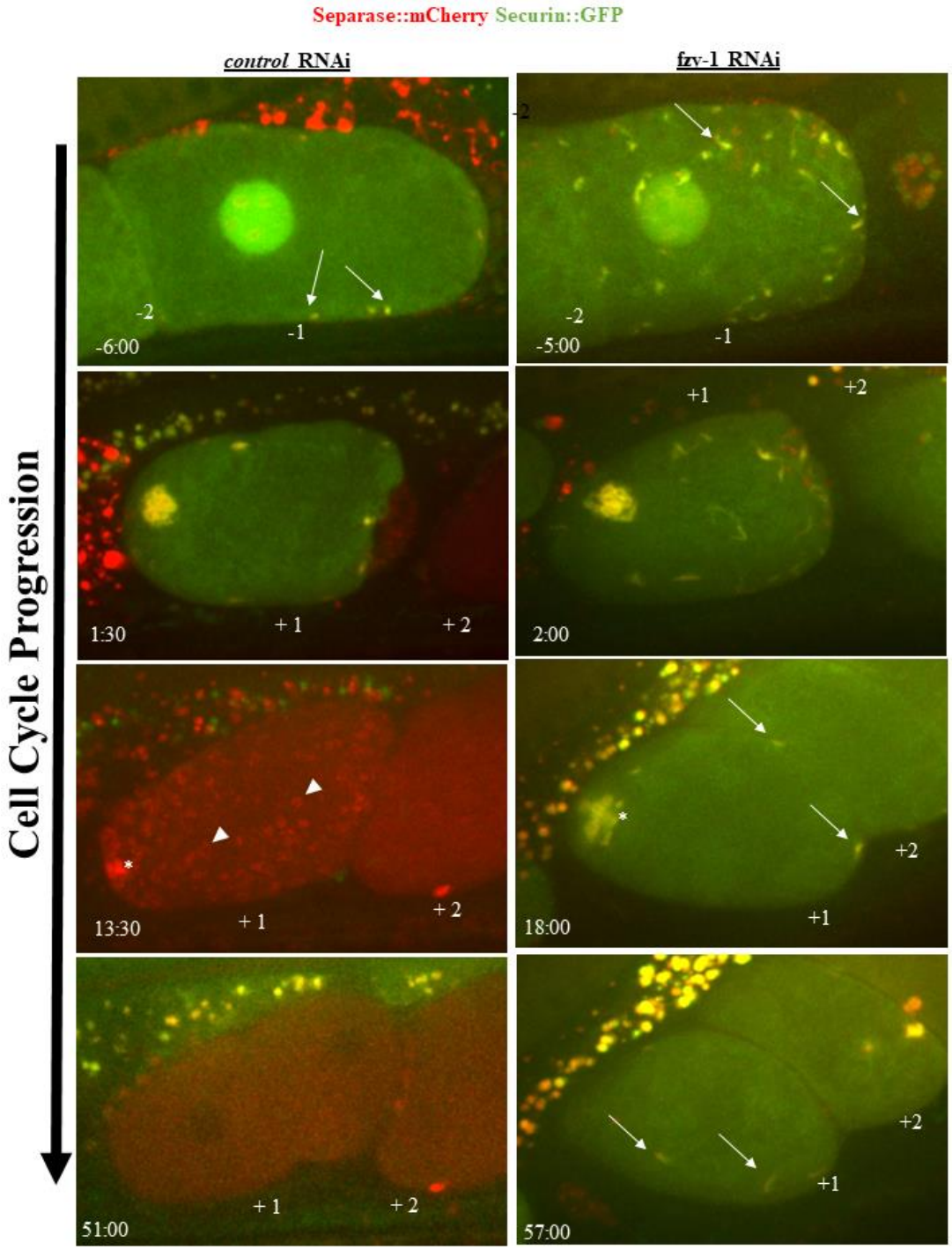
CGs, and to other sites throughout the cell cycle is a completely unexplored aspect of the biology of Separase. Understanding how Separase localizes near sites of action (for instance the spindle and KCs) may be relevant to understanding mechanisms of aneuploidy. Given that Separase has emerged to be involved in many processes outside of chromosome segregation, like vesicle trafficking and telomere capping, there appears much to learn about this protease that is well documented to have its enzymatic activity tightly regulated (Kamenz & Hauf, 2017; Zhang & Pati, 2017).

I observe Separase and Securin colocalizing to KCs and KFs during prometaphase I and this localization changes drastically during anaphase I whereby Separase is largely on its documented anaphase I structures, the MR and CGs (Fig. 8; Bembenek *et al.*, 2007; Muscat *et al.*, 2015). The presence of Separase at the MR and on CGs coincides with diminished securin signal (Fig. 7F,L; Fig. 8F). Given the evidence that APC/C activity regulates aspects of CG trafficking and is important for Separase to localize to vesicles (Bembenek *et al.*, 2007; Kimura & Kimura 2012), this suggested to me that the canonical M phase checkpoint (SAC-APC/C) is involved in regulating Separase localization and functions during anaphase I. More specifically the APC/C may regulate the localization and function of Separase in anaphase I by promoting Securin degradation following satisfaction of the SAC.

After SAC satisfaction, the key E3 ubiquitin ligase responsible for chromosome segregation in anaphase, the APC/C, associates with its activator, CDC20. The APC/C-CDC20 allows Separase to become catalytically active for chromosome segregation by targeting the inhibitory chaperone of Separase, Securin, for destruction by the 26S proteosome. Interestingly, it has recently been

reported that an additional E3 ubiquitin ligase, ETC-1, can function redundantly with the APC/C during worm meiosis I (Wang *et al.,* 2013). Our initial documentation of the M phase checkpoint demonstrated that inhibition of the APC/C alone causes a strong osmotic integrity defective (OID) phenotype, with cortical granules not being exocytosed (Bembenek *et al.,* 2007). ETC-1 has not been documented to cause an OID phenotype, so it seemed unlikely to be involved in CG biology (Sönnichsen et al., 2005) We have not previously documented Separase or Securin localization in the APC/C-null background. Whether the APC/C and ETC-1 individual or together can coordinate to control Separase localization and function during anaphase I remains to be determined. Given that the absence of APC/C activity is sufficient to block cortical granule exocytosis (Bembenek *et al.,* 2007). I decided to investigate whether the APC/C-pathway regulates Separase localization and function. I hypothesized that inactivation of the APC/C will trap Separase and Securin on prometaphase-structures: the KCs and KFs. In order to address this hypothesis, I conducted *in utero* live cell imaging of meiosis I of endogenously tagged Separase::mCherry with transgenic GFP::Securin following control or *fzy-1* (*cdc20* in the worm) RNAi (Fig. 9). I chose to image worms beginning at egg activation and normalized cell cycle progression to ovulation because the cell cycle is not expected to enter anaphase following APC/C inhibition. Consistent with my hypothesis, GFP::Securin expression is stable upon APC/C inhibition. Indeed, following *fzy-1* RNAi treatment, Separase and Securin remain colocalized to KCs, KFs, and Securin signal remains detectable 18 minutes after ovulation. Nearly after an hour after ovulation, Separase and Securin remain trapped on KFs and Separase does not localize to CGs (Fig. 9). These observations of ectopic persistence on KCs and KFs suggest that APC/C activity is critical for Separase to transition from KCs and KFs to the MR and CGs in anaphase I.

Figure 9. Inhibition of APC/C activity traps Separase and Securin on prometaphase I structures at the spindle and cortex. Representative images from an *in utero* time series of meiosis in embryos expressing endogenously tagged Separase::mCherry (red) and transgenic GFP::Securin following control (right column) or RNAi of APC/C coactivator Cdc20 (*fzy-1*). Following egg activation, around NEBD, both proteins localize to KFs (-6:00). The absence and reduced number of KFs observed in wildtype can be attributed to 1) z-planes at this time were largely not cortical and 2) this timepoint being slightly earlier in the cell cycle than the corresponding mutant condition (-5:00 to ovulation). In prometaphase I, between 1:30min and 2:00min post ovulation, the general localization is the same between what we have observed earlier (Figs. 6,7). When anaphase I is expected to occur in control (13:30), Separase is localized to the MR (asterisks) AND CGs (arrowheads). By 18:00 following ovulation in *fzy-1*-treated worms, Separase and Securin remain trapped on prometaphase I structures, the KCs (asterisks) and KFs (white arrows)(e.g. KCs and KFs). In the mutant condition, at the timepoint corresponding to the completed mitotic division in wildtype, Separase and Securin remain trapped on KFs. Images are variably shown as z-projections of various depths to emphasize localization dynamics, Numbers (e.g., -1, +1, +2) correspond the standard nomenclature used to refer to the position of the oocyte or embryo if interested in the germline relative to other embryos.


Separase::mCherry Securin::GFP
control RNAi
fzv-1 RNAi
Cell Cycle Progression
-2
-1
-6:00
-2
-5:00
-1
1:30
+ 1
+ 2
+1
+2
2:00
13:30
+ 1
+ 2
*
18:00
+2
+1
*
51:00
+ 1
+ 2
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Securin Depletion Causes Precocious Separase CG localization

The APC/C targets numerous substrates during the cell cycle, and probably the best characterized are Securin and Cyclin B, which are targeted for degradation to promote chromosome segregation in anaphase and mitotic exit, respectively (Lindon, 2008). Emerging evidence suggests that the APC/C becomes active in oocyte before anaphase onset to degrade Cyclin B and Securin to allow meiotic M phase events to progress faithfully, including to avoid chromosome segregation defects (Levasseur *et al.,* 2019; Thomas *et al.,* 2021). It was previously reported that Securin is degraded before anaphase I onset in worms (Wang *et al.*, 2013). We also observe that cytoplasmic GFP::Securin signal is lost as the oocyte progresses towards anaphase I onset (Figs. 6,7). Indeed, it appears that Securin is present in excess to Separase, as there are two populations of GFP::Securin in prophase I arrest: one population in the nucleus and one population in the cytoplasm (Fig 6G; Fig. 7A); in contrast, Separase exists solely in the cytoplasm and is excluded from the nuclease during the prophase I arrest (Fig. 6A; Fig.7 A).

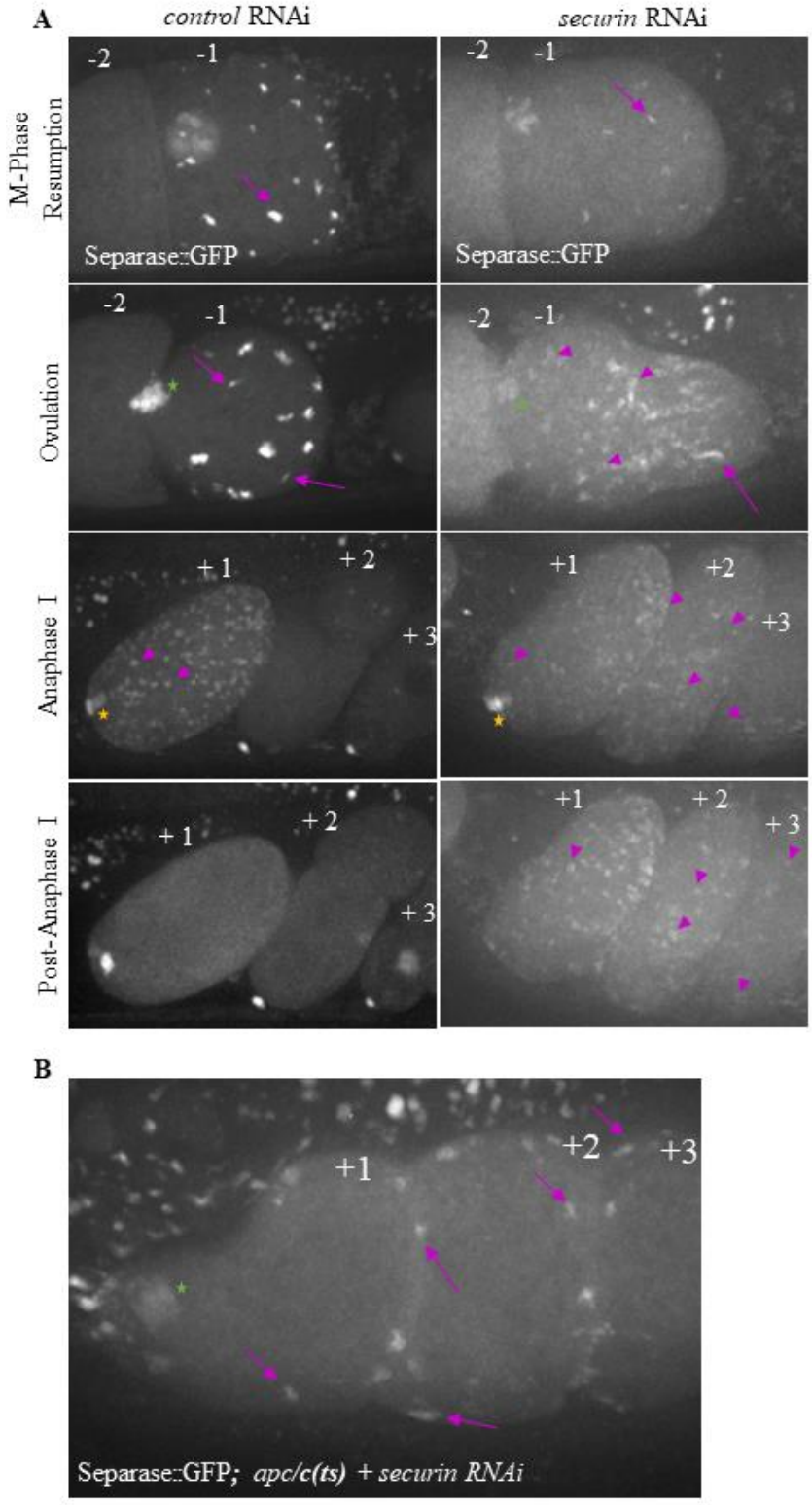
A previous study using *C. elegans* suggested that Separase is not able to localize to CGs following *securin* RNAi, which they attributed to compromised folding function of Separase which Securin endows (Kimura & Kimura, 2012). However, little background was given regarding how this experiment was performed, such as optimizing for *securin* RNAi penetrance, sample size, and image quality (e.g. reduction in GFP signal due to improper folding). Moreover, whether localization of Separase to CGs is necessary and sufficient to promote their exocytosis has not been directly tested. For these reasons, I decided to reassess the effect of *securin* RNAi on Separase localization.

Given the emerging evidence of APC/C activity initiating prior to anaphase onset to degrade excess substrates, like Securin, I hypothesized that *securin* depletion in the presence of an already active APC/C would promote early activation of the enzymatic activity of Separase. In order to test this hypothesis, I first diligently optimized image quality to account for anticipated weak Separase signal associated with compromised protein folding in a transgenic line expressing Separase::GFP, chromosome marker H2B::mCherry, and cortical granule cargo marker CPG-2::mCherry. I then carefully titrated *securin* depletion levels, documenting mild to severe *securin* phenotypes(see Materials & Methods for details). The severely penetrant *securin* RNAi phenotype resulted in diminished Separase::GFP signal and CGs were not exocytosed, consistent with compromising of the folding role of Securin. Interestingly, following mild and intermediate *securin* RNAi, I consistently noticed that Separase localized to CGs in older embryos within the uterus (Fig. 10A), which is in contrast to a previous study that claimed Separase does not localize to CGs following *securin* RNAi (Kimura & Kimura, 2012). This discrepancy is likely due to my increased sample size, careful documentation of *securin* RNAi penetrance levels, and diligent optimization of image quality. My observations suggest that the absence of Securin inhibits cortical granule exocytosis, which we previously documented (Bembenek et al., 2007) and is in agreement with Kimura & Kimura (2012).

According to my hypothesis for this experiment, which was not addressed by a previous group (Kimura & Kimura, 2012), depletion of *securin* may cause early activation of Separase. To begin to directly test this hypothesis I decided to scrutinize Separase localization following M-phase resumption in the -1 oocyte and spermatheca which are timepoints in meiosis before Separase is known to be enzymatically activated, at least for chromosome segregation. Upon initial

assessment of these stages in the intermediate *securin* depletion condition, I noticed that Separase appeared to localize to CGs in the mother's spermatheca, in 4 independent worms, which I had not previously documented with our imaging system. This is consistent with emerging evidence in the literature from mouse oocytes that aiding in APC/C substrate depletion promotes premature M phase events (Levasseur *et al.,* 2019; Thomas *et al.,* 2021), in this case CGE (Fig. 10A). To verify they this phenotype, I conduced an *in utero* movie of a line expressing Separase::GFP, H2B::mCherry, and CPG-2::mCherry following control RNAi and intermediate depletion of *securin* RNAi (Fig 10A). The cell cycle time was normalized to ovulation to account for the possibility of chromosome segregation occurring precociously. I was able to observe Separase localized precociously to CGs around ovulation, which is well before anaphase I onset and never observed in the control (Fig. 10A). I was not able to directly test my hypothesis that Separase becomes active prematurely. This is due to two primary reasons: 1) We do not possess a biosensor compatible of imaging Separase, CGs, and chromosomes together; 2) It is possible that Separase becomes active prematurely following *securin* RNAi, but because cortical granules are not properly positioned to the cortex until near the onset of anaphase I (Bembenek *et al.*, 2007; Bai & Bembenek, 2017), they failed to exocytose properly because they are not localized close enough to the plasma membrane. Nevertheless, these observations suggest two key points about how Securin regulates separase localization: 1) proper Securin degradation is important for controlling the timing of Separase localization to CGs; 2) the role of Securin as the chaperone of Separase is important, given that depletion of *securin* prevents CGE despite Separase localizing to CGs.

Figure 10. APC/C activity promotes timely Separase localization to vesicles, independent of its role in targeting Securin for destruction. (A) Representative images from an *in utero* time series of meiosis I in embryos expressing Separase::GFP following control or *securin* RNAi (due to poor image quality of z-projected red channel, H2B::mCherry and CPG-2::mCherry are not shown). At ovulation, Separase precociously localized to CGs (purple arrowheads) following intermediate *securin* depletion, which is not observed in control conditions. After anaphase I, cortical granules are no longer present in the cell in the control, and in contrast, following *securin* depletion are retained and Separase::GFP remains on cortical granules in the embryo. Numbers refer to the relative position of the oocytes/embryos in the germline. Green stars indicate prometaphase or prometaphase-like spindles; Orange stars indicate anaphase I or anaphase-like spindles. (B) Simultaneous inactivation of the APC/C and intermediate *securin* depletion trap Separase on kinetochore filaments (purple arrows). All images are z-projections of variable sizes in order to emphasize different aspects of Separase localization during the cell cycle.


A
control RNAi
securin RNAi
M-Phase Resumption
Ovulation
Anaphase I
Post-Anaphase I
-2
-1
Separase::GFP
+1
+2
+3
B
Separase::GFP; apc/c(ts) + securin RNAi

APC/C Activity Is Required to Promote Separase Localization Independently of Targeting Securin for Destruction

The data so far suggests that APC/C activity is required for Separase localization to anaphase I structures, such as MR and CGs (Fig. 9). The activation of the APC/C is required to degrade Cyclin B which inactivates CDK1 to promote exit from M phase (Lindon, 2008). It is possible that while Securin destruction might be necessary for Separase to promote CGE in anaphase I (Bembenek *et al.,* 2007), it might not be sufficient. Alternatively, the APC/C could be targeting one or multiple Cyclin B orthologs present in the worm genome (van der Voet *et al.,* 2009). The discovery of APC/C targets is an active field of research, and novel substrates are still being identified (Franks *et al.,* 2020). To address these possibilities, I decided to conduct live cell imaging of Separase::GFP in the presence of a temperature sensitive APC/C allele, *mat-1(ye121)*, which I will refer to as APC/C(ts). Upon shift to non-permissive temperatures, APC/C(ts) embryos arrest in a metaphase-I-like state and do not enter anaphase I (25°C; Shakes *et al.,* 2005). Under non-permissive conditions, I expect Separase to remain on KCs and KFs and not localize to the MR and CGs which is where I and others have observed Separase to localize in anaphase (Bembenek *et al.,* 2007; Muscat *et al.,* 2015). I hypothesized that if APC/C promotes Separase localization through targeting Securin for destruction, then Separase localization to the MR and CGs should be rescued following simultaneous inhibition of APC/C(ts) and intermediate *securin* RNAi treatment. This experiment is specifically targeting the activity of APC/C toward Securin, so it is not expected to promote exit from M phase. I Surprisingly, in a pilot experiment (N=1), after simultaneous inactivation of the APC(ts) and intermediate RNAi of *securin*. Separase remains trapped on KFs (Fig. 10B). I did not observe the spindle region during this pilot experiment. Separase could localize to the MR, but this

cannot be determined based of this preliminary assessment. I will need to repeat this experiment to address the issue of having a small sample size and to verify this phenotype, but I will tentatively conclude that APC/C activity, independent of targeting Securin for destruction, is required to promote Separase localization to the MR and CGs in anaphase I. Alternatively, the HECT E3 ligase, ETC-1 is unlikely to be directly required for controlling Separase localization through degradation of either Securin or Cyclin B, given that Separase and Securin persist on KFs persistence on KFs (Fig. 10B). Despite the small sample size of this experiment (N=1), it is not a provocative idea that the APC/C could be targeting multiple proteins during meiosis I. For instance, the APC/C positively regulates dynein-dependent spindle rotation during meiosis I in *C. elegans* by targeting Cyclin B for destruction (Ellefson & McNally, 2011; Crowder *et al.*, 2015). Furthermore, our knowledge of substrates the APC/C interacts with is increasing, along with newly appreciated motifs. Whether the APC/C can regulate Separase localization via solely targeting Securin for destruction or through additional proteins is unexplored.

Generation and Maintenance of Non-Degradable Securin (Securin^{DM}) *C. elegans* lines

Taken together, Securin does not appear necessary for Separase to localize to KFs (Fig. 9), it colocalizes with Separase during meiosis I at KCs and KFs (Fig. 8), and its degradation appears necessary but not sufficient for Separase to localize to vesicles (Fig. 10), and Securin signal is largely absent from the cytoplasm, KCs, and KFs when Separase is localized to the MR and CGs (Fig. 9F,L; Fig. 8F). Whether stabilized Securin affects Separase localization during anaphase I, as well as its function in CGE remains to be determined.. When the APC/C is inactivated, Securin is stabilized and colocalizes with Separase on KFs and KCs (Fig. 9). When Securin is

artificially depleted by RNAi, Separase precociously localizes to CGs (Fig. 10A). In this *securin* RNAi treatment, the APC/C is active and can target other substrates which may influence the localization of Separase. It one scenario, entry into anaphase throw Securin destruction and activation of Separase is sufficient for Separase to localize to CGs in anaphase. Alternatively, it is possible that some other aspect of APC/C activity, such as those directed toward exit from M-phase are important. In order to determine the role of the APC/C and by extension, Securin, on regulating the localization and function of Separase during anaphase I, we made the first worm non-degradable versions of Securin (see Material & Methods for specific details). APC/C substrates, like Securin, possess motifs that render them recognizable for ubiquitination by the APC/C (Davey & Morgan, 2016). In the case of worm Securin, the only clear APC/C motif is the **D**estruction-box **m**otif (DM). Non-degradable Securin was generated by mutating two highly conserved leucine residues in the DM of Securin. Non-degradable Securin will henceforth be referred to as Securin^{DM} (Fig. 11C; Morgan, 2014). Securin^{DM} was fused at the N-terminus to green fluorescent protein (GFP::Securin^{DM}). GFP::Securin^{DM} is expressed under control of the *pie-1* promoter to ensure hermaphrodite-specific germline expression (Praitis *et al.,* 2001). These mutations are expected to prevent the APC/C from physically recognizing Securin and targeting it for destruction (Morgan, 2014), but not its other substrates like Cyclins. Thus, once the SAC is satisfied, the APC/C cannot promote chromosome segregation as Separase protease activity remains inhibited by the bound GFP::Securin^{DM}, but initiation of its other anaphase functions will be unperturbed like the targeted destruction of Cyclin B required for mitotic exit (Fig. 11C). We have previously shown that expression of protease-dead Separase (Separase^{PD}) is dominant negative in worms (Mitchell *et al.,* 2014). Expression of Securin^{DM} causes chromosome segregation defects and lethality in other systems (Funabiki *et al.,* 1996; Zou *et al.,*

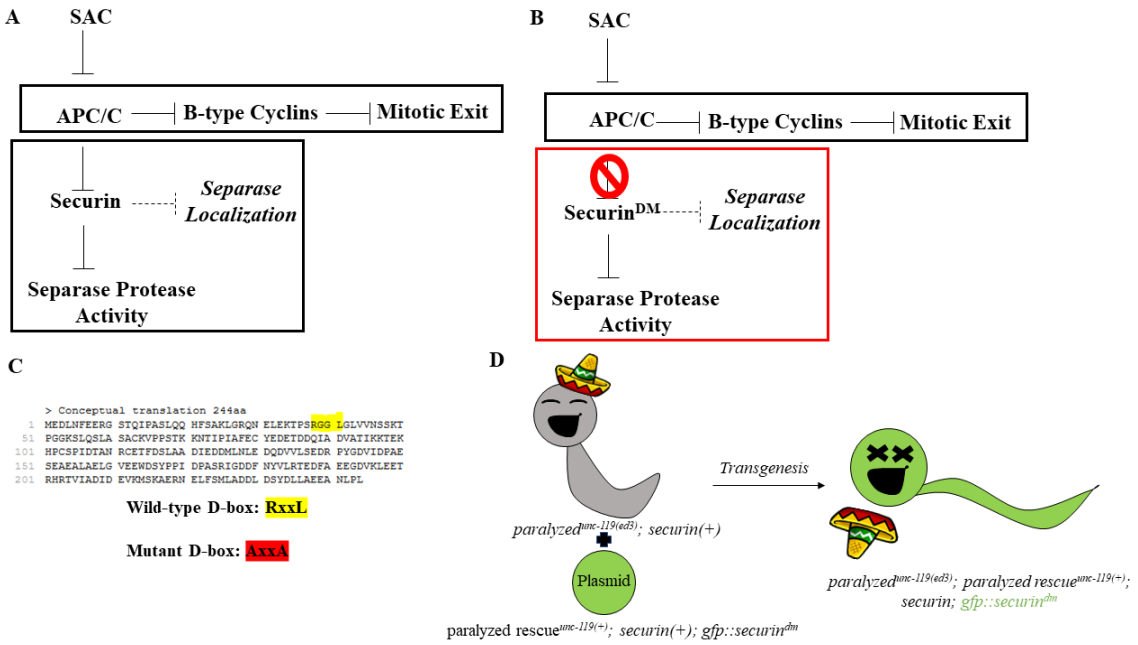


Figure 11. Generation and expression of non-degradable Securin (Securin^{DM}) worm lines. Schema of SAC-APC/C pathway and key processes it governs denoted by black boxes. (**A**). Schema of the SAC-APC/C- pathway in the context of Securin^{DM}, and the red box is the emphasized processes this pathway predicted to be affected by SecurinDM (**B**). Dashed inhibitory bar and italicized bold texts here refer a hypothesized mode of Separase regulation by Securin (**A**-**B**). Genomic sequence of worm Securin. The wildtype D-box sequence is highlighted in yellow, and the red highlighted sequence indicates mutations made to stabilize Securin (Securin^{DM}, **C**). GFP::Securin^{DM} introduced into the worm is predicted to result in lethality when expressed (**D**).

1999; Zur & Brandeis, 2001; Cohen-Fix *et al.*, 2006; Leismann *et al.*, 2006). Our mutation is expected to render Separase effectively protease-dead, and we expect expression of GFP::Securin^{DM} to be toxic when expressed (Fig. 11D).

GFP::Securin^{DM} maintenance and expression control strategies in worms

Our lab has established two robust strategies for the maintenance and controlled, induced expression of harboring toxic transgenes harbored by the worm (Mitchell *et al.,* 2014). The first strategy for controlling expression of GFP::Securin^{DM} exploits the ability of the worm germline to transgenerationally inherit siRNAs. Using this first method, maintenance of worms carrying *gfp::securin^{dm}* are raised on *gfp* RNAi, which inhibits GFP::Securin^{DM} expression. Using this method, the phenotype of the transgene can be documented in the F6 progeny as a homozygote (Mitchell *et al.,* 2014). The second method, our group developed allows us to indefinitely passage our toxic transgenes through the male germline as the *pie-1* promoter is not expressed in the males, and we can immediately analyze their sisters' toxic transgene expression as a heterozygote (Fig. 12; Mitchell *et al.,* 2014).

Characterization of GFP::Securin^{DM} Expression during Meiosis I in *C. elegans*

To initially characterize the phenotype of GFP::Securin^{DM} I used our first maintenance strategy. I hypothesized that mutations to the conserved residues with the D-box of Securin will result in stabilized expression during meiosis I and exert a dominant-negative effect over endogenous, untagged, wildtype Securin, like what we observed with Separase^{PD} (Fig. 11C,D; Mitchell *et al.,* 2014). In order to address this hypothesis, I conducted *in utero* live cell imaging of wildtype GFP::Securin and GFP::Securin^{DM} localization dynamics with chromosome marker

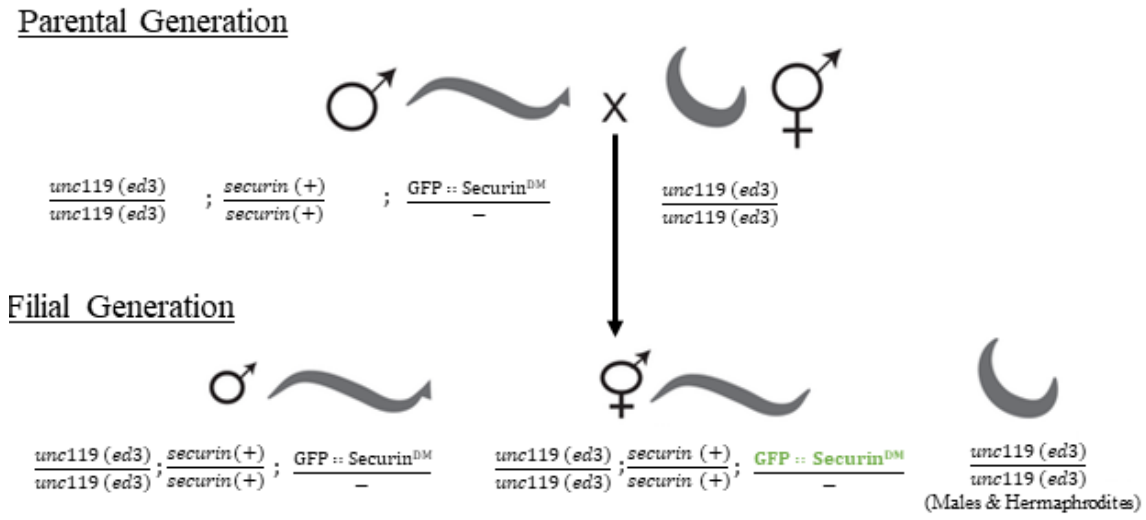


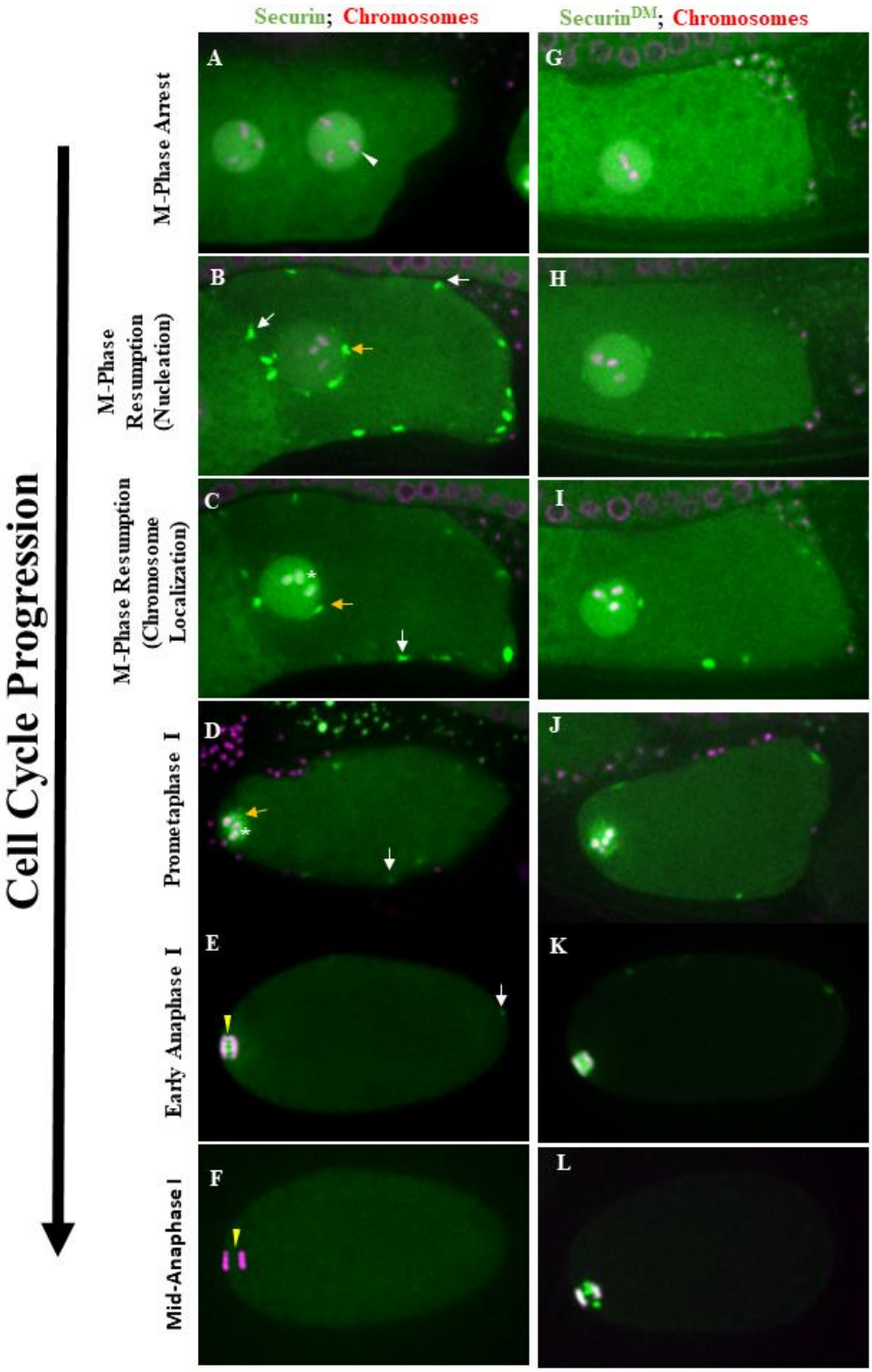
Figure 12. GFP::Securin^{DM} maintenance and expression control strategies in worms through the male germline. Our lab has established two robust strategies for the maintenance and control expression of worms harboring toxic transgenes (Fig. 11, Mitchell *et al.*, 2014). The second strategy described here exploits transcriptional differences between the male and hermaphroditic germline. Because our GFP::Securin^{DM} transgenes are driven by the *pie-1* promoter, expression of GFP::Securin^{DM} is confined to the hermaphrodite germline. Here, GFP::Securin^{DM} in black text indicates that the *gfp::securin^{dm}* locus is not expressed,

H2B::mCherry (Fig. 13). Upon inspection, wildtype Securin and Securin^{DM} display identical localization in prophase I arrest through early anaphase I, when Securin and Separase localize to the MR (Fig. 7; Fig. 8; Fig. 13A-E,G-K; Bembenek *et al.,* 2007; Muscat *et al.,* 2015). Strikingly, in contrast to wildtype GFP::Securin, and consistent with enhanced stabilization of Securin^{DM}, I noticed that GFP::Securin^{DM} ectopically persists in the spindle region on chromosomes and the MR into late anaphase, while no appreciable vesicle signal was detected in our mutant (Fig. 13L).

We did not expect negative effects prior to anaphase I onset, as distinct mechanisms are in place at chromosomes to govern early oogenesis that are independent of Separase protease activity and Securin destruction (Challa *et al.,* 2019). Additionally, we did not expect GFP::Securin^{DM} to interfere with APC/C activity as our mutations render it unrecognizable by the APC/C. More specifically, with respect to the latter expectation, meiotic spindle shortening, and rotation are not affected by stabilized GFP::Securin^{DM}, which are known to be regulated by APC/C activity toward Cyclin B in *C. elegans* (t=45sec at 20°C; $p<0.05$; N=5+ for control and mutant datasets; Ellefson & McNally, 2011). The lack of interference between the timing of these pre-anaphase I events suggest that our mutant is not capable of physically interacting with the APC/C. Based on these data, I conclude that GFP::Securin^{DM} specifically acts to inhibit chromosome segregation during anaphase I.

I also conclude that Securin does not localize to CGs, but is capable of localizing the MR, suggesting that our earlier observations of GFP::Securin at the MR could include Separase-bound signal (Figs. 7,8). Further documentation of GFP::Securin^{DM} validated its expression is stabilized in anaphase I, in contrast to multiple wildtype GFP labeled Securin reporters (Fig. 14).

Figure 13. Wildtype and GFP::Securin^{DM} localization during meiosis I in the *C. elegans* oocyte. Representative images of meiosis in embryos expressing wildtype GFP::Securin (green, **A**-**F**) and mutant GFP::Securin^{DM} (green, **A**-**L**) together with chromosome marker H2B::mCherry (magenta). During prometaphase I wildtype (**G**-**D**) and mutant GFP::Securin^{DM} (**H**-**J**) appeared identical. This remained the case in early anaphase I, with the exception of cytoplasmic wildtype GFP::Securin levels (**F,K**). Shortly before anaphase I onset, wildtype GFP::Securin is degraded in the cytoplasm but remains stably expressed in GFP::Securin^{DM} (quantified in Fib. 14). GFP::Securin^{DM} signal saturated at the spindle, so I adjusted pixel values to emphasize prolonged localization to the MR, which gives the false impression that its cytoplasmic signal as substantially decreased at these timepoints (**K**,**L**). By mid- to late-anaphase I, wildtype GFP::Securin (**F**) is largely absent from the mutant embryo, while our mutant (**L**) is noticeably present at MR and chromosomes (cytoplasmic persistence is quantified in Fig. 14). Interestingly the number of KFs decreases over time in our mutant, like wildtype. White arrowheads indicate chromosomes. Linear elements and cortical filaments are indicated by orange arrows and white arrows, respectively. Kinetochores are indicated by orange arrowheads. Images were acquired from both *in utero* and *ex utero* specimens and are variably shown as single plane or z-projections of various depths to emphasize localization dynamics.


Securin; Chromosomes
Securin^DM; Chromosomes
Cell Cycle Progression
M-Phase Arrest
M-Phase Resumption (Nucleation)
M-Phase Resumption (Chromosome Localization)
Prometaphase I
Early Anaphase I
Mid-Anaphase I
A
B
C
D
E
F
G
H
I
J
K
L

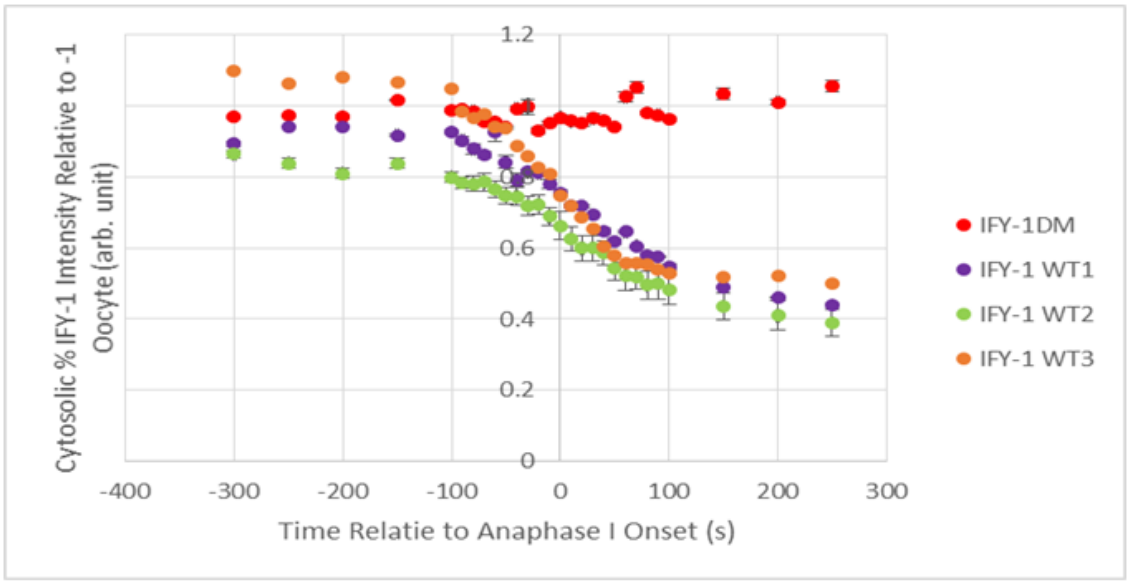


Figure 14. GFP::Securin^{DM} is stably expressed during meiosis I. Quantification of cytoplasmic wildtype and GFP::Securin^{DM} signal relative to anaphase I onset. Levels are normalized to -1 oocyte cytoplasm. Embryos were imaged 18-24hrs after shifting L4 parents to 25°C for growth and imaged *in utero* at room temperature (not recorded). Each timepoint reflects the average of at least 5 independent embryos. IFY-1DM (red dots) corresponds to the most toxic IFY-1^{DM} transgene (JAB180, see Fig. 15 for details). IFY-1 WT1-3 correspond to three independent GFP-tagged fluorescent lines (see Fig. 15 for details).

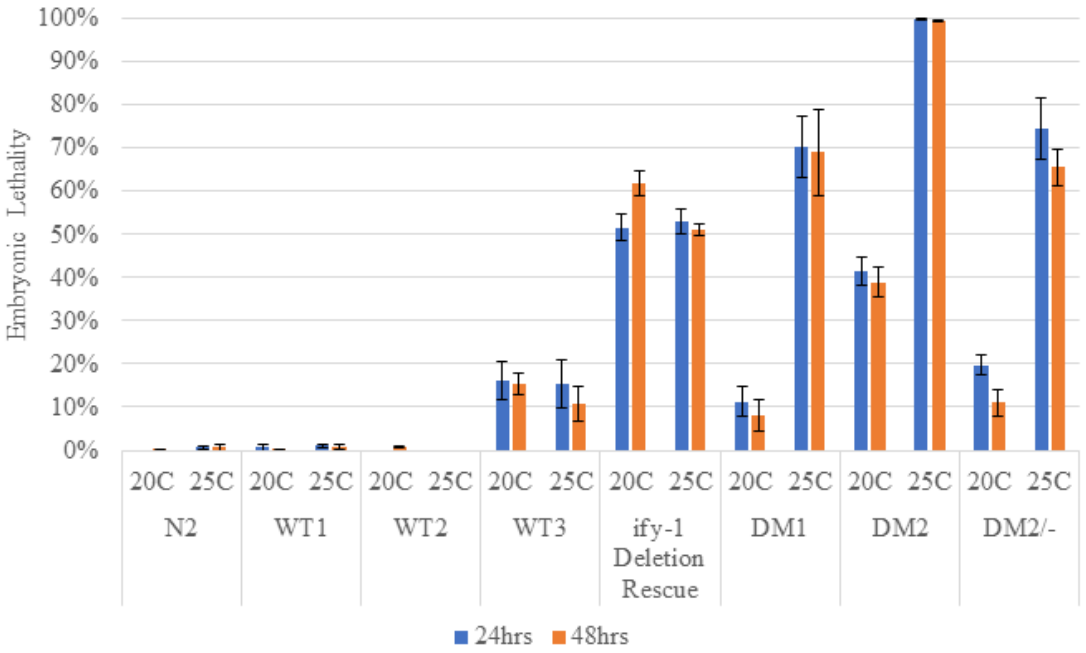
Consistent with my prediction of toxic effects of Securin stabilization, I observe an increase in embryonic lethality in the F6 generation progeny (Fig. 15). Defects in a subset of the M phase checkpoint known to control Separase for chromosome segregation, including the APC/C an Securin cause the osmotic integrity defective (OID) phenotype (Bembenek *et al.*, 2007). Because Securin is a direct target of the APC/C and I expect stabilized Securin expression to constitutively inhibit the protease activity of Separase, then I would expect Securin to interfere with the function of Separase in its documented roles in during meiosis I. This led me to the hypothesis that expression of GFP::Securin^{DM} will cause defects in chromosome segregation and eggshell formation. To address this hypothesis, I broke this hypothesis into two experiments our first toxic transgene maintenance strategy (Mitchell *et al,* 2014; Bai & Bembenek, 2017). In order to address the hypothesis that stabilized Securin expression disrupts the function of Separase during chromosome segregation in anaphase I, wildtype and GFP::Securin^{DM} were combined with chromosome marker H2B::mCherry and assessed for polar body extrusion failure.

In wildtype, there are two polar bodies present in the two-celled stage, consistent with successful anaphase and cytokinetic events of meiotic M phase (Fig. 16A). Consistent with having defects in chromosome segregation during anaphase I, I observed a spectrum of phenotypes, including cases indicative of one or both anaphase events having failed, such as embryos possessing one polar body and zero polar bodies, respectively (Fig. 16B).

With respect to the cytokinesis and vesicle trafficking functions of Separase, I hypothesized that expression of GFP::Securin^{DM} will compromise eggshell formation and exhibit an OID

Figure 15. GFP::Securin^{DM} expression cause embryonic lethality. 24 and 48hr embryonic lethality assays were conducted at 20 and 25°C (**A**) with wildtype untagged worms (N2), multiple wildtype GFP-tagged Securin lines, and our two independent GFP::Securin^{DM} lines (**B**). With the exception of N2 and DM2/-, all genetic backgrounds where homozygous for GFP-tagged Securin. Analysis of mutant GFP::Securin conditions assayed F6 embryos oviposited from their F5 embryo mothers, which corresponds to 6 and 5 generations removed from *gfp* RNAi, respectively,. Higher temperatures were associated with increased lethality. No or slightly detectable lethality was associated with GFP::IFY-1 expressed in WH491 (WT1) and WH526 (WT2), and this was not significantly different from N2 ($p=<0.05$, t-test) JAB180 exhibited the most penetrant lethality, significantly different from N2, WH491, WH526, and N2 and our mutant isolate JAB30 (DM1). JAB180 (DM2) exhibited striking lethality at 25°C as a heterozygote (DM2/-). Error bars are standard error of the mean.

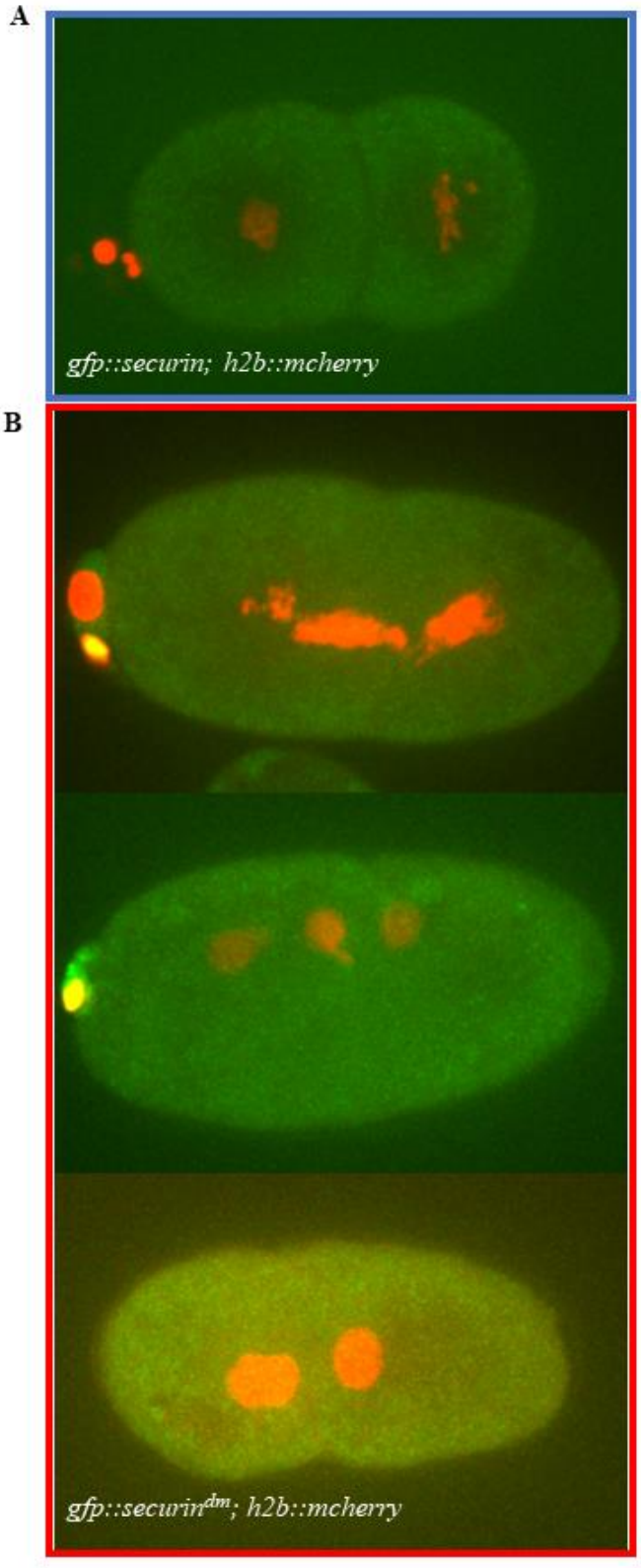
A



B

Strain	Genetic Background	Reference
N2	*wildtype*	CGC
WH491 (WT1)	*unc-119(ed3) III; pjk3[pie-1p::ify-1::gfp; unc-119(+)]*	Gift from John White
WH526 (WT2)	*unc-119(ed3) III; pjk3[pie-1p::ify-1::gfp; unc-119(+)]*	Gift from John White
TH214 (WT3)	*unc-119(ed3) III; ddIs128 [ify-1::TY1::EGFP::3xFLAG(92C12) + unc-119(+)]*	Sarov *et al.*, 2012
RQ386 (WT4)	*ify-1(tm947) unc-4(e120)II; jzIs63[pRK333; rpl-22, ify-1 ::LAP(his6+PreScission+wGFP), unc-119(+)]; ltIs37[pAA64; Ppie1::mCherry::his-58; unc-119(+)]*	Wang *et al.*, 2013
JAB30 (DM1)	*unc-119(ed3) III; pjk3(pie-1p::gfp::ify-1^{dm}; unc-119(+)]*	This Study
JAB180 (DM2)	*unc-119(ed3) III; pjk3(pie-1p::gfp::ify-1^{dm}; unc-119(+)]*	This Study
JAB180 (DM2/-)	*Heterozygous JAB180 background*	This Study

Figure 16. Pilot observations suggest GFP::Securin^{DM} causes polar body extrusion defects. Representative ex utero max projections of embryos homozygous wildtype GFP::Securin (green, A) and GFP::Securin (B) with chromosome marker H2B::mCherry. . Wildtype embryos contain two polar bodies at the 2-cell stage. Mutant embryos left the impression of a spectrum of phenotypes where 2, 1 or no polar bodies were observed. Wildtype and mutant worms were shifted to 25°C overnight before being assayed at room temperature. Mutant embryos represent those from the F6 generation (i.e. 6 generations removed from gfp RNAi). Images are depicted as z-projections of various depths to emphasize present and/or absence of polar bodies.


A
gfp::securin; h2b::mcherry
B
gfp::securin^dm; h2b::mcherry

phenotype. Consistent with this hypothesis, and in contrast to wildtype, where eggshell integrity is preserved, expression of GFP::Securin^{DM} results in an OID phenotype, and the embryo loses water when dissected in a hypertonic environment (Fig. 17). While the penetrance of phenotypes from these pilot experiments needs to be readdressed, the striking defects in polar body extrusion (Fig. 16B), which could also be due in part to defective cytokinesis rather than chromosome segregation, in addition to the stabilized GFP::Securin^{DM} signal during anaphase I (Fig. 13K-L, 14A) and defective eggshell formation (Fig. 17B) are consistent with Securin having a role in regulating Separase during its anaphase I functions by inhibiting its protease activity. One reason why we observe chromosome segregation in our Securin^{DM} background is likely explained to a combination of: 1) wildtype, untagged Securin is able to associate with Separase in the mutant background and upon APC/C activation, some populations of Separase are able to successfully proteolyze Cohesin, and allowing chromosomes to segregate; 2) related to this first point, the APC/C can activate motor proteins that promote poleward movement following SAC satisfaction, which I suspect could be aided by the successful cleavage of Cohesin we believe could still occur in our mutant background. These caveats remain to be tested. Nevertheless, these data are consistent with Securin inhibiting the functions of Separase during anaphase I.

GFP::Securin^{DM} inhibits chromosome segregation and causes polar body extrusion failure during anaphase I

During anaphase I, Separase is activated and proteolyzes the meiosis specific Cohesin subunit, REC8, liberating the homologous chromosomes for segregation (Siomos *et al.*, 2001). Our lab has demonstrated that Separase^{PD} inhibits chromosome segregation during anaphase I and the

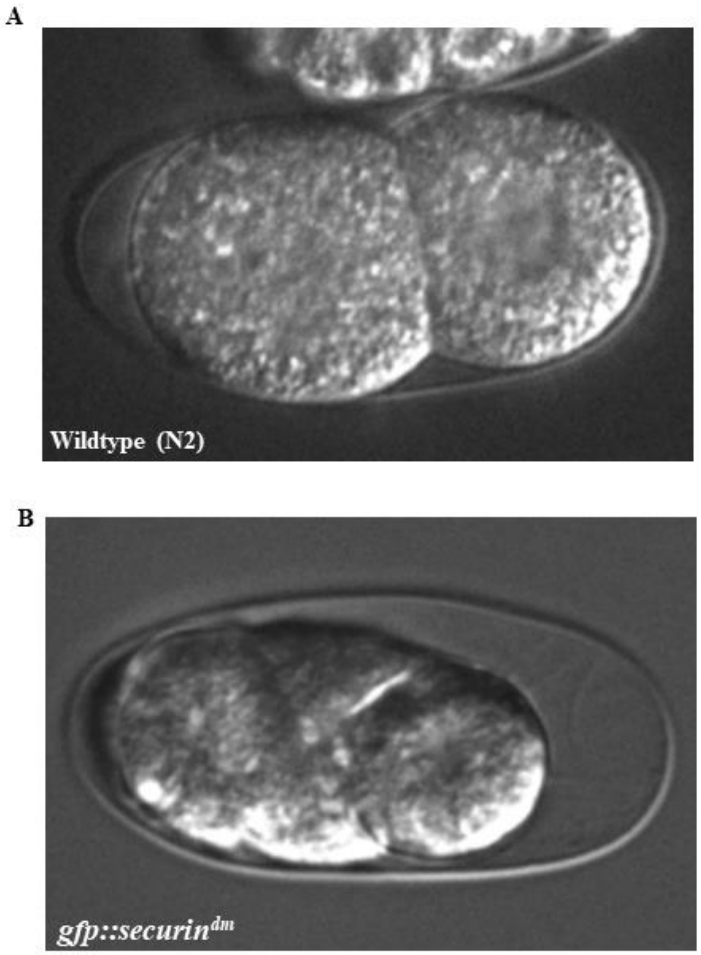


Figure 17. Pilot observations suggest GFP::Securin^{DM} has defects in eggshell formation. Representative *ex utero* DIC z-projection images of wildtype (N2, **A**) and mutant homozygous GFP::Securin^{DM} (**B**) dissected from mothers in a solution of 300mM KCl. Note that the mutant's appearance is indicative of emersion in a hypertonic environment, while the integrity in appearance of wildtype appears maintained. Wildtype and mutant worms were shifted to 25°C overnight before being assayed at room temperature. Mutant embryos represent those from the F6 generation (i.e. 6 generations removed from *gfp* RNAi). N = 10/10 for both wildtype and mutant conditions.

first mitotic anaphase in worms (Mitchell *et al.,* 2014; Bai & Bembenek, 2017). *in vitro* work has demonstrated that when Separase is bound to its canonical inhibitory chaperone Securin, it is catalytically inactive (Gorr *et al.,* 2005). It has been demonstrated in multiple systems that inhibiting Securin degradation causes chromosome segregation defects during anaphase (Funabiki *et al.,* 1996; Zou *et al.,* 1999; Zur & Brandeis, 2001; Cohen-Fix *et al.,* 2006; Leismann *et al.,* 2006), which is consistent with the idea that preventing Securin recognition for destruction locks it to Separase and effectively renders it catalytically inactive. It is also possible that the polar body extrusion defects I observed are associated with cytokinesis failure which could also be associated with defects in CGE (Figs. 16,17). To address these possibilities I conduced *ex utero* imaging of wildtype and GFP::Securin^{DM} with the chromosome marker H2B::mCherry during meiosis I at room temperature after upshifting hermaphrodites to 25^{o}C for 18-24hrs. I hypothesize that stabilized GFP::Securin^{DM} expression causes chromosome segregation defects. In this case, a delay in chromosome segregation is expected, as we have previously documented for Separase^{PD} (Bai & Bembenek, 2017).

Our lab has previously demonstrated that the cell cycle progresses normally up until anaphase of both mitosis and meiosis in worms expressing Separase^{PD}::GFP (Bembenek et al., 2010; Mitchell *et al.,* 2014). And I have documented that in the presence of GFP::Securin^{DM}, the time between the initiation of spindle rotation to anaphase I onset is identical to wildtype GFP::Securin.. This is consistent with Securin degradation not being necessary for APC/C mediated spindle shortening and rotation (see results for Fig. 13; Ellefson & McNally, 2011; Crowder *et al.,* 2015). I also reported that spindle rotation to anaphase onset using the MR as a marker, and these

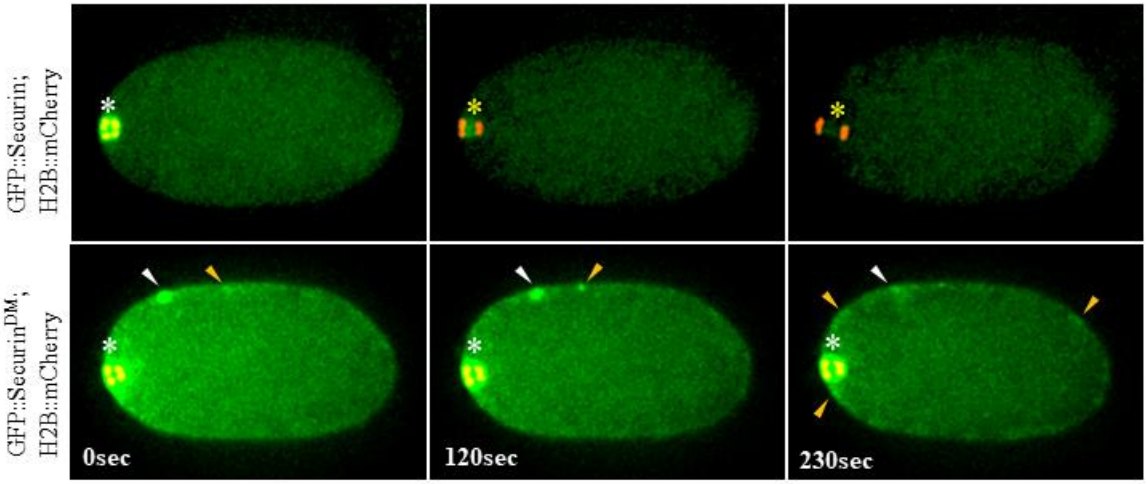
data suggest that Securin does not inhibit Separase localization as it moves from the KC to MR at anaphase I. When I observe anaphase I onset (Fig. 18), I noticed that there appeared to be delay between Securin localization to the MR and the poleward movement of chromosomes in our mutant background. In wildtype, GFP::Securin localizes to the MR approximately 2 seconds before chromosome segregation in anaphase (N=6; SD= 7.5). In contrast the first appearance of poleward movement of chromosomes following GFP::Securin^{DM} localization to the MR was delayed approximately 115 seconds (N=23; SD=42), and this delay was significantly different ($p=1.4810^{-12}$, t-test). This suggest poleward movement of chromosomes is impaired during anaphase I.

To further address this delay, I monitored chromosome segregation in wildtype and mutant background until the end of anaphase I, which I designated as the timepoint when chromosome movement becomes more variable in the wildtype and in the case of the mutant, the point at which kinetochores become distinct, which may correspond to a metaphase-II-like spindle (Fig. 18B). Based on these criteria, anaphase I took roughly three times longer to complete in the mutant condition. At comparable anaphase I time points, the distance in poleward movement became significantly different 60 seconds after wildtype and GFP::Securin^{DM} localized to the MR.

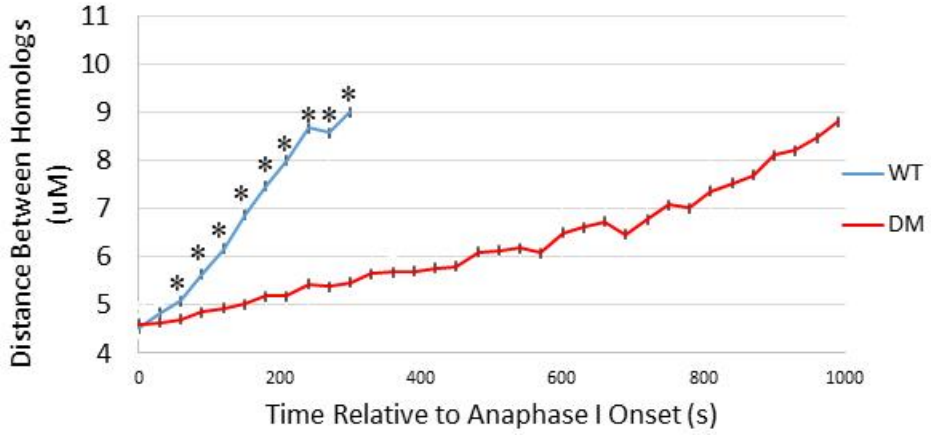
Taken together, despite previously mentioned caveats about the genetic background, these data are consistent with the hypothesis that Securin^{DM} inhibits the protease activity of Separase during chromosome segregation by interfering with its ability to bind to substrates through its proteolytic domain. However, it cannot be ruled out that Separase is definitively inactivated at

Figure 18. GFP::Securin^{DM} inhibits homologous chromosome segregation during anaphase I. Representative images from an *ex utero* time series of anaphase I embryos expressing wildtype or GFP::Securin^{DM} (green) with chromosome marker H2B::mCherry (red, **A**). Trace signal of wildtype Securin GFP is detected between homologous by mid-anaphase (120sec) albeit noticeably lower than the mutant at this timepoint. We attribute this to embryos being produced at 25˚C and *pie-1* driven gene are expressed more highly than those observed following incubation at 20°C (see wildtype or mutant Securin where applicable in Figs. 1, 2, and 6-8 for reference). Wildtype Securin signal is not detected. Filaments appear to persist into anaphase along with additional ectopic signal at the cortex. Quantifications of chromosome segregation rate (**B)**. Asterisks denote statistically significant difference in movement ($p<0.05$, t-test). Images represent a single focal plane at the spindle. White asterisks denote the spindle region with strong signal in the MB. Yellow asterisks designate diminished MR signal. White arrowheads correspond to KFs. Orange arrowheads correspond to unknown cortical signal.

A


GFP::Securin;
H2B::mCherry
GFP::Securin^DM;
H2B::mCherry
0sec
120sec
230sec

B


Distance Between Homologs (uM)
11
10
9
8
7
6
5
4
0
200
400
600
800
1000
Time Relative to Anaphase I Onset (s)
WT
DM

this time, but a chromosome-based biosensor could resolve this issue. Furthermore, we cannot exclude the possibility of Separase being able to interact with other factors while bound by Securin, or indirectly through Securin or another interacting partner. Additional structural studies will be needed to resolve this issue. Furthermore, we cannot exclude the possibility that Securin^{DM} directly interferes with meiotic cytokinesis, polar body extrusion. A more systematic assessment will be needed to be carried out. Nevertheless, these data suggest that Securin^{DM} inhibit chromosome segregation during anaphase, complementing our reports on the role of Separase^{PD} causing chromosome segregation defects.

GFP::Securin^{DM} expression blocks cortical granule exocytosis during anaphase I

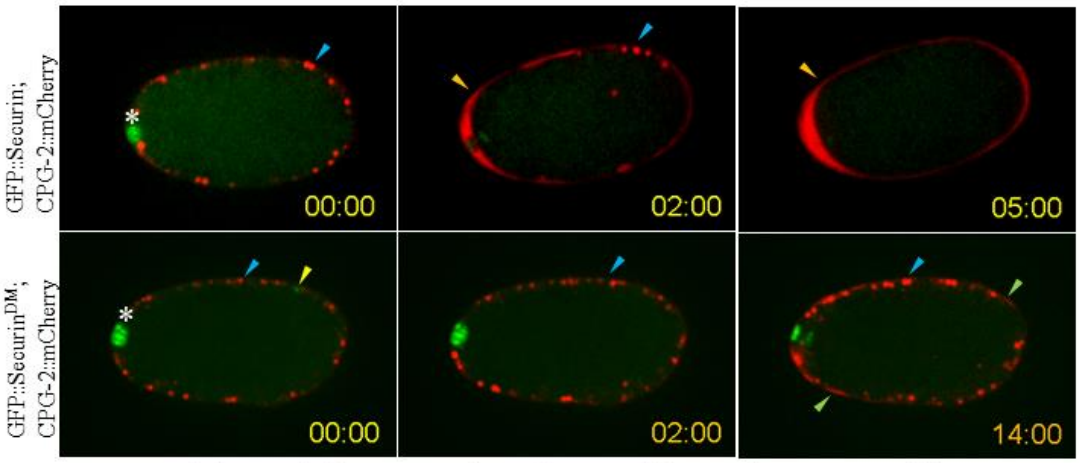
My preliminary observations of Securin^{DM} expression documented severe defects in eggshell formation (Fig. 17). I suspected that this OID phenotype could be related to the function of Separase during CGE, as we have implicated its proteolytic activity as being required for timely CGE (Bai & Bembenek, 2017) I suspected that Securin^{DM} expression may negatively affect the ability of Separase to promote CGEs. This led me to hypothesize that Securin^{DM} expression will inhibit CGE during anaphase I. To test this hypothesis, I conducted *ex utero* live cell imaging using of wildtype and GFP::Securin^{DM} with the cortical granule cargo protein CPG-2::mCherry. The mothers were upshifted to 25˚C as L4s for between 18-24hrs. Initially we began imaging at room temperature, but it turned out that momentarily downshifting the worms from 25˚C to room temperature was sufficient to delay the expected duration of CGE in wildtype (data not shown, see Bembenek *et al.,* 2007 for details). This issue was resolved my monitoring the temperature of the stage with a space heater and high precision thermometer.

I began imaging prometaphase I worm embryos through anaphase I, which was defined by midbivalent localization (Fig. 19; Bembenek *et al.,* 2007; Muscat *et al.,* 2015). In anaphase I, as previously observed, wildtype and GFP::Securin^{DM} localize to the midbivalent and no CG-associated Securin signal was detected in either condition (Fig. 19A). Consistent with CGE not initiating in early anaphase I, the number of cortical granules before and immediately following anaphase I onset remains the same and there was no statistical difference between the WT and mutant (Fig. 19B). Consistent with our initial report (Bembenek *et al.,* 2007), beginning at what I defined earlier as late anaphase, CGs are rapidly exocytosed, and by 200 seconds post-anaphase I onset, determined by Securin MR localization, the majority of CGs were exocytosed in wildtype while the number of CGs remained unchanged in the mutant, and this marks the first timepoint of statistical significance in this analysis (Fig. 19A,B). By the end of anaphase I in wildtype, CGE is complete, and the CPG-2 layer of the eggshell is established (Fig. 19A; Olson *et al.,* 2012).

These data are consistent with the hypothesis that Securin^{DM} inhibits the function of Separase during CGE. What happens to Separase and Securin in both the wildtype and mutant condition between the reduction in number of KFs, and the appearance of Separase on CGs in at least wildtype remains to be determined. These points of speculation will be elaborated in the discussion section. Taken together, our observation that Securin^{DM} blocks cortical granule exocytosis during anaphase I is consistent with the hypothesis that Securin negatively regulates Separase activity during CGE by serving as a pseudosubstrate inhibitor, though I cannot rule out the possibility of non-proteolytic functions of Separase being impeded by this assay.

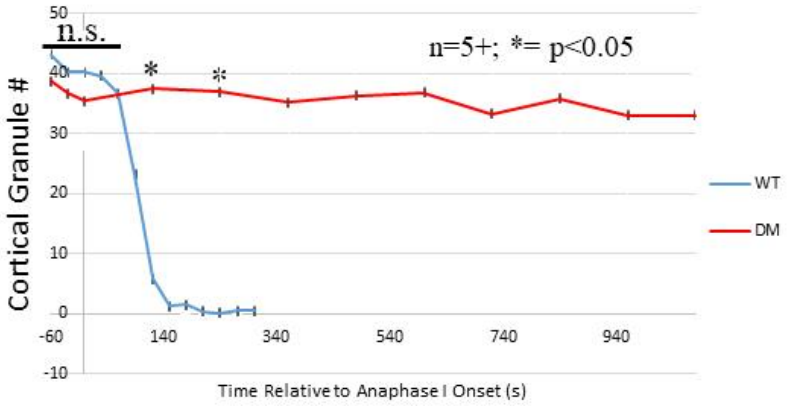
Figure 19. Cortical granule exocytosis is inhibited during anaphase I in the presence of GFP::Securin^{DM}. Representative images from an *ex utero* time series of anaphase I embryos expressing wildtype GFP::Securin or GFP::Securin^{DM} (green) with CG cargo marker CPG-2::mCherry (red, **A**). During anaphase I onset (0:00min), CGs are present at the cortex prior to their exocytosis in mid-anaphase I. Trace signal of wildtype Securin GFP remains detectable on chromosomes after the majority of CGs have been exocytosed, where the CPG-2 layer of the eggshell begins to form. At a comparable time point in the mutant GFP::Securin^{DM} visibly labels the MR and chromosomes, CGs are retained near the cortex and the presence of the CPG-2 layer is not detected. Quantifications of CPG-2::mCherry-positive CGs during anaphase I in the cytoplasm over time (**B)**. In early anaphase I, there is no statistically significant difference between vesicles at this timepoint and prior to anaphase I onset between and within conditions, consistent with CGs not being exocytosed during this time. Two minutes after anaphase I onset, I notice a significantly different number of CGs retained relative to wildtype. The number of CGs in the mutant condition before, at the start, and after anaphase I onset are not statistically different, indicating no exocytosis. By the end of mid-anaphase, I in wildtype, CGs are completely exocytosed, whereas residual CPG-2 layer appears detectable but there is no change in total vesicle number. In the mutant, no KFs are present at the selected timepoints, indicating some variation in presence. Asterisks denote statistically significant difference cortical granules (N=5+ for each timepoint and condition; $p<0.05$). Images are single plane, and to the best of my abilities the middle plane of the spindle was tracked over time. Parents were incubated at 25˚C, embryos were quickly dissected and imaged at 25˚C. White asterisks indicate MR localization; yellow arrowheads indicate filaments; blue arrowheads indicate CGs, orange arrowheads indicate CPG-2 layer of the eggshell; green arrowheads indicate apparent, residual exocytic events.

A



Time relative to anaphase I-onset (time: minutes:seconds)

B



How Separase localization is regulated during the cell cycle has been largely unexplored. Early studies demonstrated that Securin is important for Separase to concentrate within the nucleus and at the mitotic spindle (Jensen *et al.,* 2001; Hornig *et al.,* 2002), and this appears true for worms (Fig. 10A). Phosphorylation of Separase may play an important role in its localization during the cell cycle(Agarwal & Cohen-Fix, 2002). Indeed, in a screen to rescue Separase loss-of-function phenotypes, we found that protein phosphatase 5 (PPH-5) is a negative regulator of Separase localization to vesicles. For example, the Separase allele, *separase(ax110),* does not normally localize to CGs, but we observed that a mutation in PPH-5 could restore the localization of Separase(AX110) to CGs. Outside of this work, little work has been done to understand how Separase localizes to different structures in the cell.

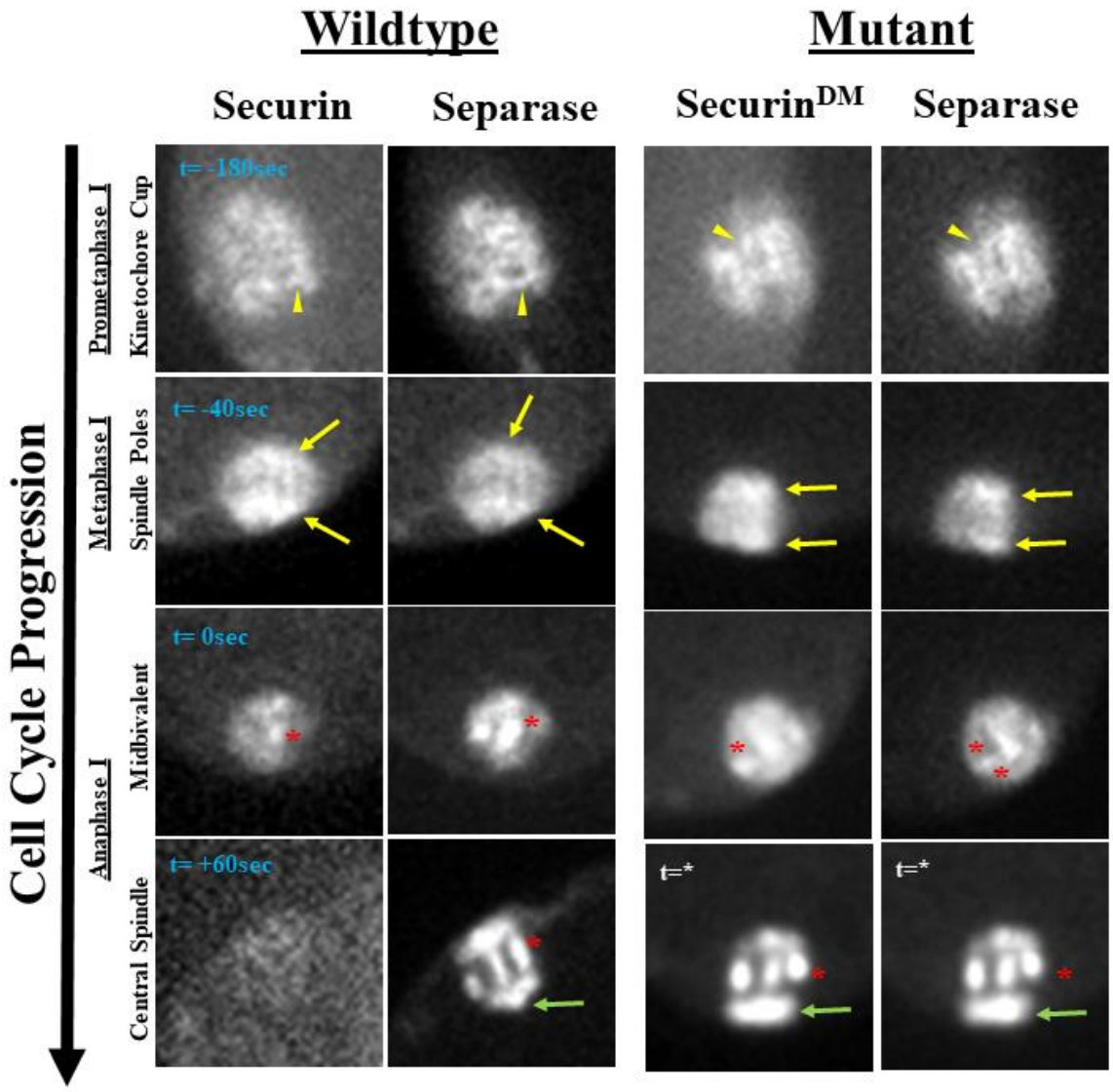
Little if any work has addressed how Separase spatiotemporally localizes from KCs in metaphase I and then to the MR in anaphase I. In *C. elegans,* it is known kinetochore proteins exhibit similar localization dynamics to what we observe of Separase as the metaphase I spindle is reorganized for during anaphase I (Dumont *et al.,* 2010). And I have shown that APC/C activity is required for this transition from KCs to the MB during anaphase I (Fig. 9), though I cannot conclude from my current observations that its activity toward Securin is required for this transition (Fig. 10B).

Our observation that APC/C activity is required for Separase to localize from KCs and KFs to MR and CGs in anaphase I suggests that the canonical SAC-APC/C pathway may also serve to regulate its localization within the cell throughout the cell cycle (Figs. 9,10). My initial observation that residual wildtype GFP::Securin signal appeared at the MR briefly in earlier

anaphase I from my *in utero* imaging suggested that Securin degradation may not be necessarily required for Separase localization to the MR (Fig. 7). This observation of GFP::Securin localization was validated using *ex utero* imaging, but in this case the signal appeared relatively more distinct than my *in utero* imaging (Figs. 18,19). This is likely attributed to the higher resolution offered by *ex utero* live cell imaging of *C. elegans*, as signal quality can be obscured by the mother's tissue (Figs. 7-10,13). However, the strong ectopic signal of GFP::Securin^{DM} led us to reconsider this initial hypothesis that Securin blocked Separase localization to the MR, since Separase and Securin are known to form a complex (Kamenz & Hauf, 2017). I hypothesize that Securin^{DM} will colocalize with Separase to the MR and remain colocalized during anaphase I. Alternatively, the localization of Separase to the MR may be delayed, though I suspect this is unlikely given that Separase and Securin form a tight complex in worms (Boland *et al.*, 2017), and I demonstrated earlier that wildtype and GFP::Securin^{DM} localize to the MR at the same time following the initiation of spindle rotation. In order to address these scenarios, and after diligent troubleshooting, I decided to conduct single plane, *ex utero* live cell imaging of endogenously tagged Separase::mScarlet with wildtype and GFP::Securin^{DM} through the central plane of the spindle, prior to spindle rotation and through anaphase I onset (Fig., using our male germline maintenance strategy (Fig. 20).

In preparing for this experiment, I observed that Separase localization on the spindle was dynamic and followed a reproducible pattern as the cell cycle approached anaphase I onset. It interested me whether this could be related to restructuring and positioning of the meiotic spindle for anaphase I. This aspect of Separase localization was previously overlooked (Bembenek *et al.*, 2007; Mitchell *et al.*, 2014; Muscat *et al.*, 2015). In order to investigate this phenomenon further,

Figure 20. Separase and Securin remain colocalized in the spindle region during anaphase I. Representative images of meiosis in embryos expressing Separase::mScarlet with wildtype GFP::Securin (left column) or mutant GFP::Securin^{DM} (right column) in prometaphase, -180 seconds before either Separase or Securin midbivalent location through clear MB/central spindle colocalization later in anaphase I. Separase and Securin localization are identical up until anaphase I onset, when differences are quickly observed. Where GFP::Securin signal is largely diminished, Separase::mScarlet begins to and remains associated with the midbivalent region as anaphase I progresses. In stark contrast, GFP::Securin^{DM} and Separase::mScarlet remain strongly colocalized on the chromosomes and MR region as anaphase I progresses. Images represent as close to the central plane of the spindle possible. Cytoplasmic GFP levels were adjusted to emphasize KC and MR localization in order to emphasize localization dynamic similarities and differences**.** Yellow arrowheads indicate KC; Yellow arrows indicate spindle poles; Red asterisks indicate MR; Green arrows indicate chromosomes.


Wildtype
Mutant
Securin
Separase
Securin^DM
Separase
Cell Cycle Progression
Prometaphase I
Kinetochore Cup
Metaphase I
Spindle Poles
Anaphase I
Midbivalent
Central Spindle
t= -180sec
t= -40sec
t= 0sec
t= +60sec
t=*
t=*

I then conducted *ex utero* love cell imaging with high spatiotemporal resolution of Separase::mScarlet in the presence of wildtype GFP::Securin or mutant GFP::Securin^{DM}.

In wildtype, during prometaphase I, approximately 180 seconds before anaphase I onset, Separase and Securin display identical localization patterns, particularly at the KCs, though not all of these structures are in the focal plane of the microscope. After spindle rotation, around -40 seconds before anaphase I onset, Separase and Securin appears to preferentially localize to the polar regions of the spindle. Roughly -20 seconds before anaphase I onset, as marked by Separase or Securin localization to the MR, there appears to be a transition period, which is not shown as it is difficult to assign a discrete category due to the polar signal beginning to make the gap between the spindle poles less resolvable. Near the end of this 20 second transition period, Separase and Securin rapidly relocalizes to the MR at anaphase I onset. After about +60 seconds into anaphase, Separase is strongly localized to the MR. Separase is known to bind the chromosomes (Sun *et al.,* 2010), and we see it localizing to the area where chromosomes are known to be present (Bembenek *et al.,* 2007; Mitchell *et al.,* 2014; Bai & Bembenek, 2017). At this timepoint, after which chromosomes have moved noticeably poleward, Securin signal does not appear to be above cytoplasmic levels. These observations are consistent with my hypothesis along with my earlier observations that Separase, wildtype Securin, and Securin^{DM}, display identical localization patterns in anaphase I. Collectively, these suggest that Securin degradation is not necessary for localization of Separase to the MR, and that stabilizing Securin negatively affects the ability of Separase to promote chromosome segregation during anaphase I.

GFP::Securin^{DM} Blocks Separase Localization to CGs during Anaphase I

When meiosis I resumes, Separase and Securin localize to mysterious structures we refer to as KFs (Figs. 7,8). As the cell cycle progresses, Separase and Securin are lost from these structures, coincident with Securin degradation from the cytoplasm apparent in prometaphase I (Fig. 14). In early anaphase I, Separase localizes to the MB and CGs near the spindle in the anterior of the cell, while Separase and Securin are confined to KF and absent from vesicles in the posterior (Fig. 7E,K). This localization pattern of Separase localization is reminiscent of the wave of exocytosis that initiates at the beginning of what I designate as late anaphase I, near the spindle in the anterior, and progresses towards the posterior (Bembenek *et al.*, 2007). By mid-anaphase at the cortex, before CGE, Separase has transferred entirely from KFs to CGs. The function of these KFs is elusive. Following an extensive literature search, in addition to novel observations of worm strains in our lab, I was able to determine that these KFs are composed of members of outer kinetochore, SAC, motor proteins, an assortment of Nucleoporins, in addition to Separase and Securin (Table 1). Earlier, I demonstrated that APC/C inactivation traps Separase on kinetochores at the spindle (KCs) and cortex (KFs; Fig. 9). Satisfaction of the SAC triggers APC/C activity that enables the reorganization of the metaphase I spindle into the anaphase I spindle (Lara-Gonzalez *et al.,* 2021). As we and others have reported (Bembenek *et al.,* 2007; Muscat *et al.,* 2015), Separase localizes from form KCs to the MR, and I have shown that this movement is dependent upon APC/C activity, and this appears to coincide with APC/C-dependent removal of the SAC from the spindle (Lara-Gonzalez *et al.,* 2021). Whether KFs are an extension of the SAC required for coordination of chromosome segregation with vesicle trafficking is at the moment speculation, and this will be addressed further in chapter 4.

Table 1. Functional Classification of Kinetochore Filament Resident Proteins. All genes/proteins are as documented from *C. elegans*, unless otherwise mentioned. Additional components may have been overlooked do to being overlooked or not specifically shown, as in the case of additional *C. elegans* Y-complex components (Hattersley *et al.*, 2016).

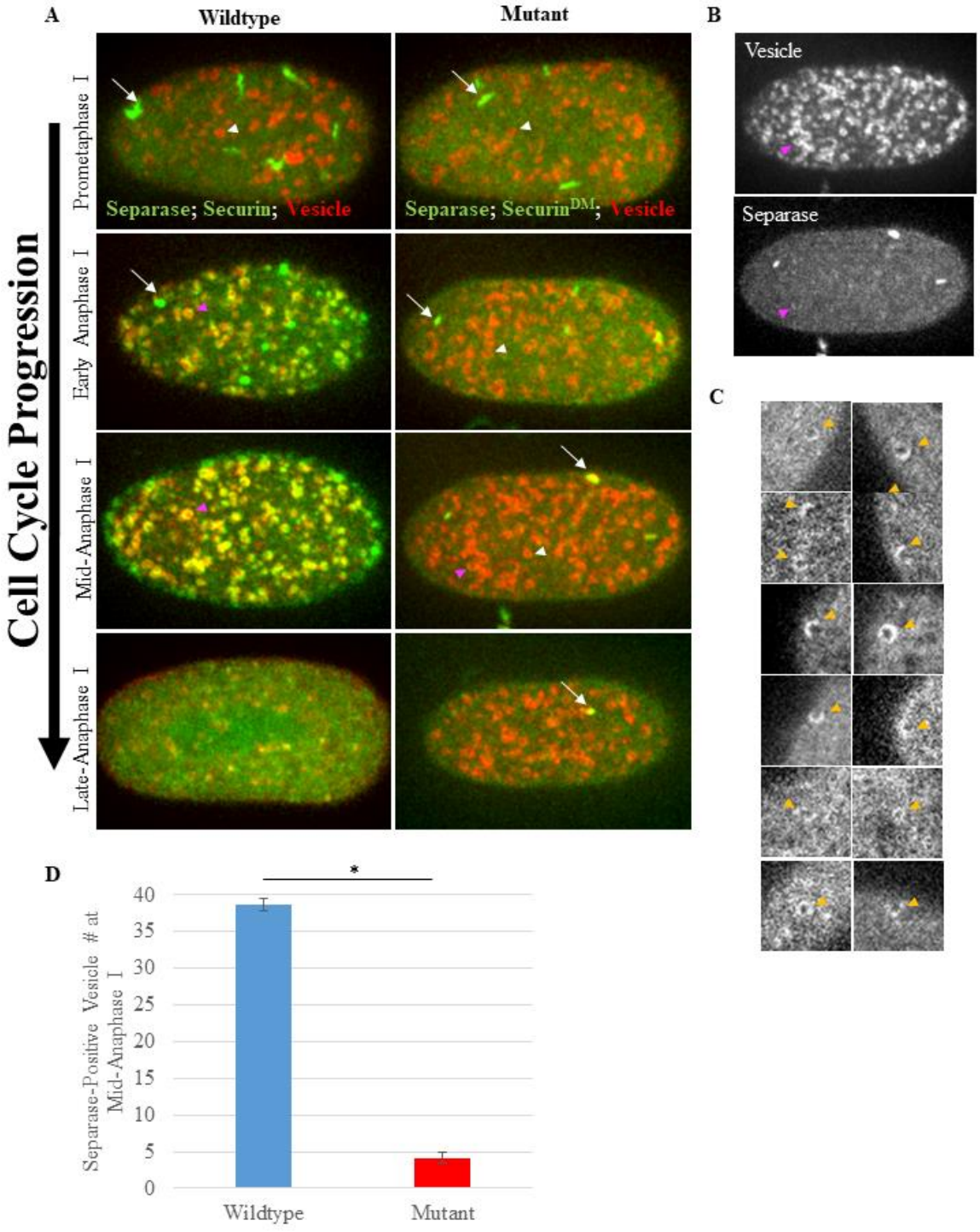
*****There is no ortholog of MPS-1 present in *C. elegans*. In *C. elegans*, PLK-1 appears to substitute for MPS-1 function (Espuet *et al.*, 2015), but we have not attempted to determine if PLK-1 localizes to cortical filaments.

Classification	Gene	Comments	Reference
Inhibitory chaperone	ify-1		Wang et al. (2013)
Protease	*sep-1*		Bembenek et al. (2007)
SAC	*czw-1*	RZZ Complex	(This Study)
SAC	*rod-1*	RZZ Complex	Pereira *et al.* (2018)
SAC	*bub-1*	Important for MCC formation	This Study
SAC	*dnc-1*	Dynactin	This Study
Outer Kinetochore	*him-10*		Howe *et al.* (2001)
Outer Kinetochore	*knl-1*		Monen *et al.* (2005)
Outer Kinetochore	*klp-19*		Pelisch *et al.* (2017)
Outer Kinetochore	*mel-28*	A kinetochore-localized nucleoporin component and member of the nucleoporin Y-complex, which is an important core component of animal nucleoporins	Hattersley *et al.* (2016)
Outer Kinetochore	*ndc-80*		Monen *et al.* (2005)
Outer Kinetochore	*mps-1**	Observed in fly oocytes	Gilliland *et al.* (2007)
Outer Kinetochore	*csl-2*	A CLASP, which is important for microtubule stabilization at kinetochores	Schlientz & Bowerman (2020)

I suspected Securin^{DM} interferes with the ability of Separase to localize to CGs during anaphase I. I hypothesize that GFP::Securin^{DM} will inhibit cortical granule exocytosis by blocking Separase vesicle localization. To address this hypothesis clarifying the relationship between Separase CG localization and Securin, I conducted *ex utero* live cell imaging of the cortical-most cytoplasm using our wildtype or GFP::Securin^{DM} expressed with endogenous Separase::mScarlet and CG marker CAV1::GFP (Fig. 21A). Anaphase I was subcategorized to reflect novel insight gained into wildtype localization dynamics of Separase (refer back to Fig. 7 for details). Consistent with our observations that GFP::Securin^{DM} exerts its effects in anaphase, Separase::mScarlet localization with respect to KFs and CGS in prometaphase I were identical in the presence of wildtype GFP::Securin and GFP::Securin^{DM} (Fig. 21A).

At the onset of anaphase I, Separase::mScarlet begins to display radically different localization patterns when in the presence of mutant GFP::Securin^{DM} compared to wildtype GFP::Securin. During early anaphase I in wildtype, Separase localizes to both CGs and KFs in the cortex (Figs. 7F,21A). In contract, in the presence of GFP::Securin^{DM}, Separase does not appear to localize to CGs (Fig. 21A). By mid-anaphase I in wildtype, Separase is no longer on filaments and has completely transferred from them onto CGs (Fig. 21B). Interestingly, Separase localization to CGs appears perturbed and reduced in the mutant at this timepoint, (Fig. 21B,C). By mid-anaphase I, Separase::mScarlet signal is entirely vesicular at the cortex, and only residual signal is picked up at the same timepoint in the mutant. By late anaphase I, cortical granules have been exoctyosed in wildtype, though some unknown signal resides in the cytoplasm which could be endosomes (Fig. 21A; Sato *et al.,* 2006). And in stark contrast, and consistent with our observations that GFP::Securin^{DM} blocking cortical granules exocytosis, CAV1::GFP is retained

Figure 21. Separase localization to cortical granules is perturbed during anaphase I the presence of GFP::Securin^{DM}. Representative cortical z-projections of meiosis I (**A**) in embryos expressing Separase (green) and the CG marker Caveolin (red) with wildtype GFP::Securin (red, left column) and mutant GFP::Securin^{DM} (red, right column). In prometaphase I, Separase localizes to KFs (white arrows) and is not present on CGs (white arrowheads) in both conditions. In early anaphase I, KFs in both conditions become distorted from the prometaphase I appearance (white arrows). In wildtype, Separase begins to accumulate on CGs in the posterior of the cell (purple arrowhead), whereas in the mutant no detectible Separase signal is observed (white arrowhead). Interestingly, Separase and Securin colocalization to KFs becomes more noticeable beginning at this timepoint in the mutant. By mid-anaphase, in wildtype Separase is localized to CGs throughout the cortex and absent from KFs. Residual Separase signal is detected on vesicles (**A**, right column; **B**). Examples of Separase localized to CGs in the presence of Securin^{DM} (**C**); Securin^{DM} does not localize to CG (not shown).By late anaphase I CGs have been largely exocytosed whereas are retained in the mutant condition. Quantification of Separase-positive CG signal in wildtype vs. mutant GFP::Securin^{DM} (**D**). The number of Separase-labeled CGs is significantly reduced in the mutant condition compared to wildtype ($p= 3.065x10\text{-}16$, t-test). Asterisk denotes statistically significant difference ($p<0.05$).


A
Wildtype
Mutant
Cell Cycle Progression
Prometaphase I
Early Anaphase I
Mid-Anaphase I
Late-Anaphase I
Separase; Securin; Vesicle
Separase; Securin^DM; Vesicle
B
Vesicle
Separase
C
D
Separase-Positive Vesicle # at Mid-Anaphase I
40
35
30
25
20
15
10
5
0
*
Wildtype
Mutant

in the embryo in the presence of GFP::Securin^{DM} in late anaphase (Fig. 21A). The observation that Separase::mScarlet-GFP::Securin^{DM} filaments persist in our mutant background is reminiscent of severe APC/C RNAi phenotype I have previously observed, locking Separase and Securin on kinetochore structures in the spindle and cortical regions (data not shown), and this is a phenotype desirable to revisit. I conclude that Securin degradation is required for Separase to localize to CGs. Taken together, these results suggest that the M phase checkpoint, specifically the APC/C pathway through Securin degradation promotes Separase function during both chromosome segregation and cortical granule exocytosis during meiosis I. Furthermore, while APC/C activity is globally required for Separase localization to its sites of action at the spindle and cortex in anaphase I, Securin degradation is not necessary for localization of Separase to MR, but Securin degradation appears necessary for proper localization of Separase to GCs. This points to an interesting, complex regulatory regime by which chromosome segregation and vesicle trafficking are coordinated through the SAC-APC/C to ensure successful chromosome segregation, cytokinesis, and vesicle trafficking.

Chapter 4

Discussion

The regulatory pathway governing the activation of the protease activity of Separase required for chromosome segregation in both mitotic and meiotic M phase is well established. Additionally, the primary mechanism by which Separase is rendered enzymatically inactive is also well understood to be conferred by its chaperone, Securin, which binds the protease domain of Separase and acts as a pseudosubstrate inhibitor Nagao & Yanagida, 2006; Luo & Tong, 2018). From *in vitro* work, it is understood that Separase becomes enzymatically active when Separase is liberated from Securin by APC/C- mediated destruction of Securin.

Previous work in our lab established several lines of novel insights (Bembenek *et al.,* 2007; Bembenek *et al.,* 2010; Mitchell *et al.,* 2014; Bai & Bembenek, 2017): First, we established the *C. elegans* meiotic embryo as the first system to visualize Separase localization in real time at high spatiotemporal resolution, making our system especially unique in its ability to precisely correlate its localization with enzymatic activity and function. Second, Separase localization is dynamic and appears to correspond to cell cycle progression. During prometaphase I when, biochemically, the APC/C is considered inactive, Separase is associated with kinetochore-based structures in the spindle region and the cortex, kinetochore cups and kinetochore filaments, respectively. During anaphase I, Separase transfers from kinetochore cups to the midbivalent where it will promote chromosome segregation. At the same time, at the cortex, Separase transfers from the kinetochore filaments to cortical granules to promote their exocytosis. Third, we have characterized a separation-of-function allele of Separase, *sep-1(e2406),* which has reduced localization to cortical granules but minimal chromosome segregation defects (Siomos *et al.,* 2001; Bembenek *et al.,* 2007). One implication of this separation-of-function allele of Separase is that its localization to cortical granules and spindle are regulated by distinct mechanisms. Fourth, Separase^{PD} behaves dominant negatively over wildtype Separase during

anaphase I, inhibiting both chromosome segregation and cortical granule exocytosis (Mitchell *et al.,* 2014; Bai & Bembenek, 2017). Lastly, we showed that a subset of cycle regulators that function during the M phase checkpoint to control proper activation of Separase for chromosome segregation, differentially affect cortical granule trafficking. Securin stood out among these, whose depletion by RNAi phenocopied the retention of cortical granules seen following *sep-1* RNAi. Given the striking contrast in Separase localization in metaphase I where it is biochemically defined as inactive and anaphase I where it is defined as catalytically active, we hypothesized that the regulatory pathway first described to control the protease activity of Separase also controls its localization from kinetochore-based structures in metaphase to sites of action at the midbivalent and at the cortex in anaphase I.

If Securin is regulating Separase localization and function during meiosis I, we would expect Securin and Separase to display similar localization patterns prior to anaphase I. Securin localization was previously reported in worms (Wang *et al.,* 2013), but whether Securin localizes to similar structures to Separase was not reported. After carefully scrutinizing Securin localization during meiosis I, we determined that Separase and Securin colocalize and remain associated with each other on kinetochore-based structures up until anaphase I: kinetochore cups at the spindle and kinetochore filaments at the cortex.

In anaphase, it has been reported that Separase localizes to the midbivalent and cortical granules (Bembenek *et al.,* 2007; Muscat *et al.,* 2015). Strikingly, and previously unreported and coincident with chromosome segregation, Separase and Securin signal rapidly move from the kinetochore cups to the midbivalent at anaphase I onset. The midbivalent is where Cohesin is localized, which Separase must destroy to promote chromosome segregation (Severson &

Meyer, 2014). We observed wildtype and Securin^{DM} signal persisting at the midbivalent at anaphase I onset. In wildtype, the time delay between Separase-Securin localization and chromosome segregation is statistically insignificant and dependent upon APC/C activity. To date, the molecular transition states between the time APC/C-CDC20 recognizes Securin and when Separase becomes proteolytically active toward Cohesin has not been addressed. The nature of these transition states is elusive. Addressing these molecular transition states leading up to chromosome segregation are beyond the scope of my project. Addressing these transition states would likely yield fundamental insights into the ubiquitin-proteosome system and how it operates at the same time in different regions of the cell to help ensure faithful chromosome segregation and vesicle exocytosis. It is interesting to speculate that the *C. elegans* meiosis I embryo could be used as a powerful model to address discrepancies in the literature regarding the *in vitro* kinetics of Separase activity. For instance, Sun *et al.,* (2009) found that Separase becomes catalytically more efficient towards the mitotic Cohesin subunit SCC1, with cleavage efficiency increasing in a DNA and DNA sequence length-dependent manner. It was argued by Sun *et al.,* (2009) that Separase cleavage of SCC1 cleavage requires DNA. It is unlikely that DNA itself directly plays a role in cortical granule exocytosis. Proteases contain exosites, which are distinct from their proteolytic domain, and are important for interacting for substrates and enhancing substrate cleavage (Jabaiah *et al.,* 2012). Using human cells, Rosen *et al.,* (2019) showed that the Kleisin subunit SCC1, destruction of which is required for mitotic chromosome segregation, has a motif distinct from its Separase cleavage site. This site on Cohesin is important for its destruction by Separase. This suggests that Separase contains a substrate docking sequence (exosite) outside of its protease domain. Securin impedes this putative exosite on Separase, which is required for enhanced Cohesin cleavage (Rosen *et al.,* 2019). Whether

exosites are present in *C. elegans* Separase is unknown. Our previous work has shown that Separase^{PD} can associate with cortical granules (Mitchell *et al.,* 2014; Bai & Bembenek *et al.,* 2017). My work suggests that Securin^{DM} blocks Separase from associating with cortical granules during anaphase I (Fig. 21). Whether Separase associates with cortical granules through its protease domain, through a putative exosite, or through a combination of both sites remains to be determined. It would be interesting to eliminate the active protease domain of Separase to determine whether it is still able to associate with cortical granules. Such an experiment would help clarify whether or not its protease domain or some other site is required for association with vesicles.

Following anaphase I onset, early anaphase I is marked by a period of previously undescribed Separase and Securin localization dynamics. Early anaphase I appears to involve a transition period, the beginning of which coincides with anaphase I onset and lasts approximately one minute at 20°C. The early anaphase I transition period is characterized by the localization of Separase overlapping with its previously documented prometaphase I and anaphase I localization pattern (Bembenek *et al.*, 2007; Bai *et al.*, 2017; Muscat *et al.*, 2015). At the beginning of this transition period, Separase localizes to the midbivalent region and cortical granules in the anterior portion of the cell where the spindle is located (Fig. 7E). On the opposite end of the cell, on the posterior side where fertilization occurs, Separase remains on kinetochore filaments, as does Securin (Fig. 7E,K). As anaphase I progresses, at the cortex, Separase localizes to cortical granules coincidently with loss from the kinetochore filaments in an anterior to posterior polarity. The end of this transition period is marked by the absence of Securin signal from the spindle region and kinetochore filaments (Fig. 7L; Fig. 8F). Importantly, the end of this

transition period in early anaphase I is marked by Separase becoming localized to where it needs to function at the midbivalent region and cortical granules (Fig. 7F; Fig. 8F). Interestingly the localization pattern of Separase reported here as it transitions from early to mid-anaphase structures in an anterior to posterior manner is reminiscent of the wave of cortical granule exocytosis that initiates in the spindle region and propagates through the cell cortex in an anterior to posterior polarity. Collectively these observations suggest that by the time Separase is active at the spindle and cortex, Securin has been degraded. Because Securin is a direct target of the APC/C, which governs the metaphase-to-anaphase transition following SAC satisfaction, we hypothesized that APC/C activity is required for Separase to localize to the midbivalent and cortical granules. Following inhibition of the APC/C, we noticed that Separase and Securin are retained on kinetochore-based structures at the spindle and cortex. This implicates the APC/C in a completely novel role in positively regulating Separase localization, in addition to its activity. Entry into anaphase I is thus required for Separase to transition from kinetochore-based structures to sites of function during anaphase I. Interestingly, during anaphase I while we do observe Separase-Securin colocalization on the midbivalent, we do not observe Separase and Securin colocalizing to cortical granules. In contrast to the spindle region during anaphase I, Separase transfers from kinetochore filaments, without Securin, to cortical granules. This suggests an intriguing structural relationship whereby some interface of Separase-Securin is capable of associating with kinetochore proteins, though this Separase-Securin renders Separase incapable of localizing to cortical granules. One possibility is that Securin directly interferes with Separase associating with vesicles via the catalytic domain. In line with this logic, we have previously reported that Separase^{PD} is able to localize to vesicles when Securin is degraded, though the exocytosis of these vesicles is delayed (Bai & Bembenek, 2017).

Our results strongly implicate the APC/C beyond its role in promoting the activation of the catalytic activity of protease domain of Separase, but also its localization to sites of action. The precise mechanism downstream of the APC/C involved in recruiting Separase to the midbivalent and cortical granules is elusive. My data suggest that Securin degradation is necessary, but not sufficient for Separase localization to vesicles (Fig. 10B). The substrate the APC/C targets for this transfer process is unknown. From studies in yeast, it is known that Securin is required to recruit Separase to spindle poles, but much less is known about how it localizes to the spindle in metazoa, kinetochores, and kinetochore filaments in the case of worm oocytes. There is a dearth of information regarding mechanisms governing Separase localization in all systems, including all the recent documented activities Separase is now known to engage with (Zhang & Pati, 2017). We now implicate the APC/C in regulating the localization of Separase, specifically by promoting the transfer of Separase from kinetochore-containing structures to sites of action during anaphase I.

We have also documented members of the spindle assembly checkpoint, an effector of the motor protein Dynein, and kinetochore proteins to localize to kinetochore filaments (Table 1). To date, we found no reports of inner kinetochore proteins localizing to kinetochore filaments. A more a more systematic analysis should be done to delineate the origins of these filaments, as kinetochores are composed of over 100 proteins in vertebrates (Samejima *et al.,* 2015). Kinetochore formation depends on DNA sequence as in yeast, or the centromeric the centromeric histone variant, CENP-A (McKinley & Cheeseman, 2015; Musacchio & Desai, 2017). Currently, CENP-A has not been reported or observed at kinetochore filaments (Monen *et al.,* 2005; our unpublished observations). Whether these kinetochore filaments can elicit the formation of the mitotic checkpoint complex remains to be determined. We do observe that BUB-1 localizes to

cortical filaments, including those associated with the nuclear envelope, and BUB-1 is important for facilitating MCC formation (Musacchio & Salmon, 2007; Overlack *et al.,* 2015). The outer-most region of the kinetochore is the diffuse corona, which expands to promote microtubule capture and proper chromosome orientation during mitosis (Sacristan *et al.,* 2018). In *C. elegans*, BUB-1 has been shown to shown to associate with RZZ, and RZZ is capable of polymerizing into filaments to promote the expansion of the kinetochore when microtubules are not attached to kinetochores (Pereira *et al.,* 2018; Rodriguez-Rodriguez *et al.,* 2018). The reason why kinetochore filaments are present at the same time as kinetochore cups in meiosis I remains to be determined. One hypothesis is that these kinetochore filaments serve as means by which chromosome segregation and vesicle exocytosis are coordinated at the same time to ensure faithful cell division. While not studied in detail, kinetochore filaments are localized near cortical microtubules, though whether populations of cortical microtubules are capable of association with these filaments remains to be determined.

How could these cortical filaments form in absence of inner kinetochore structures? The fact that kinetochore formation is documented to depend on DNA sequence or protein sequences suggests some flexibility in the formation of structures that could theoretically generate the MCC signal. Intriguingly, it has recently been documented that Kinetoplastid *Trypanosoma brucei* builds its kinetochores without CENP-A, so there is clearly much to learn about how kinetochores or kinetochore protein-containing structures can form in the cell (Akiyoshi & Kull, 2014). Even more recently, and consistent with the emergence of non-canonical kinetochore assembly, it was found that kinetochores can also form without CENP-A in invertebrates (Cortes-Silva *et al.,* 2020). The exact composition of kinetochore filaments and how they form is unknown. My unpublished observations about the biogenesis and dynamic nature of kinetochore filaments may

provide clues to their origins. In worm oocytes, kinetochore filaments appear to form after egg activation but before nuclear envelope breakdown (NEBD) in meiosis I. Initially, there are many small puncta that appear in the cytoplasm, and they rapidly mature into larger filaments. There is also similarity between what we call kinetochore filaments in the worm oocyte and structures called cytoophidium, which were first documented in bacteria (Liu, 2016). Cytoophidium, or "cellular snakes" are filamentous aggregates of the metabolic enzyme cytidine triphosphate synthase (CTPS). This enzyme is required for *de novo* CTP synthesis, which was recently reported by multiple groups to form cytoophidium in fly oocytes (Liu, 2016). Interestingly, it has been reported that the outer kinetochore protein MPS1 forms filamentous assemblies in fly oocytes, and these appear similar to CTPS and what we observe as kinetochore filaments in worms (Liu, 2010; Gilliland *et al.,* 2007). These CTPS filaments have been reported across phyla: bacteria, fungi, and eukaryotes (yeast, flies, mice, and human cell culture (Liu, 2016). The functions of these structures are largely unknown. With respect to CTPS, as reviewed by Liu (2016), it is believed to serve roles related to 1) cytoskeleton function/cell shape, 2) metabolic control, 3) metabolism buffering, 4) protein stabilization, 5) cell proliferation, 6) developmental switches, 7) stress coping, 8) metabolic channeling, 9) intracellular transport, and 10) nuclear compartmentalization. The kinetochore filaments could be based on the RZZ aggregates. It was reported recently that ROD-1, a member of the SAC, polymerizes into cytoplasmic aggregates in worms (Pereira *et al.,* 2018). Cytoplasmic compartmentalization of kinetochore and more broadly mitotic checkpoint proteins is an unexplored phenomenon. It is speculated that ROD-1 protein aggregates in the cytoplasm could be related to the expansion of the kinetochore corona, which is important for microtubule capture and general SAC signaling (Table 1; Suzuki & Varma, 2018). Based on our studies, the kinetochore filaments may regulate membrane events in

the cytoplasm. Interestingly, we observe the RZZ component, CZW-1 (Zw10 in flies) on kinetochore filaments (Table 1). Preliminary, unpublished observations suggest that Separase does not localize to kinetochore filaments following depletion of *czw-1* by RNAi, though it is retained on kinetochore cups in worm oocytes (data not shown). Interestingly, of eight SAC proteins I knocked down, only CZW-1 prevented Separase from localizing to cortical filaments. Future work will be needed to clarify and address the relevance of these observations. The RZZ complex could recruit Separase and Securin localize to kinetochore filaments and have a role in moving separase to cortical granules. Microtubules are closely associated with kinetochore filaments by staining (Monen *et al.,* 2005), though their dynamic behavior relative to each other has not been studied. We have observed a co-factor of Dynein, Dynactin, at kinetochore filaments during prometaphase (Table 1). Our unpublished observations of Separase structure suggest it could be farnesylated (Boland *et al.,* 2017). In the context of the SAC, farnesylation is important for allowing Dynein to recognized RZZ and subsequently silence SAC signal. Our separation-of-function allele, e2406, does not localize to cortical granules efficiently, and the mutation in this allele is near a residue capable of being farnesylated. Perhaps this residue is important for interaction with Dynein and recruitment to cortical filaments. These preliminary observations suggest that an active motor-dependent transport mechanism could be involved in Separase localization and movement between kinetochore filaments and cortical granules.

Nevertheless, our implication of the APC/C in controlling Separase localization suggests the possibility that Securin degradation is necessary for Separase to localize from kinetochore structures of sites of function. A previous study using *C. elegans* suggested that Separase is not able to localize to cortical granules following *securin* RNAi, which they attributed to compromised folding of Separase (Kimura & Kimura, 2012). However, little background was

given regarding how this experiment was performed, such as optimizing for *securin* RNAi, sample size, and image quality. We were surprised to find that under mild and intermediate *securin* RNAi conditions that Separase localizes to and remains on cortical granules that are not exocytosed. In preliminary observations from egg activation following intermediate *securin* depletion we noted that Separase was able to localize to the spindle region, though it was difficult to determine which structures these were, and we were struck that Separase showed strong localization to kinetochore filaments. While it was shown that Securin is required for Separase to localize to the spindle pole bodies in yeast, our observations suggest that Securin is not required to localize to kinetochore filaments in the cortex. In independent oocytes from four individual worms, we observed that Separase transferred from kinetochore filaments to vesicular structures prematurely at ovulation, which never occurs in wildtype (Fig. 10A). Whether Separase becomes catalytically active at this time remains to be determined. Nevertheless, this observation is consistent with recent studies from mouse oocytes, where APC/C degrades its substrates in prometaphase I before anaphase onset. It was found that the pre-anaphase I degradation of excess APC/C substrates is important for the timely execution of anaphase I events (Levasseur *et al.,* 2019; Thomas *et al.,* 2021). Our observation fits a substrate competition model, whereby partial depletion of APC/C substrates results in advance destruction of all substrates and precocious cell cycle events. Based on these data, I conclude that there is strong reason to believe that Securin degradation controls the timing of Separase localization and function in meiosis.

APC/C associates with its co-activator, CDC20, to promote the destruction of Securin and Cyclin B during anaphase I. In this way, APC/C activity leads to chromosome segregation, the hallmark of anaphase, and exit from the cell cycle, respectively. In the absence of APC/C activity,

Separase and Securin remained trapped on two important types of kinetochore-based structures: the kinetochore cups at the spindle and kinetochore filaments at the cortex. This led us to the hypothesis that the APC/C may target Securin for destruction in order to promote the localization of Separase to the midbivalent and cortical granules so that it can carry out its anaphase I functions. Unexpectedly, this did not appear to be the case as simultaneous inhibition of the APC/C and depleting *securin* using intermediate RNAi conditions did not enable Separase localization to vesicles. We conclude that the APC/C has a positive role in promoting Separase localization from the filaments to vesicles in anaphase independently of Securin.

APC/C also associates with Cyclin B; however, there are multiple Cyclin B homologs in worms which function partially redundantly with one another. As a consequence this makes teasing apart these interactions difficult. Given that Separase localization and its association with activity in the cell cycle is a relatively novel area of study, not only finding substrates Separase acts upon will be of high interest, but so will finding what factor or factors responsible for Separase to localize to different areas of the cell will be of high interest and further elucidate the biology of this important protease. A recent *in silico* study expanded the pool of APC/C substrates, implicating this ligase in variety of cellular phenomena, such as chromatin remodeling (Frank *et al.*, 2020). Of interest, one of these substrates, a RhoGTPase, has been implicated in cytokinesis in human cells, suggesting a potential role of the APC/C in vesicle trafficking (David *et al.*, 2014).

We have established the novel connection of the APC/C in promoting Separase localization and subsequent function in anaphase I. This would not be the first time the APC/C has been documented to have a positive role in the cell cycle, as it is known for promoting anaphase and as promoting meiotic spindle rotation via suppression of CDK1-Cyclin B inhibitory

phosphorylation of Dynein in worm oocytes (Ellefson & McNally, 2011; Crowder *et al.*, 2015), and an established conserved roll in exit from M phase. Securin itself has been established itself as a dual regulator of Separase in a positive role in protein folding and negative role in pseudosubstrate inhibition (Horning *et al.*, 2002). Our observations suggest that Securin provides an additional cellular function related to cell cycle progression. During prophase I arrest, we observe Securin existing in two compartments in the oocyte: the cytoplasm and nucleus (Fig. 7G). This is in contrast to Separase, which is present in a single compartment, the cytoplasm (Fig. 7A), and this suggests the possibility that Securin is present in molar excess to Separase. In this context, Securin could function as a competitive substrate with other known APC/C substrates. It has been observed that in mouse oocytes, APC/C substrates like Cyclin B and Securin are expressed in excesses to their bound counterparts (e.g. the Securin-Separase complex; Levasseur *et al.*, 2019; Thomas *et al.*, 2021). In the context of mouse oocytes meiosis, Cyclin B and Securin are degraded before anaphase I-onset, and this is important for the fidelity of cell division by preventing aneuploidy. Consistent with these mouse studies, wildtype Securin begins to be degraded before anaphase I onset in *C. elegans* (Wang *et al.*, 2013; Fig. 14).

Through careful analysis we established that expression of our GFP::Securin^{DM} does not exert any negative effects on the cell cycle until the onset of anaphase I. Securin began exerting its effect during anaphase I, in which there was significant delay in chromosome segregation compared to wildtype (Fig. 18). We also observed that cortical granule exocytosis is significantly blocked during anaphase I when GFP::Securin^{DM} is expressed (Fig. 19). We were particularly struck by the stark contrast between Separase and Securin^{DM} localization dynamics at the spindle and cortex. We noticed that Securin and Separase colocalized to the midbivalent at the same time following spindle rotation. This indicates that while Securin interferes with the protease activity

of Separase, at least at the spindle, the Separase-Securin complex can localize to the midbivalent. In contrast, we did not observe appreciable colocalized signal of Separase::mScarlet with GFP::Securin^{DM} on vesicles.

Ultimately, how Separase can localize to the midbivalent in the presence of Securin but not localize to vesicles, is an open question. Recent work from our lab has identified a novel motif in the N-terminus of Separase, which is conserved in metazoa, though its functional significance remains unexplored (Melesse *et al.,* 2018). Perhaps this motif corresponds to an exosite, in which case mutating these conserved residues could perturb Separase localization to the vesicles, the midbivalent region, or kinetochores-containing structures. We now have near-atomically resolved structures of Separase in multiple systems, including worms and humans (Boland *et al.,* 2017; Yu *et al.,* 2021). Interestingly, novel autoinhibitory loops intrinsic to human Separase are induced by its binding to CDK1-Cyclin B. Whether such autoinhibitory loops are present in worm Separase remains to be determined.

The novel insights we are gaining from structural, genetic, and biochemical studies on Separase suggests we have much more to learn about this critical enzyme (Boland *et al.,* 2017; Melesse *et al.,* 2018; Rosen *et al.,* 2019; Yu *et al.,* 2021) It is of clinical relevance to find ways to control Separase, and our novel insight of a well-defined molecular pathway controlling its localization provides additional ammunition in the fight against infertility and other human maladies, such as epithelial cancer, in which Separase mutations are known to be associated with genomic instability (Shepard *et al.,* 2007). The *C. elegans* oocyte/early embryo offers itself as a unique model for addressing the localization relative to enzyme kinetics of this protease. Important insight into cell division and human maladies is surely going to be gained through further studies

of the molecular pathway controlling the localization of Separase. This work provides a foundation for further work into understanding how the localization of Separase is regulated for chromosome segregation and vesicle trafficking, in addition to how the localization of Separase may be regulated for its emerging cellular roles (Luo & Tong, 2021). Equally as important, this work will provide an important platform for gaining insight into how cell cycle progression, chromosome segregation, and vesicle trafficking are coordinated through a shared pathway to ensure that cell division is executed with high fidelity. It is interesting to speculate that the SAC-APC/C pathway coordinates a chromosome segregation checkpoint with a vesicle exocytosis checkpoint to ensure through tight control of Separase localization and function.

Chapter 5

Materials & Methods

Cloning, Mutagenesis of Securin, and Transgenesis of Worms with GFP::Securin^{DM}

Genomic, wildtype *securin* was cloned from worm genomic DNA via Gibson Cloning (Gibson, 2011). The cloned wildtype *securin* sequence was integrated into the pJK3 plasmid. The pJK3 plasmid allows N-terminal GFP fusion proteins expression via the *pie-1* promoter. Mutant, Non-degradable Securin (Securin^{DM}) was generated from the wildtype *securin* sequence cloned into the pJK3 promoter using the Quickchange mutagenesis kit (Stratagene, La Jolla, CA). The following primers were used for the mutagenesis:

Forward primer (5'->3'):

GGT**GCG**GGGCTGGTTGTAAACTCGTCA

Reverse primer (5'->3'): AGTCGAGTTTACAACCAGCCC**CGC**ACCGCC**AGC**AGAAGG.

Bold, underlined sequences correspond to alanine mutations made within the D-box. Mutant worm lines were transformed with the pJK3 plasmid carrying *pie-1::gfp::securin^{dm}* using microparticle bombardment as previously described (Praitis *et al.*, 2001). Multiple independent GFP::Securin^{DM} lines were generated. Two integrated mutant lines were chosen for analysis: JAB30 and JAB180. The JAB180 strain produced reproducibly high lethality compared to JAB30 (Fig. 15), so JAB180 was used to characterize its effect on meiosis I in worms.

CRISPR/Cas9

Dillon Sloan generated endogenously tagged, wildtype Separase::mScarlet, Separase::GFP, and Separase::mCherry using CRISPR/Cas9 following established protocol (Paix et al., 2015).

I used CRISPR/Cas9 to generate endogenously tagged, wildtype Securin fused to GFP as previously described (Paix et al., 2015). I failed to isolate homozygous N-terminal GFP::Securin fusion, suggesting compromised function when Securin is modifications are made to the N-terminus. However, I was able to isolate a viable homozygous C-terminal fusion: Securin::GFP.

The repair template, which includes a 5 amino acid flexible linker between *securin* and *gfp*, was amplified from pDD282, a gift from Bob Goldstein (Addgene plasmid #66823).

The primer sequences use:

IFY-1::GFP Forward:

ACGACCTCCTCGCCGAAGAAGCCAACCTTCCCCTGGGAGCATCGGGAGCCGGAGCA

TCGGGAGCC

IFY-1::GFP Reverse:

AAACAGGTAGAAGAGGCTGACGTCGTGGGAAATCACTTGTAGAGCTCGTCCATTC

The *ify-1* guide RNA: GACGUCGUGGGAAAUCACAGGUUUUAGAGCUAUGCUGUUUUG

Caenorhabditis elegans Maintenance

Routine *Caenorhabditis elegans* Maintenance

Routine *Caenorhabditis elegans* strains were maintained following standard protocol (Brenner, 1974), except where specified for maintenance of toxic transgenes (see the section "Propagation of Non-degradable Securin Strains" for details). The temperature sensitive line WH495 was maintained at 15°C and upshifted to 25°C for 36 hours for intermediate *securin* RNAi (see "RNAi" section below). Strains used for this study are listed in Table 2. Some strains were provided by the *Caenorhabditis* Genetics Center (CGC). Worm strains were maintained at 20°C,

Table 2. Stains used in this study.

Strain	Genotype
DP38	*unc-119(ed3)III*
JAB13	unc-119(ed3) III; ItIs151 [pSO33; Pcpg-2::cpg-1SigSeq::mCherry-TEV-STag::cpg-2; unc-119(+)] crossed with unc-119(ed3) III; ltIs37 [pAA64: pie-1p::mCHERRY::his-58 + unc-119 (+)]
JAB20	*ItIs151 [pSO33; Pcpg-2::cpg-1SigSeq::mCherry-TEV-STag::cpg-2; unc-119(+)]; unc-119(ed3) III, ojIs58[SEP-1::GFP unc119(+)]; ojIs37[[Ppie-1::H2B::mCherry; unc-119(+)]]*
JAB30	*ify-1(dm)::gfp (pjk3); unc-119(ed3)*
JAB157	*ojEx75(ify-1::gfp); Itls37[pAA64: pie-1p::mCherry::his-59 + unc119 (+)]*
JAB161	*unc-119(ed3) III; ddIs128[ify-1::TY1::EGFP::3xFLAG(92C12) + unc-119(+)]; ltIs37 [pAA64: pie-1p::mCHERRY::his-58 + unc-119 (+)]*
JAB180	*ify-1(dm)::gfp (pjk3); unc-119(ed3)*
JAB181	*unc-119(ed3); ify-1(dm)::gfp (pjk3); ltIs37 [pAA64: pie-1p::mCHERRY::his-58 + unc-119 (+)]*
JAB189	*sep-1(erb-75[sep-1::linker::mCh])*
JAB195	*sep-1(erb-75[sep-1::linker::mCh]); unc-119(ed3) III, ojEx75[IFY-1::GFP unc119(+)]*
JAB197	*sep-1(erb-84[sep-1::linker::mScarlet])*
JAB212	*ify-1(erb-82[ify-1::linker::GFP])*
JAB221	*sep-1(erb-74[sep-1::linker::GFP]); ItIs37 [Ppie-1::mCherry::his-58 (pAA64); unc-119(+)] iv; unc-119(ed3)???*
JAB222	*ify-1(erb-82[ify-1::linker::GFP]); ItIs37 [Ppie-1::mCherry::his-58 (pAA64); unc-119(+)] iv; unc-119(ed3)???*
JAB248	*sep-1(erb-84[sep-1::linker::mScarlet]); ify-1(erb-82[ify-1::linker::GFP])*
JAB259	*sep-1(erb-84[sep-1::linker::mScarlet]); unc-119(ed3), pwIs281[Ppie-1:cav-1:GFP;unc-119(+)]*

Table 2 Continued

Strain	Genotype
N2	*Bristol (wildtype)*
OCF1	ojIs37[[Ppie-1::H2B::mCherry; unc-119(+)]]
OD366	*unc-119(ed3) III; ItIs151 [pSO33; Pcpg-2::cpg-1SigSeq::mCherry-TEV-STag::cpg-2; unc-119(+)]*
RQ372	*unc-119(ed3)III, ojIs58[SEP-1::GFP; unc-119(+)]; 1tIs37{pAA64(Ppie-1::mCherry::his-58); unc-119(+)]}IV*
RQ386	*ify-1 & rpl-22(tm947) unc-4(e120)(II);jzIs63{pRK333{rpl-22.ify-1::LAP)his6+PreScission+wGFP),unc-119+}];1tIs37[pAA64(Ppie-1::mCherry::his-58)]*
TH214	*unc-119(ed3) III; ddIs128 [ify-1::TY1::EGFP::3xFLAG(92C12) + unc-119(+)].*
WH491	*unc-119(ed3) III, ojEx75[IFY-1::GFP unc119(+)]*
WH496	*mat-1(ye121); unc-119(ed3); ojIs58[SEP-1::GFP unc-119(+)]*
WH526	*unc-119(ed3) III, ojIs76[GFP::IFY-1 unc119(+)]*

unless otherwise specified for experiments involving temperature upshifts, toxic transgenes (see subsection "Propagation of Non-degradable Securin Strains" below) and maintenance of temperature-sensitive strains, the latter of which were maintained at 15°C (WH495).

Propagation of Non-degradable Securin Strains

Our lab has devised two methods for controlling the expression of toxic transgenes in worms (Mitchel *et al.,* 2014). The first method involves feeding worms *gfp* RNAi to silence transgene expression. Removal of worms from the *gfp* RNAi-inducing bacteria to the standard feeding *E. coli* for at least 5 generations results in the expression of GFP::Securin^{DM} due to the loss of transgenerational RNAi inheritance. Using this first method, the toxic transgene is expressed as a homozygote when the transgenerational suppression of GFP is lost. All toxic transgenes (JAB30, JAB180, and JAB181) were initially maintained using this strategy. The first method was used to document part of the localization dynamics of GFP::Securin^{DM} during meiosis I (Fig. 13G-J), the stability of GFP::Securin^{DM} during anaphase I (Fig. 14), embryonic lethality (Fig. 15), polar body extrusion defects (Fig. 16B), and eggshell permeability defects (Fig 17B).

The second strategy relies on passaging toxic transgenes through the male germline. Toxic transgenes can be propagated through the male germline for at least 56 generations without deleterious effects using this approach (Mitchell *et al.*, 2014). The pJK3 plasmid containing our mutant includes a selectable marker expressed in males and hermaphrodites allowing the identification of animals with transgenes regardless of expression. This method was used to characterize heterozygous animals for part of the localization dynamics of GFP::Securin^{DM} during meiosis I (Fig. 13K-L), in addition to embryonic lethality (Fig. 15). It was noticed that embryonic lethality associated with heterozygous GFP::Securin^{DM} expression increased to nearly 100% around the 13th generation removed *gfp* RNAi feeding. Once 100% lethality was achieved,

this level of lethality was maintained in proceeding generations. Unfortunately, this phenomenon was not characterized in detail. While unreported here, the trend of increased and then stabilized severe lethality beginning by the 13th generation removed from *gfp* RNAi feeding was reproducible in independently generated lineages passaging the toxic transgene through the male germline. Considering this observation, we used the second strategy to characterize the effects of GFP::Securin^{DM} expression after the 13th generation for our analysis of chromosome segregation (Fig. 16), cortical granule exocytosis (Fig. 19), and Separase localization (Figs. 20,21).

Embryonic Lethality Assays

Embryonic lethality assays were performed for control (N2), multiple wild type GFP::Securin lines, and mutant GFP::Securin^{DM} lines (JAB30 and JAB180). Parental L4 hermaphrodites were maintained at 20°C or upshifted to 25°C and assessed for embryonic lethality after 24 hours and 48hrs. The average embryonic lethality for each strain corresponds to the average embryonic lethality observed in at least three independent experiments. For each independent lethality experiment, lethality corresponds to the average embryonic lethality in a brood produced by 15 worms. Importantly, assessment of homozygous JAB30 and JAB180 embryonic lethality was performed using our first toxic transgene maintenance strategy (see below). Assessment of JAB180 as a heterozygote was performed using our second toxic transgene maintenance strategy (see below). For assessment of homozygous and heterozygous transgene expression, mutant L4 hermaphrodites that were 5 generations removed from *gfp* RNAi were allowed to lay broods in which lethality was assessed, as previously described (Mitchell *et al.*, 2014).

Live Cell Imaging

Live cell imaging data was collected using a spinning disk confocal system with an inverted Nikon Eclipse microscope with a 60 X 1.33 oil objective, a CSU-22 spinning disc system, and a Photometrics EM-CCD camera from Visitech International operated by MetaMorph software (Molecular Devices). Image analysis and manipulation was performed in Fiji (National Institutes of Health) and Adobe Photoshop (Adobe). A combination of *in utero* and *ex utero* live cell imaging was used throughout this project.

Live Cell Imaging of the Hermaphrodite Germline *in utero*

For *in utero* live cell imaging, multiple strategies were systematically compared before we used the optimized nanoparticle-mediated immobilization technique adapted from Kim *et al.,* (2013). Unless otherwise noted (see the RNAi Treatments section), all *in utero* imaging was conducted at room temperature on young adult worms at peak ovulation, which typically happens after L4 hermaphrodites grow for 18-24hrs at 20C°. This technique was used for the acquisition of wild type Separase and Securin localization (Figs. 7,8), the RNAi experiments (Figs. 9, 10) the characterization of wildtype vs. GFP::Securin^{DM} localization during meiosis (Fig. 13), and the Securin degradation curve (Fig. 14). The following is the mounting protocol:

Prepare 8.88% agarose stock tubes

1) Combine 0.08g agarose with 900uL of 1X M9 into 1.5uL centrifuge tube
2) Homogenize solution with vortexer.
    a. This may take a few seconds because agarose tends to clump at the bottom of the tube after you add the M9
3) Dissolve agarose into solution by incubating on a heat block for about 10-15 minutes at 95°C and then set aside for later use.

Important: Do not leave 8.88% agarose solution sitting in the 95°C heat block for a long period of time as the agarose will break down (tip from the Bowerman lab). I tend to remelt my 8.88% solution before I mount worms. It takes about 10minutes to melt. I will then scoop out the agarose I need and set the tube aside in a tube rack at room temperature.

Prepare Agarose Pad

1) Melt 8.88% agarose stock at 95C in a heat block for about 10 minutes
    a. Sometimes there is variation between the stock tubes: some have bubbles in them, and others appear as uniform in texture. I have not noticed an impact on worm immobilization and the appearance of the melted state of the molten agarose. If the agarose turns gold/brown, discard the tube because worms will not immobilize.
2) Scoop 8.88% agarose out of the stock tube, place a pea-sized blob in the center of the slide
3) Compress/flatten by placing a second slide on top of it propped up with a spacer made of a stack of four slices of lab tape wrapped around two supporting slides placed on each side of the slide containing the agarose.
4) Wait to cool (~30 seconds) and remove the compressing slide

Mounting Worms

1) Place equal volumes of polystyrene beads (2.5% by volume, 0.1um diameter) and 1X M9 onto the center of the agarose pad

a. I usually use 3uL of beads and 3uL of M9
b. I get the best mounts when first applying the beads AND THEN the M9 (application of M9 disperses the beads better).

2) Place worms in the 1:1 polystyrene bead:M9 solution
    a. Add worms quickly, before the solution dries up.
        i. If the solution becomes dark brown, then a new agarose pad should be prepared (i.e. start over at step 1).
        ii. I usually place at between 10-15 worms in the solution on the pad
            1. Try to make sure worms are spaced apart and not touching each other. I have not documented this, but if worms are laying on top of each other, immobilization may be partially compromised when the coverslip is applied (see step 3).
3) Place coverslip over agarose pad
    a. I rest the tip of part of a needle on part of the agar pad, then I place the coverslip on the agar pad (the needle prevents the worms from being compressed/immobilized). I then watch the worms wiggle around and when most of them appear to be aligned straight, I quickly slide the needle out from under the coverslip. Worms are now immobilized. This has the added benefit of more gentling immobilizing the worms than just dropping a coverslip on the agarose pad.
4) Seal slides with Vaseline

Live Cell Imaging *Meiosis I* Embryos *ex utero*

Before the formation of the eggshell, meiotic embryos are especially fragile to the chemical environment and mechanical perturbations (Stein & Golden, 2018). To image meiotic embryos and those with an OID phenotype, like GFP::Securin^{DM} (Fig. 18B), chemical and mechanical and osmotic pressure must be reduced. This was achieved by dissecting embryos from hermaphrodites in blastomere culture media using the hanging drop mounting technique (Edgar & Goldstein, 2012). For all *ex utero* imaging reported here, L4 hermaphrodites were shifted from 20°C to 25°C for 18-24hrs until they reached peak ovulation in young adulthood. Documentation of chromosome segregation defects associated with GFP::Securin^{DM} was conducted at room temperature as previously described (Fig. 16; Bai & Bembenek, 2017). However, documentation of GFP::Securin^{DM} during cortical granule exocytosis (Fig. 19) and Separase localization (Figs. 20,21) was conducted at 25°C. This change in approach was made after realizing that downshifting worms from 25°C to room temperature significantly affected cell cycle timing. Downshifting worms from 25°C to room temperature was sufficient to delay the timing cortical granule exocytosis in wild type, compared to what we initially observed to be its duration at 25°C (not reported here; Bembenek *et al.*, 2007). Despite the initial downshift associated with dissecting embryos at room temperature, the cell cycle was restored to normal timing when the *ex utero* mount was placed on the microscope stage maintained at 25°C.

During *ex utero* imaging, cortical granules exhibit autofluorescence in the green channel when we expressed our wild type and mutant transgenic GFP::Securin lines (JAB180 and WH526, respectively). Using settings we determined to be optimal for resolving GFP::Securin^{DM} on kinetochore cups and filaments during prometaphase I, the GFP autofluorescence signal could be eliminated by photobleaching with approximately 13 pre-exposures with the 488 laser. This was

especially important for documentation of the effects of wildtype and GFP::Securin^{DM} during cortical granule exocytosis (Fig. 19) and Separase localization during anaphase (Figs 20,21) because failure to eliminate GFP autofluorescence would have led to the false conclusion that wildtype and GFP::Securin^{DM} localize to cortical granules. Importantly, the autofluorescent signal appears to fill the lumen of the vesicles, while the GFP signal of our relevant markers is likely on the cytosolic side of the membrane, which appears as hollow donut-shaped vesicle structures.

RNAi Treatments

RNAi of *gfp* was induced by feeding as we have previously described (Mitchell *et al.*, 2014). To knockdown the target genes used in this study (*gfp, fzy*-1, and *ify-1*), L4 hermaphrodites were picked to plates containing the desired IPTG-induced RNAi-expressing bacteria. To ensure optimal RNAi feeding, RNAi bacterial strains were grown until they entered log-phase growth. The bacteria were then spread on plates containing 1 mM IPTG and left to grow for 48hrs at X°C prior to use because this allows the T7 promoter that drives RNAi to become optimally induced (Grishok *et al.,* 2005). The penetrance of *fzy-1* and *ify-1* RNAi induction by feeding was carried out at 20°C and 25°C, and the phenotypes were scored after 24, 36 and 48hrs after L4 hermaphrodites had been placed on the RNAi feeding plates.

Documentation of terminal phenotype of APC/C arrested embryos has been described in worms (Shakes *et al.,* 2003). One indicator of a severe APC/C-null phenotype is the presence of an entire uterus containing single-celled embryos arrested in a prometaphase I-like state. Using *fyz-1* RNAi, I noticed that I could achieve this phenotype between 24hr-36hrs of feeding worms

using the *fzy-1* RNAi vector at 25°C. *fzy-1* RNAi feeding was thus conducted after a 24-36hr RNAi upshift to 25°C and imaged at room temperature by *in utero* live cell imaging.

Knockdown of *ify-1* (*securin* in worms) by RNAi has been conducted previously in worms and a terminal phenotype is associated with a uterus full of single, multinucleated cells (Sönnichsen *et al.*, 2005). I assessed the penetrance of *ify-1* RNAi using the JAB20 strain (Separase::GFP; H2B::mCherry; CPG-2::mCherry). In order to account for the possibility of Separase becoming prematurely activated following *ify-1* RNAi, I analyzed oocytes and embryos using the *in utero* mounting method. In assessing *ify-1* RNAi penetrance, I noticed that I was able to achieve a more penetrant *ify-1* RNAi phenotype when upshifting and feeding worms at 25°C. I decided to characterize the penetrance of *ify-1* more systematically at 25°C. I was able to document three categories of severity: *mild*, *intermediate*, and *severe*.

Mildly penetrant *ify-1* RNAi (24hrs of RNAi feeding at 25°C) was associated with high but not 100% embryonic lethality on the feeding plate, the presence of multicellular and unicellular embryos within the uterus, and the presence of Separase on cortical granules that failed to exocytose in 1-2 of the youngest fertilized embryos. Based on CPG-2::mCherry, the older multicellular and some unicellular embryos had a CPG-2 layer of the eggshell, indicating that Separase was still capable of being activated under this condition (not quantified).

The *intermediately* penetrant *ify-1* RNAi phenotype (36hrs of RNAi feeding at 25°C) was associated with early 100% embryonic lethality, the majority of embryos in the uterus being unicellular and multinucleated, and Separase-labeled cortical granules could be found in 1-4 of the youngest fertilized embryos (data not shown). Based on CPG-2::mCherry localization, few if any older embryos possessed a CPG-2 layer, indicating that the function of Separase is significantly compromised under this condition.

The *severe ify-1* RNAi phenotype was associated with 100% embryonic lethality, decreased Separase cytoplasmic signal and no clear indication of Separase localization to cortical granules (data not shown). The latter two observations suggest that the chaperone function of Securin has been sufficiently compromised by this time. Due to the severity of this phenotype, the *intermediate* RNAi conditions were chosen as severe enough to gain insights into the effect of *ify-1* RNAi on Separase localization.

Pilot Experiments: Polar Body Extrusion and Osmotic Sensitivity Assays

The polar body extrusion assay was performed using embryos dissected from mothers homozygous for H2B::mCherry and wildtype or GFP::Securin^{DM}. In the case of the mutant L4 F5 hermaphrodites were upshifted to 25°C and their embryos were dissected into M9 24hrs later. For the control, L4 hermaphrodites were also upshifted to 25°C and their embryos were dissected into M9 24hrs later. For both conditions, the number of polar bodies present in at least 5 embryos for each condition was determined. We also selected embryos only at the two-cell stage to avoid confusion generated by the fact that the second polar body is internalized and degraded in older embryos (Fazeli *et al.,* 2018).

The osmotic sensitivity assay was performed by dissecting wildtype (N2) or mutant embryos expressing GFP::Securin^{DM} as a homozygote in a hypertonic solution of 100mM KCl, following a previously described protocol (Knight *et al.,* 2012). A single multiplane image of both wildtype and mutant was acquired.

Imaging Parameters & Statistical Analysis

For construction of the cytoplasmic Securin degradation curve, *in utero* imaging was conducted at 10 second intervals beginning after egg activation through entry into anaphase I. At each timepoint, three random regions of cytoplasmic GFP intensity (arbitrary units) present in the embryo of interest were averaged and normalized to average of three random regions of cytoplasm in the -1 oocyte. The -1 oocyte serves as the point of normalization because it is arrested in prophase I, and the Securin levels are not expected to change during this time window as documented, and also serves to confirm the signal is not lost due to photobleaching (Fig. 14). Each timepoint in Fig. 14 represents the normalized ratio of at least 5 embryos per time point for each strain analyzed. Imaging was conducted using hermaphrodites at peak ovulation for each stain at room temperature, following incubation at 20°C.

To determine the effects of Securin^{DM} on chromosome segregation during anaphase I, wildtype and GFP::Securin^{DM} males were crossed with hermaphrodites carrying the chromosome marker H2B::mCherry. The resulting F1 progeny were heterozygote for H2B::mCherry and the GFP-tagged Securin locus. L4 heterozygote hermaphrodites from the F1 generation were upshifted overnight at 25°C until they reached peak ovulation in early adulthood (18-24hrs post-upshift). Single plane *ex utero* imaging was carried out beginning in prometaphase I at room temperature using 10 second acquisition intervals. Anaphase I onset is marked by wildtype and GFP::Securin^{DM} appearing at the midbivalent region, which coincides with poleward movement of chromosomes at anaphase onset in wild type embryos. Chromosome segregation distance was measured by recording the maximum distance between the polar-most regions of the segregating

chromosomes, as labeled by H2B::mCherry; along the plane of the spindle. Anaphase I lasts approximately 3 minutes in wild type, the end of which is characterized by the chromosomes beginning to float randomly in the cytoplasm. In the presence of GFP::Securin^{DM} anaphase I takes approximately 9 minutes before random chromosome movement in the cytoplasm. The distance recorded at each time point between the beginning of anaphase I to the end of anaphase I (300 seconds) corresponds to the average distance between homologs of at least 5 independent anaphase events in wild type and the mutant.

To determine the effects of Securin^{DM} on cortical granule exocytosis during anaphase I, wildtype and GFP::Securin^{DM} males were crossed with hermaphrodites carrying the cortical granule cargo marker CPG-2::mCherry. The resulting F1 progeny were heterozygote for CPG-2::mCherry and the GFP-tagged Securin locus. L4 heterozygote hermaphrodites from the F1 generation were upshifted overnight at 25°C until they reached peak ovulation in early adulthood (18-24hrs post-upshift). Single plane *ex utero* imaging through the spindle plane was carried out beginning in prometaphase I at 25°C using 10 second acquisition intervals. The imaging duration was similar to those used for the analysis of chromosome segregation. The number of CPG-2::mCherry vesicles was quantified throughout anaphase I in wild type and mutant GFP::Securin^{DM}. Each time point between late prometaphase I and the end of anaphase I onset (300 seconds in wild type) represents the average number of cortical granules in at least 5 independent experiments for both wildtype and mutant conditions.

To determine the effects of Securin^{DM} on Separase localization during anaphase I, wildtype and GFP::Securin^{DM} were crossed with Separase::mScarlet. Homozygous Separase::mScarlet combined with the mutant GFP::Securin^{DM} did not give the expected phenotype but displayed

abnormal ovulation and hypersensitive cell division failures potentially due to combining endogenously tagged separase with mutant securin. We ultimately succeeded in imaging Separase::mScarlet with wildtype and GFP::Securin^{DM} as heterozygotes using our male germline maintenance strategy. This required extensive troubleshooting, as Separase::mScarlet as a heterozygote is dim and photobleached quickly (data not shown). Following a cross between wild type and mutant Securin males with Separase::mScarlet hermaphrodites, L4 heterozygote hermaphrodites from the F1 generation were upshifted overnight at 25°C until they reached peak ovulation in early adulthood (18-24hrs post-upshift). To document the effect of wild type and GFP::Securin^{DM} on Separase localization at the spindle, single plane *ex utero* imaging through the spindle plane was carried out beginning in prometaphase I through shortly after anaphase I onset. Imaging was conducted at 25°C using 20 second acquisition intervals. Anaphase I onset was determined by Separase and wildtype or GFP::Securin^{DM} localization to the midbivalent region.

To document the effect of wildtype and GFP::Securin^{DM} on Separase localization at the cortex, I crossed wildtype and mutant GFP::Securin^{DM} males with Separase::mScarlet hermaphrodites. L4 heterozygote hermaphrodites from the F1 generation were upshifted overnight at 25°C until they reached peak ovulation in early adulthood (18-24hrs post-upshift). Upon first inspection, using *ex utero* live cell imaging through the spindle plane, Separase::mScarlet localization to vesicles appeared reduced and this signal appeared at random intervals following anaphase I onset (data not show). Upon further inspection, it appeared that Separase appeared to localize to a maximum, but reduced number of vesicles compared to wild type at a much later time than normal. To systematically address the time point at which Separase::mScarlet localized to the

maximum number of vesicles in the presence of mutant GFP::Securin^{DM}, I conducted two *ex utero* live cell imaging movies for each wild type and mutant embryo used in this analysis. The first movie was a single plane, single channel (GFP) movie, which I used to monitor anaphase I onset timing. For the first movie the time intervals were 10 seconds. In addition to serving as a monitor for anaphase I onset, it also photobleached the autofluorescent cortical granule signal. After anaphase I onset, I switch to dual channel (mScarlet and GFP), multiplane imaging of the cortex that varied from 20 second intervals for wildtype and between 30-60 second intervals for the mutant. Peak vesicle localization for wildtype corresponded to the frame before cortical granules begin to exocytose in anaphase I. Peak vesicle localization for the mutant corresponds to the time point at which Separase localizes to the maximum number of cortical granules, which has approximately 5-7 minutes after anaphase I onset. The average number of Separase positive vesicles within a single cortical plane of approximately equal circumference were compared to each other. The sample size was at least 5 for wildtype and mutant conditions. Cortical planes of approximately the same circumference, at peak vesicle localization, served as a proxy for focal planes of approximately the same number of vesicles around the perimeter of the cortex.

To verify the structures that Separase localized to in the mutant background were cortical granules, I crossed wild type or mutant GFP::Securin^{DM} into a line carrying Separase::mScarlet with the cortical granule marker GFP::Caveolin-1. L4 heterozygote hermaphrodites from the F1 generation were upshifted overnight at 25°C until they reached peak ovulation in early adulthood (18-24hrs post-upshift). I used one single plane movie with 10 second acquisition intervals using on the GFP channel to photobleach cortical granule autofluorescence and monitor for anaphase I onset. After cortical granules autofluorescence disappeared and anaphase I onset occurred, I

switch to multiplane still images corresponding to the first 5um of the cortical most cytoplasm at timepoints corresponding to early-, mid-, and late-anaphase I time points for both wildtype and mutant conditions.

Microsoft Excel was utilized to perform T-tests (two-tailed, assuming unequal variance) to determine statistical significance.

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Vita

Chris Turpin grew up in a rural area along the Central Oregon Coast where he helped his parents operate the family business. His parents helped him discover his knack for something he would later learn to be “aseptic technique.”

In middle school, Chris wrote a scientific protocol about assembling a PB&J sandwich, which his 6th grade science teacher intended to use to assemble and eat the sandwich. His teacher tried to assemble the sandwich and apparently he didn’t write the protocol too well because his science teacher had sandwich all over his face. Chris thought science was pretty neat after that moment.

When Chris was in the 10th grade, he went on a field trip to collect water from a stream. Back in the classroom his biology teacher asked him what he saw under the microscope. Chris obviously didn’t know what he was looking at, but he did remember looking at the 1997 Hercules movie and some strange shapes in the stream sample reminded him of a dragon. Impulsively, he answered his teacher, “Uh, a hydra.” She took a look, backed up in surprised and said, “How’d you know that!?” All Chris remembers from this moment is that he smiled awkwardly, didn’t say anything, and figured he might as well become a biologist. One thing led to another, and Chris was given some worms to play with (along with a cool question related to Separase and cell division, which manifested in the dissertation you just read/glossed over).

Chris has accomplished a few things in life, and his proudest accomplishment during his graduate studies was when he figured out how to get N2 to ovulate once every 23 minutes… we know that answer has something to do with Fisher-brand tape. He would like to figure out why this method appears to work efficiently. He has some ideas about a connection between worm movement and ovulation that stemmed from his struggles getting worms to ovulate routinely/as expected.

Chris was diagnosed with ADHD at 33, on the first day of October 2022. October happens to be ADHD awareness month and the ribbon is orange. He was a little unsettled by this information, especially because UT is known as the "Big Orange." At lot of weird things happened during his time at UT, and he has a gut feeling that investigating mental health is important. He would like to use the knowledge and skills acquired during his graduate studies to use worms to model human neurodevelopmental disorders. Specifically, he'd like to model schizophrenia using worms because that idea sounds bizarre. He also likes bizarre ideas and spurious correlations that may or may not mean something. He has a list of ideas. It's a good list, not the best list, but it's definitely a list. Before he latches onto any of these ideas, he intends to hone his skills a bit more with Dr. Bembenek.

Ultimately, Chris wants to do some more science, learn a bit more, and one day convince at least one of the uncannily large number of libertarian friends he knows that "maybe publicly funding science is a good idea."

On a last note, Chris thinks it would be cool to start his own lab one day using worms and ants to address rare and neglective human diseases. He also thinks he could expand this to integrated pest management: Imagine what would happen if you made a whole caste of ants have "ADHD." Chris hopes someone runs with this idea: "let's use gene drive to make the worker ant caste of the invasive Argentine ants have a neurodevelopmental disorder whenever they decide to leave the colony and forage. Maybe that would decrease their competitive advantage." Weirder things have been reported (Kern *et al.*, 2021), and don't get him started about the Bruce effect and his idea for a nasal contraceptive.