Chapter 3

Cell Cycle Regulators in Female Meiosis of *Drosophila melanogaster*

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Additional information is available at the end of the chapter

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Abstract

Meiosis is a highly regulated and complex variation on the canonical cell cycle. It depends on the activity of most of the known mitotic cell cycle regulators, as well as many meiosis-specific factors that interact with and modify the activities of this core cell cycle machinery. This review will examine the roles of known mitotic cell cycle regulators and meiosis-specific factors in *Drosophila* female meiosis, focusing on three important meiotic events: nuclear envelope breakdown or maturation, establishment of the meiosis I spindle, and release from metaphase I arrest at ovulation. Many meiotic processes are controlled by the mitotic kinase, Cdk1 with its cyclin partners, cyclins A, B, and B3. Other major mitotic kinases, including Polo and Aurora B have been found to play multiple roles in *Drosophila* meiosis. The Anaphase Promoting Complex or Cyclosome (APC/C) controls many meiotic processes through regulation of Cdk1, the sister chromatid cohesion regulator, Separase and other targets. This review will focus on these and other meiotic regulators, emphasizing some of the technical advances that have driven the field forward in recent years, and highlighting gaps that need to be filled to achieve a more complete picture of how meiosis is regulated in *Drosophila*.

Keywords: APC/C, Aurora B, Cdk1, cohesin, Cort, cyclin, *Drosophila*, meiosis, oogenesis, Polo, Separase

1. Introduction

The major events of meiosis are conserved throughout eukaryotes, and as with all cell biology, knowledge gained in one model system informs our understanding of meiosis in other organisms. On the other hand, as researchers gain a better understanding of how meiosis is controlled at the molecular level, it becomes clear that there are major differences between model systems and even between males and females in the same organism. This review

discusses the regulation of meiosis specifically in one model system, females of *Drosophila melanogaster*. Mitosis and meiosis in other model systems will be brought into the discussion, both to highlight areas of divergence, and to suggest explanations for events that are not yet well understood in this model system.

2. Oogenesis and meiosis in *Drosophila* females

Female meiosis takes place in the context of oogenesis. *Drosophila* females have two ovaries, each containing approximately 18 ovarioles, chains of progressively developing egg chambers, each consisting of an oocyte and associated support cells. Ovarioles are organized from anterior to posterior. The anterior contains the germarium, the location of germline stem cells and somatic stem cells that give rise to the egg and the follicle cells respectively. The posterior end contains mature developed eggs. Egg development has been divided into 14 distinct stages, based on major morphological events [1, 2].

In the anterior tip of the germarium, germline stem cells divide asymmetrically to give rise to a stem cell and a daughter cell. The daughter cell undergoes 4 incomplete divisions to generate a cyst of 16 cells that remain connected via cytoplasmic bridges called ring canals [1, 2].

From this cyst of 16 cells, one of the two cells with the most cytoplasmic bridges will differentiate into the oocyte [1]. Oocyte determination occurs while the oocyte is still within the germarium. The oocyte enters meiotic prophase, assembling synaptonemal complexes between homologs and undergoing crossing over. Throughout prophase, which lasts until stage 13 of oogenesis, the chromatin is compacted within the nucleus in a structure referred to as the karyosome. The other 15 germline cells of the cyst enter the endocycle concurrent with entry of the oocyte into meiotic prophase. These polyploid cells, called nurse cells, generate proteins and mRNAs important for meiosis progression, egg maturation and early embryonic development. The nurse cells use the cytoplasmic bridges to transfer their contents into the egg, prior to undergoing apoptosis in late oogenesis [1, 2].

As mentioned, the oocyte is arrested in prophase I until stage 13, at which point oocyte maturation occurs, highlighted by nuclear envelope breakdown (NEB). Spindle formation occurs in the absence of centrosomes, via microtubule polymerization around the karyosome, and a bipolar spindle assembles. Meiosis arrests at stage 14, the final stage of oogenesis, and the arrest is maintained until ovulation triggers egg activation, marked by the resumption of meiosis [2, 3]. Upon egg activation, the meiosis I spindle rotates and undergoes anaphase I. At the completion of the first anaphase, the two meiosis II spindles form around the separated homologs. These spindles are arranged perpendicular to the egg length and are held together by an aster of microtubules. At the completion of the 2nd meiotic division the 4 meiotic products enter a post-meiotic interphase. They then appear to undergo DNA replication in synchrony with the male pronucleus that entered the egg during fertilization. One of the 4 female meiotic products, usually the most interior, migrates towards the male pronucleus, apparently along microtubules that radiate out from the male aster. The male and female pronuclei enter the first mitosis together. The remaining female haploid products come together,

undergo nuclear envelope breakdown, and arrest in a mitotic-like state with condensed chromatin arranged on an aster-like array of microtubules called the polar body [4].

3. Cell cycle regulation and oocyte maturation

Oocyte development in most metazoans has two arrest points, a primary arrest at prophase, and a secondary arrest in metaphase. In insects, the secondary arrest is at metaphase I, while in many vertebrates it is at metaphase II. The long prophase arrest allows synapsis and crossing over to occur and at the same time, allows for oocyte growth. The secondary arrest facilitates the coordination of completion of meiosis with fertilization and the transition from oogenesis to embryogenesis [5].

The primary arrest in prophase is broken by nuclear envelope breakdown, a process that in most, if not all, eukaryotes is dependent on the mitotic cyclin-dependent kinase (Cdk), Cdk1. The Cdks are the core regulators of the cell cycle. They are activated by phosphorylation on their T-loop via a Cdk activating kinase or CAK, and by dephosphorylation of a Thr and Tyr at 14 and 15, respectively, by the Cdc25 phosphatase. Cdks also require association with cyclin partners, which themselves are subject to both transcriptional control and ubiquitin/mediated destruction.

The importance of Cdk1 in *Drosophila* female meiosis was first established through analysis of Twine, a germline-specific Cdc25 homologue. *Twine* mutants are viable but females produce eggs that do not hatch. In a study focusing on Polo and its regulator Matrimony (Mtrm, discussed below), it was found that *twine* mutant oocytes undergo NEB in stage 14 instead of in mid-stage 13 [6]. This finding was supported by the finding that a temperature-sensitive allele of *Cdk1* also produces a delay in NEB [7].

These studies illustrate a major challenge in studying meiosis in a genetic system such as *Drosophila*: classical genetics is limited to the study of genes that are non-essential for viability, such as *twine*; or the study of hypomorphic or conditional mutants as with *Cdk1*. The development of transgenic RNAi for the female germline has allowed researchers in the last few years to overcome these limitations and study the meiosis-specific requirements for otherwise essential genes. The Transgenic RNAi Project (TRiP) out of Harvard University has generated a genome-wide collection of transgenic RNAi lines that are driven by the *UAS/Gal4* system [8]. While the earlier collections were not effective in the germline, a micro-RNA based collection is now available that can be very effective. In most studies of meiosis RNAi is expressed using the female germline-specific mat-α-Tubulin Gal4 driver which expresses just after the premeiotic divisions in the germarium, thus not affecting mitotic divisions that are necessary for oocyte formation [9]. Using transgenic RNAi, it was found that loss of Cdk1 in meiosis leads to a complete failure of NEB in most oocytes, indicating that Cdk1 is indeed essential for NEB [10].

Drosophila, like other metazoans has three mitotic cyclins, Cyclin A, B and B3, though unlike vertebrates and other animals, *Drosophila* has only a single representative of each subtype. Cyclin A is the only one that is essential for viability. *Cyclin B* and *B3* mutants are viable but female sterile, though *Cyclin B/B3* double mutants are lethal [11]. The identity of the cyclin

partner for Cdk1 in oocyte maturation was investigated using transgenic RNAi, as well as a conditional mutant for *Cyclin B* and female sterile alleles of *Cyclin B3* [10]. While classic studies of meiosis in *Xenopus* and other vertebrate models have revealed Cyclin B to be the major Cdk1 partner in meiotic maturation, RNAi knockdown and conditional mutants of *Cyclin B* had no effect on NEB timing in *Drosophila* female meiosis [10]. Loss of Cyclin B3 also had no effect on NEB timing, either alone or when combined with *Cyclin B* knockdown. Knockdown of *Cyclin A* resulted in a slight delay in NEB timing. This delay was not enhanced by simultaneous loss of *Cyclin B* or *B3*. However, simultaneous knockdown of all three mitotic cyclins produced a prolonged delay or complete block in NEB, similar to *Cdk1* knockdown. Therefore, in *Drosophila* female meiosis, all three mitotic cyclins function in NEB, with Cyclin A apparently playing the most important role [10] (**Figure 1**).

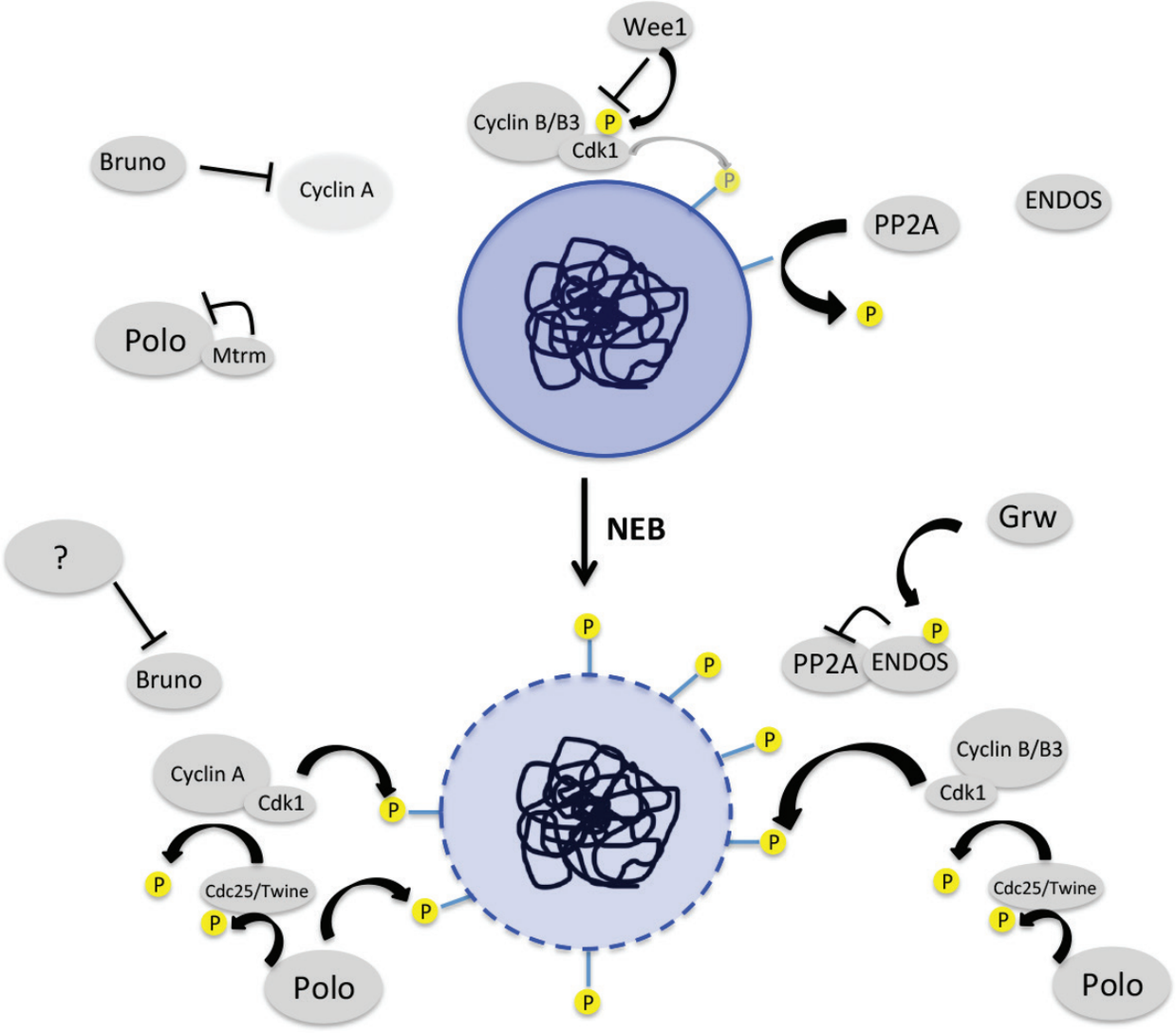

Figure 1. Model for nuclear envelope breakdown in *Drosophila* female meiosis. Phosphorylation events that drive nuclear envelope breakdown are here depicted as occurring on the nuclear envelope. The nuclear envelope is depicted in the lower panel as a dashed line to indicate NEB. See text for details and references.

The regulation of Cyclin A expression may contribute to the timing of NEB in *Drosophila*. Cyclin A is translationally repressed during meiotic prophase by the mRNA binding protein Bruno [12]. Bruno levels fall dramatically in stages 12 and 13 of oogenesis by an as yet unknown mechanism. This coincides with a dramatic increase in Cyclin A levels, possibly driving oocyte maturation [13] (**Figure 1**). Therefore, the regulation of Cyclin A translation appears to be critical for NEB timing in *Drosophila*. If this is the case, the forced expression of Cyclin A prior to stage 12 of oogenesis may be expected to result in premature NEB.

In addition to cyclin binding, Cdk1 activity appears to be regulated in many other ways that may contribute to the timing of NEB. The *Endos* gene was found to be required for proper NEB timing [7]. In vertebrates, Endos binds to the B55/Twins subunit of Protein phosphatase 2A (PP2A) to inhibit PP2A activity [14, 15]. This activity appears to be conserved for *Drosophila* Endos [16]. PP2A has been shown to recognize and dephosphorylate Cdk phosphorylated proteins, and as such is a major negative regulator of Cdk-dependent processes.

The ability of Endos to bind and inhibit PP2A appears to in turn be regulated by the phosphorylation of Endos by Greatwall kinase (Gwl) [16, 17]. Gwl was initially discovered in *Drosophila* by the dominant Scant allele, and was found to play multiple roles in meiosis beyond NEB [18]. Both genetic and biochemical evidence points to a simple linear pathway in which Gwl phosphorylation of Endos allows Endos to bind and inhibit PP2A [16, 17, 19, 20]. While Gwl has not yet been directly implicated in oocyte maturation, transgenic Endos with a S68A mutation, abolishing the Gwl phosphorylation site, is unable to rescue the delayed NEB phenotype of a *Endos* null mutant [17]. Thus Gwl-mediated phosphorylation likely plays a role in the Endos-mediated inhibition of PP2A to promote NEB (**Figure 1**).

The activity of Gwl itself is subject to regulation via phosphorylation via both Cdk1 and Polo kinases [21, 22]. Both kinases phosphorylate Gwl in the central region of the protein, disrupting the function of two nuclear localization sequences, thus promoting the cytoplasmic accumulation of Gwl. This may allow it to efficiently inhibit PP2A, which is predominantly cytoplasmic [21, 22].

In mitotic cells, Polo kinase plays multiple roles throughout the cell cycle, regulating centrosome dynamics, chromosome cohesion, and events at cytokinesis [23]. Polo promotes Cdk1 activity through the activation of Cdc25, while also targeting common substrates of Cdk1, such as the APC/C component Cdc27. Polo also recognizes many of its substrates depending on their prior phosphorylation by Cdk1 [23]. Therefore, the activities of Polo and Cdk1 are closely coordinated and often synergistic.

Studies of the Polo-binding protein, Matrimony (Mtrm), suggest a critical role for Polo in the timing of NEB in *Drosophila* female meiosis [6]. Matrimony is a maternally expressed protein that acts as a physical inhibitor of Polo. *Mtrm* mutants, even when heterozygous, display precocious NEB. This is likely due to precocious activity of Polo, since the simultaneous reduction of *Polo* gene dose leads to suppression of this phenotype [6]. Furthermore, a mutation in the Polo-interacting domain of Mtm results in a loss-of-function phenotype, suggesting that the antagonistic relationship between Polo and Mtm reflects inhibition of Polo by Mtm and not the other way around.

The timing of Mtrm expression is consistent with it having a role in NEB timing. Matrimony expression in the oocyte starts to increase in stage 10 and peak levels are reached at stage 11–12, when it localizes to the nucleus and cytoplasm [6]. Levels of Polo start to rise above Matrimony levels in stage 12, possibly allowing Polo to escape inhibition and help to promote NEB in stage 13 [6] (**Figure 1**). Recently, a strong RNAi knockdown allele of *Polo* has been characterized. Surprisingly, NEB still occurs in these oocytes [24], but a possible effect on the timing of NEB remains to be determined.

4. Meiosis I spindle assembly and chromosome orientation on the spindle

Meiotic spindles in many organisms, including humans, frogs and *Drosophila*, differ from mitotic spindles in that they are acentrosomal. Meiotic spindle microtubules do not originate from centrosomes but instead appear to nucleate from the chromosomes, and become tapered into bipolar spindles. Another major difference is in the behavior of kinetochores. In metaphase of meiosis I, the kinetochores of sister chromatids contact microtubules from the same pole, referred to as co-orientation. Homologous chromosomes, on the other hand, contact microtubules from opposite poles. This is referred to as biorientation of homologs. Tension created by pulling forces of kinetochore microtubules results in a stereotypic arrangement of chromosomes in metaphase I in which centromeres for each homolog are oriented on either side of the spindle midzone [25]. Assembly of this meiotic spindle and proper orientation of chromosomes requires the actions of many cell cycle regulators.

The problem of how to build a spindle without centrosomes appears to be dealt with differently in different organisms. In Xenopus egg extracts the small GTPase, Ran accumulates in its active GTP-bound form in the vicinity of chromosomes as a result of its chromatin-bound activating GEF, Rcc1. This Ran gradient promotes acentrosomal spindle assembly [26]. Rcc1 associates with the karyosome in *Drosophila* female meiosis, suggesting a similar role in flies [27]. The importance of the Ran gradient was investigated by over-expression of a Ran mutant that is unable to exchange GDP for GTP. This dominant negative allele produced only mild defects in spindle pole formation [27]. Therefore it appears that Ran does not play a central role in spindle assembly in *Drosophila* female meiosis. As discussed below, it appears that Aurora B and the chromosome passenger complex takes on this role.

The chromosome passenger complex (CPC), composed of Aurora B kinase, Incenp, Survivin and Borealin has multiple functions in mitotic cells [28]. The CPC promotes chromatin condensation in prophase. It accumulates at kinetochores in prometaphase, where Aurora B promotes the breakage of kinetochore-microtubule contacts. Importantly, the CPC is sensitive to tension. Attachment of spindle microtubules to a kinetochore results in a pulling of that chromatid toward the spindle pole. When the kinetochores of a pair of attached sister chromatids each make attachments to opposite poles (known as an amphitelic attachment), pulling forces from either pole generate tension across the kinetochores. This is sensed by the CPC, leading to inactivation of Aurora B. As a result, bi-polar attachments are stabilized. Aurora B and

other CPC components are also required at kinetochores for activation of the spindle assembly checkpoint (SAC). At anaphase onset the CPC relocates to the spindle midzone where it then plays a role in establishing the cleavage furrow [28].

In *Drosophila* meiosis the CPC has multiple roles, though surprisingly, these are quite different than CPC roles in mitosis. CPC proteins appear to associate with the karyosome immediately after NEB, coincident with the first detectable microtubules forming around the chromatin. CPC localization is independent of microtubules, suggesting that the CPC directly associates with chromatin [29]. Hypomorphic mutations in *Incenp* were found to produce a delay in microtubule polymerization around the karyosome following NEB [30]. Subsequent analysis of strong RNAi knockdown alleles of *Incenp* and *Aurora B* revealed a near complete failure to nucleate microtubules around the chromatin [29]. Therefore the CPC localizes to chromatin following NEB and is required for the first step in meiotic spindle formation, the recruitment of microtubules to the karyosome.

Unlike the situation in mitotic cells, the CPC does not appear to associate with kinetochores in *Drosophila* female meiosis. Nonetheless, core kinetochore proteins fail to assemble in strong Aurora B knockdown oocytes, suggesting that the CPC is necessary for kinetochore assembly [31]. Instead of accumulating on kinetochores in prometaphase, the CPC accumulates on the interpolar microtubules of the spindle midzone early in prometaphase [32]. Partial loss of function mutations in CPC genes permit meiotic spindle formation but these spindles often have a poorly formed spindle midzone. Many spindles appear to have more than two poles, reflecting the importance of interpolar microtubules in maintaining spindle bipolarity. In terms of protein localization and mutant phenotype, CPC hypomorphs resemble *Subito* mutants. *Subito* encodes the *Drosophila* MKLP2 homolog, and is a major organizer of the meiotic spindle midzone [32]. The CPC is required for Subito localization to the spindle midzone [29, 32].

Weaker CPC alleles that permit bipolar spindle formation display defects in chromosome orientation on the meiotic spindle. This failure of biorientation is also observed upon knockdown of *Subito*, suggesting that interpolar microtubules are important for chromosome movements that establish biorientation. Knockdown of the essential kinetochore component *Spc105R* also results in biorientation defects [31]. Interestingly, Ndc80, a kinetochore component required for head-on attachment of spindle microtubules to kinetochores, has only a subtle role in biorientation of homologs [31]. Taken together the results support the idea that side-on interactions between kinetochores and interpolar microtubules lead to chromosome movements that then lead to establishment of end-on attachments. The CPC appears to be important for both the formation of interpolar microtubules and for assembly of the kinetochore [31].

In mitotic cells, the Centralspindlin complex that includes the kinesin-like protein, MKLP1 (Pavarotti in *Drosophila*), is a major regulator of cleavage furrow formation at cytokinesis. MKLP1 at the central spindle recruits the GEF for RhoA [33]. This leads to the local activation of RhoA on overlying cell membrane, leading to actin/myosin recruitment, contractility and cleavage furrow formation [33]. Recently it was found that Centralspindlin components, and surprisingly, Rho and its GEF, play a role in biorientation of homologs in *Drosophila* female meiosis. This implies a novel role for this cytokinesis pathway in organizing the acentrosomal meiotic spindle [24].

In many cell types, Polo kinase plays an important role at the central spindle. In *Drosophila* male meiosis for example, Polo is required for CPC localization to the central spindle, and for localization of Shugoshin to centromeric chromatin where it protects centromeric cohesin complexes during the first meiotic division [34–36]. In *Drosophila* female meiosis, Polo does not specifically accumulate on the central spindle. It is not required for central spindle formation or for the localization of the CPC to the central spindle [24, 32]. *Polo* knockdown results in bi-orientation failure and instead of a compact karyosome in metaphase I, each homolog pair is randomly dispersed on the meiosis I spindle, often appearing as discrete chromatin masses [24]. Interestingly, a similar phenotype is observed in mutants or RNAi knockdown of the major CENP-E homolog in *Drosophila* (*cmet*), suggesting a possible function for Polo in regulation of this plus-end directed microtubule motor that may function to oppose poleward forces during prometaphase [31].

Polo accumulates in a punctate pattern on chromosomes in metaphase I oocytes, likely at kinetochores. It may function at kinetochores to stabilize kinetochore microtubules, as loss of Polo leads to the apparent hollowing out of the meiotic spindle, possibly due to a reduction in the number of kinetochore microtubules [24].

Given the close functional relationship between Polo and Cdk1, it is interesting that biorientation of homologs in meiosis is also dependent on Cyclin A-Cdk1 [10]. Unlike Polo, Cyclin A does not appear to be required for maintaining a compact chromatin mass or for proper spindle morphology, though *twine* mutant oocytes and *Cyclin A*, *Cyclin B3* double knockdown oocytes display a scattered chromatin phenotype that may be similar to that seen in Polo [7, 10]. As yet it is not known how Cyclin A-Cdk1 promotes biorientation. In other systems Cdk1 can phosphorylate Incenp, thereby activating the CPC [37]. Cyclin A-Cdk1 has also been found to promote proper head-on attachment of kinetochore microtubules in prometaphase of mitosis [38].

5. The APC/C and control of meiotic anaphase

Mature eggs are maintained in a metaphase I arrest that can be stable for long periods of time. This arrest is maintained by multiple forces, many of which are focused on the inhibition of Anaphase Promoting Complex/Cyclosome (APC/C) activity. The APC/C is a multi-subunit E3 ubiquitin ligase that catalyzes the addition of ubiquitin chains to target proteins, marking them for degradation by the proteasome. This section will first introduce the APC/C in general and in *Drosophila* oocytes more specifically. This will be followed by a discussion of how the APC/C is kept inactive in metaphase I arrested mature oocytes, how it is activated upon egg activation, and how its activity leads to the completion of meiosis and other processes that occur upon egg activation.

Ubiquitination by the APC/C, as with other E3 Ubiquitin ligases, depends on the activity of E1 and E2 enzymes. The E1 activates Ubiquitin and transfers it to an E2. In the case of the APC/C, the E2 appears to mediate Ubiquitin transfer to the substrate, with the APC/C serving to bring E2 and substrate together [39, 40]. The core of the APC/C is composed of APC2, APC11, DOC1 and an activator, CDC20 or Cdh1. Doc1 and CDC20/Cdh1 facilitate the substrate recognition component of the APC/C, whereas APC2 allows for binding of the E2 [39]

The APC/C activators Cdc20 and Cdh1 (known as Fzy and Fzr respectively in *Drosophila*) recognize well-conserved degron motifs on target proteins. Cdc20 can recognize the destruction box (D-Box), which has the consensus sequence RXXLXXXXN. Cdh1 recognizes the D-Box, the KEN box and a small number of other degron motifs [40].

In vertebrate mitosis, the two APC/C activators function in tandem to control cyclin levels. APC/C^{Cdc20} is activated by Cdk1 phosphorylation in mitosis, and drives anaphase by targeting mitotic cyclins and Securin for destruction. Destruction of Securin results in activation of Separase, a protease that cleaves the klesin subunit of the cohesin complexes, thereby releasing sister chromatids. APC/C^{Cdh1} is inhibited by Cdk-mediated phosphorylation and is thus activated following cyclin destruction in anaphase. APC/C^{Cdh1} remains active through G1 and maintains low Cdk activity. It is then inactivated as Cdk activity rises at S-phase, and it remains inactive through G2. Both APC/C activators are important in vertebrate meiosis. In the mouse APC/C^{Cdh1} is active in G2 and prophase to maintain low cyclin levels to prevent precocious NEB [41]. In Xenopus, by contrast, it promotes NEB, targeting Protein Phosphatase 6 for destruction, thereby maintaining Cdk1 phosphorylations that promote NEB [42]. APC/C^{Fzy} is the primary driver of anaphase in vertebrate meiosis, as it is in mitosis [40].

In *Drosophila*, Fzr (Cdh1) is expressed at very low levels in early stage embryos, suggesting that it may not be present during late stages of meiosis [43]. As yet, no role has been described for Fzr in meiosis. Meanwhile, two APC/C complexes cooperate in meiotic anaphase, APC/C^{Fzy} (Cdc20) and the female germline-specific APC/C^{Cort} [40, 44].

6. Inhibition of APC/C during meiosis I arrest

Classic studies in Xenopus oocytes and other vertebrate models identified Cytostatic Factor (CSF) as the key to inhibition of APC/C activity in the meiosis II arrest. While the molecular identity of CSF remained unknown for many years, it now seems that the APC/C^{Fzy} inhibitor, Emi2 is responsible for CSF activity. Emi2 functions by competing with APC/C^{Fzy} for interaction with the E2, Ube2S [45]. Emi2 is related to another APC/C inhibitor, Emi1, that functions in meiotic prophase and in mitotic cells. *Drosophila* has a single Emi homolog, Rca1. Rca1, like Emi1, has a clear mitotic role. Its role, if any, in meiosis has not yet been determined. It does not appear capable of interacting with and inhibiting Fzy [46], and no interaction with APC/C^{Cort} has been described.

Emi2 stability and its ability to interact with APC/C depend on phosphorylation by the Rsk kinase, acting downstream of a Map kinase pathway that has Mos as the upstream kinase. This phosphorylation recruits PP2A which in turn reverses the Cdk1-mediated phosphorylation of Emi2 that leads to its inactivation and destruction [47]. The role of Mos and Mapk have been investigated in *Drosophila* and neither appears to be necessary for maintaining the meiosis I arrest [48].

The spindle assembly checkpoint (SAC) plays a key role in assuring faithful chromosome segregation in mitosis by inhibiting anaphase initiation until all chromosomes have made bipolar attachments to the mitotic spindle. During prometaphase, kinetochores that are not yet connected to spindle microtubules act as sites for recruitment of SAC proteins including

the core SAC component, Mad2. Mad2 is converted to an active form that can diffuse away to assemble the mitotic checkpoint complex (MCC), which binds and inhibits APC/C^{Cdc20}. In some organisms such as yeast and *Drosophila*, the SAC is not essential for normal mitosis, but becomes essential under conditions in which mitotic spindle assembly or chromosome attachment to the spindle is disrupted. In vertebrates, the SAC plays an essential role even in the absence of spindle disruption [49].

A role for the SAC in meiosis has been most clearly established in the mouse where it is important for delaying anaphase I under normal conditions and under conditions in which the spindle is disrupted [50]. A role for the SAC in *Drosophila* is suggested by the protein localization and mutant phenotype for the essential SAC component, Mps1 (also known as Ald) [51]. Mps1 is a kinase that is recruited to unoccupied kinetochores where it functions to recruit other SAC components. In *Drosophila* female meiosis, Mps1 accumulates on kinetochores starting in prometaphase of meiosis. Mutants display a precocious anaphase phenotype, consistent with a SAC function in meiosis I [51]. Another essential SAC component, BubR1, also displays a precocious sister chromatid separation and/or missegregation phenotype in meiosis that could be attributed to a meiotic SAC role [52]. On the other hand, both Mps1 and BubR1 clearly have non-SAC roles in meiosis that could underlie these phenotypes [51, 52].

Unlike many SAC proteins, Mad2 appears to have no function outside of the SAC [53] and, importantly, null alleles of *Mad2*, do not result in precocious anaphase in meiosis I [54]. Furthermore, loss of Mad2 as well as BubR1, Mps1 and another SAC gene, Zwilch, do not result in reduced levels of the APC/C^{Fzy} target, Cyclin B, either globally or locally on the meiosis I spindle, as would be expected if the APC/C were activated [54]. Genetic evidence also argues against a role for the SAC in inhibiting APC/C activity in the 2nd meiotic division [54]. This apparent lack of a requirement for the SAC in female meiosis is also seen in Xenopus female meiosis [55].

Cyclin B-Cdk1 may play a role in inhibiting APC/C activity in *Drosophila* female meiosis, as *Cyclin B* knockdown and conditional mutants of *Cyclin B* result in precocious homolog segregation [10]. In Xenopus oocytes, Cyclin B-Cdk1 has been shown to bind to and inhibit the activity of Separase [56], and the above phenotype may indicate a similar role in *Drosophila* oocytes. In such a model, Cyclin B loss results in APC/C-independent activation of Separase. This could be tested genetically, since it predicts that precocious anaphase observed in the Cyclin B knockdown would still occur in an APC/C knockdown background. While this experiment has not been performed, it was found that precocious anaphase in the *Cyclin B* knockdown oocyte is suppressed by loss of *Cyclin B3* [10]. As we discuss below, Cyclin B3 appears to activate the APC/C in meiosis. Therefore, the precocious anaphase resulting from Cyclin B loss appears to depend on the APC/C. This would better fit with a model in which Cyclin B-Cdk1 inhibits APC/C activity rather than Separase activity (**Figure 2**).

Loss of a single copy of the *Mtrm* gene results in precocious anaphase [6]. As for its role in NEB, Mtrm appears to function in metaphase I primarily as an inhibitor of Polo. Therefore Cyclin B-Cdk1 activity and Polo inhibition are both necessary for maintaining a metaphase I arrest.

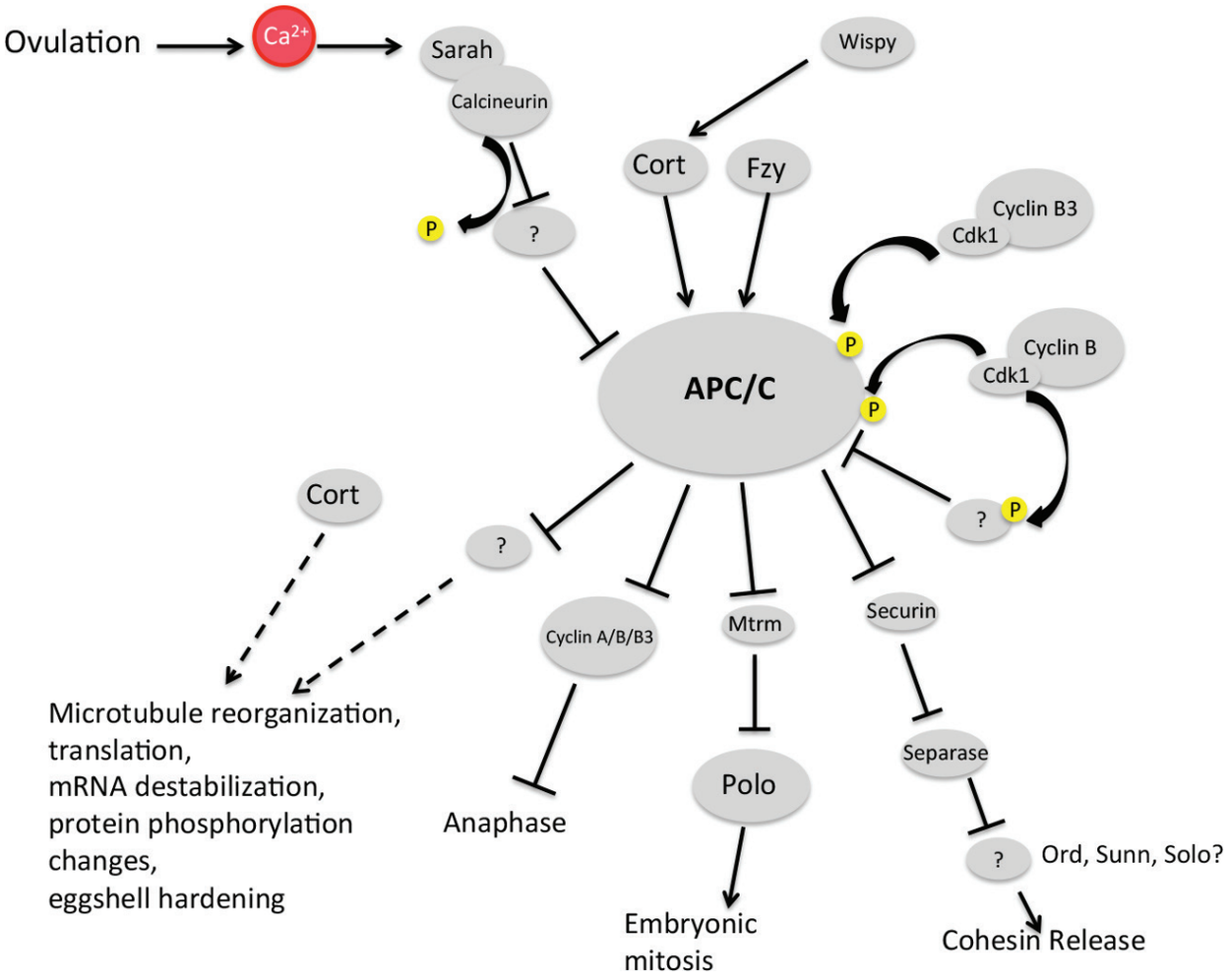

Figure 2. Model for regulation of and roles of the anaphase-promoting complex in *Drosophila* female meiosis. See text for details and references.

7. Egg activation and the resumption of meiosis

In *Drosophila*, ovulation is the trigger for egg activation, one of the major transition points in development. At activation, the egg undergoes multiple changes that set the stage for embryonic development. These include the completion of meiosis, global changes in mRNA stability, translation, protein phosphorylation, as well as changes in cytoskeletal organization and the completion of eggshell formation. All of these changes appear to require the *Cort* gene [40] (**Figure 2**).

Cort was originally discovered in a screen for maternal effect lethal mutations [57]. *Cort* mutants undergo normal metaphase I arrest at stage 14. Anaphase I is generally normal, though some eggs arrest at this point. The vast majority of *Cort* mutant eggs arrest in metaphase II [58, 59]. The cloning of *Cort* revealed it to encode a member of the Cdc20/Cdh1 family of APC/C activators. Cort interacts with the APC/C core and is required for the destruction of mitotic cyclins and Securin in the female germline [44, 60]. APC/C^{Cort} appears to function

in a partially redundant manner with the canonical APC/C^{Fzy}. *Fzy* hypomorphs arrest in anaphase II, with elevated cyclin levels, while *cort*, *fzy* double mutants arrest in meiosis I, with further elevated cyclins levels [44]. Though both APC/Cs target Cyclin B for destruction, they appear to have distinct sites of activity: Cort is responsible for spindle midzone degradation of Cyclin B, whereas Fzy is responsible for degradation of Cyclin B all along the spindle [44]. In addition to cyclins and Securin, APC/C^{Cort} targets Mtrm for destruction, thereby promoting Polo activity that appears to be important for proper mitosis in the early embryo [61] (**Figure 2**). Cort recognizes a degron sequence on Mtrm that is related to but distinct from the D-box, found on Fzy and Fzr targeted proteins [61]. Given that APC/C^{Cort} targets the mitotic cyclins for destruction, it is likely that it also recognizes canonical D-box and possibly KEN box degrons on these targets, but this has not been directly tested as yet.

As mentioned, Cort functions in multiple processes that depend on egg activation. Wild type oocytes contain arrays of microtubules around the cortex of the egg. These are broken down into shorter filaments at egg activation. *Cort* mutants fail to undergo this change in microtubule organization [58, 59]. How Cort affects this change in microtubule behavior is not known.

Cort is also implicated in the translation of specific mRNAs at egg activation. These include mRNA for patterning genes *Bicoid*, and possibly *Toll* and *Torso*. Reduced translation of *bicoid* mRNA correlates with reduced polyA tail length, suggesting that Cort promotes translation by promoting polyadenylation of specific mRNAs [59].

Cort is required for the destabilization of many mRNAs at the mid-blastula transition in cycle 14 of embryogenesis. At this transition, many maternal mRNAs are degraded and zygotic transcription is upregulated. Egg activation leads to a pathway in which the Pan gu (Png) kinase is activated and promotes the translation of the RNA-binding protein Smaug. Smaug is responsible for the destabilization of mRNAs at the transition to zygotic development [62]. It is currently unknown at what level in this pathway Cort functions.

Egg activation also involves global changes in protein phosphorylation, and Cort is implicated in a subset of these [63]. One protein that is dephosphorylated at egg activation dependent on Cort is Gnu, a component of the Png complex that is implicated in translational control at egg activation [63]. While the significance of this dephosphorylation is not clear, one possibility is that APC/C^{Cort} activates the Png kinase complex by promoting dephosphorylation of Gnu, possibly by targeting for destruction a Gnu kinase. Png then promotes the translation of Smaug, leading to transcript destabilization. The identification of specific Cort targets at egg activation will be necessary to sort out the relationship amongst these Cort-dependent functions. It will also be important to determine if these functions of Cort depend on its role as an APC/C activator or if they represent novel functions of Cort.

8. Activation of the APC/C at ovulation

In many species, fertilization is the signal for egg activation, but in *Drosophila*, ovulation triggers this process (reviewed in [64]). Even though egg activation is not directly coupled

to fertilization, the events are linked, as both depend on passage of the egg through the oviduct. When the egg enters the uterus it is positioned to allow sperm that is stored by the female after mating to enter the egg through an opening at the egg anterior called the micropyle. Meanwhile, ovulation triggers egg activation. The experimental application of physical pressure on a mature oocyte can induce egg activation, suggesting that physical pressure encountered during passage through the oviduct triggers egg activation [65]. Supporting this idea, inhibiter studies implicate mechanosensitive calcium channels, presumably activated during squeezing of the egg in the oviduct, in mediating an initial increase in calcium levels upon ovulation. This is followed by the IP3-mediated release of intracellular calcium stores in the egg. The result is a wave of calcium that passes through the oocyte cytoplasm [66]. The increase in calcium appears to then lead to activation of calcium-dependent enzymes such as Calcineurin (**Figure 2**).

The central role of Calcineurin in *Drosophila* egg activation was first revealed through the study of its interacting partner, Sarah [67–69]. Sarah is a positive regulator of Calcineurin in *Drosophila* meiosis, though it is a negative regulator in other contexts. *Sarah* mutants display a metaphase I arrest, and elevated Cyclin B, suggesting lack of APC/C activity. In addition, other events of egg activation are impaired, such as the translation of *bicoid* mRNA, and reorganization of cortical microtubules. *Calcineurin (CanB2)* mutants were subsequently found to have similar defects, indicating that *sarah* phenotypes are a result of loss of Calcineurin activity [67]. Phosphorylation of Sarah by Glycogen synthase kinase-3 (GSK-3/Shaggy) and by Polo, are necessary for Calcineurin activity, implicating these kinases as potential upstream regulators of Calcineurin, in addition to calcium [70].

In Xenopus, Calcineurin appears to promote anaphase by relieving APC/C^{Fzy} of Emi2 inhibition [71, 72]. As discussed earlier, it is not yet clear if the *Drosophila* Emi, Rca1, is important for meiosis and therefore it is not clear if Calcineurin targets this protein or another to mediate APC/C activation (**Figure 2**).

The similarity between *CanB2* and *sarah* mutants on one hand and *cort* mutants on the other, support the idea that Calcineurin acts in a pathway with APC/C^{Cort} (presumably upstream, as in Xenopus) to promote egg activation. The main difference between *calcineurin* and *cort* mutants is the timing of the meiotic arrest: meiosis II for *cort* mutants and meiosis I for Calcineurin mutants. As mentioned *cort*, *fzy* double mutants arrest in meiosis I [44], suggesting that Calcineurin is required upstream of both APC/C complexes.

While Cort regulates translation, possibly by affecting polyadenylation, Cort itself is subject to this form of regulation. Wispy is a female specific PolyA Polymerase required for translation, and transcript destabilization in *Drosophila* embryos [73, 74]. *Wispy* mutants arrest in meiosis I, at least in part due to failure to translate Cort [74]. A role in Fzy translation has not yet been reported (**Figure 2**).

In mitotic cells, APC/C^{Fzy} activity is dependent on phosphorylation by Cdk1. Based on the mutant phenotype for *Cyclin B3*, Cyclin B3-Cdk1 may play this APC/C activating role in meiosis. *Cyclin B3* mutant females undergo meiotic arrest in metaphase or early anaphase of either meiosis I or II [10, 75]. Similarly, Cyclin B3 RNAi injection into early embryos results in an

anaphase arrest, suggesting a role in APC/C activation in the mitotic divisions of the early embryo [75]. If Cyclin B3-Cdk1 activates the APC/C in meiosis, it remains to be determined if this involves direct phosphorylation of APC/C subunits, and whether APC/C^{Cort} or APC/C^{Fzy} or both are activated (**Figure 2**).

9. Chromosome cohesion and its release in meiosis

The key event of meiotic anaphase is the separation of attached homologs in meiosis I, and then sister chromatids in meiosis II. In mitotic cells in *Drosophila* and possibly all eukaryotes, sister chromatids are held together by the ring-like cohesin complex. At anaphase, the APC/C dependent cleavage the kleisin component of the cohesin complex (called Rad21), leads to chromosome segregation. In diverse organisms, from yeast to mammals, the cohesin complex is modified for meiosis. Most notably, a meiotic kleisin, Rec8, takes the place of Rad21. APC/C^{Cdc20} activation at anaphase triggers the degradation of Securin, leading to Separase activation and consequent Separase-mediated cleavage of Rec8 (reviewed in [76]). Rec8 cleavage and cohesin disassembly in meiosis occurs in two steps. Prior to anaphase I, homologs are kept together by the combined effect of crossing over between homologs and sister chromatid cohesion distal to crossovers. At anaphase I, cohesion along chromatid arms is released to allow homolog segregation. At anaphase II, centromere-proximal cohesion is released to allow sister chromatids to separate [77].

As described above, the APC/C activator, Cort is required for anaphase II of female meiosis. FISH using an X-chromosome centromere-proximal probe revealed that these meiosis II figures each contain a single dot, indicating that centromere cohesion is maintained in *cort* mutants, implying that APC/C^{Cort} is necessary for the release of centromere-proximal cohesion in anaphase II [44]. *Cort*, *fzy* double mutants as well as *Calcineurin* mutants produce an arrest in meiosis I [44], but it is not known if this arrest occurs prior to the release of arm cohesion. This could be easily tested using arm-specific FISH probes described below.

The roles of Securin and Separase in *Drosophila* meiosis were investigated, employing centromere-proximal and arm-specific FISH probes to monitor cohesion release in meiosis I and II [78]. The expression of a D-box, KEN-box mutant version of Securin in *Drosophila* oocytes that were depleted of endogenous Securin by RNAi, produced a delay or failure of homolog segregation in meiosis I and sister segregation in meiosis II. In the same study it was found that RNAi knockdown of the *Drosophila Separase* gene leads to an identical phenotype [78] (**Figure 2**). Interestingly, neither *Separase* knockdown nor stabilized Securin lead to a complete failure of cohesion release in meiosis, though both produced a complete and stable failure of cohesion release in the post-meiotic polar body chromosomes [78]. While it is possible that incomplete effects are due to a failure to completely inactivate Separase in these experiments, it could also be that a 2nd pathway for cohesion release operates in parallel with and partially redundant with the Securin/Separase pathway.

Knockdown of cohesin component *SMC3* leads to precocious homolog segregation (though not sister segregation) [78], implying a role for the cohesin complex in arm cohesion in *Drosophila*. Complicating this interpretation is the finding that many cohesin components, including SMC1 and SMC3 are important for synaptonemal complex (SC) assembly or maintenance [79]. The SC brings homologs into register in meiotic prophase and thus it is necessary for homolog pairing in meiosis I. Recent evidence has shown that core cohesin components are indeed required for cohesion, independent of their roles in SC assembly or maintenance. First, it was found that knockdown of either *SMC1* or the cohesin loading protein, *Eco*, leads to non-disjunction even in cases where a cross-over occurred [80]. Therefore, the non-disjunction is not due to failed synapsis and is presumably due to a failure of cohesion. Direct cytological evidence also supports an essential role for the core cohesin complex in meiotic cohesion. Using an arm-specific FISH probe, it was found that *SMC3* knockdown, but not knockdown of *Rad21*, results in absence of chromosome arm cohesion in meiotic prophase [78]. Interestingly, centromere-proximal cohesion appears to persist in these oocytes, either because of incomplete loss of cohesin at these sites or perhaps because a 2nd mechanism contributes to centromeric cohesion [78].

While the core components of the cohesin complex are required for meiotic cohesion, the identity of the Separase-cleavable component remains unknown. Fruit flies lack an obvious Rec8 homolog. A distant relative of Rec8, C(2)M, was found to function in the SC, but it appears to be released from chromosomes well before anaphase I. Furthermore, a form of C(2)M lacking putative Separase cleavage sites does not prevent anaphase, suggesting that C(2)M is not the cleavable kleisin [81]. Meanwhile, several lines of evidence demonstrate that the mitotic kleisin, Rad21, is also not the cleavable cohesin complex component in meiosis. First, a Separase cleavage site mutation in Rad21 fails to prevent anaphase in meiosis. Second, when the *Rad21* gene was replaced with a TEV-cleavable *Rad21* transgene, TEV cleavage prior to anaphase did not result in precocious anaphase [82]. Finally, knockdown of *Rad21* in meiosis did not lead to precocious release of arm cohesion [78].

While the Separase target in *Drosophila* meiosis is not known, 3 likely candidates have been identified, Ord, Sunn and Solo (**Figure 2**). All three genes were found to be required for proper homolog and sister chromatid disjunction in meiosis, and they each encode novel proteins that have been found to localize to meiotic chromosomes and to core cohesin complexes [83–87]. It will be important to assess these proteins for Separase-mediated cleavage, either by identifying and mutating putative Separase cleavage sites or by direct identification of cleavage products in post-meiotic eggs.

10. Conclusion

The ability to study mutant and knockdown phenotypes in female meiosis in recent years has led to a great advancement in our understanding of how cell cycle regulators work together to regulate meiosis. One of the big challenges for the future will be in discovering specific

substrates for Cdk1 and other kinases, and for the APC/C. There is also a need to better understand how these central meiotic regulators are themselves regulated in meiosis.

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