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The Cdc20 (Fzy)/Cdh1-related protein, Cort, cooperates with Fzy in cyclin destruction and anaphase progression in meiosis I and II in *Drosophila*

Andrew Swan and Trudi Schüpbach*

Meiosis is a highly specialized cell division that requires significant reorganization of the canonical cell-cycle machinery and the use of meiosis-specific cell-cycle regulators. The anaphase-promoting complex (APC) and a conserved APC adaptor, Cdc20 (also known as Fzy), are required for anaphase progression in mitotic cells. The APC has also been implicated in meiosis, although it is not yet understood how it mediates these non-canonical divisions. Cortex (Cort) is a diverged Fzy homologue that is expressed in the female germline of *Drosophila*, where it functions with the Cdk1-interacting protein Cks30A to drive anaphase in meiosis II. Here, we show that Cort functions together with the canonical mitotic APC adaptor Fzy to target the three mitotic cyclins (A, B and B3) for destruction in the egg and drive anaphase progression in both meiotic divisions. In addition to controlling cyclin destruction globally in the egg, Cort and Fzy appear to both be required for the local destruction of cyclin B on spindles. We find that cyclin B associates with spindle microtubules throughout meiosis I and meiosis II, and dissociates from the meiotic spindle in anaphase II. Fzy and Cort are required for this loss of cyclin B from the meiotic spindle. Our results lead to a model in which the germline-specific APC^{Cort} cooperates with the more general APC^{Fzy}, both locally on the meiotic spindle and globally in the egg cytoplasm, to target cyclins for destruction and drive progression through the two meiotic divisions.

KEY WORDS: Fzy, Cort, Cks, APC, *Drosophila*, Cell cycle, Meiosis

INTRODUCTION

The cell divisions of female meiosis and the ensuing mitotic cycles of early embryogenesis represent two examples of non-canonical cell cycles. Meiosis differs from the typical mitotic cycle in several respects. Most notably, two divisions occur in sequence without an intervening S-phase, resulting in the production of four haploid gametes. Additionally, the first meiotic division involves the segregation of homologous chromosomes and occurs without sister chromatid segregation, whereas the second meiotic division involves the segregation of sister chromatids, as occurs in mitosis. The regulation of meiosis requires a significant reorganization of the canonical cell-cycle machinery and the use of a number of meiosis-specific cell-cycle regulators (reviewed in Marston and Amon, 2004). One example is in the regulation of anaphase – the coordinated series of events that results in the segregation of chromosomes to produce two daughter nuclei. In mitotically dividing cells, anaphase progression crucially depends on the inactivation of the mitotic kinase Cdk1 (also known as Cdc2) and on the subsequent release of sister chromatid cohesion through the destruction of cohesin complexes. These events are controlled by an E3 ubiquitin ligase – the anaphase-promoting complex (APC) – in association with an adaptor protein, Fzy, and this complex targets mitotic cyclins and securin for destruction (reviewed in Peters, 2002). The role of the APC in meiosis appears to be more complex than in mitotic cells. For example, the APC only partially inhibits Cdk1 activity between meiotic divisions (Gross et al., 2000) and sister chromatid cohesion persists at centromeres through anaphase

I (Katis et al., 2004; Kitajima et al., 2004). It is not yet clear how the activity of the APC is modified in these specialized cell divisions.

In most eukaryotes, the meiotic cell cycle is followed by another atypical cell cycle – the cleavage divisions of early embryogenesis. In *Drosophila*, these cleavage cycles occur as a series of synchronized, rapid nuclear divisions and are referred to as syncytial divisions. The female meiotic cell cycle is not only closely linked to the syncytial mitotic cell cycle in time, but it also occurs within a shared cytoplasm – that of the egg. Therefore, these two distinct cell cycles share a common pool of cell-cycle regulators, and may share common strategies for spatially and temporally regulating cell-cycle progression within a syncytium.

One way in which the syncytial cell cycle is modified appears to be in the limited destruction of mitotic cyclins in each cell cycle, apparently by restricting their destruction to the area of the mitotic nuclei. Although there is evidence that cyclin destruction is spatially regulated in somatic cells (Kallio et al., 1998; Rieder et al., 1997), this strategy appears to be of particular importance in the syncytial embryo of *Drosophila* as a means to conserve mitotic cyclins for the duration of the rapid syncytial divisions. Several lines of evidence suggest that at least one cyclin, cyclin B, undergoes limited local destruction on mitotic spindles in the syncytial embryo (Edgar et al., 1994; Huang and Raff, 1999; Raff et al., 2002; Su et al., 1998). It is not yet known what mediates this local cyclin B destruction, and it is also not known whether this is unique to the syncytial mitotic cell cycle or if it occurs in the preceding meiotic divisions.

Drosophila represents an excellent model system for understanding how the canonical cell-cycle machinery is developmentally modified, and how novel cell-cycle regulators are used to control meiosis and syncytial divisions. *cortex* (*cort*) encodes a Cdc20/Cdh1 (Cdh1 is also known as Fzr and Rap)-related protein, which appears to be required specifically in female meiosis (Chu et

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al., 2001; Lieberfarb et al., 1996; Page and Orr-Weaver, 1996) and functions with a germline-specific Cks gene, *Cks30A*, to mediate the destruction of cyclin A (Swan et al., 2005; Swan and Schupbach, 2005). Here, we show that the canonical APC adaptor Fzy functions together with Cort to target mitotic cyclins for destruction, and to drive anaphase in both meiosis I and meiosis II. Female meiosis, like the subsequent syncytial mitotic cell cycles, appears to involve the local destruction of cyclin B, and we find that both Cort and Fzy are required for this process.

MATERIALS AND METHODS
Drosophila stocks
The two *cort* mutants (*cort^{QW55}* and *cort^{RH65}*) (Schupbach and Wieschaus, 1989) have similar meiotic phenotypes (Page and Orr-Weaver, 1996) and were analyzed as transheterozygotes. *cort^{QW55}* has a conserved Y303C change and *cort^{RH65}* encodes a truncated protein lacking the seventh WD repeat (Chu et al., 2001). *Cks30A* corresponds to the remnants gene, and *Cks30A^{KO}* was generated by site-directed mutagenesis and is a molecular null, lacking a start codon (Swan et al., 2005). *fzy^6* and *fzy^7* (Dawson et al., 1995) are temperature-sensitive lethal alleles that are female sterile at 22°C. These were analyzed as transheterozygotes. Double mutant *fzy; cort* chromosomes were generated by recombining *cort^{QW55}* with *fzy^6* and recombining *cort^{RH65}* with *fzy^7*. All experiments with *fzy* mutants and *fzy; cort* double mutants were performed on eggs from females kept at 29°C for 3-5 days. *UAS-HA-cort* was made by PCR amplification of genomic *cort* (including introns), followed by cloning into pUASp with a 2× hemaglutin (HA) tag at the N-terminus. Expression of UAS-HA-Cort using the *nosGal4-VP16* driver resulted in the rescue of the female sterility of *cort* mutants (data not shown). UAS-Fzy and UAS-Cdh1 were obtained from Christian Lehner (Sigrist and Lehner, 1997). UAS-CyclinB-TPM-GFP was obtained from Jordan Raff (Raff et al., 2002). Wing expression of UAS-HA-Cort, UAS-Fzy and UAS-Cdh1 was driven by ptcGal4 and enGal4. With these drivers, UAS-Fzy and UAS-Cdh1 expression results in pupal lethality (data not shown). To observe the wing phenotype in enGal4-UAS-Fzy, flies were raised at 18°C to reduce expression levels of this protein and permit survival to adult.

Antibody staining
To observe early meiotic events in wild type, mature eggs were activated to undergo meiosis in vitro, as previously described (Page and Orr-Weaver, 1997). To observe later meiotic events in wild type, eggs from 0 to 20 minute egg-lay collections were used. To detect cyclin B, eggs from 0-2 hour collections or from activated oocytes were fixed in 100% methanol, rehydrated gradually, blocked in PBST, 1% BSA and incubated with rabbit anti-cyclin B antiserum (from Jordan Raff) at 1/500. Rat anti-α-Tubulin (Cappel) was used at 1/500 and DNA was labeled either with mouse anti-Histones (Chemicon) at 1/1000 or OliGreen (Molecular Probes) at 1/500. Rat anti-Subito (anti-Sub) antibody (Jang et al., 2005) was used at 1/3000. FISH was performed on 0-2-hour-old eggs or dissected oocytes using a probe to a repeated 359 bp repeat sequence unique to the centromeric region of the X-chromosome (Dernburg, 2000). For immunostaining of wing discs, third instar larvae were collected from crosses of UAS-HA-Cort, UAS-Cdh1 or UAS-Fzy to ptcGal4. Discs were fixed for 30 minutes in 3.7% formaldehyde/PBST, extracted for 1 hour in PBST +0.3% Triton X-100 and labeled with rabbit anti-cyclin B, B3 (from Christian Lehner, University of Bayreuth, Germany) at 1/500 or with rabbit anti-cyclin A (from David Glover, Cambridge University, England) at 1/500. Discs were also labeled with rat anti-HA antiserum (Roche) at 1/500 and mouse anti-β-gal antiserum (Promega) at 1/500.

Western analysis
Extracts were prepared in 2× sample buffer from wild-type and mutant eggs collected over a 2-hour period. Wild-type eggs were derived from unfertilized females (crossed to XO males). Western blotting was performed by standard techniques. Antibodies were mouse anti-cyclin A and mouse anti-cyclin B (both from Developmental Studies Hybridoma Bank), rabbit anti-cyclin B3 (Sigrist et al., 1995), rabbit anti-Pim (Stratmann and Lehner, 1996) and rabbit anti-PSTAIR (Santa Cruz).

RESULTS
Cort and Fzy are required for the completion of meiosis I and meiosis II
The *Drosophila* genome contains four Cdc20/Cdh1 genes (Jacobs et al., 2002). Fzr2 appears to be exclusively transcribed in the male germline (Jacobs et al., 2002), whereas Cdh1 is transcribed in the female germline (Sigrist and Lehner, 1997), but the protein is not detectable in early embryos, either by western blot analysis or by in vivo functional assays (Jacobs et al., 2002; Raff et al., 2002). To determine the role of APC complexes in female meiosis, we focused on the canonical *Cdc20* (*fzy*), and a female-specific *Cdc20/Cdh1* homologue, *cort*, both of which are highly expressed in the female germline (Chu et al., 2001; Dawson et al., 1995). We re-examined the meiotic phenotypes of *cort* and *fzy* mutants separately and in double-mutant combinations by observing spindles and DNA, and by following chromosome segregation using FISH against an X-chromosome probe. Temperature-sensitive *fzy* mutants were analyzed at 29°C and, to control for temperature effects, wild-type and *cort* mutants were therefore examined at both room temperature and at 29°C. In female *Drosophila*, meiosis arrests in metaphase of the first meiotic division until ovulation (for a review see McKim et al., 2002). At this stage, the egg contains a single spindle near the anterior cortex; this spindle contains two X-chromosome signals representing the two pairs of sister chromatids (Fig. 1A,A'). Upon ovulation, meiosis resumes. In metaphase of meiosis II, two tandemly arranged spindles form around the products of the first meiotic division. Both metaphase spindles contain a single sister chromatid pair (Fig. 1B,B'). In anaphase II, sister chromatids separate, resulting in four meiotic products, each with a single X-chromosome (Fig. 1C,C'). Meiosis is completed very rapidly after ovulation and, at 22°C, only 1% (*n*=220) of eggs from a 0-2-hour-old collection were still in meiosis. The remainder of eggs contained arrested meiotic products (polar bodies). Similarly, in eggs from females kept at 29°C, only 4% (*n*=113) were in meiosis. In addition, 3% of eggs contained aberrant spindles near the cortex, suggesting low-level disruption of meiosis at this temperature. As previously described, eggs from *cort*-mutant females (hereafter referred to as *cort* eggs) contain two spindles near the anterior cortex of the egg, indicative of an arrest in meiosis II (Chu et al., 2001; Lieberfarb et al., 1996; Page and Orr-Weaver, 1996) (Fig. 1D). Similarly, at 29°C, 90% (*n*=78) of *cort* eggs contained two meiotic spindles. Both of the spindles contained a single X-chromosome signal (Fig. 1D'), indicating an arrest in metaphase, prior to sister chromatid separation.

Cks30A, like *cort*, is required for the proper completion of meiosis II, consistent with a model in which Cks promotes the activation of APC^{Cort} (Swan et al., 2005). However, whereas *cort* mutants invariably arrest in the second meiosis, in *Cks30* mutants, most oocytes eventually complete meiosis, although they are delayed in doing so (Swan et al., 2005). In 0-2-hour-old collections of *Cks30A^{KO}* eggs, 26% were in meiosis II (*n*=46). In 58% of these, both spindles had a single X-chromosome signal and were therefore in metaphase of meiosis II (Fig. 1E,E'), while the remaining 42% had two X-chromosomes per spindle and were therefore in anaphase of meiosis II (Fig. 1F,F'). Therefore, loss of *Cks30A* results in a meiotic phenotype similar to, but weaker than, *cort*, suggesting that Cks30A activity enhances but is not essential for the function of the APC^{Cort}.

In *Drosophila*, as in most eukaryotes, Fzy is the crucial APC adaptor in mitosis, and is essential for anaphase progression in most cell types (Dawson et al., 1993; Dawson et al., 1995; Sigrist et al., 1995). It is not yet known if Fzy is also required for anaphase
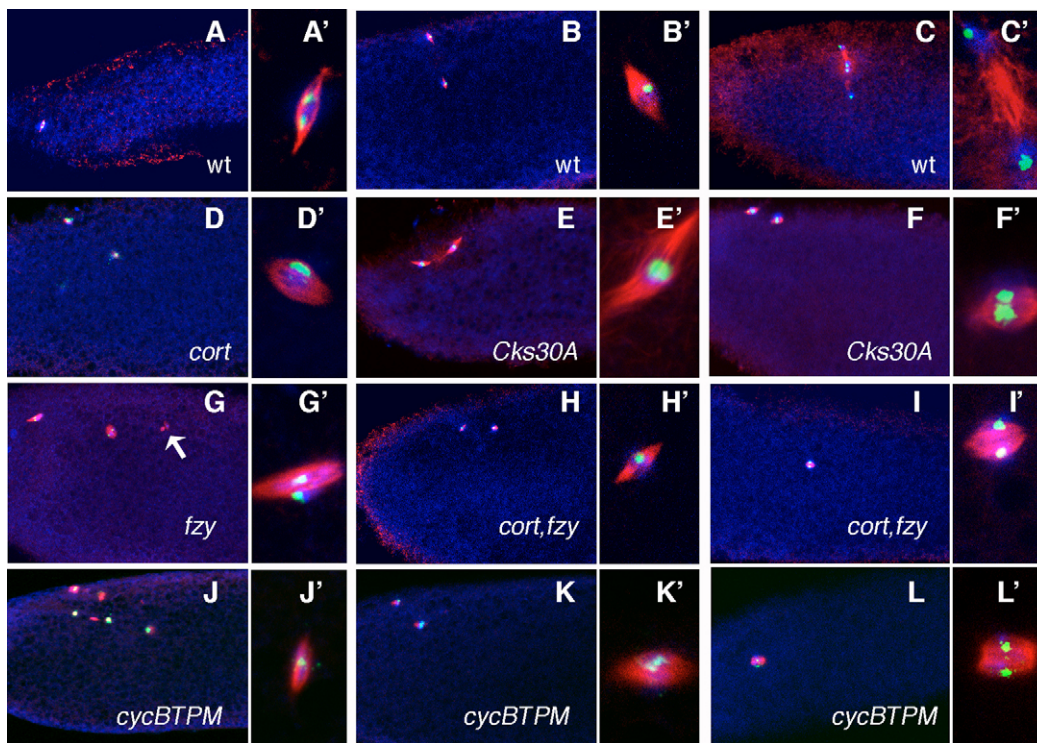
Fig. 1. Cort and Fzy are required for the completion of meiosis I and meiosis II. (A-L′) Microtubules are red, a 359 bp centromeric repeat unique to the X-chromosome is labeled green and DNA is blue. Smaller panels (A′-L′) are higher-magnification views of one of the spindles in A-L, respectively. Mitotic spindles derived from the male pronucleus (when present) are more internal and are not shown in these images. (A-C) Meiosis in wild-type eggs obtained by in vitro activation (A) or from 0-20 minute egg collections (B,C). In metaphase of meiosis I (A), the egg contains a single spindle with two discrete X-chromosome signals representing two pairs of sister chromatids. In metaphase II (B), there are two tandemly arranged spindles, each containing a single X-chromosome signal. In anaphase II (C), sister chromatids separate and both spindles contain two discrete X-chromosome signals. (D-L) Eggs at 0-2 hours from females of the genotypes indicated. (D) In *cort^{QW55}/cort^{RH65}* (females kept at 29°C for 3-5 days), eggs contain two spindles, each with one X-chromosome, indicating arrest in metaphase II. (E,F) *Cks30A^{KO}/Cks30A^{KO}* eggs with two spindles and either one (E) or two (F) X-chromosomes, indicating a delay as early as metaphase of meiosis II. (G) In *fzy^6/fzy^7* kept at 29°C, the majority of eggs contain two spindles, both with two X-chromosome signals, indicating arrest in anaphase II. Arrow indicates a small spindle and associated chromatin. (H,I) In *cort^{QW55},fzy^6/cort^{RH65},fzy^7* double mutants kept at 29°C, eggs contain either two spindles with one X-chromosome each (H), indicating a metaphase II arrest, or a single spindle with two X-chromosomes (I), indicating a metaphase I arrest. (J-L) Eggs from nosGal4VP16/UAS-cyclinB-TPM-GFP arrest with multiple spindles (J) or with two spindles (K), suggesting a meiosis II arrest, or with a single spindle (L), indicative of a meiosis I arrest.

progression in the meiotic divisions. To address this question, we analyzed female meiosis in eggs produced by *fzy* females. *fzy*, unlike *cort* or *Cks30A*, is essential for viability, and germline clones of a null allele did not produce eggs (data not shown). However, temperature-sensitive allele combinations raised at a permissive temperature are viable and have been used to study the role of *fzy* in early embryogenesis (Dawson et al., 1995). *fzy^6/fzy^7* mutants raised at the permissive temperature of 22°C are female-sterile and embryos arrest in the first mitosis (Dawson et al., 1993). Meiosis appeared to be unaffected in these eggs (data not shown). To achieve a stronger phenotype, we shifted *fzy^6/fzy^7* females to the restrictive temperature of 29°C. In addition to the mitotic arrest, eggs from *fzy^6/fzy^7* females kept at 29°C (hereafter referred to as *fzy* eggs) displayed defects in meiosis. 74% (*n*=78) of *fzy* eggs contained two spindles near the cortex (Fig. 1G), indicative of a delay or arrest in meiosis II. In most cases, both spindles contained two X-chromosome signals (Fig. 1G′), indicating that sister chromatid separation had occurred and that they were therefore in anaphase of meiosis II. Often, as shown in Fig. 1G′, the two X-chromosomes were not properly aligned along the spindle axis, probably as a result of prolonged arrest. In rare cases, we detected more than two X-

chromosome signals per spindle (data not shown), suggesting that DNA replication can occur during the aberrant meiosis in *fzy* eggs. We did not observe meiotic spindles with only a single X-chromosome, indicating that meiosis did not detectably delay or arrest in metaphase of meiosis II in these eggs. Eggs often contained, near the two major spindles, one or more smaller spindles with associated chromatin (arrow, Fig. 1G), possibly resulting from chromosome loss at the first meiotic division. In total, 13% of embryos contained one or more spindles at the anterior cortex in addition to a polar body, suggesting a partial completion of meiosis, whereas 6% of embryos contained only polar bodies at the anterior cortex, and therefore appear to have completed meiosis.

In total, 8% of *fzy* eggs contained only a single spindle near the cortex, possibly indicative of a meiosis I arrest. The same percentage of eggs from *cort* mutants raised at 29°C also arrested with a single meiotic spindle [in agreement with previous findings (Page and Orr-Weaver, 1996)], suggesting the possibility that *cort* and *fzy* play partially redundant roles in meiosis I. To test this possibility, we analyzed the phenotype of a *fzy; cort* double mutant raised at 29°C. In total, 74% (*n*=57) of *fzy; cort* double-mutant eggs contained two spindles, each with a single X-chromosome signal (Fig. 1H,H′),

indicating that they arrested in metaphase of the second meiotic division. The remaining 26% of the eggs contained only a single spindle containing two X-chromosome signals (Fig. 1I,I′), indicating an arrest in meiosis I. We conclude that the two APC adaptors Cort and Fzy are necessary for anaphase progression in both meiotic divisions, performing partially redundant roles in meiosis I and non-redundant roles in meiosis II.

In addition to its role in anaphase, Cks30A is required earlier in meiosis, for the assembly or maintenance of the first meiotic spindle (Pearson et al., 2005; Swan et al., 2005). To determine whether spindle assembly or metaphase I arrest is affected in *cort* or *fzy* mutants, we analyzed chromosome alignment in unactivated oocytes using the X-chromosome FISH probe. In metaphase I in wild type, the autosomes are aligned at the spindle equator while the X-chromosomes are typically precociously segregated to either pole (McKim et al., 2002). We found that chromosomes were properly aligned in both *cort* and *fzy* mutants, as well as in *fzy; cort* double mutants (see Fig. S1 in the supplementary material). Therefore, with the caveat that we are not able to study null alleles of *cort* and *fzy*, we conclude that the first requirement for *cort* and *fzy* in meiosis is in anaphase of meiosis I.

Cyclin destruction is necessary for the completion of meiosis in *Drosophila*

In mitotic cells of most eukaryotes, the APC[Fzy] promotes anaphase by targeting cyclins and other mitotic regulators for destruction (Peters, 2002). The importance of cyclin destruction in the two meiotic divisions is less clear. To determine whether cyclin destruction is necessary for female meiosis in *Drosophila*, we examined meiotic progression in eggs from females expressing a destruction-box (D-box) mutated form of cyclin B – cyclin B-TPM-GFP (Raff et al., 2002). When expressed in the female germline, cyclin B-TPM-GFP results in mitotic arrest at a variable stage of the syncytial mitotic cycle in the majority of embryos, indicating that cyclin B destruction is necessary for anaphase progression in these cell cycles (Raff et al., 2002). To determine whether a failure to destroy cyclin B also disrupts meiosis, we expressed cyclin B-TPM-GFP with the strong germline driver nosGal4VP16 at 29°C (to induce higher expression). Under these conditions, almost all embryos arrested in the first mitotic division (data not shown). In addition to this mitotic arrest, only 38% (*n*=51) appeared to complete female meiosis, as judged by the presence of polar bodies and the absence of spindles at the dorsal anterior of the egg. A total of 50% of eggs contained multiple small spindles in the dorsal anterior, possibly as a result of meiotic spindle breakdown and/or chromosome mis-segregation (Fig. 1J,J′). The remaining 14% of eggs appeared to arrest in meiosis. A small proportion of the eggs (4%) had two spindles with either one or two X-chromosomes, indicative of an arrest in either metaphase or anaphase of meiosis II (Fig. 1K,K′). In addition, 10% of the eggs contained a single spindle at the dorsal anterior, typically with two X-chromosome signals, indicative of a meiosis I arrest (Fig. 1L,L′). Therefore, cyclin B destruction is necessary for the proper completion of female meiosis in *Drosophila*.

Cort and Fzy are required for the destruction of mitotic cyclins in the egg

The above results suggest the possibility that the meiotic arrest in *cort* and *fzy* eggs could be caused by a failure to destroy mitotic cyclins. In *Drosophila*, it is not known whether the APC[Fzy] has any role in cyclin destruction during meiosis. On the other hand, the APC[Cort] has been implicated with *Cks30A* in cyclin A destruction

in the female germline (Swan et al., 2005). To determine the respective roles of *cort* and *fzy* in cyclin destruction in female meiosis, we compared cyclin levels in egg extracts from *cort*, *fzy* and *Cks30A* single mutants, and from *fzy; cort* double mutants. All of these mutants arrest at or before entry into the first mitotic cell cycle and we therefore used unfertilized, and therefore non-cycling, wild-type eggs for control extracts. As previously reported, *Cks30A* and *cort* eggs contain high levels of cyclin A protein (Swan et al., 2005) (Fig. 2). Cyclin A levels were not elevated in egg extracts from *fzy* mutants raised at 22°C (data not shown). However, eggs from *fzy* females kept at 29°C showed a clear elevation in cyclin A levels, and *fzy; cort* double mutants had an even-greater elevation in cyclin A levels (Fig. 2). Therefore, *fzy* and *cort* are both required for cyclin A destruction in the *Drosophila* egg. Cyclin B and cyclin B3 levels were also elevated in *fzy* and *cort* single mutants, and more so in *fzy; cort* double mutants (Fig. 2), indicating that Cort and Fzy cooperate in the destruction of all three mitotic cyclins. Comparing the relative effects of *cort* and *fzy* mutants on the different cyclins suggests that Cort is more important for cyclin A and cyclin B3 destruction, whereas Fzy is more important for cyclin B destruction. Therefore, the two APC adaptors may have different target preferences

In *Xenopus* and mice, Cks2 is necessary for the activation of the APC[Fzy] complex by associating with Cdk1 and promoting its phosphorylation of the APC subunits Cdc27 and Cdc16 (Patra and Dunphy, 1998; Spruck et al., 2003). In *Drosophila*, Cks30A interacts with Cdk1 in the germline and is required for cyclin A destruction (Swan et al., 2005). *Cks30A* eggs also have elevated cyclin B3 levels, and both cyclin A and cyclin B3 were at levels higher than in *cort* or *fzy* single mutants, and were approaching levels observed in *fzy; cort* double mutants (Fig. 2). This could be explained if Cks30A activity is required for the function of both APC[Fzy] and APC[Cort] complexes. Cyclin B, by contrast, is not strongly affected in *Cks30A* mutants (Fig. 2), indicating that Cks30A plays a lesser role in promoting the activity of APC[Fzy] and APC[Cort] in cyclin B destruction.

The above results indicate that Cort, like other Fzy/Cdh1-family proteins, functions in the targeting of mitotic cyclins for destruction. To further test the ability of Cort to target cyclins for destruction, we expressed HA-tagged Cort in a stripe of cells in the wing imaginal disc using the Gal4-UAS system and then looked at cyclin levels by immunolocalization. The expression of HA-Cort resulted in a
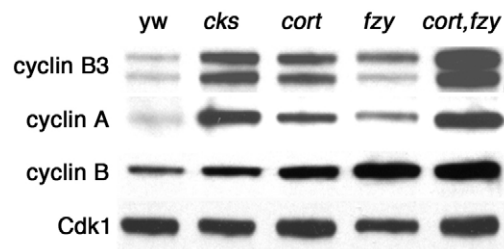

Fig. 2. Cks30A, Cort and Fzy regulate overall cyclin levels in the egg. Western blots of eggs aged 0-2 hours from unfertilized (females crossed to X0 males) wild type (*yw*), *Cks30A^{KO}/Cks30A^{KO}* (*cks*), *cort^{QW55}/cort^{RH65}* (*cort*), *fzy^6/fzy^7* (*fzy*), and *cort^{QW55},fzy^6/cort^{RH65},fzy^7* (*cort,fzy*) kept at 29°C and probed for cyclin B3, cyclin A and cyclin B. Cdk1, a stable cell-cycle regulator, serves as a loading control. Cyclin B is slightly elevated in *Cks30A*, more so in *fzy* and *cort*, and is highly elevated in *fzy; cort* double mutants. Cyclin A and cyclin B3 are both elevated in *cort* and *fzy*, but elevation is higher in *cks30A* and *fzy; cort* double mutants.

corresponding decrease in cyclin A, cyclin B and cyclin B3 (Fig. 3A-C), consistent with these cyclins being targeted for destruction by Cort. A similar effect was observed upon the overexpression of Fzy or Cdh1 (Fig. 3D and data not shown). Therefore, Cort is able to target all of the mitotic cyclins for destruction, consistent with a proposed role as an APC adaptor.

The reduction of cyclin levels would be expected to inhibit mitosis in the wing imaginal disc. Each cell in the wing secretes a single bristle, and mitotic failure results in fewer, but larger, cells; consequently, there are fewer wing hairs (Weigmann et al., 1997). Indeed, the expression of Fzy or HA-Cort in the posterior compartment of the wing disc, using the enGal4 driver, led to fewer but larger cells, as judged by an increase in the spacing between the wing hairs (Fig. 3E,F). To test the possibility that Cks30A is required for the activation of the APC^{Cort}, we used enGal4 to express HA-Cort in *Drosophila* that also lacked zygotic expression of *Cks30A*. In the *Cks30A* background, the wing-hair-spacing phenotype was suppressed (Fig. 3G). It was largely restored if Flag-Cks30A is co-expressed with HA-Cort in the *Cks30A*-mutant background (Fig. 3H), whereas the expression of Flag-Cks30A alone had no effect (Fig. 3I). Therefore, Cks30A is required for Cort activity.

Cyclin B associates dynamically with the meiotic spindle

Cyclin B undergoes incomplete destruction in the syncytial mitotic cycles, apparently as a result of localized destruction restricted to spindles (Edgar et al., 1994; Huang and Raff, 1999; Raff et al., 2002;

Su et al., 1998). It is not known how this local destruction is mediated, or whether localized cyclin B destruction is unique to the syncytial mitotic cycles or whether it also occurs in the preceding meiotic divisions. To determine if cyclin B is subject to localized destruction in female meiosis, we first determined the localization of cyclin B in wild-type meiosis. In *Drosophila* females, meiosis is arrested in metaphase of the first meiotic division until ovulation and cyclin B accumulates at high levels on the metaphase I spindle (Fig. 4A) (Pearson et al., 2005). This cyclin B accumulation is non-uniform and appears to be focused at the meiotic spindle mid-zone – the region of the meiotic spindle where non-kinetochore microtubules from either pole overlap. The meiotic spindle mid-zone (or meiotic metaphase central spindle) appears to play a specialized role in establishing spindle bipolarity and in recruiting chromosomal passenger proteins to the meiotic spindle (Jang et al., 2005). To confirm that cyclin B associates with the spindle mid-
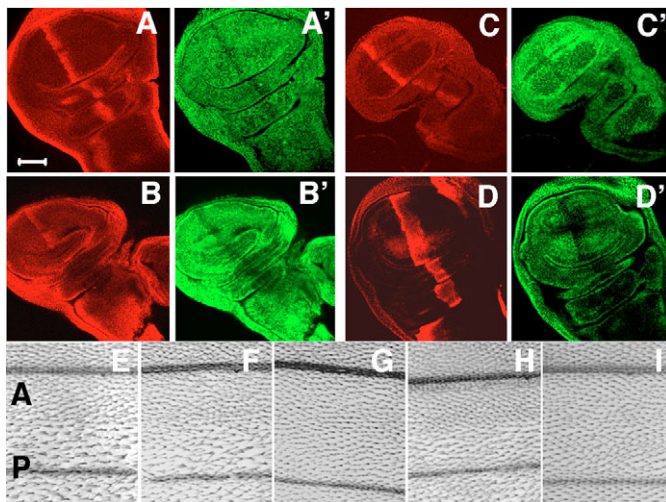

Fig. 3. Cort can mediate the destruction of mitotic regulators and its activity depends on Cks30A. (**A-C'**) UAS-HA-Cort expressed under *ptcGal4* and detected with anti-HA antibodies. Cyclin A (A'), cyclin B (B') and cyclin B3 (C') are reduced in the stripe, corresponding to Cort expression. (**D,D'**) UAS-Fzy expressed under *ptcGal4* (detected with anti-β-gal antibodies; D) results in a corresponding reduction in cyclin A levels (D'). (**E-H**) Magnified area of an adult wing from *Drosophila* expressing HA-Cort (E,G,H) or UAS-Fzy (F) in the posterior-half of the wing (P) using *enGal4*. The anterior-half of the wing (A) serves as a control. Expression of UAS-HA-Cort (E) or UAS-Fzy (F) in the posterior wing results in a wider spacing of wing hairs. (G) Wing-hair-spacing phenotype is suppressed when HA-Cort is expressed in a *Cks30A^{KO}/Cks30A^{KO}* background. (H) Co-expression of UAS-Flag-Cks30A with HA-Cort in a *Cks30A^{KO}/Cks30A^{KO}* background restores the wing-hair-spacing phenotype. (**I**) Expression of Flag-Cks30A alone does not affect bristle spacing. Scale bar: 20 μm.
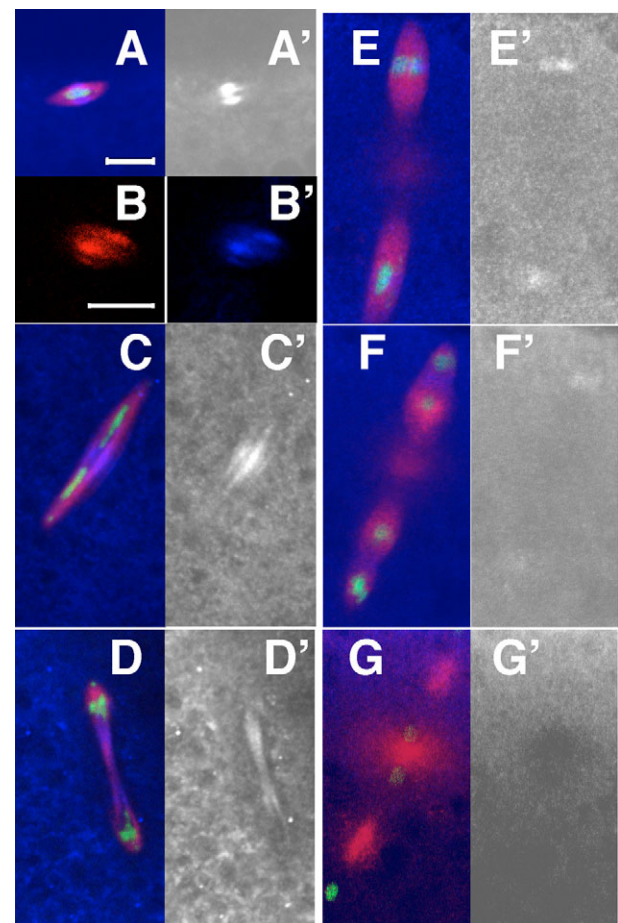

Fig. 4. Cyclin B associates with the spindle in female meiosis. Wild-type (yw) eggs obtained by in vitro activation of oocytes (**A-D'**) or from 0-20 minute egg collections (**E-G'**). In all panels except B,B', microtubules are red, DNA is green and cyclin B is blue. The cyclin B channel is shown by itself in grayscale in right-hand panels (labeled with '). (A) In metaphase I, high levels of cyclin B accumulate on the spindle mid-zone. (B,B') Metaphase I spindle labeled with antibodies to Sub (B) and cyclin B (B') reveals colocalization at the spindle mid-zone. (C,D) In anaphase I, cyclin B persists at the spindle mid-zone. (E) In metaphase II, cyclin B accumulates at the mid-zone on both spindles. (F) Cyclin B persists at lower levels at the mid-zone in anaphase II. (G) Late in anaphase II, cyclin B is no longer detected on the meiotic spindle. Scale bars: 5 μm in A for A,A',C-G'; 2 μm in B for B,B'.

zone, we double-labeled oocytes for both cyclin B and the spindle mid-zone component Subito (Sub) (Jang et al., 2005). Cyclin B and Sub appeared to colocalize precisely (Fig. 4B), confirming that cyclin B specifically associates with the spindle mid-zone in metaphase of meiosis I. In anaphase of meiosis I, the spindle mid-zone extends as the spindle elongates, and chromosomes segregate to either pole (Jang et al., 2005). Cyclin B persisted on the spindle mid-zone throughout anaphase I (Fig. 4C,D). Upon assembly of the second meiotic spindle, cyclin B appeared to redistribute to the spindle mid-zone of the newly formed meiosis II spindles (Fig. 4E). The protein persisted at the spindle mid-zone after the onset of anaphase (Fig. 4F), but was no longer detected later in anaphase (Fig. 4G). Therefore, cyclin B is associated with the meiotic spindle mid-zone throughout meiosis, and dissociates from the spindle late in anaphase II. This pattern of accumulation suggests that cyclin B, presumably in complex with Cdk1, plays a unique role at the meiotic mid-zone in meiosis I and meiosis II, and that it is targeted for destruction at this site in anaphase II.
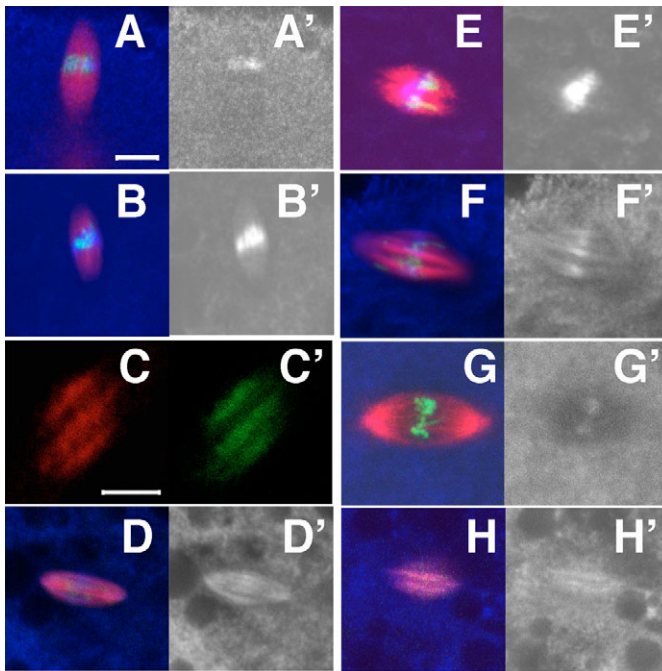

Fig. 5. Cyclin B is stabilized on the meiotic spindle in *cort*, *fzy* and *Cks30A*. (A-B',D-H') Tubulin is red, DNA is green and cyclin B is blue. Cyclin B is shown by itself in grayscale in right hand panels (labeled with '). (**A**) In wild type, cyclin B accumulates on the spindle mid-zone in metaphase of meiosis II. (**B**) Arrested meiosis II spindle from *cort^{QW55}/cort^{RH65}* at 29°C, with high levels of cyclin B associated with the mid-zone of the spindle. (**C,C'**) Co-labeling of a metaphase II spindle from *cort^{QW55}/cort^{RH65}* with antibodies to Sub (C) and cyclin B (C') reveals colocalization at the spindle mid-zone. (**D**) Meiosis II spindle from *fzy^6/fzy^7*, showing the accumulation of cyclin B along the length of the spindle. (**E**) Metaphase-arrested meiotic spindle from *cort^{QW55},fzy^6/cort^{RH65},fzy^7* with a high level of cyclin B at the spindle mid-zone. (**F**) Meiotic spindle from *Cks30A^{KO}/Cks30A^{KO}*, with elevated levels of cyclin B on the spindle mid-zone and along the spindle. (**G,H**) Mitotic spindles in *yw* (G) and *fzy^6/fzy^7* (H). In wild type, cyclin B associates with the spindle mid-zone late in metaphase, before disappearing from the spindle in anaphase. In *fzy*, cyclin B accumulates along the length of the arrested mitotic spindle. Scale bars: 5 μm in A for A-B',D-H';. 2 μm in C for C,C'.

Cort, Fzy and Cks30A are required for the local destruction of cyclin B

To determine if the dissociation of cyclin B from meiotic and mitotic spindles in anaphase reflects its local destruction by the APC^{Cort} or APC^{Fzy}, we compared cyclin B distribution in wild-type eggs (Fig. 5A) with eggs from *cort* and *fzy* single-mutant females at 29°C. In *cort*, cyclin B accumulated on the arrested meiotic spindles (Fig. 5B, only one of the two meiosis II spindles is shown). This accumulation was significantly higher than that detected in wild-type metaphase II, suggesting that cyclin B is stabilized on the arrested spindle. In *cort*, as in wild type, cyclin B specifically associated with the overlapping microtubules of the spindle mid-zone, co-localizing with the mid-zone component Sub (Fig. 5C). *fzy* eggs also arrested, with elevated levels of cyclin B on the meiosis II spindles (Fig. 5D). However, rather than exclusively accumulating at the spindle mid-zone, cyclin B was at lower levels more uniformly along the spindle. The finding that mutations in *cort* and *fzy* result in a stable association of cyclin B with the meiotic spindle strongly supports a model in which the loss of cyclin B from the meiotic spindle in anaphase is a result of localized destruction by the APC^{Cort} and APC^{Fzy} complexes.

The difference in site of cyclin B accumulation on the meiotic spindle between *cort* and *fzy* could be a result of Cort and Fzy having distinct sites of activity. In this model, Cort would mediate cyclin B destruction at the spindle mid-zone while Fzy targeted cyclin B along the length of the spindle. One consequence of this model would be that *fzy; cort* double mutants might have a cyclin B accumulation that is the sum of that of the two single mutants. Alternatively, Cort and Fzy may mediate cyclin B destruction at different stages of meiosis. In this model, Cort would mediate cyclin B destruction in metaphase when cyclin B is primarily at the mid-zone, and Fzy would function in anaphase along the entire spindle. This model fits with the time of arrest of *cort* and *fzy* in metaphase and anaphase, respectively (Fig. 1), and it predicts that *fzy; cort* double mutants would arrest in metaphase, with cyclin B localized at the mid-zone. We find that *fzy; cort* double mutants do indeed accumulate cyclin B largely at the spindle mid-zone and not along the length of the spindle (Fig. 5E), and are therefore identical to *cort* single mutants. Therefore, the different site of accumulation of cyclin B in *cort* and *fzy* may reflect different temporal requirements for the APC^{Cort} and APC^{Fzy} in meiosis.

Analysis by western blot showed that *Cks30A* has little effect on overall cyclin B levels (Fig. 3). However, the immunostaining of eggs from *Cks30A* revealed that cyclin B was enriched on meiotic spindles (Fig. 5F). Therefore, Cks30A is also required for the destruction of cyclin B on spindles in female meiosis, consistent with a role in the activation of the APC^{Cort} and APC^{Fzy} complexes.

In the syncytial embryonic cell cycles, cyclin B associates with the mitotic spindle at metaphase (Huang and Raff, 1999) (Fig. 5G), and its destruction on spindles may play a role in anaphase progression. Given that the APC^{Cort} and APC^{Fzy} are both required for the destruction of cyclin B on the meiotic spindle, it seems likely that either or both APC complexes would also be involved in local cyclin B destruction on mitotic spindles. *cort* mutants arrested prior to the assembly of a mitotic spindle and, therefore, the role of Cort in localized cyclin B destruction in mitosis could not be determined. *Fzy* and *Cks30A*, however, enter into, and arrest in, the first mitosis. In both of these mutants, the mitotic arrest is associated with a failure to locally destroy cyclin B (Fig. 5H and data not shown), arguing that Cks30A and Fzy are necessary for the local destruction of cyclin B in syncytial mitosis, as well as in meiosis.

DISCUSSION

In most cell types, in both *Drosophila* and in other metazoans, the APC^{Fzy} drives anaphase progression by targeting mitotic cyclins and other mitotic proteins for destruction. The female germline is an exception in that the APC^{Fzy} is not sufficient. A germline-specific APC adaptor, Cort, cooperates with Fzy to mediate cyclin destruction in meiosis.

Cort is a functional Fzy/Cdh1 homologue

The *cort* gene encodes a diverged member of the Fzy/Cdh1 family (Chu et al., 2001). Fzy/Cdh1 homologues interact with the APC and with specific sequences (D-box, KEN box or A-box) found on cyclins and on other APC targets. As such, Fzy/Cdh1 proteins act as specificity factors to target proteins for ubiquitination and eventual destruction. Cort protein, like all Fzy/Cdh1-family proteins, contains seven WD domains in the C-terminal-half of the protein, implicated in substrate recognition (Pfleger et al., 2001). We also found that Cort has an N-terminal C-box (amino acids 482, 483) and a C-terminal IR tail (amino acids 54-60), both implicated in binding to the APC (Passmore et al., 2003; Schwab et al., 2001; Vodermaier et al., 2003). In addition to containing these conserved functional domains, Cort displays a conserved ability to mediate cyclin destruction. *cort* mutations result in the overaccumulation of cyclin A, cyclin B and cyclin B3 in the egg (Swan et al., 2005) (Fig. 2), whereas the ectopic expression of Cort in the wing disc leads to a reduction in the levels of these mitotic cyclins (Fig. 3). Taken together, these results indicate that Cortex encodes a functional member of the Fzy/Cdh1 family.

Fzy and Cort cooperate to promote cyclin destruction and meiotic progression

Although the *Drosophila* genome has four genes that encode Fzy/Cdh1 proteins, only two of these proteins, Fzy and Cort, are expressed at detectable levels in the female germline (Raff et al., 2002; Jacobs et al., 2002; Chu et al., 2001). We have studied the role of these two APC adaptors both individually and in double mutants, and have found that they function together to promote anaphase in both the first and second meiotic divisions of female meiosis. In most cell types in *Drosophila* and other eukaryotes, a single APC complex, APC^{Fzy}, is responsible for cyclin destruction and anaphase progression. It is therefore surprising that, in the female germline of *Drosophila*, two APC adaptors are necessary for meiotic progression. In the case of meiosis I, Cort and Fzy appear to play largely redundant roles, as only removing both genes results in a significant block in meiosis I. The two APC complexes may also be functionally redundant with respect to global cyclin levels. Mutations in either *fzy* or *cort* result in an increase in the levels of cyclin A, cyclin B and cyclin B3, whereas mutation in both genes results in even-further increases in cyclin levels.

Although Cort and Fzy have overlapping roles in promoting anaphase I, both are essential for meiosis II. This could simply reflect a greater quantitative requirement for APC activity in meiosis II. Alternatively, the two APC complexes could have distinct roles in the second meiotic division. Consistent with this latter possibility, mutations in either *cort* or *fzy* both result in arrest at different stages of meiosis II: *cort* mutants arrest with the sister chromatids associated, and therefore in metaphase, whereas *fzy* mutants almost invariably arrest with separated sister chromatids, and are therefore in anaphase. *cort* and *fzy* also result in different patterns of cyclin B stabilization on the arrested spindles, suggesting roles in metaphase and anaphase, respectively. Therefore, Cort may function to initiate sister chromatid separation at the onset of anaphase II and Fzy may

primarily function later, in anaphase II. Alternatively, the later arrest observed in *fzy* could simply reflect the fact that the *fzy* alleles that we have used are not nulls, and it is possible that a complete loss of Fzy activity would also result in a metaphase arrest, as seen in *cort*. However, comparing the meiosis II phenotypes of *fzy* with *Cks30A*-null mutants suggests that the later arrest in *fzy* is not simply due to residual activity. *Cks30A*-null mutants have a weaker meiotic arrest than *fzy*, as they complete meiosis at high frequency (Swan et al., 2005), but they display a higher frequency of metaphase arrest or delay. The fact that *fzy* does not similarly cause a delay in metaphase of meiosis II suggests that it is only required at anaphase. Therefore, it is possible that Fzy is crucial at anaphase, whereas Cort is necessary for the metaphase to anaphase transition.

The different temporal requirements for Cort and Fzy prior to and after sister chromatid separation, respectively, could be related to their apparent differences in substrate specificity. Western analysis (Fig. 2) reveals that Cort is more important for the destruction of cyclin A and cyclin B3, whereas Fzy appears to play a greater role in cyclin B destruction in the egg. In mitotic cells, cyclin destruction occurs sequentially. Cyclin A is destroyed first, in prometaphase, and this is a prerequisite for sister chromatid separation. Cyclin B destruction occurs at anaphase onset and is necessary for later anaphase events, subsequent to sister chromatid separation (Sigrist et al., 1995). Therefore, it is possible that Cort promotes the early stages of meiotic anaphase by targeting cyclin A for destruction, whereas Fzy is more crucial later, through its targeting of cyclin B for destruction.

Role of the APC in meiosis

The meiotic cell cycle differs in many respects from the standard mitotic cycle. Whereas APC-mediated destruction of mitotic regulators appears to be required for anaphase progression in most or all mitotic cells, the role of the APC and cyclin destruction in meiosis is not as well-understood. Our analysis of the two APC adaptors Cort and Fzy has permitted an evaluation of the role of the APC complex in female meiosis in *Drosophila*. We found that the APC is required for anaphase progression in both meiotic divisions. Correlating with its requirement for the completion of meiosis, the APC is required for the destruction of mitotic cyclins. At least one of these cyclins, cyclin B, is a crucial substrate in meiosis, because the expression of a stabilized form of cyclin B disrupts this process (Fig. 1). Therefore, APC activity and cyclin destruction are required for anaphase progression in both meiotic divisions, in addition to in mitosis. APC activity has been implicated in both meiotic divisions in *C. elegans* (Furuta et al., 2000; Golden et al., 2000) and in the mouse (Salah and Nasmyth, 2000; Terret et al., 2003), and in the second, but not the first, meiotic division in *Xenopus* (Peter et al., 2001; Taieb et al., 2001). In yeast, two APC complexes, the mitotic APC^{Fzy} and a meiosis-specific complex (APC^{Ama1} in *S. cerevisiae* and APC^{Mfr1} in *S. pombe*) function together to mediate protein destruction in meiosis (Asakawa et al., 2001; Blanco et al., 2001; Izawa et al., 2005; Salah and Nasmyth, 2000). It now appears that *Drosophila* also uses two APC complexes in female meiosis, and this may turn out to be a common strategy in other eukaryotes.

The role of Cks30A in activating the APC

Cks30A belongs to a highly conserved family of proteins that bind to and stimulate the activity of the mitotic kinase Cdk1. In *Xenopus*, the Cks30A homologue Xep9 stimulates the Cdk-dependent phosphorylation of APC subunits, and thereby promotes the activation of the APC^{Fzy} complex (Patra and Dunphy, 1998). Our results suggest that Cks30A may have a similar role in stimulating

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both the APC^{Fzy} and APC^{Cort} in female meiosis in *Drosophila*. First, *Cks30A*, as are *cort* and *fzy*, is required for the completion of meiosis II and, like *fzy*, it is required for the completion of the first mitotic division of embryogenesis (this study, Fig. 1) (Lieberfarb et al., 1996; Page and Orr-Weaver, 1996; Swan et al., 2005). Second, Cks30A, as are Cort and Fzy, is necessary for global cyclin destruction in the *Drosophila* egg and for local cyclin B destruction on the meiotic spindle (Figs 2, 5). Global levels of cyclin A and cyclin B3 are elevated to a greater extent in *Cks30A* mutants than in single mutants for *cort* or *fzy*, consistent with the idea of Cks30A activating both Cort and Fzy. Third, we have shown that Cks30A is necessary for the activity of ectopically expressed Cort in the adult wing (Fig. 3). Cks30A may also play a role in activating APC^{Fzy} in mitotic cells. We have found that the temperature-sensitive *fzy⁶* allele is lethal at all temperatures in a *Cks30A*-null background (A.S. and T.S., unpublished), suggesting that the Cks30A-dependent activation of APC^{Fzy} becomes essential when Fzy activity is compromised.

Although Cks30A appears to promote the activity of the APC^{Cort} and the APC^{Fzy}, these complexes seems to retain some activity in the absence of Cks30A. Whereas *cort* and *fzy* cause an arrest in meiosis II, *Cks30A*-null mutants are typically delayed only in meiosis II (Swan et al., 2005). Also, although cyclin A and cyclin B3 levels are elevated more in Cks30A eggs than in either *fzy* or *cort*, their levels are still not as high as in *fzy; cort* double mutants, indicating that Fzy and Cort can destroy cyclin A and cyclin B3 to some degree in the absence of Cks30A. Cyclin B destruction is even less dependent on Cks30A, because cyclin B levels are affected less in *Cks30A* mutants than in either *cort* or *fzy* single mutants. Therefore, Cks30A may be more crucial for the activity of APC^{Cort} and APC^{Fzy} complexes on cyclin A and cyclin B3, and less crucial for their activity on cyclin B. The relatively weaker meiotic arrest in *Cks30A* mutants compared to *fzy; cort* double mutants may also indicate that the APC has other meiotic targets that can be destroyed in the absence of Cks30A.

Localized cyclin destruction in *Drosophila* meiosis

Cyclin B undergoes local oscillations in its association with mitotic spindles in syncytial embryos, appearing transiently along the full length of the mitotic spindle in early metaphase and gradually disappearing from the spindle starting at the centrosomes and ending at the kinetochores (Huang and Raff, 1999). The timing of this loss of cyclin B from the spindle, at the onset of anaphase, corresponds with the timing of cyclin B destruction in other cell types, suggesting the possibility that cyclin B is locally destroyed on the spindle in anaphase. We now show that cyclin B is subject to similar local oscillations in the female meiotic cycles (Fig. 4), and that cyclin B destruction is necessary for the completion of female meiosis (Fig. 1J-L). Importantly, we demonstrate that the local loss of cyclin B from the spindle in meiosis is dependent on the APC adaptors Cort and Fzy, and that the local loss of cyclin B from the spindle in mitosis depends on Fzy (Fig. 5). These results strongly suggest that the local loss of cyclin B from the spindle in anaphase of meiosis II and anaphase of mitosis is actually due to its local destruction.

The pattern of accumulation and loss of cyclin B from the spindle in meiosis differs in some respects compared to syncytial mitotic cycles. First, in metaphase of mitosis, cyclin B initially accumulates throughout the spindle microtubules (Huang and Raff, 1999), whereas, in metaphase of the meiotic divisions, cyclin B first appears exclusively at the spindle mid-zone. This difference may reflect the fact that the meiotic spindle does not contain centrosomes and cyclin B may, therefore, not load onto spindles from centrosomes and progress along the spindles to the kinetochores, as has been proposed for mitosis (Huang and Raff, 1999). Second, the timing of

cyclin B destruction appears to be different between the meiotic and mitotic cycles. Most strikingly, there is no loss of cyclin B from the spindle in anaphase of meiosis I, implying that local cyclin B destruction is not necessary for the completion of the first meiotic division. In addition, the loss of cyclin B from the spindle following meiosis II only occurs late in anaphase rather than at the onset of anaphase, as occurs in the syncytial mitotic cycles. We do not yet know how cyclin B destruction is prevented in anaphase I and early in anaphase of meiosis II. One possibility is that the spindle-assembly checkpoint is locally active during these stages. This checkpoint is required for the proper completion of female meiosis in *Drosophila* (Fischer et al., 2004; Gilliland et al., 2005), and it will be interesting to see if this requirement reflects a role in inhibiting either APC^{Fzy} or APC^{Cort} activity.

The specific accumulation of cyclin B at the spindle mid-zone in meiosis may reflect the unique properties of the meiotic spindle. The mid-zone microtubules or central spindle microtubules are a subset of spindle microtubules that do not end in kinetochores, but instead overlap at the mid-zone with microtubules from the other pole. In dividing cells, the central spindle is crucial for cytokinesis, but, in female meiosis, it appears to have a role in spindle assembly (Jang et al., 2005). Along with cyclin B, the chromosomal passenger proteins Aurora B and Incenp are recruited to the spindle mid-zone. It will be of great interest to determine what these proteins do at the mid-zone and how cyclin B destruction at this site may be important for anaphase in meiosis. It will also be important to determine how the APC^{Cort} targets cyclin B at the spindle mid-zone. We have not been able to detect any specific localization of GFP or HA-tagged Cortex in meiosis or in the syncytial embryo (A.S. and T.S., unpublished), but it is possible that its activity is spatially regulated.

In conclusion, our results support a model in which two APC complexes, APC^{Fzy} and APC^{Cort}, cooperate to mediate the destruction of meiotic cyclins and allow progression through female meiosis

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