

Drosophila Cks30A interacts with Cdk1 to target Cyclin A for destruction in the female germline

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Summary

Cks is a small highly conserved protein that plays an important role in cell cycle control in different eukaryotes. Cks proteins have been implicated in entry into and exit from mitosis, by promoting Cyclin-dependent kinase (Cdk) activity on mitotic substrates. In yeast, Cks can promote exit from mitosis by transcriptional regulation of cell cycle regulators. Cks proteins have also been found to promote S-phase via an interaction with the SCF^{Skp2} Ubiquitination complex. We have characterized the *Drosophila* Cks gene, *Cks30A* and we find that it is required for progression through female meiosis and the mitotic divisions of the early embryo through an interaction with Cdk1. *Cks30A*

mutants are compromised for Cyclin A destruction, resulting in an arrest or delay at the metaphase/anaphase transition, both in female meiosis and in the early syncytial embryo. *Cks30A* appears to regulate Cyclin A levels through the activity of a female germline-specific anaphase-promoting complex, CDC20-Cortex. We also find that a second closely related Cks gene, *Cks85A*, plays a distinct, non-overlapping role in *Drosophila*, and the two genes cannot functionally replace each other.

Key words: Cks, *Drosophila*, Cell cycle, Meiosis

Introduction

Passage through the cell cycle must be precisely regulated during development. A number of conserved cell cycle regulators have been shown to act at important developmental transitions in various organisms, including *Drosophila* (for a review, see Lee and Orr-Weaver, 2003). Cks, or Suc1, is a small cell cycle regulator that was first identified by its ability to interact genetically and physically with the Cyclin-dependent kinases (Cdks) in yeasts (Hadwiger et al., 1989; Hayles et al., 1986). Members of this family were subsequently found in many multicellular organisms, including *Xenopus*, *Caenorhabditis elegans* and humans (reviewed by Bartek and Lukas, 2001; Pines, 1996). In addition to interacting with Cdks, Cks proteins share an anion-binding domain implicated in binding to the phospho-epitope Ser/pSer/pThr/X, found on many mitotic proteins; and a domain that mediates a conformational switch between a monomeric and a dimeric form in vitro.

Despite the strong conservation of these proteins, studies in different animal models have revealed a surprising number of distinct roles at different points in the cell cycle. Studies of the Cks2 homolog in *Xenopus* (Xe-p9) indicate a role in the G2-M transition and the metaphase/anaphase transition, possibly by linking the Cdk to its substrates (Patra and Dunphy, 1996; Patra and Dunphy, 1998; Patra et al., 1999; Spruck et al., 2003). Mammalian Cks2 and *C. elegans* CKS-1 are also required for the metaphase/anaphase transition (Polinko and Strome, 2000; Spruck et al., 2003). Cks1 in mammals plays a seemingly unrelated role in promoting S-phase progression as an adaptor protein that links the SCF^{Skp2} complex to one of its substrates,

the Cdk inhibitor p27 (Ganoth et al., 2001; Spruck et al., 2001). Recent work in yeast has revealed yet another function for Cks. *Saccharomyces cerevisiae* Cks1 was found to recruit the 19S and 20S proteasome to the promoter of the CDC20 gene to promote its transcription late in mitosis (Morris et al., 2003). In metazoans, which all appear to have two Cks genes, it is not yet clear how the two Cks genes carry out these diverse roles, and to what degree there is functional redundancy between them.

Female meiosis in *Drosophila* represents an excellent system for studying the developmental control of cell cycle progression. Female meiosis progresses through well-characterized transitions that are linked to development of the oocyte. With the aim of identifying genes required for completion of female meiosis in *Drosophila*, we analyzed a collection of maternal effect mutants (Schupbach and Wieschaus, 1989) that arrest early in embryogenesis. We found that the *remnants* (*rem*) gene is required for female meiosis and for mitosis in the early embryo, and that this gene corresponds to the *Drosophila* Cks gene at cytological location 30A (*Cks30A*). In addition to its maternal role, *Cks30A* appears to function with Cdk1 to prevent S-phase in G2-arrested histoblasts in the larva. *Cks30A* interacts with Cdk1/cyclin complexes, and this interaction is necessary for its function. One of the key functions of *Cks30A* is to mediate the destruction of Cyclin A, and we present evidence that this is through an effect on the activity of Cortex, a germline-specific adaptor for the Anaphase-Promoting Complex (APC). We also found that a closely related Cks gene, *Cks85A*, plays a distinct role in *Drosophila*, and the two genes cannot substitute for each other in vivo.

Materials and methods

Genetics and molecular biology

rem^{RA74} and *rem^{HG24}* were generated in our laboratory (Schupbach and Wieschaus, 1989). *Cdk1* alleles *Cdk1^{E1-23}* and *Cdk1^{E1-24}* (Stern et al., 1993) were provided by Christian Lehner. The distal breakpoint of *Df(2L)30AC* was mapped between *CG9582* (2L:9017635 and *Toll-4* (2L:9081671) by PCR on homozygous embryos. By the same method, the proximal breakpoint of *Df(2L)gamma25* was mapped between *G-gamma30A* (2L:9248035) and *CG13111* (9365826). This allowed mapping of *rem* to a region of 348 kb, containing 19 genes. *Cks30A^{KO}* was made by homologous recombination (Rong et al., 2002), removing the start codon from *Cks30A*. No alternative start sites exist upstream or downstream. Cks rescue constructs were all made by PCR amplifying wild-type genomic DNA corresponding to the coding region and inserting downstream of a sequence encoding the FLAG epitope tag in pUASp. Mutant forms of *Cks30A* were made using QuikChange Site-Directed Mutagenesis Kit (Stratagene). *Cks85A* (CG9790) null mutants were generated by imprecise excision of the P-element EP2346, which is upstream of the first exon. *Cks85A^{ex15}* is deleted for all of the promoter sequence and most of the coding sequence of the gene. Northern blots were performed on mRNA purified by Trizol (Sigma) followed by polyAtract (Promega). RT-PCR to *cyclin A* was performed for 15 cycles on total RNA, using radiolabeled primers to allow detection of products.

Immunoprecipitations and western blots

Immunoprecipitations were performed on frozen 0-8 hour embryos or 0-4 hour embryos from females carrying a single copy of *nanos-Gal4VP16* (Van Doren et al., 1998) and a single copy of a given FLAG-tagged Cks in 10 volumes of 50 mmol/l Tris (pH 8), 150 mmol/l NaCl, 1 mmol/l CaCl₂ and 0.1% Tween 20 supplemented with complete mini protease inhibitor (Roche) and type I and II phosphatase inhibitors (Sigma). Anti-FLAG beads and FLAG peptide for elution were from Sigma. Antiserum against *Drosophila* Cks30A was raised in guinea pig (Pocono Rabbit Farms) against full-length His-tagged Cks30A expressed in bacteria, and was affinity purified against GST-tagged Cks30A. This was used at 1/100 on western blots. Other antibodies for western blots were rabbit α -Cdk2 at 1/500, rabbit α -Fzr (1/500) and rabbit α -Cyclin B3 (1/1000) (all gifts from Christian Lehner), mouse α -Fzy (from Ian Dawson), mouse α -Cyclin B and α -Cyclin A (both from Developmental Studies Hybridoma Bank) at 1/10, mouse α -FLAG M5 (Sigma) at 1/20 and rabbit α -PSTAIR (Santa Cruz) at 1/1000. Relative Cyclin A levels were determined by loading 1 \times , 4 \times , 8 \times , 12 \times and 16 \times dilutions of *Cks30A^{KO}* extracts and comparing band intensity (using IP Image software) to 1 \times of wild-type extract.

Antibody staining and fluorescence in-situ hybridization

To visualize early events in female meiosis, mature oocytes from hand-dissected ovaries were activated in vitro (Page and Orr-Weaver, 1997). Later stages of meiosis were observed in fixed embryos from 0-20 minute collections. For most embryo stainings, timed collections were dechorionated in bleach, fixed in 1:1 heptane/methanol. For phospho-histone H3 staining, embryos were fixed in 3.7% formaldehyde in PBST/heptane. Embryos and ovaries were mounted in tetrahydronaphthalene after incubating in methanol followed by isopropanol. Antibodies were rat α -Tubulin (Cappel) at 1/200, rabbit α -Cnn (gift of Thomas Kaufman) at 1/500, mouse α -Lamin Dmo (Development Studies Hybridoma Bank) at 1/20, rabbit α -phospho-histone H3 (Upstate) at 1/500, rabbit α -Caspase 3 (Cell Signaling Technologies) at 1/500. DNA was visualized with Oligreen (Molecular Probes) at 1/500. Fluorescence in-situ

hybridization (FISH) against X- and Y-chromosome-specific repeat sequences was performed according to Dernburg (Dernburg, 2000).

Results

Remnants is required for female meiosis and early embryogenesis

The first alleles of *rem* were isolated in a screen for female sterile mutations (Schupbach and Wieschaus, 1989). Embryos derived from females homozygous or hemizygous for *rem^{HG24}* or *rem^{RA74}* arrest development at the onset of embryogenesis, typically with a single metaphase mitotic spindle (Fig. 1A,B; Table 1, 40-80 minutes after egg-laying). Some embryos progress through a limited number of divisions before arresting, again in a metaphase-like state (Fig. 1C; Table 1). Several observations indicated that these metaphase-arrested spindles were zygotic and not derived from the female meiotic spindle. First, they were positioned in the egg interior, where the zygotic nuclei are typically found. Second, these spindles contained centrosomes (Fig. 1B,C), suggesting that they were organized by the male pronucleus, because centrosomes in the embryo

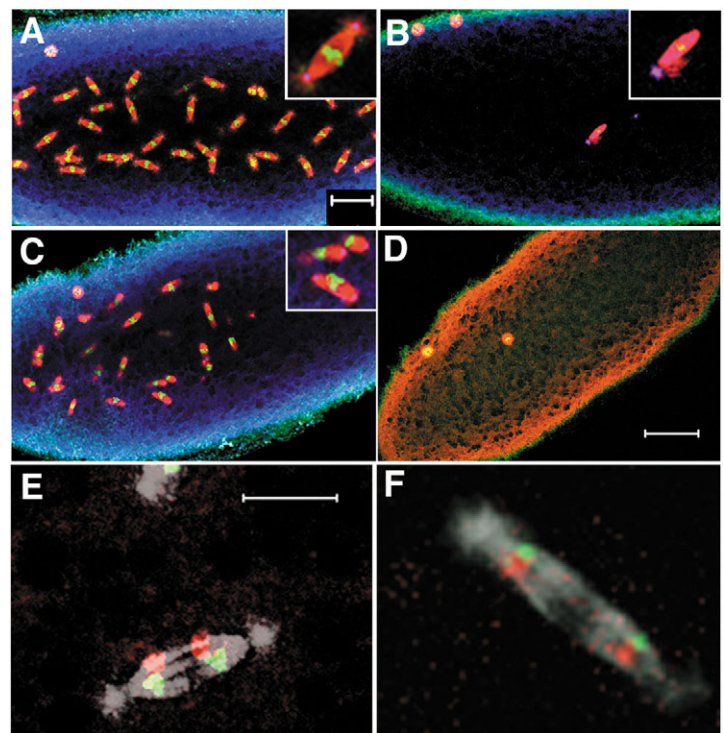


Fig. 1. Embryos from *remnants* mutant females arrest in early embryogenesis. (A-C) Embryos labeled for DNA (green), microtubules (red) and Cnn to label centrosomes (blue). (A) Wild-type embryo in metaphase of syncytial cycle. (B,C) Embryos from *rem^{HG24}/rem^{HG24}* arrested with one metaphase spindle (B) and with multiple metaphase spindles (C). Nuclei fail to migrate and accumulate at the anterior of the embryo. Spindles often lack centrosomes and/or are unipolar (insets B,C). (D) Unfertilized egg from *rem^{RA74}/Df(2L)30AC* possessing polar bodies but no mitotic spindles. (E,F) Microtubules (white) and FISH against X chromosome (green), and Y chromosome (red). (E) Wild-type embryo in which all mitotic spindles label for both X and Y chromosomes. (F) *rem^{HG24}/rem^{HG24}* embryo in which mitotic spindles contain both X and Y chromosomes. Scale bars: in A, 20 μ m for A,B,C; in D, 50 μ m; in E, 10 μ m for E,F.

Table 1. *cks30A* meiotic and mitotic phenotypes

Genotype	Minutes AEL	Female meiosis			Embryo mitosis		
		Metaphase/anaphase	Interphase	Polar bodies	Centrosomes; no spindle	One spindle	More than one spindle
Wild type	0-20	7	25	64	26	26	48
	20-40	0	0	100	0	0	100
	40-80	0	0	100	0	0	100
<i>cks30A^{HG24}</i>	0-20	30	15	55	37	58	5
	20-40	18	0	82	27	59	14
	40-80	0	0	100	4	41	56
<i>cks30A^{KO}</i>	0-20	57	10	33	13	29	0
	20-40	45	10	45	46	54	0
	40-80	0	10	90	19	77	4

Embryos were collected at different times after egg lay (AEL) at 22°C, fixed and immunostained to reveal microtubules and DNA. All embryos were then classified with respect to stage of female meiosis and embryonic cell cycle, and expressed as a percentage (mean $n=28$, $n \geq 19$). For mutant embryos, development did not continue significantly beyond 40-80 minutes AEL, and therefore this represents the terminal arrest phenotype.

derive from the male sperm aster. Third, spindles were not detected in eggs from unfertilized *rem* mutant females (Fig. 1D). To confirm that *rem* eggs were fertilized and to determine if pronuclear fusion occurred before arrest, we performed FISH against X- and Y-chromosome sequences. In wild type, as expected, half the embryos were positive for the Y-probe, and all nuclei in these embryos also labeled positively for the X-probe (Fig. 1E). In eggs from *rem^{HG24}* homozygous mutants, approximately 50% contained Y-signal (17/38), indicating that embryos were successfully fertilized. Of the embryos fertilized by Y-carrying males, the majority (13/17) had spindles containing both X and Y chromosomes (Fig. 1F), indicating that most, although not all, embryos completed pronuclear fusion.

The partial failure of pronuclear fusion could be due to a failure to complete meiosis. In meiosis in *Drosophila* females (reviewed by Foe et al., 1993), the oocyte nucleus is arrested in prophase for most of oogenesis. This arrest is broken in stage 12: nuclear envelope breakdown (NEB) occurs and the meiosis I spindle is assembled. Ovulation triggers resumption of meiosis. The meiosis I spindle rotates 90° and then rapidly enters anaphase and assembles the tandem meiosis II spindle (Fig. 2C,E). While female meiosis is being completed, the male

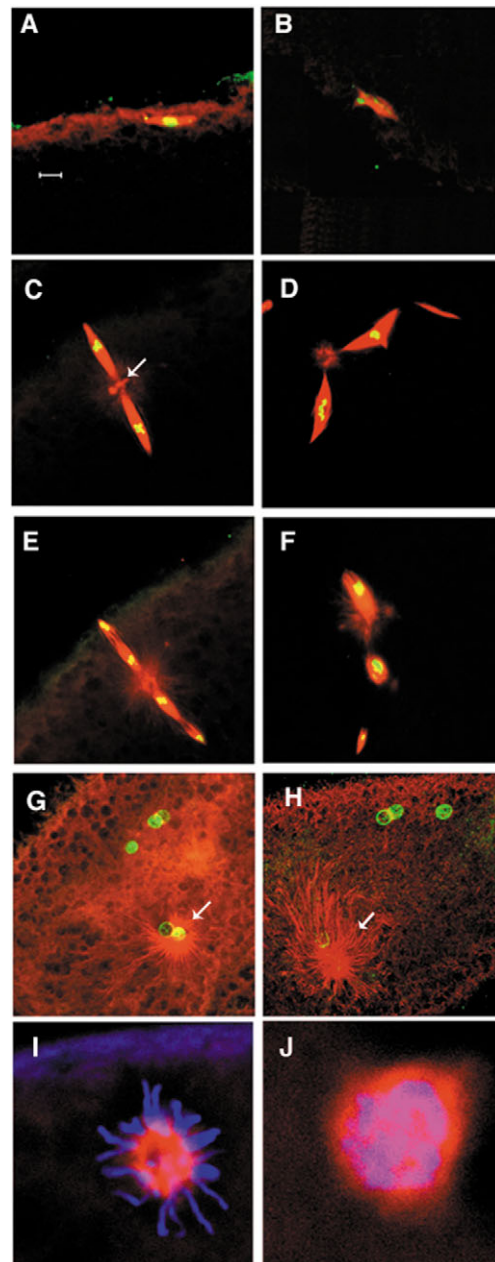


Fig. 2. Female meiosis is disrupted in *rem* mutants. (A,B,D,F) *rem^{HG24}/rem^{HG24}*, (C,E,G) wild type and (H) *rem^{RA74}/Df(2L)30AC*, labeled for DNA (green) and microtubules (red). (A) Metaphase I of meiosis is typically not affected in *rem* mutants, but 7% display disorganized spindles as in B. (C,D) Metaphase of meiosis II. In wild type, the meiosis II spindles are arrayed in tandem and connected by the spindle mid-body (arrow), while in *rem*, the spindles are kinked or separated. (E,F) Anaphase II. In wild type, four meiotic products with equal DNA content can be distinguished. *rem* spindles frequently break down and mis-segregation occurs. In the lower spindle, the two meiotic products are of different sizes, while the upper spindle has not entered anaphase. Pronuclear fusion in wild type (G) and *rem* (H). All nuclei are in interphase, and the male pronucleus and female pronucleus are in apposition and associated with the male sperm aster (arrows in G,H). The male pronucleus is largely obscured by the sperm aster in H. (I,J) Polar bodies from wild type (I) and *rem^{HG24}/rem^{RA74}* (J) stained for microtubules (red) and the prophase/metaphase marker phospho-histone H3 (blue). Overlap appears pink. In wild type and in *rem*, DNA labels with phospho-histone H3, although in *rem* the chromatin is surrounded by microtubules within a shell, while in wild type the chromatin radiates out from a microtubule array. Scale bar: in A, 5 μ m for A-H.

aster forms. At completion of meiosis, the four meiotic products and the male pronucleus synchronously reform nuclear envelopes and one female pronucleus (typically the one closest to the male pronucleus) moves towards the male aster (Fig. 2G). When the nuclei are closely apposed, the male centrosome divides, migrates around the two pronuclei, and assembles the first zygotic spindle.

In *rem*, the first events of female meiosis are unaffected. The karyosome appeared normal, the timing of NEB and appearance of this spindle was also unaffected in the majority of mutant ovaries (Fig. 2A), although approximately 7% had abnormal spindles and failed chromosome congression on the spindle (Fig. 2B). Consistent meiotic defects were always detected upon ovulation and entry into the second meiotic division. Rotation of the spindle failed in the majority of oocytes (data not shown) and assembly of the meiosis II spindle was abnormal. The most common defect in *rem* mutants was a kink and/or separation of the two tandem spindles (Fig. 2D). In more severe cases, the meiotic spindle broke down and chromosome segregation was severely disrupted (Fig. 2F). Despite these errors, the female meiotic products appeared to exit meiosis, reforming nuclear envelopes, and pronuclear fusion occurred (Fig. 2H), consistent with the FISH results.

In wild type, after completion of meiosis, the three post-meiotic nuclei that do not join with the male pronucleus undergo nuclear envelope breakdown and arrest in a metaphase-like state. Chromosomes are condensed and label for the prophase/metaphase marker Phospho-Histone H3, and are associated with a radial aster of microtubules (Fig. 2I).

In *rem*, chromosomes condensed and stained positive for phospho-histone H3, but the polar body microtubule aster did not form, and instead a shell of microtubules surrounded the chromatin (Fig. 2J).

Remnants is a *Drosophila* Cks gene

Rem mutants fail to complement *Df(2L)30AC* but complement *Df(2L)gamma 27* (Schupbach and Wieschaus, 1989; Lane and Kalderon, 1993). We refined the location of these deficiency breakpoints molecularly, and this allowed us to map *rem* to a region containing 19 genes (see Materials and methods). One probable candidate in the region is the cell cycle regulator, Cks, located in genomic position 30A (*Cks30A*). We found that both *rem* alleles contained a mutation in *Cks30A*, remarkably both disrupting the same conserved amino acid residue, proline 61 (P61) (Fig. 3A). *rem^{HG24}* had a P61S substitution, while *rem^{RA74}* resulted in a P61L change. Both alleles behaved similarly in all phenotypes tested, and therefore we will refer to them both as *Cks30A^{P61}* mutants. We confirmed that *rem* indeed corresponded to *Cks30A* by rescuing the *rem* mutant phenotype with a UAS-FLAG-tagged *Cks30A* transgene under control of the maternal driver, *nanos*-Gal4VP16 (Table 2).

***Cks30A* null mutants are delayed in mitotic spindle assembly**

As both existing *Cks30A* alleles possess the same single amino acid substitution, these may not represent the loss-of-function phenotype for this gene. To establish the null phenotype of *Cks30A*, we created a knockout allele by homologous recombination (Rong et al., 2002). As expected, this allele,

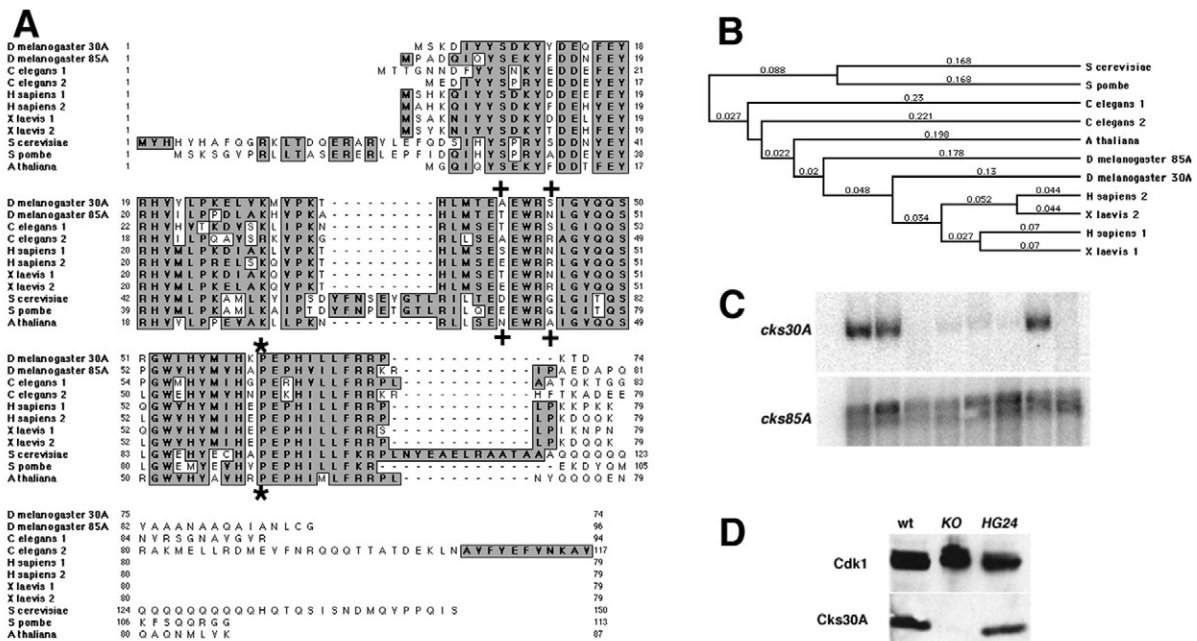


Fig. 3. *rem* encodes one of two *Drosophila* Cks gene homologs. (A) Alignment of *Drosophila* Cks30A (Remnants) with Cks from other organisms. * indicates residue P61 altered in *Cks30A^{RA74}* and *Cks30A^{HG24}*. + indicates residues implicated in Skp2 binding (S/T41,N45) for human Cks1. (B) Phylogenetic reconstruction (MacVector Neighbor Building Best Tree) does not permit assignment of *Drosophila* Cks proteins as orthologs of vertebrate Cks1 and Cks2, respectively. (C) Developmental northern blot probed for *Cks30A* and *Cks85A* reveals that *Cks30A* is mainly expressed in ovaries and embryos. Low concentrations are detected in third instar larvae and pupae. *Cks85A* is found at all stages examined. (D) Western blot from 0-2 embryos from wild type, *Cks30A^{KO}/Cks30A^{KO}* and *Cks30A^{HG24}/Cks30A^{HG24}* probed with anti-Cks30A antiserum reveals a single band in wild-type and *Cks30A^{HG24}* mutants, but no protein in *Cks30A^{KO}*.

Table 2. Rescue of *cks30A* mutants

Genotype	Hatching rate
<i>cks30A^{KO}/cks30A^{KO}</i>	0
<i>cks30A^{KO}/cks30A^{KO}; FLAG-cks30A⁺</i>	90
<i>cks30A^{KO}/cks30A^{KO}; FLAG-cks85A⁺</i>	0
<i>cks30A^{HG24}/cks30A^{HG24}; FLAG-cks85A⁺</i>	0
<i>cks30A^{KO}/cks30A^{KO}; FLAG-cks30A^{P61L}</i>	0
<i>cks30A^{KO}/cks30A^{HG24}; FLAG-cks30A^{P61L}</i>	0
<i>cks30A^{KO}/cks30A^{KO}; FLAG-cks30A^{K10ES50E}</i>	0

Females of the genotypes described were mated to CyO siblings, and eggs were collected at 22°C and scored for hatching. Mean $n=167$, $n \geq 81$.

Cks30A^{KO}, made no detectable protein on western blots probed against an affinity-purified Cks30A-specific antiserum (Fig. 3D). Like *Cks30A^{P61}* mutants, *Cks30A^{KO}* are homozygous viable and male fertile, but maternal effect lethal. Whereas *Cks30A^{P61}* frequently progressed through a number of aberrant cell cycles, most *Cks30A^{KO}* embryos arrested with a single spindle (Fig. 4A, Table 1), indicating a more severe arrest phenotype.

Cks30A^{KO} embryos also displayed a pronounced delay in entry into mitosis. In a 0-20 minute collection of embryos, only 29% had mitotic spindles, compared with 74% in wild type (Table 1). Instead, they typically contained two separated microtubule asters, one of which was associated with condensed chromatin (Fig. 4B, arrow; Table 1). In many cases, a thin spindle connected the two asters (data not shown), suggesting that these were centrosomes that had duplicated in advance of mitosis but had not yet assembled a mitotic spindle. In 20-40 minute collections, 54% of embryos had assembled a mitotic spindle, and in 40-80 minute collections, 80% of embryos had a single mitotic spindle. The others appeared to arrest with separated centrosomes that underwent a small number of extra divisions (Fig. 4C). Therefore, *Cks30A^{KO}* is delayed in, or arrests before, mitotic spindle assembly. This delay is apparent but less pronounced in *Cks30A^{P61}* embryos (Table 1).

***Cks30A^{KO}* causes a delay in exit from metaphase II of meiosis**

As in *Cks30A^{P61}*, meiosis in *Cks30A^{KO}* progressed normally up until the resumption of meiosis following egg activation, and a similar phenotype was observed in which the tandem meiosis II spindle was kinked or separated. In addition, *Cks30A^{KO}* mutants displayed a pronounced delay in metaphase of meiosis II (Fig. 4B, Table 1). *Cks30A^{P61}* mutants also displayed a slight delay in completing meiosis (Table 1). The delayed exit from female meiosis in *Cks30A^{KO}* embryos would be predicted to result in a failure to complete nuclear fusion. Indeed, FISH to X and Y chromosomes revealed that most embryos (11/12) did not complete joining of the male and female pronuclei (Fig. 4D). The occasional failure to complete nuclear fusion in *Cks30A^{P61}* embryos could similarly be explained by the observed partial delay in exit from female meiosis (Table 1).

***Cks30A* interacts genetically with *Cdk1* in histoblast cells**

In addition to a maternal effect lethal phenotype, all *cks30A* mutants have a defect in abdominal cuticle deposition

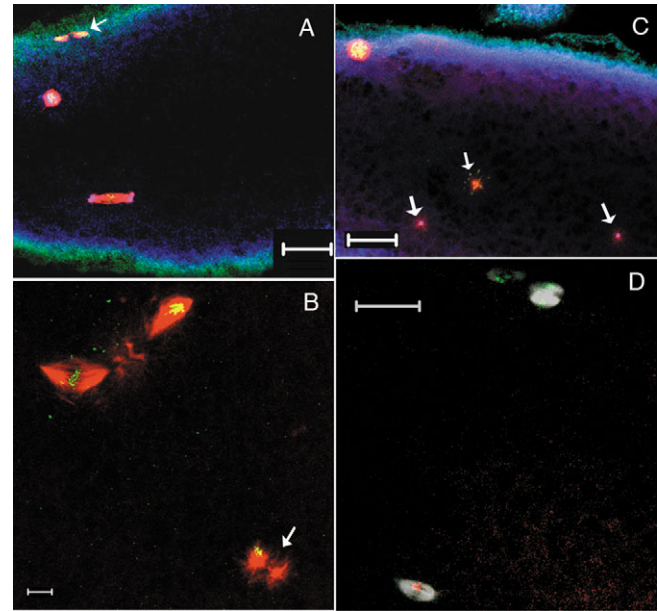


Fig. 4. *Cks30A^{KO}* homozygous embryos (40-80 minutes after egg-laying) arrest either with a single metaphase spindle (A) or without assembling a spindle (C). The arrow in A indicates a part of a meiotic spindle that has failed to disassemble. Arrows in C point to separated centrosomes. Embryos are labeled for DNA (green), microtubules (red) and Cnn (blue). (B) Early embryo (0-20 minutes after egg-laying) labeled for DNA (green) and microtubules (red), contains two closely apposed centrosomes, one of which is associated with the paternal chromatin (arrow). This embryo also shows the typical delay in metaphase of meiosis II. (D) FISH labeling of the X chromosome (green) and Y chromosome (red) and microtubules (white) reveals that the metaphase spindle (lower) in *Cks30A^{KO}* homozygous embryos contains only the male DNA complement, indicating that pronuclear fusion has not occurred. Polar bodies with only X chromosome signal (green) are at the top. Scale bars: 20 μ m.

indicative of a partial disruption of larval abdominal histoblast development (Fig. 5A,B). This phenotype was variable, but was expressed in all alleles, and at similar strengths. In wild type, the abdominal histoblasts arrest in G2 of the cell cycle through most of larval development and then re-enter the cell cycle after puparium formation. In *Cdk1* and *escargot* mutants, histoblasts fail to arrest in G2 and instead enter an endocycle (Hayashi, 1996; Hayashi et al., 1993). *Myb* mutants produce a similar phenotype, although these specifically disrupt resumption of mitosis in pupae (Katzen et al., 1998). To determine if either process is disrupted in *Cks30A*, we looked for genetic interactions between *Cks30A* and *Cdk1* and between *Cks30A* and *myb*. While reducing *myb* activity in a *Cks30A* mutant background did not produce a stronger phenotype (data not shown), *Cks30A* in combination with loss of a single copy of *Cdk1* produced a very strong effect (Fig. 5C). Similarly, a weak allele of *Cdk1* displayed a mild abdominal phenotype at 18°C, but this was strongly enhanced by loss of a single copy of *Cks30A* (data not shown). These genetic interactions suggest that Cks30A interacts with Cdk1 to prevent polyploidy in larval histoblasts.

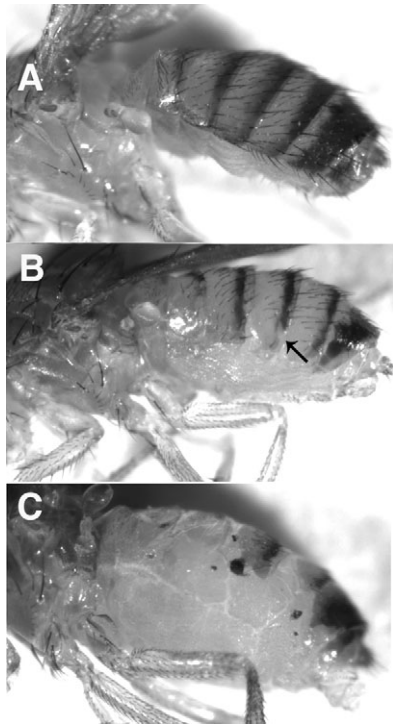


Fig. 5. Abdominal cuticle phenotypes of (A) wild type, (B) *Cks30A^{HG24}/Cks30A^{RA74}*, (C) *Cks30A^{HG24}/Cks30A^{RA74},cdk1^{E1-23}*. Arrow in B indicates a minor cuticle defect.

***Drosophila* Cks genes are functionally distinct**

All multicellular organisms that have been sequenced to date have two Cks genes. We identified a second *Drosophila* Cks gene at chromosomal location 85A (*Cks85A*) (Fig. 3A,B) that encodes a protein with 61% identity and 76% similarity to Cks30A, and has an additional C terminal 21 amino acids. While *Cks30A* is expressed mainly in the female germline and early embryo, *Cks85A* is expressed throughout development (Fig. 3C). To determine the function of *Cks85A*, we generated a null allele by imprecise excision of a nearby P-element (see Materials and methods). This mutant (*cks85A^{ex15}*) grew more slowly than wild type and was lethal at the third instar/pupal transition. This lethality was rescued by a *UAS-FLAG-Cks85A* transgene under control of the zygotic driver, *daughterless-Gal4* (data not shown), indicating that the lethal phenotype is due to loss of Cks85A activity. Using the FRT-ovoD system to generate *Cks85A* germline clones, we found that *Cks85A* was not required maternally, and double mutants with *Cks30A* in the germline produced a *Cks30A* mutant phenotype (data not shown). *Cks85A* also did not appear to have any obvious mitotic defects in zygotic tissues, either alone or in double mutants with *Cks30A* (data not shown). *Cks85A* also failed to dominantly enhance the abdominal phenotype of *Cks30A* mutations (data not shown). To determine whether the two Cks proteins can functionally replace each other, we attempted to rescue either mutant by constitutively expressing the other. While *UAS-FLAG-Cks30A* expressed with the *nanos-Gal4VP16* driver could rescue a *Cks30A^{KO}* mutant, *UAS-FLAG-Cks85A* could not (Table 2). Similarly, while *UAS-FLAG-Cks85A* under the control of *daughterless-Gal4* could rescue *cks85A^{ex15}*, *UAS-FLAG-Cks30A* could not (data not

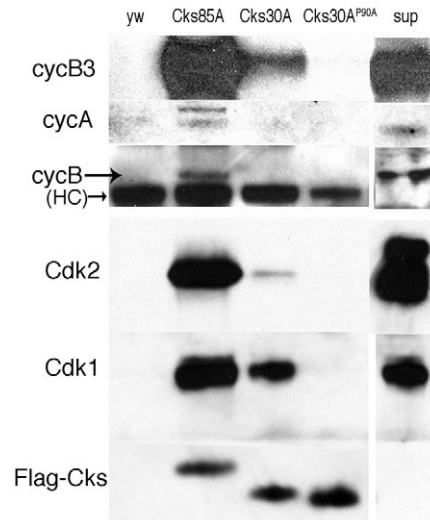


Fig. 6. *Drosophila* Cks30A interacts with Cdks. Western blots from FLAG-Cks immunoprecipitations probed with antibodies to Cdk1, Cdk2, Cyclin A, Cyclin B and Cyclin B3. The lower band in the Cyclin B lane is antibody heavy chain (HC). FLAG-Cks85A strongly co-immunoprecipitates Cdk1, Cdk2 and the mitotic cyclins, while FLAG-Cks30A co-immunoprecipitates Cdk1 and very weakly Cdk2. Cyclin B and Cyclin B3 are detected only in longer exposures. Cyclin B3 is overexposed here. FLAG-Cks30A^{P90A} fails to co-immunoprecipitate Cdk1 or mitotic cyclins. Sup (last lane) is IP supernatant.

shown). These observations indicate that the two Cks proteins are functionally distinct and cannot compensate for each other.

Cks85A and Cks30A interact with Cyclin-dependent kinases

In histoblasts, *ck30A* interacts genetically with *Cdk1* and both the meiotic phenotype and early mitotic arrest could reflect a role in regulating Cdk1 activity. To determine whether Cks30A and Cks85A interact physically with Cdks in *Drosophila*, we performed co-immunoprecipitation experiments with FLAG-tagged Cks30A and Cks85A proteins expressed in embryos (Fig. 6). FLAG-Cks85A interacted strongly with Cdk1 and Cdk2, and also co-immunoprecipitated the mitotic cyclins, A, B and B3 (Fig. 6). FLAG-Cks30A also co-precipitated Cdk1, although much less efficiently than FLAG-Cks85A, and very weakly co-precipitated Cdk2 in embryos (Fig. 6). Cyclin A was not detected in FLAG-Cks30A immunoprecipitates, while Cyclin B and B3 were found at low concentrations (Fig. 6). Therefore, Cks85A was found in complexes with Cdk1 and the mitotic cyclins, as well as the S-phase kinase, Cdk2; while Cks30A associated at lower levels with Cdk1-Cyclin B and Cdk1-Cyclin B3 complexes.

The two mutant alleles, *Cks30A^{HG24}* and *Cks30A^{RA74}* disrupt an amino acid, P61, that lies in a region of the protein implicated in Cdk binding (Bourne et al., 1996). To determine if the loss of function phenotype in these alleles could be due to a failure to bind to Cdks, we constructed a FLAG-Cks30A^{P61L} mutant (corresponding to the mutation in *Cks30A^{RA74}*). As expected, this failed to rescue the *Cks30A^{KO}* mutant (Table 2). We then performed immunoprecipitations with FLAG-Cks30A^{P61L} and found that it was strongly

impaired for Cdk binding, supporting the idea that *Drosophila* Cks30A functions with Cdks to promote female meiotic progression and mitotic exit in embryos.

Cks proteins all share an anion-binding domain that can interact with the phospho-epitope Ser/pSer/pThr/X. This domain can interact with partially phosphorylated Cdk substrates and has been suggested to recruit them to the Cdk to allow more phosphorylations to occur. To test the significance of this domain, we altered two residues in the domain (Bourne et al., 2000). The mutant *FLAG-Cks30A^{K10E550E}* completely failed to rescue a *Cks30A^{KO}* mutant (Table 2), indicating that the anion-binding domain is essential for Cks30A function. As expected, this mutation had no effect on Cdk interaction (data not shown).

Cks30A is required for Cyclin A destruction

Cdk1 activity during mitosis is necessary for the activation of the APC. In the cellularized *Drosophila* embryo, the APC targets the mitotic Cyclins A, B and B3 sequentially for degradation, and this degradation is necessary for mitotic exit (Sigrist et al., 1995). APC-mediated Cyclin degradation has also been implicated in anaphase progression in the early syncytial divisions (Su et al., 1998; Huang and Raff, 1999). Therefore, the metaphase arrest in *Cks30A* mutants could be due to a requirement for *Cks30A* in the Cdk1-dependent activation of the APC. To test this possibility, we examined cyclin levels in *Cks30A^{KO}* mutants. Cyclins B and B3 occurred at wild-type levels in *Cks30A* mutants, but the levels of Cyclin A were much higher than in wild type (Fig. 7A). This was not due to a secondary effect of cell cycle arrest, as it was also observed in ovaries from *Cks30A* mutants (Fig. 7A). It was also not due to increased transcription, as *cyclin A* mRNA levels were equivalent in wild-type and *Cks30A* mutants (Fig. 7B). In cellularized embryos, the introduction of non-degradable Cyclin A results in a metaphase delay or arrest (Sigrist et al., 1995), and therefore it is likely that the metaphase arrest in *Cks30A* mutants was a result of the failure to degrade Cyclin A. If so, it may be possible to relieve the requirement for *Cks30A* by reducing Cyclin A levels. Indeed, when *Cyclin A* gene dosage was halved, we saw a significant rescue of *Cks30A* mutants (Fig. 7C).

The destruction of mitotic cyclins is mediated by the APC and an associated subunit, CDC20, that is thought to target the APC to its substrates (reviewed by Peters, 2002). In *Drosophila*, three CDC20 homologs, Fzy, Fzr (Rap) and Fzr2 have known roles in cyclin degradation in vivo (Dawson et al., 1995; Jacobs et al., 2002; Sigrist et al., 1995; Sigrist and Lehner, 1997). Of these, only Fzy appears to be active in the syncytial embryo (Jacobs et al., 2002; Raff et al., 2002). Like *Cks30A* mutants, hypomorphic alleles of *fzy* result in a metaphase arrest in the first mitotic division of the embryo (Dawson et al., 1993), suggesting the possibility that Cks30A regulates Cyclin A levels through the APC^{Fzy}. However, we found that Cyclin A levels were not affected in ovaries or embryos from *fzy* mutant females (data not shown). Based on homology, the *cortex* gene encodes a fourth, distantly related *Drosophila* CDC20 protein (Chu et al., 2001). Mutations in *cortex* result in a meiotic phenotype similar to that in *Cks30A*, in which meiosis is arrested at metaphase of the second division (Lieberfarb et al., 1996; Page and Orr-Weaver, 1996). We examined cyclin levels in *cortex* mutant ovaries and found

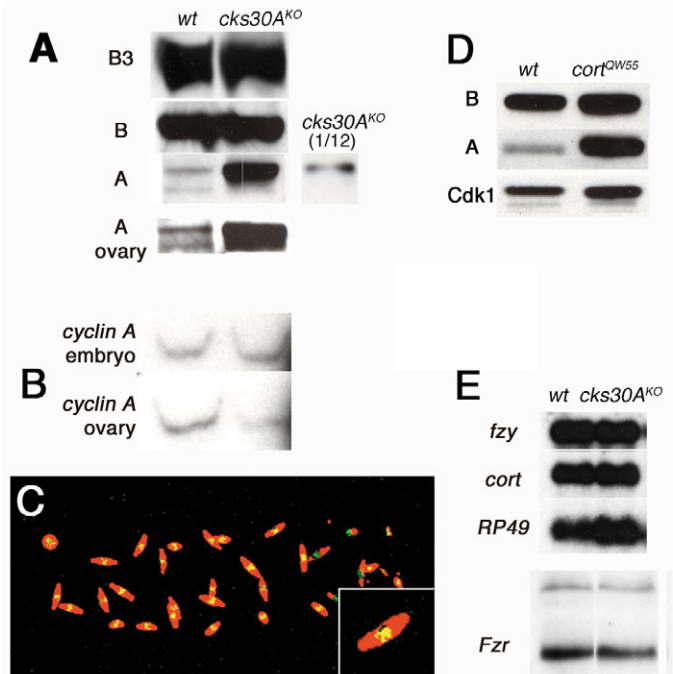


Fig. 7. Cks30A and Cortex mediate Cyclin A destruction.

(A) Western blot from *Cks30A^{KO}/Cks30A^{KO}* and unfertilized wild-type embryos (0-2 hours after egg-laying) and ovaries probed for Cyclin A, B and B3, revealing excess Cyclin A in *Cks30A* mutants. The upper (active) form is particularly enriched. Far lane, *Cks30A^{KO}* extract at 1/12 dilution contains approximately equal Cyclin A concentrations to wild type (see Materials and methods), indicating a 12-fold excess of Cyclin A in the mutant. (B) RT-PCR on wild-type and *Cks30A^{KO}/Cks30A^{KO}* ovaries and embryos (0-2 hours after egg-laying) reveals similar *cyclin A* mRNA concentrations. (C) Embryo from *Cks30A^{RA74}/Cks30A^{KO}; cycA^{C5LRI}/+* female (0-2 hours after egg-laying). Reduction of *cyclin A* dosage partially suppresses the *Cks30A* mutant phenotype, allowing some embryos to develop normally through several syncytial divisions. Mitotic spindles in this embryo are bipolar and are evenly spaced (compare to Fig. 1C – both embryos are at a similar stage, with approximately 32 nuclei). (D) Western blot from wild-type and *cortex^{QW55}* ovaries probed for Cyclin A and B reveals an elevated concentration of Cyclin A. (E) Northern blot from wild-type and *Cks30A^{HG24}/Cks30A^{HG24}* ovaries probed for *fzy* and *cortex* mRNA shows equal transcript concentrations, and (below) western blot from wild-type and *Cks30A^{KO}/Cks30A^{KO}* ovaries probed for Fzr protein, showing wild-type concentrations of Fzr protein. The upper band is non-specific and serves as a loading control.

that, as in *Cks30A*, those of Cyclin A but not Cyclins B and B3 were elevated (Fig. 7D and data not shown). Therefore, *Cks30A* may regulate maternal Cyclin A levels through an effect on the activity of APC^{Cortex}.

In *S. cerevisiae*, the role of Cks1 in mitotic exit appears to be primarily as a transcription factor, functioning with the 19S and 20S proteasome to promote *CDC20* transcription in metaphase (Morris et al., 2003). To determine if *Cks30A* is required for the transcription of *cortex* or other *Cdc20* genes, we performed northern analysis on *Cks30A* mutant ovaries with probes for *cortex* and *fzy*, and western analysis using a Fzr-specific antiserum. All three *Cdc20* genes were expressed

at normal concentrations (Fig. 7E), and therefore *Cks30A* does not function at the level of transcription of maternal *Drosophila Cdc20* genes but more likely affects the activity of the APC^{Cortex}.

Discussion

We have found that the maternal effect lethal gene, *remnants*, corresponds to one of the two *Drosophila* Cks genes (*Cks30A*). Analysis of two hypomorphic alleles and a null allele made by homologous recombination confirmed that *Cks30A* is not essential for cell cycle regulation in most tissue types. Rather, *Cks30A* functions in specialized cell cycles: the abdominal histoblast divisions, female meiosis and the syncytial divisions of the early embryo.

Cks30A interacts with Cdk1 to promote spindle assembly and anaphase progression

Cks30A mutants displayed a strikingly simple mitotic phenotype: most embryos from mutant females arrested in metaphase of the first mitotic division. Similarly, *Cks30A* mutants displayed a pronounced delay or arrest in metaphase of female meiosis II. Therefore, there is a common requirement for *Cks30A* in metaphase to anaphase progression in female meiosis II and in early embryonic mitosis. The second meiotic division is similar to a mitotic division in that it involves the segregation of sister chromatids, and therefore *Cks30A* may be part of a conserved machinery that is required for both of these processes.

An alternative explanation for the mitotic arrest in *Cks30A* mutants is that it is a secondary effect of a prior failure in meiosis or pronuclear fusion. However, our FISH experiments indicate that this mitotic arrest occurs even in embryos that successfully underwent pronuclear fusion. Also, mutations in *α-Tubulin67C* that block pronuclear fusion do not lead to a mitotic arrest in the embryo (Komma and Endow, 1995), indicating that these events are not coupled.

In addition to a crucial role in exit from mitosis, *Cks30A* is important in at least one aspect of entry into mitosis: spindle formation. *Cks30A*^{KO} mutants were severely delayed in assembly of the first mitotic spindle. *Cks30A* was also required for proper assembly of the female meiotic spindle and the specialized spindle-like microtubule aster of the polar bodies. Therefore, *Cks30A* appears to be required at two points in the mitotic (or meiotic) cell cycle: in prometaphase for spindle assembly and at the metaphase-to-anaphase transition.

These dual roles for *Cks30A* in meiosis appear to be at least partially conserved in other metazoans. *Xenopus* Cks2 (Xe-p9), like *Cks30A*, is required for the metaphase-to-anaphase transition in meiosis II (Patra and Dunphy, 1998). *Xenopus* Cks2 is also required in vitro for entry into mitosis (Patra and Dunphy, 1996), and this may be related to the in vivo requirement for *Cks30A* in spindle assembly in meiosis and mitosis. Cks genes in other eukaryotes also appear to have related but distinct functions in meiosis. Mouse *cks2* is essential for anaphase progression in meiosis I of both male and female meiosis (Spruck et al., 2003), while in *C. elegans* *cks-1* is not required for entry into anaphase, but is necessary for proper chromosome segregation in meiosis I (Polinko and Strome, 2000), possibly reflecting a role in meiotic spindle

assembly. Therefore, Cks genes appear to share a common requirement in entry into and exit from meiosis in different eukaryotes.

The two roles for *Cks30A* appear to reflect a conserved role in promoting Cdk1 activity (reviewed by Pines, 1996). Cdk1 is the central mitotic Cdk, and its kinase activity on specific mitotic proteins is required for entry into mitosis, including spindle assembly, and in maintaining the metaphase state. Cdk1 activity is also required for exit from mitosis through activation of the APC, which in turn promotes anaphase progression by targeting mitotic cyclins for destruction (reviewed by Nigg, 2001). We have found that in *Drosophila* *Cks30A* and Cdk1 interact in vivo, and that mutations in *Cks30A* that disrupt Cdk1 binding are compromised for activity in vivo. *Cks30A* also interacts genetically with *Cdk1* in another cell type, the abdominal histoblasts. Therefore, our genetic and physical evidence supports the conclusion that the observed interaction with Cdk1 is required for *Cks30A* function.

Cks30A-Cdk1 may activate the Cortex-APC-mediated destruction of Cyclin A

Cks30A is required for the destruction of Cyclin A in the ovary and in the syncytial embryo and two observations argue that it is this failure to degrade Cyclin A that results in the observed delay or arrest in metaphase of meiosis II and in mitosis. First, a similar metaphase arrest is seen in cellularized embryos expressing non-degradable Cyclin A (Sigrist et al., 1995), while syncytial embryos with a slight excess of Cyclin A (approximately 1-3× wild type) due to mutations in *grapes* display a metaphase delay (Su et al., 1999). Second, the *Cks30A* mutant phenotype can be partially rescued by lowering Cyclin A levels. The CDC20 homolog, *cortex*, is also required for exit from meiosis II (Page and Orr-Weaver, 1996; Lieberfarb et al., 1996), and we have found that *cortex* is also required for Cyclin A destruction in the ovary. These results argue that *Cks30A* and the APC^{Cortex} function in the same pathway leading to Cyclin A destruction, although we cannot rule out the possibility that *Cks30A* and Cortex act in independent pathways to promote Cyclin A destruction.

In vitro studies of Cks2 in *Xenopus* have led to a model in which Cks bound to Cdk1 recruits phosphorylated Cdk1 substrates to the kinase, allowing these substrates to be more efficiently recognized and thereby further phosphorylated by Cdk1 (Patra and Dunphy, 1996; Patra and Dunphy, 1998; Patra et al., 1999). The CDC27 and CDC16 components of the APC are key targets of Cks-Cdk1 phosphorylation in *Xenopus*. While it is not yet clear how APC phosphorylation leads to its activation, there is evidence that one of the effects of phosphorylation is to stimulate CDC20 binding to the APC (Kraft et al., 2003). Therefore it is possible that in *Drosophila* *Cks30A*-Cdk1 phosphorylates the APC, and this phosphorylation specifically stimulates the association of Cortex with the APC. Alternatively, *Cks30A*-Cdk1 may directly phosphorylate and activate Cortex.

Cyclin destruction in nuclear divisions versus cell divisions

In mammalian cells and in the cellularized embryo, the completion of mitosis depends on the sequential destruction of the three mitotic cyclins by the APC^{Fzy} (Sigrist et al., 1995).

Cyclin A is destroyed first in prometaphase, dependent on APC^{Fzy} activity. Although the APC^{Fzy} is active, the spindle checkpoint is thought to inhibit its activity on Cyclin B. Upon spindle assembly, the checkpoint is relieved and APC^{Fzy} can mediate Cyclin B destruction (Peters, 2002).

It now appears that some but not all aspects of anaphase progression are conserved in the second meiotic division and in the nuclear divisions of the syncytial embryo. Although overall Cyclin B levels do not oscillate during the early syncytial divisions (cycles 1 to 8) (Edgar et al., 1994), Cyclin B appears to undergo local degradation on the mitotic spindle at anaphase, and the injection of stabilized Cyclin B into syncytial embryos results in an early anaphase arrest (Huang and Raff, 1999; Su et al., 1998). By contrast to the early anaphase arrest upon Cyclin B stabilization, the injection of an APC-inhibiting peptide into early embryos results in a metaphase arrest (Su et al., 1998). Our results suggest that this metaphase arrest is due to the failure of APC^{Cortex}-mediated Cyclin A destruction. Like Cyclin B levels, Cyclin A levels do not oscillate detectably in cycles 1 to 7, although unlike Cyclin B, this appears to be due to a balance between constant destruction and new protein synthesis (Edgar et al., 1994). Despite this difference, it remains possible that local oscillations in Cyclin A and Cyclin B could drive these syncytial cell cycles.

While the importance of cyclin destruction may be conserved in the early embryo, the means by which the cyclins are destroyed appears to be different. In cellularized embryos, the APC^{Fzy} is responsible for the sequential destruction of all three mitotic cyclins (Dawson et al., 1995; Sigrist et al., 1995). In the syncytial embryo, Fzy is not required for Cyclin A destruction. Cortex, a diverged, female germline-specific CDC20, targets Cyclin A for destruction, but has no detectable effect on Cyclin B or B3 levels in the syncytial embryo. It remains possible that Cortex is responsible for the destruction of local pools of maternal Cyclin B (and possibly B3). Alternatively, the known maternal requirement for *fzy* may reflect a role in the local destruction of these cyclins. This would suggest a model in which the germline utilizes two CDC20 homologs, Cortex and Fzy, to mediate the sequential destruction of Cyclins A, B and possibly B3 in the syncytial embryo. Further work will be needed to test this model. It is also not clear if Cyclin A is the only target of the APC^{Cortex} and if the APC^{Cortex} is the only target of Cks30A-Cdk1. In addition to metaphase arrest, *Cks30A* mutants have spindle assembly delays or defects, a phenotype that has not been observed in other cell types to result from a failure to degrade Cyclin A. Interestingly, *cortex* mutants also have abnormal meiosis II spindles and fail to assemble a mitotic spindle around the male pronucleus (Lieberfarb et al., 1996; Page and Orr-Weaver, 1996), suggesting the possibility that the sole function of Cks30A in the female germline is to activate Cortex. Like *Cks30A*, *C. elegans cks-1* and mouse *cks2* appear to be predominantly required for meiosis (Polinko and Strome, 2000; Spruck et al., 2003), and this may also reflect specific roles in activating meiosis-specific APC complexes. The histoblast requirement for *Cks30A*, in contrast, is unlikely to represent a role in Cortex activation (or subsequent Cyclin A destruction), as *cortex* mutants, either alone or in combination with *Cks30A*, have no effect on abdominal development (Page and Orr-Weaver, 1996; Lieberfarb et al., 1996) (A.S. and T.S., unpublished).

Cks proteins in specialized cell cycles

A specific requirement for Cks30A in activation of the maternal-specific APC^{Cortex} would explain why *Cks30A* is essential for anaphase progression in female meiosis and the syncytial embryo but not in most cell types. We can rule out an alternative possibility, that Cks30A is functionally redundant with the other *Drosophila* Cks, Cks85A. *Cks85A* mutants alone or in combination with *Cks30A*, do not have obvious defects in exit from mitosis (A.S. and T.S., unpublished). Furthermore, while closely related to *Cks30A*, *Cks85A* cannot replace *Cks30A* when expressed in the female germline, and *Cks30A* cannot replace *Cks85A* when expressed zygotically. Therefore, we conclude that the two *Drosophila* Cks genes have distinct and non-overlapping functions. Recently, Cks85A was found to interact with a *Drosophila* Skp2 homolog in a genome-wide yeast two-hybrid screen (Giot et al., 2003). Two residues on Cks1 have recently been found to be crucial for Skp2 binding in vitro (Seeliger et al., 2003), and these residues are conserved or similar in *Drosophila* Cks85A (see Fig. 3A). If indeed Cks85A represents the *Drosophila* Cks1 ortholog, it is perhaps not surprising that Cks30A cannot functionally replace Cks85A, as it has been found that Cks2 orthologs cannot bind Skp2 in vitro (Ganoth et al., 2001; Spruck et al., 2001). However, it is unexpected that Cks85A cannot substitute for Cks30A in vivo. To date all Cks proteins tested can stimulate the Cdk-dependent phosphorylation of mitotic proteins in vitro, and the mouse Cks1 can functionally replace Cks2 in vivo (Spruck et al., 2003). The failure to rescue *Cks30A* mutant phenotypes cannot be due to an inability of Cks85A to interact with Cdks, as Cks85A binds Cdks with even greater affinity than does Cks30A. It is possible that, analogous to the Cks1/Skp2 interaction, Cks30A has an as-yet-to-be-identified partner that is necessary for its mitotic activities. Cks85A would, therefore, be unable to carry out the mitotic activities because of an inability to bind this putative Cks30A partner.

In conclusion, we find that *Drosophila* Cks30A is crucial for Cdk1 activity in spindle assembly and anaphase progression in female meiosis and early embryonic mitosis, and at least part of this activity appears to be to regulate Cyclin A levels. Cks30A functions non-redundantly with another closely related *Drosophila* cks, Cks85A.

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