The Developmental Role and Regulation of the Anaphase-Promoting Complex/Cyclosome in Drosophila Meiosis

by

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ABSTRACT

Meiosis is a modified cell cycle in which two round of chromosome segregation occur without an intervening DNA synthesis phase. As in mitosis, these cell divisions are driven in part by proteolysis mediated by the Anaphase-Promoting Complex/ Cyclosome (APC/C). During oogenesis of multicellular organisms, molecular control of meiosis has an added complexity because these divisions must be coordinated with the growth and development of the oocyte as well as its fertilization. The Drosophila gene *cortex* (*cort*), a putative female meiosis-specific APC/C activator, provides a unique opportunity to study the role of the APC/C in metazoan meiosis. We demonstrate that CORT protein associates with the core APC/C in ovaries, confirming its function as an activator of the APC/C. CORT triggers the sequential degradation of mitotic Cyclins in meiosis, as well as causing the degradation of PIMPLES, the Drosophila homolog of Securin. Both post-transcriptional and post-translational regulation of *cort* result in expression of CORT protein being restricted to the meiotic divisions. Cytoplasmic polyadenylation of *cort* mRNA is tightly correlated with appearance of the protein in mature oocytes. At the end of meiosis, CORT is rapidly degraded in an APC/C and D-box-dependent manner. We initiated a genetic screen to identify substrates of APC/C^{CORT} using *grauzone* (*grau*) mutants, a gene encoding a transcriptional activator of *cort*. We have identified at least one deficiency on the third chromosome, *Df(3R)p-XT103*, that dominantly suppresses *grau*. Levels of BEL, a protein encoded within this deficiency, are elevated in *cort* mutants. Our analysis of the regulation of CORT protein levels reveals one mechanism that may be important for developmental control of meiotic progression and the transition from meiosis to embryonic mitotic divisions. In addition, our screen to identify meiosis-specific substrates of APC/C^{CORT} has the potential to uncover novel regulators of meiosis in a multicellular organism.

Thesis Supervisor: Terry L. Orr-Weaver
Title: Professor of Biology

Dedicated to my parents,
Madgelyn and Richard Pesin

and

In loving memory of my grandmother,
Ethel Pesin

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Chapter One

Introduction

I. The Role of Proteolysis in Mitotic Events

Mitosis is a highly regulated process by which a cell divides to produce two daughter cells, each containing the same chromosomal complement as the parent cell. Proper mitotic cell division is crucial for the development of a multicellular organism, and its regulation is key, as deregulation of mitosis and chromosome segregation can lead to aneuploidy and unrestrained proliferation, hallmarks of cancer. Mitosis can be separated into several discrete stages based on the changing morphology of the cell as it divides. First, in prophase the newly replicated chromosomes condense and the nuclear envelope breaks down. During prometaphase "search and capture" events occur as microtubules bind to kinetochores and create an attachment between chromosomes and the spindle. Once stable bipolar attachments of kinetochores lead to alignment of chromosomes on the metaphase plate, metaphase begins. During metaphase a particular set of proteins is degraded (see below) that allows for rapid separation of sister chromatids in anaphase. Telophase begins once the sister chromatids have reached opposite ends of the spindle. The chromatids start to decondense, the nuclear envelope re-forms, and the spindle disassembles. During anaphase and telophase, the cell goes through cytokinesis to divide its cytoplasm into two cells. All of these events contribute to solving a critical problem for the cell when it divides: each daughter cell must receive an equal and identical set of genetic material.

Many studies of the regulation of the cell cycle have revealed that proteolysis is a key mechanism that drives the events of mitosis. Cyclin-dependent kinases and their activating subunits, cyclins, control many cell cycle events. Kinase activity is controlled by the availability of cyclin subunits, whose levels fluctuate during the cell cycle. Tim Hunt and colleagues discovered and gave cyclin its name after observing a protein in sea

urchin eggs that accumulates prior to mitosis and is destroyed each time the cell divides (Evans et al. 1983). Their hypothesis that the synthesis of cyclin drives entry into mitosis and its destruction drives exit from mitosis has turned out to be true (Murray and Kirschner 1989; Murray et al. 1989). In addition to cyclin, another key target of proteolysis, Securin, must be destroyed for anaphase to be initiated (Cohen-Fix et al. 1996). Thus, proteolysis is crucial for the progression of mitosis and provides an irreversible and directional switch to restart the cell cycle.

In this chapter, we will review the role of the anaphase-promoting complex/ cyclosome during mitosis and meiosis. We will focus on the activating subunits of the complex and discuss both their mechanism of action and their regulation. We will consider the specific regulation of Cdc20 by the spindle checkpoint, and look in depth at unique function and regulation of the APC/C in the developmental context of meiosis.

II. The Anaphase-Promoting Complex/ Cyclosome in Mitosis

A. The Ubiquitin-Proteasome Pathway

The ubiquitin-proteasome pathway is a widely used mechanism that conjugates ubiquitin to proteins for various purposes, the best understood of which is degradation by the proteasome (for review, see Pickart 2001). Three enzymes act sequentially to add ubiquitin and polyubiquitin chains onto substrates. In an ATP-dependent reaction, E1, the ubiquitin-activating enzyme, forms a high energy thioester bond between an active-site cysteine on E1 with a C-terminal glycine on ubiquitin. The activated ubiquitin is then transferred to a cysteine in E2, the ubiquitin-conjugating enzyme. Finally, the E3 ligase transfers the ubiquitin from the E2 to a lysine residue on the substrate. This 3-step reaction is repeated multiple times to attach subsequent ubiquitins to specific lysines on

the ubiquitin already bound to the substrate to generate polyubiquitin chains. A chain length of at least four ubiquitins is a strong signal to target a substrate for degradation by the 26S proteasome (Thrower et al. 2000).

The number of enzymes available for each step of the reaction increases as the reaction proceeds. Most organisms contain just one E1 enzyme that acts for all downstream ubiquitin-conjugating events, although recently a second E1 enzyme was identified in vertebrates and sea urchin (Jin et al. 2007). There are a limited number of E2 enzymes, and each E2 may act with several different E3 enzymes. Each E3 cooperates with one or more E2s and targets a specific set of substrates. In *S. cerevisiae* the APC/C E3 seems to act with both Ubc4 and Ubc1, two different E2s that have sequential roles in chain assembly (Rodrigo-Brenni and Morgan 2007). Ubc4 acts in monoubiquitination of target proteins that provides a substrate for assembly of polyubiquitin chains by Ubc1. In vertebrates the APC/C E3 can act with two E2 enzymes, UBCH5 and UBCH10/ UbcX/ E2-C *in vitro*, but their *in vivo* role is not entirely clear (Aristarkhov et al. 1996; Yu et al. 1996; Kirkpatrick et al. 2006;). However, in Drosophila, genetic and RNAi analysis has revealed a role for *vihar*, the gene encoding the E2-C homolog, in spatiotemporal control of Cyclin B degradation at spindle poles (Máthé et al. 2004).

B. Discovery of the APC/C

Biochemical and genetic studies converged for discovery of the enzyme that catalyzes the degradation of cyclin. Fractionation of extracts from clam oocytes led to the identification of three components that are required for ubiquitination of Cyclin B, an E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme specific to cyclin,

and a possible E3 ubiquitin ligase specific to cyclin (Hershko et al. 1994). The E3 fraction was only active when isolated from mitotic extracts but not from interphase extracts. This E3 activity was found to be associated with a 1500 kDa complex called the "cyclosome" (Sudakin et al. 1995). At the same time, a 20S complex was identified from fractionation of Xenopus egg extracts and called the "anaphase-promoting complex" (APC) (King et al. 1995). The APC was able to trigger ubiquitination of a cyclin B substrate when complemented with interphase extract or a mixture of recombinant E1 and an E2, UBC4.

In parallel, a genetic screen was carried out in *S. cerevisiae* to identify genes required for proteolysis of Cyclin B (Irniger et al. 1995). Conditional mutants of two essential genes, *cdc16* and *cdc23*, were isolated, and these genes were shown to be required for entering and exiting anaphase. The identification of two of the proteins in the Xenopus APC complex as homologs of *S. cerevisiae cdc16* and *cdc23* linked the biochemical and genetic studies together (King et al. 1995). Finally, antibodies were raised against the human homologs of Cdc16 and Cdc27, a protein that had been shown to associate with Cdc16 and Cdc23 (Lamb et al. 1994; Tugendreich et al. 1995). These two proteins cosedimented as a 20S complex from mammalian cell extracts, and when antibodies against Cdc27 were injected into HeLa cells, the cell cycle arrested in metaphase, suggesting a conservation of function for these proteins in mammals.

D. Structure of the APC/C

The APC/C is a large multisubunit complex comprised of at least 12 core subunits (Table 1-1). An understanding of the structure of the APC/C has been hindered due to its large and complex nature, lack of a crystal structure, and difficulty in reconstituting the

Table 1-1. APC/C Subunits*

S. cerevisiae	Drosophila	Vertebrates	Structural Motif
Core subunits			
Apc1	Shattered	APC1/TSG24	homology to Rpn1/2, subunits of the 26S proteasome
Apc2	Morula	APC2	Cullin homology
Cdc27	Makos	CDC27/APC3	TPRs
Apc4	APC4/CG32707	APC4	WD40 repeats
Apc5	Ida	APC5	TPRs
Cdc16	cdc16/ CG6759	CDC16/APC6	TPRs
-	APC7/ CG14444	APC7	TPRs
Cdc23	cdc23/ CG2508	CDC23/APC8	TPRs
Doc1/Apc10	-	DOC1/APC10	Doc domain, IR motif
Apc11	Lemming	APC11	RING-H2 finger
Cdc26	-	CDC26	
Swm1/Apc13	-	SWM1/APC13	
Apc9	-	-	
Mnd2	-	-	
Co-activators			
Cdc20	Fizzy	CDC20/FZY	C-box, WD40 repeats, IR-tail
Cdh1	Fizzy-related	CDH1/FZR	C-box, WD40 repeats, IR-tail
Ama1	-	-	C-box, WD40 repeats, IR-tail
-	Cortex	-	C-box, WD40 repeats, IR-tail
-	Fizzy-related 2	-	C-box, WD40 repeats, IR-tail

* See text for references

complex from purified subunits. Purification of the APC/C from budding yeast and from vertebrate cells and tissues has facilitated the development of *in vitro* assays to elucidate the mechanisms of APC/C activity (Carroll and Morgan 2005; Herzog and Peters 2005). A budding yeast strain that renders the APC/C nonessential has allowed for a detailed analysis of the architecture of the complex (Thornton and Toczyski 2003; Thornton et al. 2006). Additionally, electron microscopy studies have revealed important structural information and generated a 3-dimensional model of the enzyme (Gieffers et al. 2001; Passmore et al. 2005; Dube et al. 2005). The APC/C can be divided into four parts: an arm containing tetratricopeptide repeat (TPR) proteins, Cdc23, Cdc16, Cdc27, and Swm1; a scaffolding complex, Apc1, Apc4, and Apc5; a catalytic arm, Apc2, Apc11, and Doc1; and the non-core substrate adaptor subunits, Cdc20 and Cdh1 (Figure 1-1).

The TPR subunits form a separable subcomplex, which is thought to interact with substrate adaptors to facilitate ubiquitination (discussed below) (Passmore et al. 2003; Vodermaier et al. 2003). The TPR arm contains several copies of each TPR-containing protein, Cdc16, Cdc23, and Cdc27 (Lamb et al. 1994; Dube et al. 2005; Passmore et al. 2005). Vertebrate cells contain an additional TPR-containing protein, Apc7. The TPR arm also contains non-TPR proteins Cdc26, Swm1/Apc13, and Apc9 (only in *S. cerevisiae*), small, nonessential subunits that are important for the stability of this subcomplex (Zachariae et al. 1998; Schwickart et al. 2004).

The TPR arm is tethered to Apc1, a large scaffolding subunit, through stabilizing associations with Apc4 and Apc5 (Thornton et al. 2006). Cdc23 appears to hold the TPR arm to Apc1 by being most closely associated with the scaffold. Cdc16 is bound to Cdc23, and Cdc27 is bound to Cdc16 and farthest away from the scaffold (Schwickart et al. 2004; Kraft et al. 2005; Thornton et al. 2006).

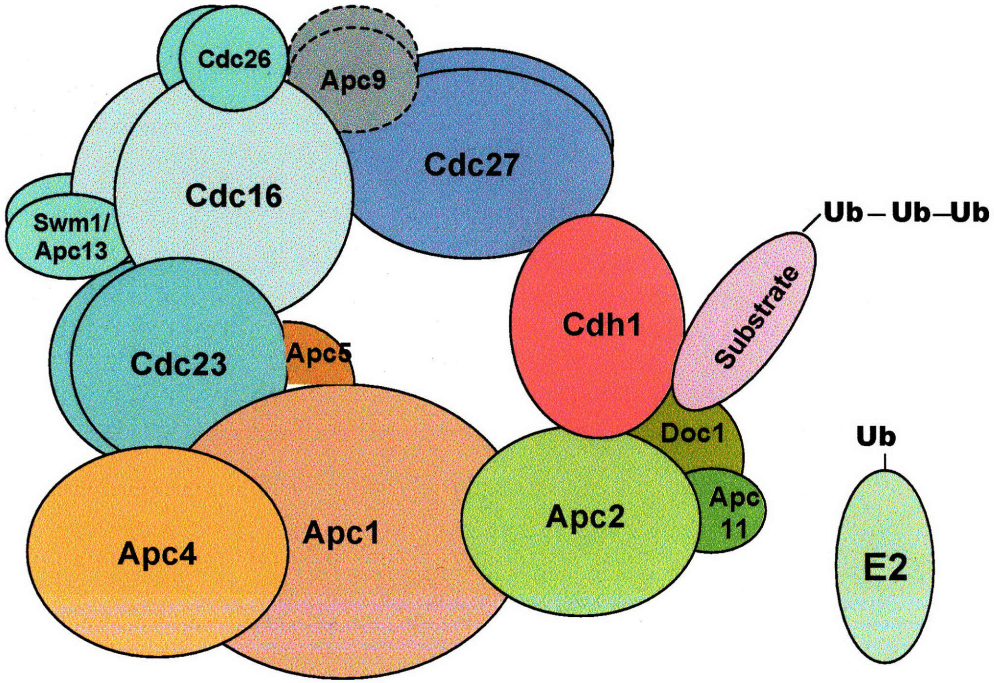

Figure 1-1. Architectural Map of the Anaphase-Promoting Complex/ Cyclosome
A map of the subunits of the APC/C based on binding and association data. Subunits of the TPR arm are represented in shades of blue, those of the scaffolding complex in shades of brown, and those of the catalytic arm in green. Adapted from Thornton and Toczyski (2006).

The APC/C is a member of the Cullin-RING finger family of E3 ligases. The catalytic arm of the APC/C that contains the Cullin and RING finger proteins binds the other side of the scaffold. Apc2 is a member of the cullin family of proteins (Yu et al. 1998; Zachariae et al. 1998). The cullin domain of Apc2 binds the RING finger domain of Apc11 (Tang et al. 2001; Vodermaier et al. 2003). The RING finger domain of Apc11 recruits E2 enzymes to the complex. Together, these two proteins can catalyze the ubiquitination of substrates in the absence of any other APC/C subunits, although with little substrate specificity (Gmachl et al. 2000; Leverson et al. 2000; Tang et al. 2001). Doc1 is a small globular protein that is thought to contribute to processivity of ubiquitination through enhancing substrate recognition by the APC/C (Carroll and Morgan 2002; Carroll et al. 2005).

The APC/C has an overall asymmetric triangular shape with two flexible arms and an inner cavity (Gieffers et al. 2001; Passmore et al. 2005; Dube et al. 2005). Although the cavity was once thought to be the site of ubiquitination, cryo-electron microscopy has mapped the locations of Apc2 and Cdh1 to the same outside wall of the complex, suggesting that ubiquitination of substrates occurs in this location (Dube et al. 2005).

E. APC/C Activators and Substrate Adaptors: Cdc20/Fzy and Cdh1/FzrF

1. Interactions with Core APC/C and Substrates

Cdc20/Fzy and Cdh1/Fzr comprise the non-core subunits of the APC/C and activate and confer substrate specificity to the complex (Dawson et al. 1995; Schwab et al. 1997; Visintin et al. 1997; Sigrist and Lehner 1997). Cdc20 directs the ubiquitination

of securin, mitotic cyclins, and other substrates for anaphase onset, while Cdh1 targets mitotic cyclins and additional substrates for degradation in mitotic exit and G1. Cdc20 and Cdh1 are members of the Cdc20 protein family whose members contain seven WD-40 repeats in their C-terminus (for a review, see Smith et al. 1999). These repeats form a seven-bladed propeller structure that mediates protein-protein interactions.

Substrate adaptors bind directly to the APC/C, although this association is not as stable as the binding of APC/C core subunits to each other (Fang et al. 1998a; Yamano et al. 2004). Direct binding to the APC/C appears to occur through two motifs that are conserved among substrate adaptor proteins (Figure 1-2). The C-box is a short motif, DR(F/Y)IPXRX~45–75(K/R)XL, in the N-terminal half of these proteins and is required for association of Cdh1 with the APC/C (Schwab et al. 2001; Pfleger et al. 2001; Thornton et al. 2006). Analysis of the ability of C-box mutant Cdh1 to bind and activate wild-type and mutant APC/C complexes has suggested that the C-box mediates an interaction between Cdh1 and Apc2 (Thornton et al. 2006). Additionally, substrate adaptor proteins, as well as Doc1, contain a conserved isoleucine arginine (IR) tail in the C-terminus that is proposed to bind directly to the TPR domain of Cdc27 (Wendt et al. 2001; Vodermaier et al. 2003). C-terminal peptides of Cdc20 and Cdh1 specifically bind Cdc27, and the TPR domain of Cdc27 is sufficient for this binding (Vodermaier et al. 2003). Furthermore, Cdh1 specifically crosslinks to Cdc27 through its IR tail, and an APC/C complex that is missing Cdc27 fails to bind Cdh1 (Kraft et al. 2005; Thornton et al. 2006). In summary, both the C-box and IR tail conserved regions of the substrate adaptor proteins contribute to their binding of the APC/C, and Cdc27 and Apc2 are thought to be the APC/C subunits that are directly bound.

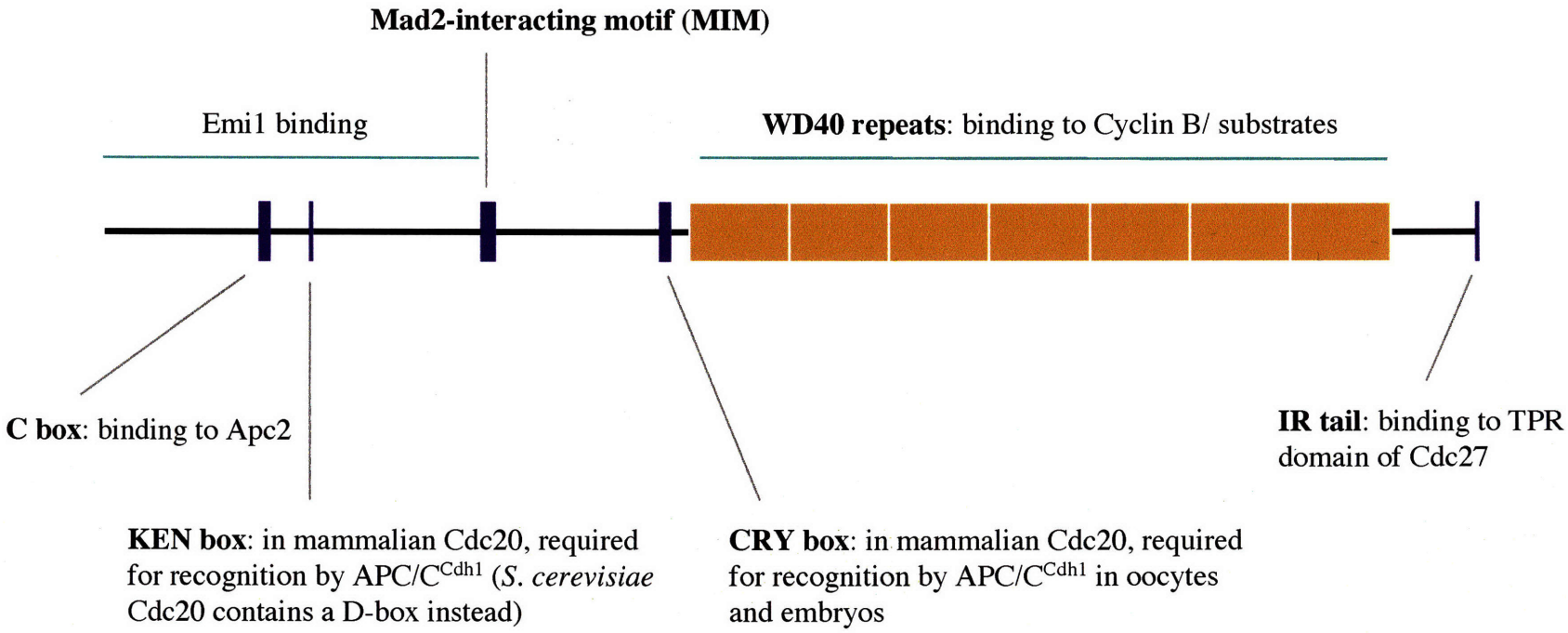

Figure 1-2. Domains and motifs of Cdc20 and Cdh1
A summary of the domains and motifs in Cdc20 and Cdh1 that are crucial for their interactions with core APC/C, substrates, and inhibitors/ regulators. See text for details and references.

Phosphorylation plays a role in regulating the association of substrate adaptors with the APC/C and contributes to the differential timing of Cdc20 and Cdh1 binding and activation of the complex. Association of Cdc20 with the APC/C requires mitotic phosphorylation of the APC/C (Fang et al. 1998a; Kramer et al. 2000). This phosphorylation is thought to be carried out by Cdk1-Cyclin B and Plk1/ Polo-like kinase on Apc1 and TPR proteins (Shteinberg et al. 1999; Rudner and Murray 2000; Golan et al. 2002; Kraft et al. 2003).

In contrast, mitotic phosphorylation acts to inhibit association of Cdh1 with the APC/C. Cdh1 itself is phosphorylated by cyclin-dependent kinases during S, G2, and M-phase, and only upon dephosphorylation in G1 is it able to bind and activate APC/C (Zachariae et al. 1998; Jaspersen et al. 1999; Lukas et al. 1999; Blanco et al. 2000; Kramer et al. 2000; Yamaguchi et al. 2000; Huang et al. 2001; Sorensen et al. 2001; Keck et al. 2007). Thus, mitotic phosphorylation directs the temporal differences in activity of APC/C^{Cdc20} and APC/C^{Cdh1}. As cells enter mitosis and mitotic kinases become active, APC/C subunits are phosphorylated and allow for Cdc20 binding and activation. At the same time Cdh1 is phosphorylated and inhibited from binding APC/C. Once APC/C^{Cdc20} targets mitotic cyclins for degradation, a decrease in mitotic Cdk-cyclin activity leads to a decrease in inhibitory phosphorylation of Cdh1, as well as dephosphorylation of Cdh1 by Cdc14 phosphatase, allowing for binding to and activation of the APC/C by Cdh1 in G1 of the cell cycle (Jaspersen et al. 1999).

In addition to binding directly to the APC/C, Cdc20 and Cdh1 are thought to bind directly to APC/C substrates. Two conserved motifs in substrates act as the main recognition sequences for the APC/C. In general, Cdc20-specific substrates contain a

destruction box (D-box), R-x-x-L-x-x-x-x-N, which is sufficient to target a protein for degradation, whereas APC/C^{Cdh1} recognizes substrates containing an additional motif, K-E-N, called the KEN box (Glotzer et al. 1991; Pfleger and Kirschner 2000).

A long-standing hypothesis has been that Cdc20 and Cdh1 recruit substrates to the APC/C by binding them directly. Many groups have demonstrated binding of Cdc20 and Cdh1 to substrates by yeast two-hybrid analysis, co-immunoprecipitation, and *in vitro* binding assays, although the details of these results are sometimes contradictory. It is clear that the D-box and KEN box are required for binding of substrates to activators (Ohtoshi et al. 2000; Pfleger et al. 2001; Burton and Solomon 2001; Hilioti et al. 2001; Kraft et al. 2005). However, three groups reported a dependence on the WD repeat region of Cdc20 and Cdh1 for binding to substrates, whereas Pfleger et al. showed a requirement of the C-box for this interaction (Ohtoshi et al. 2000; Sorensen et al. 2001; Pfleger et al. 2001; Hilioti et al. 2001).

A more recent study using a photocrosslinking approach may offer a more definitive answer (Kraft et al. 2005). Peptides from human Securin and Cyclin B containing the D-box were incubated with Xenopus extracts and crosslinked to interacting proteins to identify D-box receptors. Cdc20 and Cdh1 crosslinked to Cyclin B and Securin peptides in a D-box dependent manner, and crosslinking of mutant Cdh1 to Cyclin B peptides revealed that a conserved surface on the WD40 propeller of Cdh1 is required for this interaction. Furthermore, Cdh1 mutants unable to crosslink to Cyclin B peptides were also unable to activate APC/C in an ubiquitination assay. The C-box and IR tail of Cdh1 were not required for crosslinking to Cyclin B peptides (Figure 1-2).

Studies in the past few years have questioned the direct binding of activators with substrates and revealed a role for the core APC/C in substrate binding. Yamano et al. ran Xenopus egg extracts over a D-box affinity column and found that the column depleted the majority of core APC/C in the extract but only a small fraction of Fzy, suggesting that the APC/C core binds substrate more tightly than the activator (Yamano et al. 2004). Doc1 appears to be the core subunit that may be responsible for an interaction with substrates. It is required for D-box dependent substrate recognition by APC/C^{Cdh1} and APC/C^{Cdc20}, although a direct interaction between Doc1 and substrates has not been shown (Passmore et al. 2003; Carroll et al. 2005; Burton et al. 2005; Passmore and Barford 2005). Finally, "isotope trapping" assays demonstrated that both core APC/C and Cdc20 are required for functional substrate binding (Eytan et al. 2006). A model is emerging in which substrates may bind to both core APC/C and Cdc20 or Cdh1 at once or only after a conformational change in APC/C has been induced by activator binding for a functional interaction between all three components (Dube et al. 1995).

The APC/C recognizes a range of substrates during mitosis, and, certainly, many remain to be identified (Table 1-2). In prometaphase and metaphase, APC/C^{Cdc20} targets mitotic cyclins and securin for degradation to allow for anaphase onset and chromosome disjunction. In anaphase, APC/C^{Cdh1} takes over to target Cdc20, mitotic cyclins, and mitotic regulators such as Polo kinase, Aurora A, and Aurora B. In late anaphase, APC/C^{Cdh1} targets proteins that are involved in spindle function and cytokinesis.

Cdc20 generally recognizes substrates containing a D-box, whereas Cdh1 generally recognizes substrates with both a D-box and KEN box. However, in addition to these key recognition sequences, additional motifs have been identified. In many cases,

Table 1-2. Mitotic Substrates of APC/C

Substrate	Start of degradation	Activator	Function	References
CycA	prometaphase	Cdc20	mitotic cyclin	Geley et al. 2001
Nek2A	prometaphase	Cdc20	centrosomal kinase	Hames et al. 2001; Hayes et al. 2006
HoxC10	prometaphase	Cdc20	transcription factor	Gabellini et al. 2003
p21	prometaphase	Cdc20	CDK inhibitor	Amador et al. 2007
CycB	metaphase	Cdc20/Cdh1	mitotic cyclin	King et al. 1995; Peters 1999; Jeganathan et al. 2005
Securin	metaphase	Cdc20/Cdh1	separase inhibitor	Zur and Brandeis 2001; Hagting et al. 2002; Jeganathan et al. 2005
Xkid	metaphase	Cdc20/Cdh1	chromokinesin	Antonio et al. 2000; Funabiki and Murray 2000; Castro et al. 2003
Geminin	metaphase	Cdh1	replication inhibitor	McGarry and Kirschner 1998
Kip1	anaphase	Cdc20	kinesin	Gordon and Roof 2001
Cin8	anaphase	Cdh1	kinesin	Hildebrandt et al. 2001
Cdc20	anaphase	Cdh1	APC/C activator	Prinz et al. 1998; Shirayama et al. 1998; Pfleger and Kirschner 2000
Tpx2	anaphase	Cdh1	spindle-associated protein	Stewart and Fang 2005a
Plk1	anaphase	Cdh1	mitotic kinase	Lindon and Pines 2004
Mps1	anaphase	Cdh1	spindle-checkpoint protein	Palframan et al. 2006
Bub1	anaphase	Cdh1	spindle-checkpoint protein	Qi and Yu 2007
Mad3	anaphase	Cdh1	spindle-checkpoint protein	King et al. 2007
Aurora A	late anaphase	Cdh1	mitotic kinase	Littlepage and Ruderman 2002; Castro et al. 2002a; Castro et al. 2002b
Aurora B	late anaphase	Cdh1	mitotic kinase	Stewart and Fang 2005b
Ase1/Prc1	late anaphase/mitotic exit	Cdh1	spindle-associated protein	Juang et al. 1997; Visintin et al. 1997
Tome-1	late anaphase/mitotic exit	Cdh1	F-box protein	Ayad et al. 2003
Anillin	mitotic exit	Cdh1	actin-binding protein	Zhao and Fang 2005
Fin1	mitotic exit	Cdh1	spindle-associated protein	Woodbury and Morgan 2007
Orc1	mitotic exit	Cdh1	replication factor	Araki et al. 2003
Cdc6	G1	Cdh1	replication factor	Petersen et al. 2000

D-boxes or KEN boxes that are present in the APC/C substrate sequence are actually not required for their degradation. Xkid, a chromokinesin in Xenopus, contains 5 putative D-boxes, none of which are required for its degradation. Instead, a novel recognition sequence, GxEN, in Xkid's C-terminus is recognized by both Cdc20 and Cdh1 and is sufficient to cause degradation (Castro et al. 2003). Both Aurora A and Aurora B contain an A box, QrxL, which is required for their degradation. The KEN box in Xenopus Aurora A is not used, but one of the D-boxes in Xenopus Aurora B contributes to its degradation (Littlepage and Ruderman 2002; Crane et al. 2004). In contrast, the D-boxes in human Aurora B are not used, whereas the KEN box contributes to its degradation along with its A-box (Nguyen et al. 2005). Mammalian Cdc20 contains another novel recognition sequence, the CRY box, CRYxPS, which is required for its degradation in oocytes and embryos (Reis et al. 2006). Drosophila Orc1 contains a KEN box and 3 D-boxes, all of which are dispensable for its degradation by the APC/C. A sequence termed the O-box is used instead (Araki et al. 2003).

2. Regulation of Cdc20/Fzy

One important way in which APC/C activity is governed is through the regulation of the substrate adaptors. The activity of APC/C^{Cdc20} generally parallels its protein expression profile. Cdc20 accumulates in S-phase, peaks in mitosis, and drops in G1. A combination of transcriptional upregulation in mitosis and protein degradation in G1 contributes to this profile (Fang et al. 1998a; Prinz et al. 1998). Control of Cdc20 transcription is best understood in *S. cerevisiae* where Cks1, a small Cdk-associated protein, binds to the Cdc20 promoter and recruits Cdc28 and the proteasome to induce

Cdc20 transcription (Morris et al. 2003). It is not known whether this mechanism controls Cdc20 transcription in higher eukaryotes.

Cdc20 protein is one of several targets of APC/C^{Cdh1} when APC/C^{Cdh1} becomes active in G1 (Prinz et al. 1998). Degradation of *S. cerevisiae* Cdc20 is dependent on the presence of a D-box in its N-terminus (Prinz et al 1998). Vertebrate Cdc20 sequences do not contain a D-box, and analysis of human Cdc20 led to the identification of the KEN box motif (Figure 1-2) (Pfleger and Kirschner 2000). As previously noted, in mammalian oocytes and embryos, the degradation of Cdc20 is mediated through an additional motif called the CRY box (Reis et al. 2006).

Another major player contributing to the presence of functional Cdc20 in cells is the CCT chaperonin (chaperonin-containing TCP1). In *S. cerevisiae* CCT is required for the proper folding of Cdc20 in an ATP-dependent manner. CCT-dependent folding of Cdc20 is required for its associations and activity with the APC/C and for its regulation by the spindle checkpoint (Camasses et al. 2003). A strong dependence on CCT for the generation of functional Cdc20 may be why it has been difficult to produce large amounts of purified recombinant Cdc20 for biochemical and *in vitro* assays. For example, in the production of *in vitro* transcribed and translated Cdc20 in reticulocyte lysate, the bulk of Cdc20 remains associated with CCT, perhaps because it is not folded efficiently by the rabbit CCT (Passmore et al. 2003).

In addition to regulation of levels of Cdc20 protein in the cell, the activity of APC/C^{Cdc20} is controlled by several inhibitors, which are discussed below.

A. Emi1

Xenopus Emi1 was isolated in a yeast two-hybrid screen for Skp1 binding proteins, the F-box protein of the E3 SCF complex (Reimann et al. 2001a). It contains an F-box and a C-terminal Zn^{2+} binding region (ZBR) (Figure 1-3). In cycling embryos, levels of Emi1 increase in S-phase and decrease in mitosis, and addition of recombinant Emi1 to cycling egg extracts stabilizes APC/C substrates and prevents their ubiquitination. Emi1 associates with Cdc20 in interphase egg extracts, and inhibition of APC/C^{Cdc20}-mediated ubiquitination of mitotic substrates requires the ZBR domain in Emi1 (Reimann et al. 2001a).

The levels of Emi1 itself are regulated in a cell-cycle dependent manner by several mechanisms. Emi1 is a substrate of the E3 ubiquitin ligase $SCF^{\beta\text{-TrCP}}$ (Guardavaccaro et al. 2003; Margottin-Goguet et al. 2003). Phosphorylation by both Cdk and Plk1 contribute to recognition and ubiquitination of Emi1 by $SCF^{\beta\text{-TrCP}}$ in late prophase (Margottin-Goguet et al. 2003; Moshe et al. 2004; Hansen et al. 2004). Two mechanisms protect Emi1 from SCF-mediated degradation at other stages of the cell cycle. In S/G2 phase, the Evi5 oncogene binds directly to Emi1 and blocks phosphorylation of Emi1 by Plk1, thus preventing its recognition by $SCF^{\beta\text{-TrCP}}$ (Eldridge et al. 2006). Emi1's degradation also seems to be inhibited by Pin1, a peptidyl-prolyl *cis/trans* isomerase during G2. Emi1 associates with Pin1 *in vivo* during G2 and is stabilized by Pin1 in an isomerization-dependent pathway (Bernis et al. 2007).

Emi1 degradation in late prophase immediately precedes Cyclin A degradation by the APC/C, suggesting a requirement for Emi1 destruction for APC/C activation in mitosis. In addition, overexpression of non-degradable Emi1 in mammalian cells causes a prometaphase block (Margottin-Goguet et al. 2003). These results, combined with

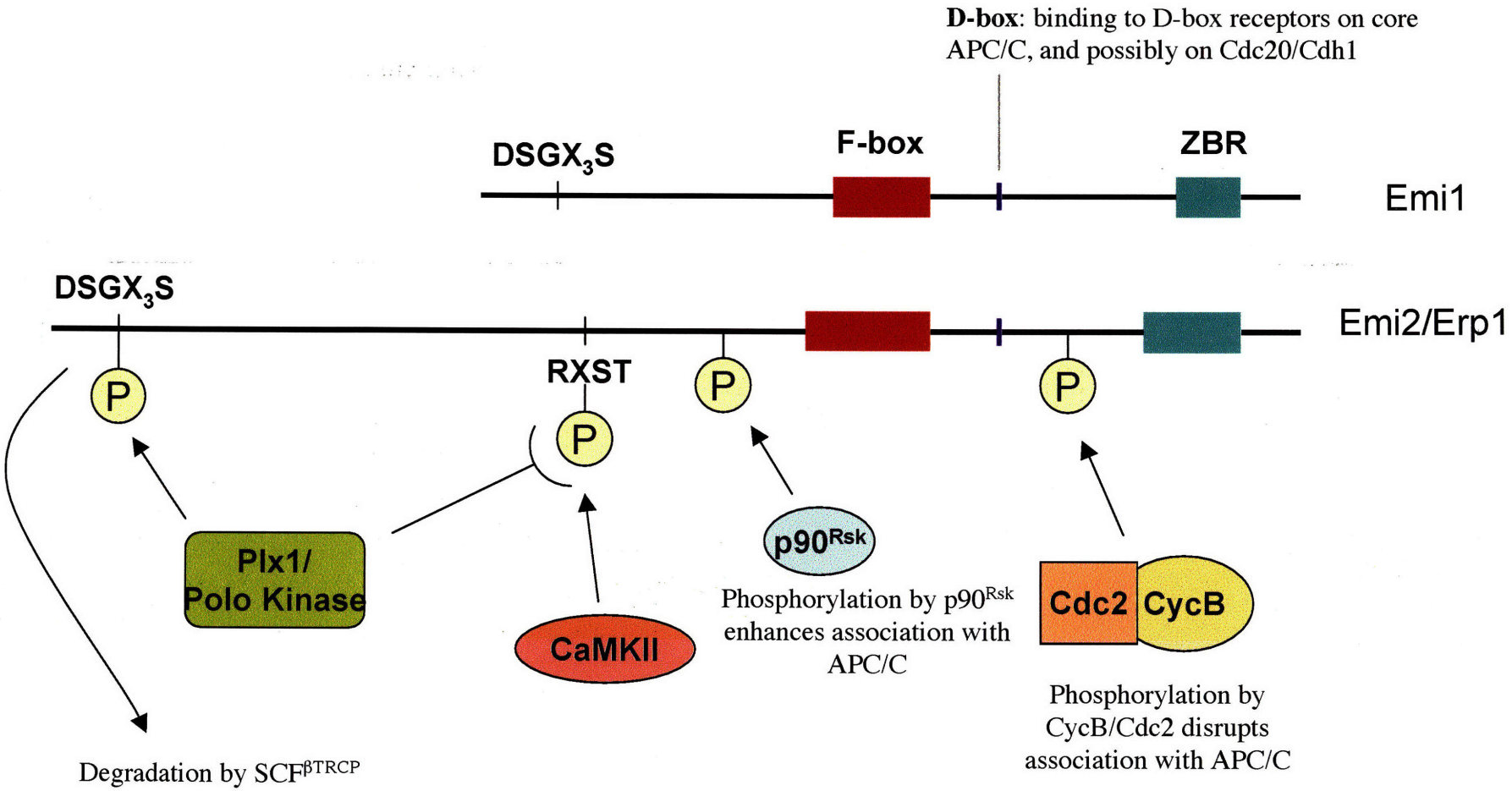

Figure 1-3. Domains and regulatory modifications of Emi1 and Emi2/Erp1
Conserved domains in Emi1 and Emi2/Erp1 include a D-box, which is important for their association with the APC/C, and an F-box, Zinc Binding Region (ZBR), and a $DSGX_3S$ motif, which mediate binding to and subsequent degradation via $SCF^{\beta TRCP}$. Regulatory phosphorylation of Emi2/Erp1 is shown. Please see text for details and references.

Reimann et al.'s demonstration of a direct interaction between Emi1 and Cdc20, has led to a model in which degradation of Emi1 is essential for activation of APC/C^{Cdc20} in mitosis. A recent study by Di Fiore and Pines questions this model (Di Fiore and Pines 2007). They timed degradation of fluorescently labeled Emi1 and Cyclin A in HeLa cells and found that Emi1 is degraded between 6 and 45 minutes before Cyclin A, whereas the timing observed by Margottin-Goguet et al. was just 5 to 10 minutes. Additionally, injection of RNA encoding non-degradable Emi1 into HeLa cells did not affect the timing of initiation of Cyclin A, Cyclin B, or Securin degradation (Di Fiore and Pines 2007). These recent findings require further investigation to determine their significance. Technical differences in the experiments performed by either group may be responsible for these contradictory results.

An additional function for Emi1 is as a member of a novel regulatory network END (Emi1/NuMA/dynein-dynactin) that restricts the activity of APC/C in early mitosis (Ban et al. 2007). A population of Emi1, along with the APC/C, localizes to spindle poles in early mitosis after the bulk of Emi1 has been degraded via $SCF^{\beta\text{-}TrCP}$ (Hansen et al. 2004; Ban et al. 2007). This localization is dependent on binding of Emi1 and APC/C to microtubules through the action of dynein-dynactin, a minus end-directed microtubule motor. APC/C, Emi1, and NuMA, a nuclear matrix and spindle assembly protein, form a complex in mitosis that spatially restricts APC/C activity. Emi1 promotes NuMA-dependent formation of microtubule asters through its inhibition of Cyclin B degradation by the APC/C at spindle poles (Ban et al. 2007).

Emi1 was originally thought to inhibit APC/C activity simply by binding directly to Cdc20. Emi1 was shown to associate with Cdc20 through binding to a domain on

Cdc20 that is distinct from the domain through which Mad2 binds and inhibits Cdc20 (Mad2 inhibition discussed below) (Figure 1-2) (Reimann et al. 2001a; Reimann et al. 2001b). However, a recent study has revealed a more complex mechanism for inhibition of the APC/C by Emi1 (Miller et al. 2006). Emi1 was found to associate with the core APC/C by co-fractionation experiments. Assays using an Emi1 affinity column showed that Emi1 efficiently captures APC/C in the presence or absence of Cdc20 or Cdh1, suggesting that Emi1 binds to the APC/C core independently of an interaction with an activator protein. Furthermore, this direct interaction is dependent on a conserved D-box in the C-terminus of Emi1 but not on the ZBR domain (Figure 1-3). Both the D-box and the ZBR domain contribute to Emi1's ability to compete with APC/C substrates for binding to the APC/C and to inhibit APC/C's ubiquitination activity. Finally, wild-type Emi1 is a poor substrate of the APC/C itself, but mutation of the ZBR domain converts Emi1 into an efficient D-box dependent APC/C substrate. Thus, Emi1 acts as a pseudo-substrate inhibitor of the APC/C. The D-box is the domain through which Emi1 binds the D-box receptor on the core APC/C, whereas the ZBR domain seems to act to inhibit access of substrates to the complex.

B. Spindle Assembly Checkpoint

Crucial inhibition of APC/C^{Cdc20} occurs in metaphase by the spindle assembly checkpoint. The details of this regulation are discussed in the subsequent section.

C. DNA damage and PKA pathway

One output of the DNA damage checkpoint is arrest of the cell cycle in the G2-M transition and the metaphase to anaphase transition. In *S. cerevisiae* the PKA pathway inhibits the metaphase to anaphase transition in response to DNA damage (Searle et al.

2004). An accumulation of single-stranded DNA at telomeres causes a PKA-dependent stabilization of Clb2 and Pds1 and phosphorylation of Cdc20. Cdc20 contains 2 consensus sites for PKA phosphorylation that are required for this DNA damage induced modification. In the presence of DNA damage, Cdc20 does not interact with Clb2, causing a metaphase arrest. When the phosphorylation sites of Cdc20 are mutated, Cdc20 does interact with Clb2 upon DNA damage, and Pds1 and Clb2 are degraded with faster kinetics. These results suggest that DNA damage induces PKA phosphorylation of Cdc20 to inhibit the targeting of APC/C substrates in mitosis. It is not known whether Cdc20 is inhibited by DNA damage in other organisms.

D. RASSF1A

RASSF1A is a tumor suppressor gene that is silenced by promoter methylation in lung cancer patients. Overexpression of RASSF1A induces mitotic arrest and the accumulation of Cyclins A and B in HeLa cells, whereas depletion of RASSF1A by RNA interference accelerates cyclin degradation and mitotic progression (Song et al. 2004). These phenotypes led to an investigation of a role for RASSF1A in regulation of the APC/C. In cotransfection experiments, RASSF1A and Cdc20 co-immunoprecipitate, and RASSF1A inhibits APC/C^{Cdc20} but not APC/C^{Cdh1} activity in an *in vitro* ubiquitination assay (Song et al. 2004). However, in a recent report, Liu et al. failed to detect an interaction between Cdc20 and RASSF1A by several experimental approaches (Liu et al. 2007). The relationship between RASSF1A and mitotic control through regulation of the APC/C remains to be clarified.

3. Regulation of Cdh1/ Fzr

Cdh1 RNA levels are constant throughout the cell cycle, and Cdh1 protein levels

are high in mitosis, but lowered in late G1 and S-phases (Prinz et al.1998; Kramer et al. 2000; Hsu et al. 2002). The decrease in Cdh1 levels in late G1 and S-phase are thought to be due to E3 ligase-mediated protein degradation. Cdh1 contains 2 putative D-boxes that are required for its degradation in G1, suggesting that Cdh1 targets its own degradation. Furthermore, Cdh1 is ubiquitinated *in vitro* and degraded in an APC/C^{Cdh1}-dependent manner (Listovsky et al. 2004). In S-phase Cdh1 levels remain low, but APC/C^{Cdh1} cannot be responsible for maintaining low levels of Cdh1 at this stage because it is inactivated by inhibitory phosphorylation. Benmaamar and Pagano investigated a possible role for SCF in degradation of Cdh1 at this cell cycle stage (Benmaamar and Pagano 2005). They found that inactivation of SCF by expression of a dominant-negative Cul1 or by RNA interference against Cul1 results in an accumulation of Cdh1, suggesting that SCF activity is required either directly or indirectly for the degradation of Cdh1.

As discussed earlier, APC/C^{Cdh1} activity is regulated by the phosphorylation state of Cdh1. Cdh1 is phosphorylated in S, G2, and M phases, and this phosphorylation greatly reduces the ability of Cdh1 to bind to and activate APC/C (Zachariae et al. 1998; Jaspersen et al. 1999; Kramer et al. 2000).

Like Cdc20, Cdh1 is also subject to regulation by a several inhibitors, but unlike Cdc20, it seems to be crucial for the cell to modulate APC/C^{Cdh1} activity throughout the cell cycle, even at times when Cdh1 is already subject to inhibitory phosphorylation.

A. Rca1/ Emi1

The first inhibitor of Cdh1, *regulator of cyclin A* (*rca1*), was discovered in Drosophila in a screen for dominant suppressors of *roughex* mutants (Dong et al. 1997).

rca1 mutants, like *cyclin A* mutants, arrest in G2 of embryonic cell cycle 16, and ectopic expression of *rca1* drives cells into S-phase and causes an increase in Cyclin A protein levels. A subsequent study demonstrated that Rca1 acts to inhibit APC/$C^{Fzr/Cdh1}$ in G2 (Grosskortenhaus and Sprenger 2002). Although Emi1, the vertebrate ortholog of Rca1, inhibits both Cdc20 and Cdh1, Rca1 only seems to affect Cdh1. Grosskortenhaus and Sprenger failed to detect an association between FZY/ Cdc20 and Rca1, and genetic studies confirmed that the effect of Rca1 on cyclin levels is specific to Fzr (Grosskortenhaus and Sprenger 2002).

In vertebrates, the role of Emi1 seems to be important for inhibiting APC/C^{Cdh1} at the G1 to S-phase transition. Emi1 binds to Cdh1 and inhibits APC/C^{Cdh1} *in vitro* (Reimann et al. 2001b; Miller et al. 2006). An *in vivo* role for Emi1 has been investigated in HeLa cells (Hsu et al. 2002). Similar to Cyclin A, Emi1 transcription is activated by E2F at the G1 to S-phase transition. Cells that are depleted of Emi1 by RNA interference fail to accumulate Cyclin A and do not enter S-phase. Overexpression of Cdh1 causes a G1 arrest, and this arrest can be overcome by overexpression of Emi1, suggesting that Emi1 regulates S-phase entry via inhibition of APC/C^{Cdh1} (Hsu et al. 2002). It is not clear whether Rca1 also regulates S-phase entry in Drosophila embryos in addition to its role in G2 or whether Emi1 acts to inhibit Cdh1 in G2 in vertebrate cells.

B. Rae1-Nup98

Inhibitory phosphorylation of Cdh1 was thought to fully inactivate APC/C^{Cdh1} during mitosis, but an investigation of the roles of Rae1 and Nup98 revealed an additional layer of regulation of APC/C^{Cdh1} in mitosis. Rae1 is an mRNA export factor that acts by anchoring Nup98, a nucleoporin, to the nuclear pore complex (Pritchard et al. 1999). Splenocytes from $Rae1^{+/-}Nup98^{+/-}$ mice exhibit premature separation of sister

chromatids, and Rae1$^{+/-}$Nup98$^{+/-}$ murine embryonic fibroblasts contain decreased levels of securin, which suggested a role for Rae1 and Nup98 in mitotic control (Jeganathan et al. 2005). In mitotic HeLa cell extracts, Cdc27, Cdc16, and Cdh1 co-immunoprecipitate with Rae1 and Nup98, whereas Cdc20 does not associate. Furthermore, the association of Cdh1 with Rae1 and Nup98 is specific to mitosis. Rae1-Nup98 was found to inhibit ubiquitination of Securin but not Cyclin B by APC/^{Cdh1}. Interestingly, Rae1-Nup98 dissociates from APC/C^{Cdh1} at the same time that BubR1 dissociates from APC/C^{Cdc0} upon release of the spindle checkpoint-mediated metaphase arrest (Jeganathan et al. 2005). These results suggest that, contrary to what was previously thought, APC/C^{Cdh1} may indeed play a role in targeting securin for degradation upon anaphase onset. The mechanism of Rae1-Nup98-mediated inhibition of APC/C^{Cdh1} remains to be elucidated as well as an understanding of how Rae1-Nup98 is targeted to the APC/C and triggered to dissociate from the APC/C.

Finally, in addition to the Cdc20 and Cdh1 inhibitors discussed above, two transcription factors, CBP and p300, are thought to be positive regulators of the APC/C in mitosis (Turnell et al. 2005). In mammalian cells CBP and p300 are associated with an active APC/C complex in vivo, and they co-immunoprecipitate with core APC/C subunits as well as both Cdc20 and Cdh1. Knockdown of CBP by RNA interference leads to an increase of Cyclin B and Plk1 levels and an accumulation of cells in mitosis. APC/C precipitated from these CBP-depleted cells has reduced ubiquitination activity (Turnell et al. 2005). The mechanism by which CBP-p300 positively regulates mitotic APC/C is not yet clear.

It is becoming increasingly clear that regulation of APC/C activators in mitosis as well as G1, S, and G2 occurs in a complex network and at multiple levels: transcriptional control, protein stability, phosphorylation, and direct binding of inhibitors.

III. Inhibition of APC/C^{Cdc20} by the Spindle Checkpoint

The spindle-assembly checkpoint (SAC) is a surveillance mechanism that prevents premature separation of sister chromatids in mitosis by monitoring the attachment of spindles to microtubules in prometaphase (for review, see Musacchio and Salmon 2007). SAC signaling is thought to initiate at or near unattached kinetochores and diffuse to inhibit APC/C^{Cdc20}'s ability to target substrates, particularly Securin and Cyclin B, for degradation. As a result, cells arrest in metaphase until each kinetochore is bioriented on the spindle and under tension. At this point, the arrest is released, APC/C^{Cdc20} becomes active, anaphase onset occurs, and mitosis proceeds.

Two spindle checkpoint proteins, Mad2 and Mad3/BubR1, have been found to inhibit Cdc20 through direct binding. Both genes were originally identified in screens in *S. cerevisiae*, in which mutations in these genes bypassed mitotic arrest in response to spindle poisons (Hoyt et al. 1991; Li and Murray 1991). Mad2 binds directly to Cdc20 and inhibits Cdc20's ability to activate APC/C *in vitro* (Hwang et al. 1998; Kim et al. 1998; Fang et al. 1998b). Furthermore, Mad2, Cdc20, and APC/C form a ternary complex in prometaphase that is localized to kinetochores, suggesting an important role for Mad2 in APC/C inhibition (Fang et al. 1998). BubR1, the human homolog of yeast Mad3, is associated with Cdc20 in mitotic cells, and also inhibits the ubiquitination

activity of APC/C^{Cdc20} (Tang et al. 2001). *In vitro*, BubR1 and Mad2 act synergistically to inhibit APC/C^{Cdc20} (Tang et al. 2001)

The physical nature of the spindle checkpoint APC/C inhibitor was further clarified in a study by Sudakin et al. A single stable complex was purified from HeLa cell lysates that is able to inhibit the ubiquitin ligase activity of APC/C^{Cdc20} (Sudakin et al. 2001). This complex, termed the Mitotic Checkpoint Complex (MCC), contains BubR1, Mad2, Bub3, and Cdc20 in roughly equal stoichiometries (Figure 1-4). In reconstituted ubiquitination assays, MCC inhibits APC/C more efficiently than recombinant Mad2 alone, suggesting that MCC is likely the *in vivo* inhibitor of APC/C during spindle checkpoint arrest (Sudakin et al. 2001).

Furthermore, formation of the MCC is thought to be aided by phosphorylation of Cdc20 (Figure 1-4). In Xenopus egg extracts, Cdk activity is required for spindle checkpoint arrest (D'Angiolella et al. 2003). Cdk-dependent phosphorylation of Cdc20 inhibits its binding to Cdc27 and enhances its binding to Mad2 (Yudkovsky et al. 2000; D'Angiolella et al. 2003). Xenopus Cdc20 also seems to be regulated by MAPK-dependent phosphorylation during spindle checkpoint inhibition. A Cdc20 mutant with all of its MAPK phosphorylation sites mutated is unresponsive to spindle checkpoint inhibition and exhibits reduced binding to Mad2 and BubR1 (Chung and Chen 2003). It is not clear whether direct MAPK phosphorylation of Cdc20 is conserved, as human Cdc20 is not phosphorylated by MAPK *in vitro* (Tang et al. 2004).

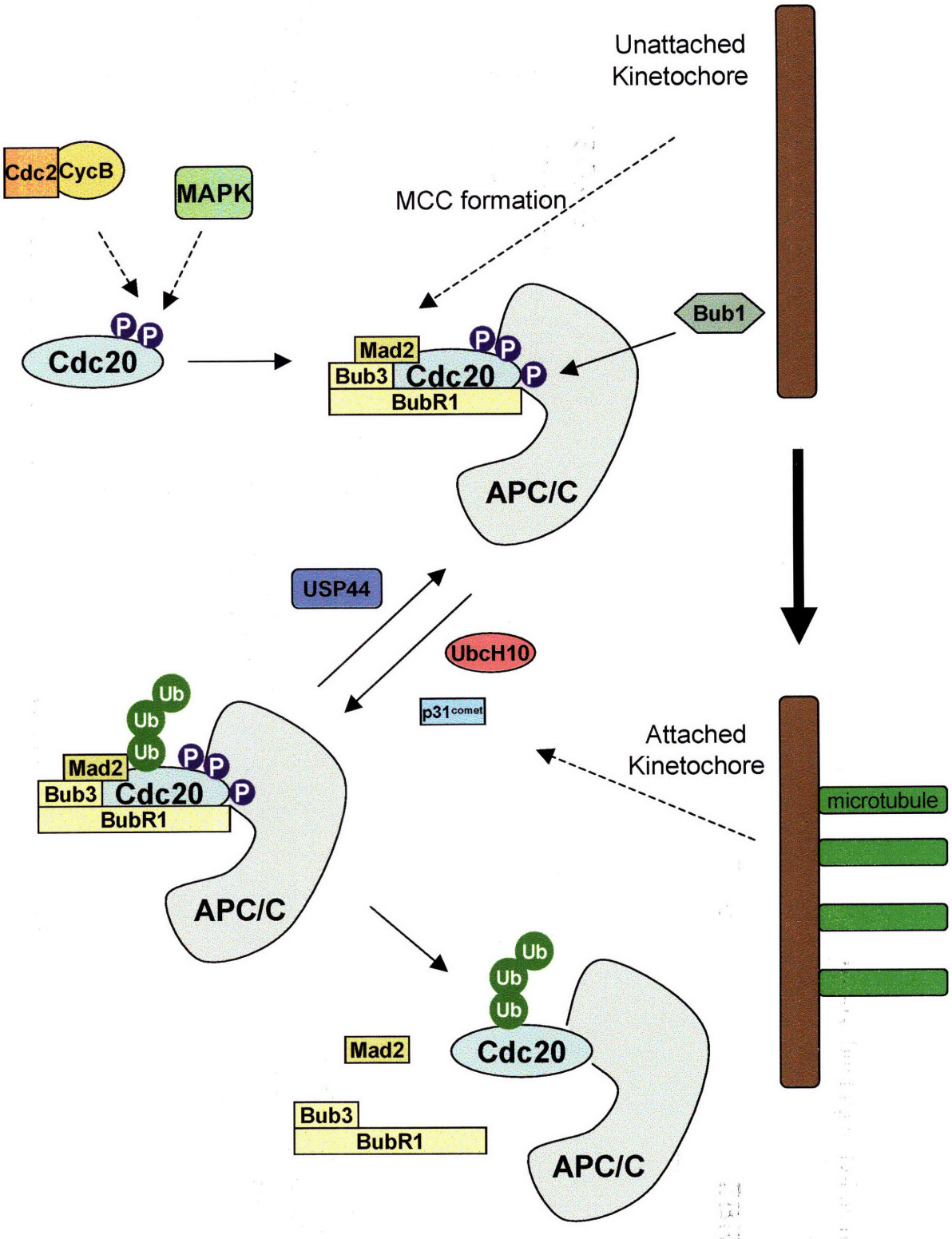

Figure 1-4. Spindle checkpoint inhibition of APC/C^{Cdc20}
Spindle checkpoint-mediated inhibition of APC/C^{Cdc20} is achieved through binding of Cdc20 to MCC (Mad2, BubR1, Bub3), phosphorylation of Cdc20 by Bub1 kinase, and deubiquitination of Cdc20 by USP44. Autoubiquitination of Cdc20 stimulated by UbcH10 and p31^{comet} leads to the dissociation of Cdc20 and MCC, inactivation of the spindle checkpoint, and activation of APC/C^{Cdc20}. Please see text for details and references.

The mechanism by which MCC inhibits APC/C^{Cdc20} is not entirely clear although several pieces of data provide clues. In an *in vitro* binding assay, Mad2 prevents release of substrates from Cdc20, which may affect turnover of substrate ubiquitination (Pfleger et al. 2001). Both Mad2 and BubR1 coimmunoprecipitate core APC/C subunits, suggesting that they do not act by preventing Cdc20 from binding APC/C (Fang et al. 1998; Sudakin et al. 2001). Finally, two recent studies in *S. cerevisiae* demonstrate that Mad3/BubR1 may act by blocking substrate access to Cdc20. Mad3 contains 2 KEN boxes that are required for spindle checkpoint function (Burton and Solomon 2007; King et al. 2007). These KEN boxes as well as a D-box in Mad3 all contribute to binding of Mad3 to Cdc20 *in vivo*, and Mad3 competes with Hsl1, an APC/C substrate, for binding to Cdc20 *in vitro* (Burton and Solomon 2007; King et al. 2007). Thus, Mad3 may act like a pseudosubstrate inhibitor of APC/C^{Cdc20}, similar to Emi1's mode of inhibition.

In addition to spindle checkpoint inhibition of Cdc20 through its association with the MCC, a recent study demonstrates that Cdc20 is independently regulated by Bub1, another spindle checkpoint protein (Figure 1-4). In HeLa cells Bub1 is required for mitotic arrest in response to spindle damage (Tang et al. 2004). Bub1, a protein kinase, phosphorylates Cdc20 *in vitro*, which reduces the ubiquitin ligase activity of APC/C^{Cdc20}. Expression of a Cdc20 mutant that is resistant to Bub1 phosphorylation causes a failure of cells to arrest in response to nocodazole, however, this form of Cdc20 is still able to bind Mad2 and BubR1 (Tang et al. 2004). In contrast to inhibition by stoichiometric binding of Cdc20 by Mad2 and BubR1, this study reveals that the spindle checkpoint also targets Cdc20 through the catalytic activity of Bub1 kinase.

Finally, direct ubiquitination of Cdc20 places yet another level of regulation on Cdc20 by the spindle checkpoint. In *S. cerevisiae*, Cdc20 protein stability is reduced upon activation of the spindle checkpoint (Pan and Chen 2004). Binding of Cdc20 to Mad2 and BubR1 is required for this APC/C-dependent degradation of Cdc20, but the D-boxes in Cdc20 and Cdh1 are not required (Pan and Chen 2004; King et al. 2007). Thus, Cdc20 is targeted for APC/C dependent degradation even at the same time that it itself is inhibited from targeting other substrates. These studies suggest that Cdc20 levels must be reduced for complete inhibition of APC/C^{Cdc20} during an active spindle checkpoint. It is not clear whether this regulatory strategy is conserved in higher eukaryotes.

Two recent studies in human cells reveal another role for ubiquitination of Cdc20 in spindle checkpoint control. In this case, autoubiquitination of Cdc20 serves to induce dissociation of Cdc20 and Mad2 and inactivation of the spindle checkpoint (Figure 1-4) (Reddy et al. 2007; Stegmeier et al. 2007). Reddy et al. showed that HeLa cells that overexpress UbcH10, the E2 enzyme that acts with APC/C, fail to arrest in response to nocodazole. UbcH10 and APC/C-dependent ubiquitination of Cdc20 is required *in vivo* for dissociation of Mad2 and Cdc20 after release from nocodazole, but surprisingly, proteasome activity is not required.

In an accompanying paper, Stegmeier et al. performed a short hairpin RNA screen in human cells to find components of the ubiquitin-proteasome pathway that are required for spindle checkpoint function. They identified USP44, an uncharacterized deubiquitinating enzyme that is required for checkpoint function. USP44 is required for stabilizing the Mad2-Cdc20 association in nocodazole arrested cells, and further, the authors showed that USP44 deubiquitinates Cdc20 *in vitro* and *in vivo*. Depletion of

Ubc10 in cells suppresses the effects of cells with reduced USP44 function by restoring spindle checkpoint arrest, strongly suggesting that USP44 acts to antagonize UbcH10-mediated release from spindle checkpoint arrest. These studies reveal an additional level of control in the spindle checkpoint pathway, which could act as a switch-like mechanism to trigger anaphase onset. It has yet to be determined how the spindle checkpoint network modulates this switch to control maintenance or release of metaphase arrest.

IV. Role of the APC/C in Female Meiosis

Meiosis is a modified cell division in which one parent cell generates four haploid cells, in contrast to the two identical diploid cells generated in a canonical mitotic cell division. Understanding the function and regulation of the APC/C in meiosis presents an interesting and complex problem given the differences of both chromosome segregation dynamics and developmental context in meiosis compared to mitosis.

A reductional division in meiosis I and an equational division in meiosis II without an intervening S phase generate haploid gametes in a meiotic cell division. Control of the meiotic divisions is coordinated by both general cell-cycle regulators as well as well as meiosis-specific proteins (for review, see Marston and Amon 2004). Meiosis I is unique because homologous chromosome pairs, as opposed to sister chromatids, must be segregated from each other. Homologous chromosomes are held together by chiasmata and arm cohesion. This segregation is achieved by loss of cohesion along chromosome arms but not in the centromeric regions. Subsequently, in meiosis II, sister chromatids are segregated through loss of cohesion at the centromeres in a process very similar to that in mitosis. Regulation of APC/C activity is likely to be

important in this process as separase must be activated twice, through APC/C-mediated degradation of securin, in one full meiotic cell division.

Furthermore, the absence of an S-phase in between meiosis I and meiosis II also likely requires specialized regulation of the APC/C. In Xenopus oocytes it has been shown that mitotic Cdk activity is maintained at a low level in between the two meiotic divisions (Figure 1-5) (Furuno et al. 1994; Iwabuchi et al. 2000). Mitotic Cdk activity must be kept at an intermediate level to satisfy the unique requirements of meiosis: Cdk activity must be low enough to allow for disassembly of the meiosis I spindle, but high enough to repress initiation of DNA replication. Presumably, APC/C-mediated degradation of mitotic and meiotic cyclins must be regulated to contribute to this modulation of Cdk activity.

Regulation of APC/C activity in meiosis is particularly crucial during oogenesis of multicellular organisms. In most animals meiosis is arrested twice to coordinate development of the oocyte with the events of meiosis (Figure 1-5) (for reviews, see Kishimoto 2003; Tunquist and Maller 2003). First, oocytes are arrested in prophase I to allow for oocyte growth and differentiation before initiation of the meiotic divisions. Then, oocytes arrest again in metaphase I or metaphase II to await egg activation or fertilization. This secondary arrest ensures that the completion of meiosis is properly coordinated with fertilization of the oocyte. During both meiotic arrests, APC/C activity must be suppressed. In prophase I, unscheduled activity of the APC/C could prevent proper maintenance of chromosome cohesion. In the secondary metaphase arrests, inhibition of the APC/C is crucial for preventing premature anaphase onset. Thus, understanding the function and regulation of the APC/C during meiosis is critical for

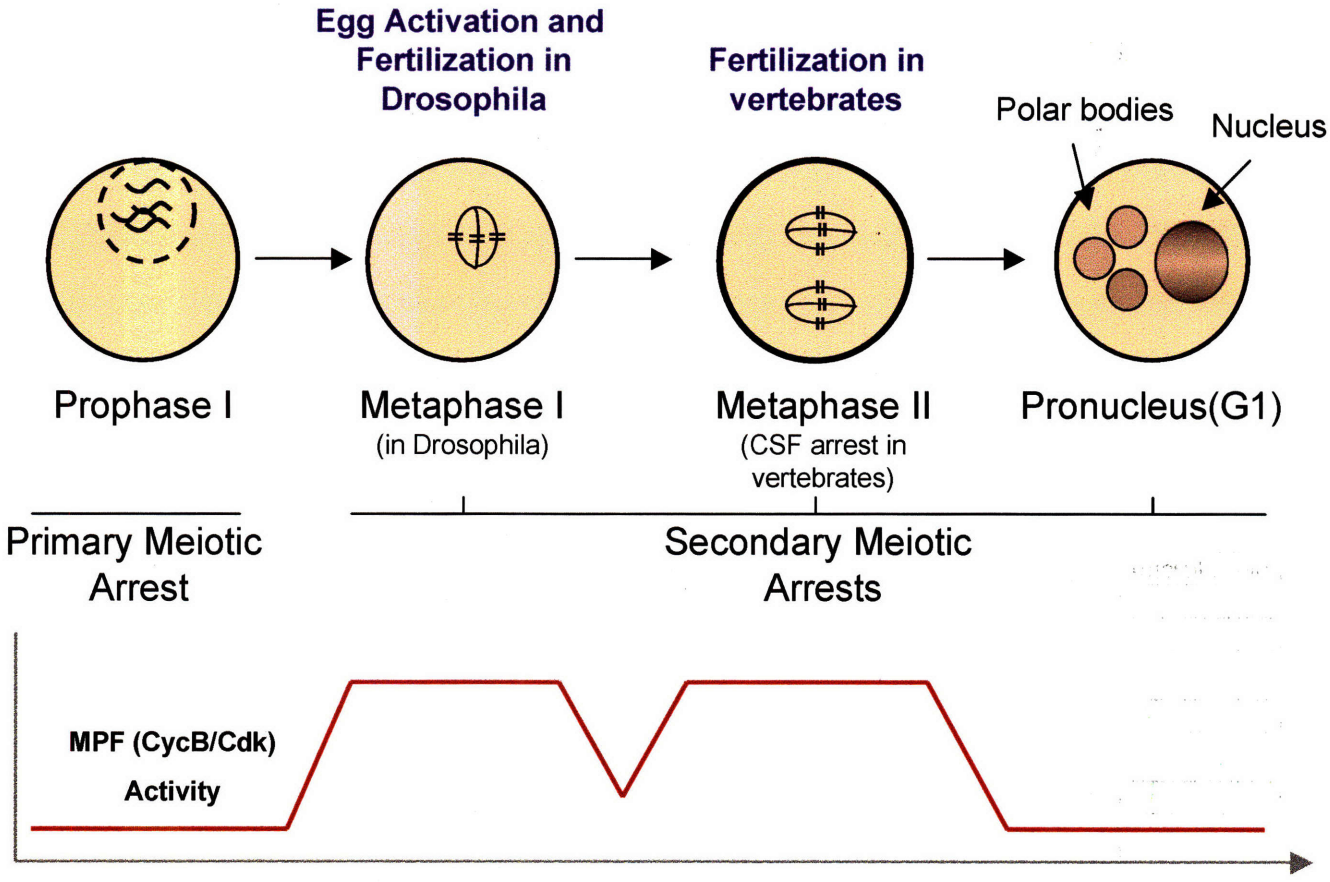

Figure 1-5. Meiotic Progression in Females
Meiosis is arrested twice during oogenesis. In most organisms the first meiotic arrest occurs during prophase I, and the second arrest time varies among organisms. Fertilization or egg activation releases these arrests in metaphase I and metaphase II. Cyclin B/ Cdk activity is elevated at each meiotic division and drops in between the divisions.

understanding control of chromosome segregation and cell cycle progression and coordination of this control with developmental signals during meiosis.

A. Requirement for APC/C in Meiosis

A requirement for APC/C activity during the meiotic divisions has been shown in all organisms tested with the exception of Xenopus oocytes. In yeast, Cdc20 is required for the degradation of Pds1/Securin and resulting activation of separase in both meiotic divisions (Salah and Nasmyth 2000). Separase activity is required for the degradation of Rec8, the meiosis-specific cohesin subunit, along chromosome arms to allow for homolog disjunction in meiosis I (Buonomo et al. 2000; Kitajima et al. 2003).

It has been more difficult to demonstrate a requirement for APC/C in meiosis in multicellular organisms. In *C. elegans*, mutations in or RNA interference against several subunits of the APC/C cause a meiotic metaphase I arrest (Furuta et al. 2000; Golden et al. 2000; Davis et al. 2002). Female sterile mutations in *fzy* cause both meiosis I and meiosis II arrests in Drosophila eggs (Swan and Schupbach 2007). Finally, several studies in mouse oocytes have demonstrated a requirement for APC/C-mediated degradation of Securin and activation of separase for Rec8 removal from chromosome arms and homolog disjunction in meiosis I (Herbert et al. 2003; Terret et al. 2003; Kudo et al. 2006).

However, two studies in Xenopus oocytes have suggested that APC/C is not required for meiosis I in this organism. Microinjection of oocytes with antibodies against Fzy or Cdc27 does not disrupt progression through meiosis I but only causes an arrest in meiosis II (Peter et al. 2001). Injection of antisense oligonucleotides against Fzy also

does not block progression through meiosis I (Taieb et al. 2001). A more recent study in Xenopus demonstrates that Securin and Cyclin B are degraded at anaphase I, reaccumulate before metaphase II, and are degraded again at anaphase II (Fan et al. 2006). These data are suggestive of an active APC/C in both meiosis I and meiosis II, although they do not demonstrate a requirement for this activity for homolog disjunction and progression through meiosis I. Additionally, misexpression of Emi2, an APC/C inhibitor that is active later in meiosis, induces a metaphase I arrest in Xenopus oocytes, again suggesting that APC/C is required for the metaphase I to anaphase I transition (Ohe et al. 2007; Tung et al. 2007). It is not clear whether the contrary results in Xenopus reveal a true biological difference in this system or are due to differences in experimental design.

B. Meiosis-Specific APC/C Activators

An intriguing development in our knowledge of meiotic cell cycle control has been the identification of meiosis-specific APC/C activators in yeast and flies. The existence of these activators suggests a unique role and set of substrates for the APC/C in meiosis that are outside of the functions of Cdc20 or Cdh1.

In yeast, meiosis-specific activators are expressed exclusively during meiosis and are required for proper spore formation (Cooper et al. 2000; Asakawa et al. 2001; Blanco et al. 2001). Ama1 in *S. cerevisiae* associates with the core APC/C throughout meiosis, becomes active after meiosis I, and is required for Clb1 and Pds1 degradation. Furthermore, it can drive the ubiquitination of Pds1 *in vitro* (Cooper et al. 2000; Oelschlaegel et al. 2005; Penkner et al. 2005). In *S. pombe* mfr1/fzr1 also associates with

the core APC/C during meiosis and is required for the degradation of M-phase cyclin Cdc13. Degradation of Cdc13 in meiosis II is required for proper spore formation at the end of meiosis (Blanco et al. 2001).

In Drosophila, *cortex* (*cort*) encodes a distant member of the Cdc20/FZY protein family that is transcribed only during oogenesis (Chu et al. 2001). *cort* mutant females lay eggs that never complete meiosis and arrest terminally in metaphase II (Lieberfarb et al. 1996; Page and Orr-Weaver 1996). *cort* seems to be required as soon as the metaphase I to anaphase I transition occurs, as meiosis II spindles often exhibit unequal numbers of chromosomes indicating aberrant chromosome segregation in meiosis I (Page and Orr-Weaver 1996). Consistent with *cort* functioning as an APC/C activator, mitotic cyclin levels are elevated in *cort* eggs, and misexpression of *cort* triggers degradation of these cyclins in wing imaginal discs (Swan and Schupbach 2007).

The Drosophila genome also contains a 4th Cdc20-related gene, *fizzy-related 2* (*fzr2*), that is expressed exclusively in male meiosis. When misexpressed, *fzr2* can rescue *fzr* function by triggering degradation of Cyclin B, which suggests a true function for *fzr2* as an APC/C activator (Jacobs et al. 2002). The use of a female and a male meiosis-specific APC/C activator in Drosophila will provide an interesting system in which to delineate the meiosis-specific roles of APC/C in the two different developmental contexts of oogenesis and spermatogenesis.

Meiosis-specific activators are not the only APC/C activators present during meiosis. In *S. cerevisiae* both Cdc20 and Ama1 are thought to contribute to APC/C function in meiosis. Ama1 protein is present beginning in pre-meiotic S-phase, although it does not become essential until late in meiosis for spore formation (Cooper et al. 2000;

Oelschlaegel et al. 2005; Penkner et al. 2005). This disparity in timing of Ama1 presence and function is explained by the action of an APC/C^{Ama1} inhibitor which will be discussed below. Cdc20, in contrast, is required in meiosis I as Cdc20 meiotic mutants arrest in metaphase I with high levels of Pds1 (Salah and Nasmyth 2000). However, both Ama1 and Cdc20 are likely to be involved in both meiotic divisions. Ama1 mutants do not arrest in meiosis I but do display a delay in spindle elongation and an increase in Pds1 and Clb5 protein levels (Oelschlaegel et al. 2005). Additionally, Cdc20 levels peak in both meiosis I and meiosis II, suggesting that it is important for both divisions (Salah and Nasmyth 2000).

In Drosophila, both *fzy* and *cort* are required for female meiotic divisions. Swan and Schupbach used double mutant genetic analysis to demonstrate that *fzy* and *cort* likely have redundant roles in meiosis I but non-redundant roles in meiosis II (Swan and Schupbach 2007). In *fzy* single mutants, a small percentage of eggs arrest with one spindle in meiosis I, and the majority of eggs arrest with 2 spindles in anaphase II. In *cort* single mutants, a similar percentage of eggs arrest in meiosis I, but the rest arrest with 2 spindles in metaphase II. Double mutants display an increased number of eggs arresting in meiosis I, consistent with a redundant role for these two genes. However, the remaining double mutant eggs arrest in metaphase II, suggesting that *cort* is epistatic to *fzy* in meiosis II. The effects of these mutations on localized Cyclin B degradation also support the idea that *cort* and *fzy* have non-redundant and different temporal roles in meiosis II. *cort* seems to be required for the degradation of Cyclin B specifically at the spindle midzone, while *fzy* seems to target Cyclin B after *cort* and along the entire spindle in meiosis II.

The role of Cdh1 in meiosis is not entirely clear. It does not appear to play any roles in the meiotic divisions but may to be important for prophase I. In *S. cerevisiae* *cdh1* mutant cells show incomplete synapsis of bivalents in prophase I (Penkner et al. 2005). This result may show a requirement for Cdh1 in meiosis, or Cdh1 may be required in the previous cell cycle for an effect in the subsequent meiotic prophase. In mouse oocytes, APC/C^{Cdh1} mediated degradation of substrates appears to be required for maintaining prophase I arrest and preventing entry into meiotic divisions (Reis et al. 2006; Marangos et al. 2007). In contrast, injection of Cdh1 antisense oligonucelotides into Xenopus oocytes inhibits oocyte maturation and induces meiotic entry (Papin et al. 2004). Finally, Cdh1/FZR appears to have no role during female meiosis in Drosophila because although *cdh1* is transcribed in the germline, the protein is not detectable in early embryos (Sigrist and Lehner 1997; Raff et al. 2002).

C. Regulation of the APC/C in Meiosis

Proper regulation of the APC/C in meiosis is crucial to prevent unscheduled APC/C activity and premature activation of Separase through Securin degradation. At both metaphase I and metaphase II, premature chromosome or sister chromatid segregation could lead to aneuploid gametes and offspring, which is a leading cause of spontaneous abortion and mental retardation (Hassold and Hunt 2001). Additionally, a unique feature of female meiosis is a prolonged prophase I arrest, up to several decades in length in humans, in which sister chromatid cohesion must be maintained for proper chromosome segregation in meiosis I.

1. Mnd2

As mentioned above, Ama1, the meiosis-specific APC/C activator in *S. cerevisiae*, is present beginning in premeiotic S-phase, yet is not required until late meiosis, suggesting that Ama1 may be inhibited in early meiosis. Two studies in *S. cerevisiae* revealed a crucial inhibitor of APC/C^{Ama1} in prophase I. Mnd2 was originally identified as being associated with the APC/C core (Hall et al. 2003; Passmore et al. 2003). Subsequently, it was found to inhibit APC/C^{Ama1} through genetic suppression and *in vitro* ubiquitination assays (Oelschlaegel et al. 2005; Penkner et al. 2005). The association of Mnd2 with APC/C from meiotic cells can inhibit the ubiquitination activity of APC/C^{Ama1} but not APC/C^{Cdc20} or APC/C^{Cdh1} (Oelschlaegel et al. 2005).

The mechanism of Mnd2 inhibition of APC/C^{Ama1} is unclear. *In vivo*, the absence of Mnd2 causes an increased association of Ama1 with the APC/C core, but *in vitro*, Ama1 binds with equal frequency to APC/C that is associated with Mnd2 or not (Oelschlaegel et al. 2005). The inhibitory actions of Mnd2 may occur after both Mnd2 and Ama1 are bound to APC/C.

The mutant phenotype of *mnd2Δ* reveals the disastrous consequences of unrestrained APC/C^{Ama1} activity in meiotic prophase. *mnd2Δ* cells fail to accumulate Pds1 in S and prophase and arrest in a prophase-like state (Oelschlaegel et al. 2005; Penkner et al. 2005). Furthermore, these cells showed premature sister chromatid separation as well as a failure to fully synapse homologs. Both groups demonstrated that these defects are the result of premature activation of separase and cleavage of Rec8 in prophase and are dependent on APC/C^{Ama1}-mediated degradation of Pds1.

Mnd2 inhibition of APC/C^{Ama1} must be relieved in late meiosis when Ama1 becomes required for spore formation. Mnd2 protein disappears from meiotic cells

during anaphase II and is thought to be one way in which APC/C^{Ama1} becomes active at this time (Oelschlaegel et al. 2005; Penkner et al. 2005). Additionally, Ama1 may be inhibited by the activity of Cdk1 kinases. Inhibition of Cdk1 in *cdc20* mutant metaphase I arrested cells triggers spindle disassembly and Ama1-dependent degradation of Pds1 (Oelschlaegel et al. 2005). This result suggests that APC/C^{Cdc20}-mediated degradation of cyclins in meiosis I is required for the activation of APC/C^{Ama1} in meiosis II.

2. Emi1

Based on Emi1's role in APC/C inhibition is mitosis, it initially seemed to be a likely candidate for an APC/C regulator in meiosis. However, after numerous studies, the role of Emi1 in meiosis is controversial. It was long thought to be a key component of cytostatic factor arrest (CSF) in metaphase II (discussed below), but this role for Emi1 has recently been called into question. Investigations of a role for Emi1 in earlier stages of meiosis suggest that it may indeed function at an earlier time point.

Tung and Jackson found that Emi1 may be required in prophase I and during the meiosis I – meiosis II transition in Xenopus oocytes. Injection of an Emi-neutralizing antibody in prophase I prior to maturation inhibited germinal vesicle breakdown (GVBD) upon progesterone stimulation and entry into the meiotic divisions (Tung and Jackson 2005). Injection of the same antibody at the onset of GVBD induced chromosome decondensation in between meiosis I and meiosis II, DNA replication, and failure to reaccumulate Cyclin B and Cdc2 activity before meiosis II. These defects were rescued by co-injection of Cdc20 oligonucleotides, suggesting they were the result of unrestrained APC/C^{Cdc20} activity. However, the authors admit that their anti-Emi1 antibody cross reacts with Emi2/Erp1 (Emi-related protein 1), and thus, these results may be due to

inhibition of Emi2 instead (discussed below). Exogenous Emi1 has been shown to be rapidly degraded during oocyte maturation in Xenopus oocytes (Ohsumi et al. 2004). Therefore, it may be more likely that Emi1 has a role in prophase and not at the meiosis I to meiosis II transition in Xenopus.

In mouse a similar story is unfolding. Emi1 protein is present in mouse oocytes but is degraded at GVBD in a $SCF^{\beta\text{-}TrCP}$ dependent manner, mirroring the timing of Emi1 destruction in mitotic cells in late prophase (Marangos et al. 2007). As in Xenopus, inhibition of Emi1 function in prophase I delays entry into the first meiotic division by preventing the accumulation of Cyclin B necessary for MPF activation and progression through meiosis I. These effects of inhibiting Emi1 function in mouse oocytes are mediated through APC/C^{Cdh1} activity (Marangos et al. 2007). Thus, a conserved role exists for Emi1 in maintaining prophase I arrest in vertebrate oocytes.

3. Mes1

The transition between meiosis I and meiosis II requires a balance between lowering Cyclin B-Cdk activity sufficiently enough to exit meiosis I and maintaining levels high enough to suppress DNA replication and promote entry into meiosis II. In *S. pombe*, Mes1 may be the solution to this problem. *mes1* is transcribed in a narrow window between late meiosis I and late meiosis II (Mata et al. 2002). *mes1* mutants arrest before meiosis II and completely degrade M-phase cyclin Cdc13 prematurely in anaphase I, instead of in anaphase II (Shimoda et al. 1985; Izawa et al. 2005). The *mes1* mutant phenotype is suppressed by *slp1*, the Cdc20 homolog in *S. pombe*, and addition of Mes1 to a Xenopus egg extract inhibits APC/C activity, both consistent with a role for

Mes1 as an APC/C inhibitor (Izawa et al. 2005). Mes1 may inhibit APC/C^{Mfr1} in addition to APC/C^{Slp1}, but this remains to be shown conclusively.

Interestingly, the mechanism of Mes1 inhibition of APC/C^{Slp1} may be similar to that of Emi1 and Mad3/BubR1. Slp1 associates with Mes1 through the same residues that it uses to bind Cdc13, and Cdc13 and Mes1 compete for binding to Mes1 *in vitro* (Izawa et al. 2005). Intriguingly, the N-terminal half of Mes1, which is sufficient for binding to and inhibiting Slp1, contains a putative KEN box and a putative D-box, suggesting a pseudosubstrate mode of inhibition like that of Emi1 and Mad3/BubR1. The evolution of APC/C substrates into APC/C inhibitors seems to be an emerging theme in the biology of APC/C regulation.

4. Emi2/Erp1

Just as Mes1 is required in *S. pombe* to inhibit APC/C activity in between meiosis I and meiosis II, Emi2/Erp1, a homolog of Emi1, is required in this role in Xenopus and mouse oocytes. A role for Emi2 in Xenopus at this time was first suggested by Tung and Jackson when they used a neutralizing Emi1 antibody that cross-reacted with Emi2 (discussed above, Tung and Jackson 2005). Two groups later found that Emi2 is not expressed until after GVBD, and its expression is coincident with polyadenylation of *emi2* mRNA (Ohe et al. 2007; Tung et al. 2007). Inhibition of Emi2 by injection of morpholinos or antisense oligonucleotides reduces Cyclin B2 reaccumulation after meiosis I, prevents entry into meiosis II, and, in the case of morpholino injection, induces DNA replication (Ohe et al. 2007; Tung et al. 2007). Injection of Emi2 morpholinos into mouse oocytes generates a very similar phenotype, suggesting that Emi2/Erp1 inhibits

APC/C-mediated degradation of Cyclin B after meiosis I to prevent DNA replication and allow entry into meiosis II (Madgwick et al. 2006).

A critical role for Emi2 in meiosis is inhibition of APC/C during cytostatic factor (CSF) arrest. Vertebrate eggs are arrested in metaphase II while they await fertilization. This arrest functions to prevent premature egg activation or completion of meiosis before fertilization occurs. Many years of research determined that establishment of CSF arrest involves the Mos/MAPK/$p90^{RSK}$ signaling pathway and Cyclin E/ Cdk2 activity through inhibition of APC/C^{Cdc20} (for review, see Tunquist and Maller 2003). Release of CSF arrest is triggered by a transient increase in free cytosolic Ca^{2+} levels induced by fertilization. An additional component contributing to CSF arrest was thought to be Emi1, although this idea has recently been questioned, as some groups report that Emi1 is not present and unstable in mature eggs, whereas others show that it is stabilized in mature eggs at low levels (Ohsumi et al. 2004; Tung et al. 2005; Shoji et al. 2006). Cross-reactivity of antibodies to both proteins has been the source of some of this confusion.

Nevertheless, Emi2/Erp1 has emerged as a crucial mediator of CSF arrest. Emi2 is present in Xenopus CSF extracts and degraded upon addition of calcium, consistent with a role as a component of CSF (Schmidt et al. 2005; Tung et al. 2005). Furthermore, depletion of Emi2 causes premature release from CSF arrest, independent of calcium addition (Schmidt et al. 2005). Conversely, addition of exogenous or non-degradable Emi2 prevents calcium-induced CSF release. These effects are likely to occur through Emi2 inhibition of APC/C^{Cdc20}, as Emi2 can inhibit ubiquitination of substrates by APC/C^{Cdc20} *in vitro* (Schmidt et al. 2005; Tung et al. 2005). In mouse oocytes, Emi2

appears to play the same role in CSF arrest. In addition, mouse Emi2 was shown to bind to Cdc20 *in vitro* (Shoji et al. 2006).

Degradation of Emi2 upon Ca^{2+} -induced CSF release is likely to be the event that relieves inhibition of APC/C^{Cdc20} and allows for passage from metaphase II to anaphase II. Plx1 kinase activity is required for activation of APC/C^{Cdc20} upon Ca^{2+} addition through its phosphorylation of Emi2 and resulting $SCF^{\beta TrCP}$-mediated degradation (Schmidt et al. 2005; Tung et al. 2005). However, the role of calcium in triggering the degradation of Emi2 still remained unknown. Furthermore, both Plx1 and SCF are active during CSF arrest, yet Emi2 remains stable until calcium addition.

Three studies elucidated the dual roles of Plx1 and calmodulin kinase II (CaMKII) in triggering Emi2 degradation at CSF release (Figure 1-3). CaMKII, like Plx1 is required for Ca^{2+}-induced CSF release and Emi2 degradation (Lorca et al. 1993; Hansen et al. 2006). CSF release triggered by a constitutively-active CaMKII (CamCat) requires Plx1, and Plx1-induced CSF release requires active CaMKII (Liu and Maller 2005). The polo-box domain (PBD) of Plx1 is thought to bind a phosphopeptide motif in target proteins generated by phosphorylation of the target by a "priming kinase." Once Plx1 docks onto the target through its PBD, its phosphorylation of the target protein is enhanced. Phosphorylation of Emi2 by CaMKII strongly enhances Plx1 binding to and phosphorylation of Emi2 *in vitro*, and degradation of Emi2 in anaphase extracts is dependent on phosphorylation by CaMKII, strongly suggesting that CaMKII is the priming kinase for Plx1 upon CSF release (Liu and Maller 2005; Rauh et al. 2005; Hansen et al. 2006). These studies have elucidated the link between calcium elevation and release of APC/C^{Cdc20} inhibition at fertilization.

Finally, recent studies in Xenopus have found additional sites of phosphorylation on Emi2, some of which directly link Emi2 to the Mos/MAPK/$p90^{RSK}$ pathway in CSF arrest (Figure 1-3). The ability of Emi2 to inhibit APC/C after meiosis I and in CSF arrest is dependent on the MAPK pathway (Inoue et al. 2007). $p90^{RSK}$ phosphorylates Emi2 *in vitro*, and these sites of phosphorylation are required *in vivo* for Emi2 stability during oocyte maturation and its CSF activity (Inoue et al. 2007; Nishiyama et al. 2007). This work strongly suggests that, at least in amphibian oocytes, Emi2 is the effector protein of Mos/MAPK/$p90^{RSK}$ signaling in CSF arrest.

Nishiyama et al. shed light on the mechanism of Emi2 inhibition of APC/C. Phosphorylation of Emi2 by $p90^{RSK}$ enhances association of Emi2 with the APC/C, consistent with its role in activating Emi2 inhibitory activity (Nishiyama et al. 2007). Like Emi1, Emi2 contains a D-box that also enhances association of Emi2 with APC/C. This association is inhibited *in vitro* by D-box peptides, suggesting that Emi2 blocks substrate access to the APC/C. In addition, phosphorylation of Emi2 by Cyclin B/Cdc2 at sites distinct from those targeted by CaMKII and $p90^{RSK}$ may disrupt the interaction between Emi2 and APC/C (Figure 1-3) (Wu et al. 2007; Hansen et al. 2007). This phosphorylation is antagonized by protein phosphatase 2A and may represent a feedback loop that serves to maintain proper levels of Cyclin B and Cdc2 kinase activity during CSF arrest.

5. Spindle Checkpoint

In a normal mitosis in the absence of spindle damage, the spindle checkpoint genes are dispensable, with the exception of *mps1*, in *S. cerevisiae*, but are essential in metazoans, although this has been questioned recently in Drosophila (Hoyt et al. 1991; Li

et al. 1991; Kitagawa et al. 1999; Dobles et al. 2000; Kalitsis et al. 2000; Buffin et al. 2007). In meiosis, these genes appear to be required not only upon spindle damage but also in a normal undisturbed meiotic division. In *S. cerevisiae*, mutations in *mad1* or *mad2* cause increased nondisjunction of homologous chromosomes in meiosis I (Shonn et al. 2000). Levels of nondisjunction are restored if anaphase is artificially delayed, suggesting that Mad1 and Mad2 are important for inducing a metaphase I delay in a normal meiosis. Loss of recombination in a *spo11* mutant, which causes a lack of tension on kinetochores, induces a Mad2-dependent suppression of APC/C activity, suggesting that the checkpoint also responds to spindle defects in meiosis I. Furthermore, Mad2 may have an additional role in promoting the biorientation of homologs perhaps through a mechanism other than inhibition of APC/C (Shonn et al. 2003). The requirement for spindle checkpoint function in a normal meiosis I division reflects the increased complexity of bi-orienting homologous chromosome pairs on the spindle compared with bi-orienting sister chromatid kinetochores in mitosis.

Higher organisms also display a requirement for spindle checkpoint genes in an undisturbed meiosis I. Mutations in spindle checkpoint genes in *C. elegans* suppress a metaphase I arrest in a *cdc23* mutant, indicating a likely role for SAC in inhibiting APC/C during meiosis I (Stein et al. 2007). In mouse oocytes, a Mad2-dependent metaphase I arrest is induced upon nocodazole treatment (Wassmann et al. 2003). Mad2 is also required for a normal meiosis I in mouse because Mad2-depleted oocytes prematurely degrade Securin and Cyclin B and missegregate their chromosomes (Homer et al. 2005). Furthermore, *bubR1* mutant female mice contain oocytes with chromosome segregation defects (Baker et al. 2004). Finally, microinjection of Bub1 antibodies into

mouse oocytes causes chromosome misalignment on the meiosis I spindle that is not corrected by delaying anaphase onset, suggesting that, like Mad2 in yeast, Bub1 has a specific role in chromosome alignment during metaphase I (Yin et al. 2006).

Control of chromosome segregation in meiosis I in Drosophila females is an interesting problem because in Drosophila the secondary meiotic arrest occurs in metaphase I, unlike CSF arrest in metaphase II. Oocytes mutant for *mps1* enter anaphase I prematurely, suggesting a role for the spindle checkpoint in mediating this arrest (Gilliland et al. 2007). Reduction of *mps1* function in these oocytes causes non-disjunction of both exchange and non-exchange chromosomes, which is likely due, in part, to defects in bi-orientation of homologous chromosomes in meiosis I (Gilliland et al. 2005; Gilliland et al. 2007).

Additionally, in female meiosis of *bubR1* mutants in Drosophila, non-disjunction of sister chromatids is elevated (Malmanche et al. 2007). Cohesin is lost from chromosomes in prophase I of these oocytes, and it is not clear whether this effect occurs through loss of an inhibitory effect on APC/C activity in the *bubR1* mutant. In *S. cerevisiae* Mad3 mediates a prophase I delay that becomes essential for chromosome segregation when chromosomes do not recombine (Cheslock et al. 2005). It is also unclear whether this defect is due to uninhibited APC/C.

A role for the spindle checkpoint in CSF arrest in meiosis II has not been shown conclusively. The spindle checkpoint was hypothesized to be a component of CSF arrest because Bub1 is phosphorylated by $p90^{RSK}$ during oocyte maturation in Xenopus oocytes, placing it downstream of the Mos/ MAPK pathway that is required for establishment of CSF arrest (Schwab et al. 2001). Immunodepletion of Bub1, Mad1, or Mad2 all prevent

establishment of CSF arrest in Xenopus oocytes, and this effect is thought to occur downstream of Mos (Tunquist et al. 2002; Tunquist et al. 2003). In contrast, inhibition of Mps1, another kinase required for spindle checkpoint-mediated arrest in mitosis, has no effect on CSF arrest (Grimison et al. 2006). Furthermore, although Bub1 and Mad2 are required for establishment of CSF arrest, they are not required for maintenance of the arrest in CSF extracts (Tunquist et al. 2002; Tunquist et al. 2003). In mouse oocytes, expression of dominant negative forms of Bub1, Mad2, or BubR1 do not affect CSF arrest, further bringing into question a role for the spindle checkpoint in inhibiting APC/C during CSF arrest (Tsurumi et al. 2004). CSF arrest is quite different from a mitotic spindle checkpoint-mediated arrest because kinetochores are already under tension and attached correctly to the spindle. If there is a role for spindle checkpoint proteins in establishment of CSF arrest, they may act while kinetochores achieve bipolar attachments, but not after, or they may have a different role or target during CSF establishment.

V. Summary of Thesis

In this thesis we analyze the function of *cort*, a putative meiosis-specific APC/C activator in Drosophila female meiosis. First we establish that *cort* is indeed an activator of the APC/C by demonstrating that it is physically associated with the core APC/C during oogenesis. Next, we analyze the timing of degradation of APC/C substrates during meiosis and demonstrate for the first time that Cyclins are degraded sequentially during meiosis in a multicellular organism. We uncover regulatory mechanisms governing levels of CORT protein during oogenesis and early embryogenesis that may be

important in developmental control of the meiotic cell cycle. Finally, we initiate a genetic screen to identify meiosis-specific substrates of the APC/C.

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Chapter Two

Developmental Role and Regulation of *cortex*, a Meiosis-Specific APC/C Activator

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ABSTRACT

During oogenesis in metazoans, the meiotic divisions must be coordinated with development of the oocyte to ensure successful fertilization and subsequent embryogenesis. The ways in which the mitotic machinery is specialized for meiosis are not fully understood. *cortex*, which encodes a putative female meiosis-specific APC/C activator, is required for proper meiosis in *Drosophila*. We demonstrate that CORT physically associates with core subunits of the APC/C in ovaries. APC/C^{CORT} targets Cyclin A for degradation prior to the metaphase I arrest, while Cyclins B and B3 are not targeted until after egg activation. We investigate the regulation of CORT and find that CORT protein is specifically expressed during the meiotic divisions in the oocyte. Polyadenylation of *cort* mRNA is correlated with appearance of CORT protein at oocyte maturation, while deadenylation of *cort* mRNA occurs in the early embryo. CORT protein is targeted for degradation by the APC/C following egg activation, and this degradation is dependent on an intact D-box in the C-terminus of CORT. Our studies reveal the mechanism for developmental regulation of an APC/C activator, and suggest it is one strategy for control of the female meiotic cell cycle in a multicellular organism.

INTRODUCTION

Developmental regulation of meiosis is crucial for generating viable eggs and sperm and, thus, a successful fertilization event. Meiosis is a modified cell cycle in which segregation of homologous chromosomes is followed by segregation of sister chromatids without an intervening S phase. These unique divisions are controlled by general mitotic cell-cycle regulators as well as specialized meiotic proteins (Marston and Amon 2004). During oogenesis in multicellular organisms, meiosis presents a particular regulatory challenge. The meiotic divisions must be coordinated tightly with growth and development of the oocyte to allow for oocyte differentiation and to ensure that the completion of meiosis is coordinated with fertilization. To achieve this coordination, oocytes arrest at prophase I and again at metaphase I or metaphase II and are released from these arrests through processes called oocyte maturation and activation, respectively (Kishimoto 2003; Tunquist and Maller 2003). Furthermore, additional specialized cell cycle regulation is required for the transition between meiosis and restart of the cell cycle in embryogenesis. In *Drosophila*, meiosis is completed without cytokinesis in the same common cytoplasm in which the rapid mitotic divisions of embryogenesis begin. Upon fertilization, the oocyte must quickly inactivate meiotic regulators to prevent interference with embryonic mitotic cycles. The ways in which general mitotic proteins act together with meiosis-specific proteins to meet the multiple regulatory challenges of meiosis in metazoans are not well understood.

The anaphase–promoting complex/cyclosome (APC/C) plays a critical role in mitosis, but much remains to be understood about its function in meiosis. The APC/C is a large E3 ubiquitin ligase, composed of at least 12 core subunits, that targets specific

substrate proteins for degradation by the 26S proteasome (Thornton and Toczyski 2006). In mitosis, the APC/C is crucial for proper cell division through targeting of key substrates. Securin, an inhibitor of separase, must be degraded to allow for cleavage of cohesin and subsequent segregation of sister chromatids, and mitotic cyclins must be degraded to allow for the metaphase to anaphase transition and events associated with mitotic exit (Cohen-Fix et al. 1996; Shirayama et al. 1999; Stemmann et al. 2001; Wasch and Cross 2002). In addition, the APC/C targets many other proteins for degradation including proteins involved in spindle function and regulators of DNA replication (Juang et al. 1997; McGarry and Kirschner 1998).

Substrate specificity is conferred to the APC/C by activator proteins Cdc20/Fizzy and Cdh1/Fizzy-related that recognize substrate proteins containing D-box or KEN box motifs (Schwab et al. 1997; Visintin et al. 1997; Fang et al. 1998; Pfleger and Kirschner 2000; Wan and Kirschner 2001). Regulation of these specificity factors is one crucial way by which APC/C activity is modulated. Cdc20 is transcribed and translated during S-phase and G2, phosphorylated in mitosis, and degraded in an APC/C^{Cdh1}-dependent manner in G1 (Fang et al. 1998; Prinz et al. 1998; Shirayama et al. 1998; Kramer et al. 2000; Huang et al. 2001). Phosphorylation of several APC/C subunits in mitosis facilitates the ability of Cdc20 to bind to and activate the APC/C (Shteinberg et al. 1999; Kramer et al. 2000; Rudner and Murray 2000; Golan et al. 2002; Kraft et al. 2003). Levels of Cdh1 are constant in mitosis and lowered in late G1 and S, but inhibitory phosphorylation of Cdh1 prevents its association with APC/C during S, G2, and M phases (Prinz et al. 1998; Kramer et al. 2000; Hsu et al. 2002). Thus, differential

regulation of Cdc20 and Cdh1 directs their transient association with the APC/C at different times during the cell cycle to target specific subsets of proteins for degradation.

In meiosis, the role of the APC/C and its regulation is less clear. An appealing hypothesis is that the meiotic divisions are driven in part by the degradation of specific meiotic APC/C substrates, and thus, the APC/C must require unique regulation during these divisions. In yeast, it is known that disjunction of homologous chromosomes in meiosis I and sister chromatids in meiosis II requires APC-mediated destruction of Pds1/Securin to release separase for cleavage of cohesin (Buonomo et al. 2000; Salah and Nasmyth 2000; Kitajima et al. 2003). In multicellular organisms, however, a requirement for the APC/C in meiosis has been more difficult to demonstrate. Mutations in or RNA interference against APC/C subunits in *C. elegans* result in a metaphase I arrest (Furuta et al. 2000; Davis et al. 2002). In *Drosophila*, mutations in *fzy* cause both meiosis I and meiosis II arrests (Swan and Schupbach 2007). Several studies in mouse oocytes have shown that APC/C-mediated protein degradation is required for homolog disjunction and polar body extrusion (Herbert et al. 2003; Terret et al. 2003; Wassmann et al. 2003; Kudo et al. 2006). However, inhibiting APC/C subunits by depletion or antibody injections in *Xenopus laevis* does not prevent the metaphase I to anaphase I transition but does cause arrest in metaphase II (Peter et al. 2001; Taieb et al. 2001). The reasons behind this apparent inconsistency in *Xenopus* remain unknown.

One way in which the APC/C may be regulated uniquely in meiosis is through its association with meiosis-specific activators. Cdc20/FZY family members have been identified in yeast that are expressed exclusively in meiosis. In *S. cerevisiae*, Ama1 activates the APC/C after meiosis I and is required for spore wall assembly (Cooper et al.

2000; Oelschlaegel et al. 2005; Penkner et al. 2005). Similarly, in *S. pombe*, fzr1/mfr1 is required for proper spore formation after the completion of the meiotic divisions (Asakawa et al. 2001; Blanco et al. 2001). The identification and study of meiosis-specific APC/C activators are starting points from which to understand the unique regulation of the APC/C during meiosis as well as to identify meiosis-specific substrates of the APC/C.

Drosophila provides the best candidate for a meiosis-specific APC/C activator in metazoans. *cortex* (*cort*) encodes a distant member of the Cdc20/FZY family and is expressed exclusively in oogenesis and early embryogenesis (Chu et al. 2001). *cort* is required for proper female meiosis. Eggs laid by *cort* mutant mothers display aberrant chromosome segregation in meiosis I and arrest terminally in metaphase II (Lieberfarb et al. 1996; Page and Orr-Weaver 1996). In addition, *cort* mutant eggs contain elevated levels of mitotic cyclins, and misexpression of *cort* causes a decrease in levels of mitotic cyclins (Swan and Schupbach 2007). *cort* presents a unique opportunity for understanding the function and the developmental regulation of the APC/C during meiosis in a multicellular organism. *Drosophila* is an ideal system for studying female meiosis because different meiotic stages can be distinguished easily by cytology and isolated by micro-dissection. In this study, we demonstrate that CORT interacts biochemically with the APC/C during female meiosis and reveal a mechanism for developmental regulation of CORT through both post-transcriptional and post-translational processes.

RESULTS

CORT protein is associated with the APC/C *in vivo*

A recent study suggested that the *cortex* gene encodes a functional activator of the APC/C (Swan and Schupbach 2007). This assertion was based on the ability of *cort* to mediate cyclin protein levels. Levels of mitotic cyclins are elevated in *cort* mutants, and misexpression of *cort* causes decreased levels of cyclins in wing imaginal discs. While these data are strong evidence of *cort*'s function as an activator of the APC/C, demonstration of a physical association between CORT and the APC/C during oogenesis is lacking. We looked for an association between CORT and the APC/C by co-immunoprecipitation. We made transgenic lines with a MYC-tagged form of *cort* under control of the *UAS* response element and drove MYC-CORT expression in the female germline using *nos-gal4*. MYC-CORT is functional because expression of this transgene rescued the meiotic arrest in progeny of $cort^{RH65}$ mutant females (Supplementary Table).

We immunoprecipitated MYC-CORT from ovary extracts using a MYC antibody. Cdc27 and Cdc16, tetratricopeptide repeat (TPR) core subunits of the APC/C, co-immunoprecipitate with MYC-CORT but not with a control mouse IgG (Fig. 2-1A and data not shown). Morula (MR), the APC2 homolog in *Drosophila*, does not co-immunoprecipitate with MYC-CORT (Fig. 2-1A). Data from *Saccharomyces cerevisiae* suggest that the APC/C exists in two distinct subcomplexes bridged together by Apc1. One subcomplex contains Apc2 and Apc11 while the other contains the TPR proteins Cdc27, Cdc16, and Cdc23 (Thornton et al. 2006). Failure of MR to co-immunoprecipitate with MYC-CORT may be explained by a tighter and more direct association of CORT with the TPR protein-containing subcomplex but not with the

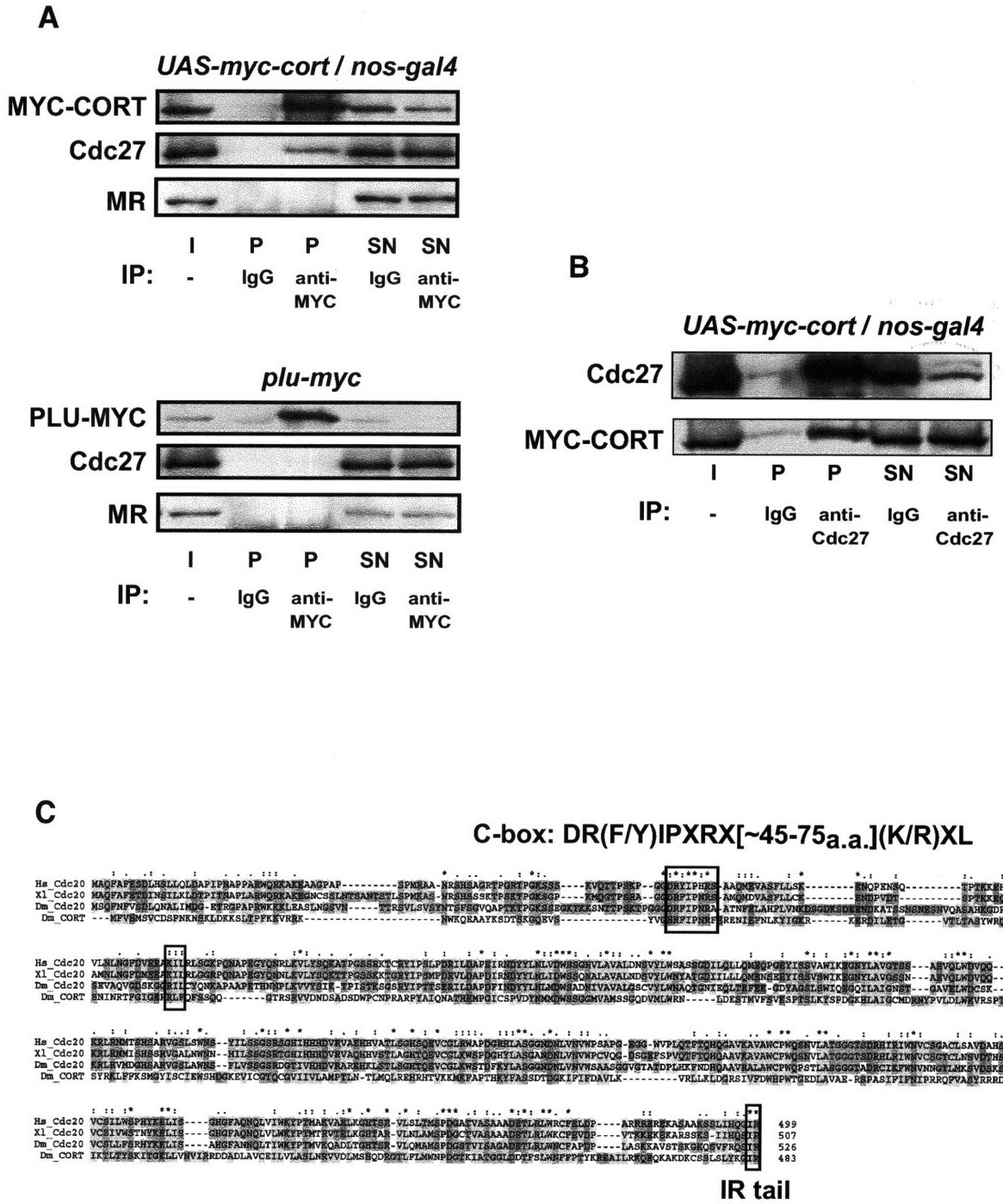
Figure 2-1. CORT protein associates with the APC/C *in vivo*

(A) Cdc27 co-immunoprecipitates with MYC-CORT. Top panel: MYC-CORT was immunoprecipitated from whole ovary extracts from *UAS-myc-cort/nos-gal4* females with anti-MYC or control mouse IgG, and immunoprecipitates were examined for the presence of APC/C subunits, Cdc27 and MR/APC2. Bottom panel: Unrelated MYC-tagged control PLU-MYC was immunoprecipitated from whole ovary extracts from *plu-myc* transgenic females with anti-MYC or control mouse IgG, and immunoprecipitates were examined for the presence of APC/C subunits. I, input, P, pellet, SN, supernatant.

(B) MYC-CORT co-immunoprecipitates with Cdc27. Cdc27 was immunoprecipitated from whole ovaries from *UAS-myc-cort/nos-gal4* females with anti-Cdc27 or a control rabbit IgG, and immunoprecipitates were examined for the presence of MYC-CORT.

(C) CORT contains motifs conserved in the Cdc20/FZY family of proteins. CORT protein sequence was hand-aligned with Cdc20/FZY protein sequences from human (Hs), Xenopus (Xl), and Drosophila (Dm). The conserved internal C-box and C-terminal IR tail are boxed. * identity, : high similarity, . some similarity.

Figure 2-1


A
UAS-myc-cort / nos-gal4
MYC-CORT
Cdc27
MR
I P P SN SN
IP: - IgG anti-MYC IgG anti-MYC
plu-myc
PLU-MYC
Cdc27
MR
I P P SN SN
IP: - IgG anti-MYC IgG anti-MYC
B
UAS-myc-cort / nos-gal4
Cdc27
MYC-CORT
I P P SN SN
IP: - IgG anti-Cdc27 IgG anti-Cdc27
C
C-box: DR(F/Y)IPXRX[~45-75a.a.](K/R)XL
Hs_Cdc20
Xl_Cdc20
Dm_Cdc20
Dm_CORT
IR tail

Apc2-containing subcomplex. Furthermore, buffer conditions may be causing CORT to dissociate from MR as extensive high-salt washes of human APC cause dissociation of Apc2 and Apc11 from the rest of the complex (Vodermaier et al. 2003). Based on previous studies of APC/C, we still think it is likely that CORT acts together with MR and Cdc27 in one complex. As an additional control, we immunoprecipitated an unrelated MYC-tagged protein, PLU-MYC, from ovary extracts to confirm that the associations of Cdc27 and Cdc16 with CORT are specific. Neither Cdc27 nor Cdc16 co-immunoprecipitate with PLU-MYC, indicating that they are associating with CORT and not with the MYC tag (Fig. 2-1A and data not shown).

In the reciprocal experiment, we immunoprecipitated Cdc27 from ovary extracts (Fig. 2-1B). MYC-CORT co-immunoprecipitates with Cdc27 but not with a control rabbit IgG, suggesting that there is a strong physical interaction between CORT and Cdc27.

In addition to demonstrating the physical association between CORT and the APC/C, we identified motifs in the CORT protein sequence that have been shown to contribute to binding of APC/C activator proteins to the APC/C (Fig. 2-1C). CORT contains an internal motif called the C-box that is important for binding to the APC/C and is conserved in Cdc20 and Cdh1 proteins throughout many species (Pfleger et al. 2001; Schwab et al. 2001). In addition, CORT contains the C-terminal IR (isoleucine-arginine) tail motif that is present in all APC/C activators as well as Doc1 and has been shown to mediate a direct interaction between Cdh1 and Cdc27 (Passmore et al. 2003; Vodermaier et al. 2003). Together, the co-immunoprecipitation of CORT with core subunits of the

APC/C in oogenesis and the presence of conserved motifs in the CORT sequence confirm CORT's identity as a meiosis-specific APC/C activator.

cortex cannot provide *fizzy* function in the early embryo

An additional way to determine if *cort* functions as an APC/C activator is to ask whether it can provide the function of another APC/C activator when over- or mis-expressed. To investigate whether *cort* acts similarly to *fzy*, we asked if *cort* can functionally substitute for *fzy* in the early embryo. fzy^6/fzy^7 females lay eggs that arrest in metaphase after a few rounds of mitotic divisions (Dawson et al. 1993). If *cort* can provide *fzy* function, we would expect these embryos to progress further in embryogenesis.

We overexpressed *myc-cort* in the germline of fzy^6/fzy^7 females, collected embryos from these females, visualized the DNA and the spindles by immunofluorescence, and counted the number of mitotic spindles. Embryos overexpressing *cort* did not contain more mitotic spindles compared to *fzy* alone, thus we did not observe any rescue of the *fzy* phenotype. In contrast, overexpression of *cort* slightly worsened the *fzy* phenotype (presented below). This result suggests either that *cort* does not function as an APC/C activator, or that CORT confers significantly different substrate specificity to the APC/C than FZY and, therefore, cannot provide its function. We cannot rule out the possibility that MYC-CORT was not present in sufficient levels for rescue due to low expression levels or protein instability (Supplemental Fig. 1).

Cortex triggers degradation of Cyclin A early in meiosis I and additional substrates later

In mitosis, cyclins are degraded sequentially by the APC/C, with Cyclin A being degraded prior to Cyclin B and Cyclin B3 (Sigrist et al. 1995; den Elzen and Pines 2001). For a more detailed analysis of CORT function in meiosis, we examined levels of putative APC/C^{CORT} substrates at different timepoints during the meiotic divisions. We can isolate different meiotic stages by dissecting egg chambers from ovaries: immature ovaries with egg chamber Stages 1-12 contain oocytes arrested in prophase I, and mature Stage 14 oocytes are arrested in metaphase I.

We performed Western blots on extracts from immature egg chamber stages and Stage 14 oocytes to assay levels of Cyclin A at these meiotic timepoints in *cort* mutants. We found that Cyclin A levels are slightly reduced in *cort* mutant immature ovaries (Stages 1-11) although the significance of this effect is not clear as we have not observed any defects in these stages of *cort* mutant ovaries. Cyclin A levels are elevated in mutant Stage 14 oocytes compared to a heterozygous control (Fig. 2-2A). The heterozygous control blots indicate that Cyclin A is normally degraded at some point between release of the prophase I arrest and establishment of the metaphase I arrest. In *cort* mutants, the failure to degrade Cyclin A by the metaphase I arrest indicates that APC/C^{CORT} is required for Cyclin A degradation at this time. In contrast, levels of Cyclin B, Cyclin B3, and PIMPLES (PIM), the Securin homolog in *Drosophila*, are not elevated in *cort* mutant Stage 14 oocytes compared to heterozygous controls, suggesting that these proteins are not subject to degradation by APC/C^{CORT} at this developmental stage (Fig. 2-2B). Upon egg activation in *Drosophila*, the metaphase I arrest is released, and meiosis is rapidly completed as the egg is ovulated and laid (Heifetz et al. 2001). Meiosis is

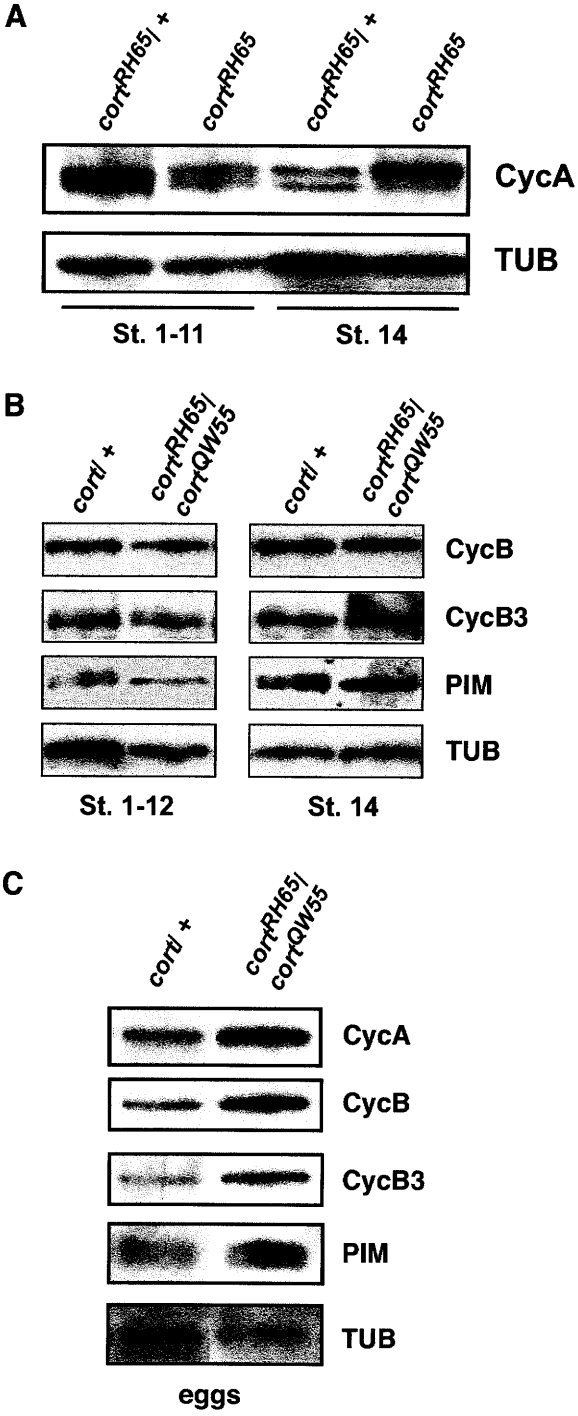
Figure 2-2. Cort targets sequential degradation of APC/C substrates in meiosis.

(A) Cyclin A levels are elevated in *cort* mutant Stage 14 oocytes. Western blot showing Cyclin A levels in immature Stage 1-11 egg chambers and mature Stage 14 oocytes from $cort^{RH65}$/ + or $cort^{RH65}$ females. The two forms of Cyclin A protein differ by phosphorylation state (Vardy, Pesin, and Orr-Weaver, unpubl.). By ImageJ analysis, the band intensity of Cyclin A in $cort^{RH65}$/ + Stage 1-11 egg chambers is approximately 1.25-fold greater than Cyclin A in $cort^{RH65}$ Stage 1-11 egg chambers. The band intensity of the more slowly migrating form of Cyclin A in $cort^{RH65}$ Stage 14 oocytes is approximately 2.1-fold greater than the same Cyclin A band in $cort^{RH65}$/ + Stage 14 oocytes. When assessing changes between developmental stages, Cyclin A band intensity in $cort^{RH65}$/ + Stage 1-11 egg chambers is approximately 4.1-fold greater than $cort^{RH65}$/ + Stage 14 oocytes. Cyclin A band intensity in $cort^{RH65}$ Stage 1-11 egg chambers is approximately 2.4-fold greater than $cort^{RH65}$ Stage 14 oocytes. Blot was probed with anti-α-Tubulin as a loading control.

(B) Cyclin B, Cyclin B3, and PIM levels are unchanged in *cort* mutant ovaries. Western blot showing levels of Cyclin B, Cyclin B3, and PIM/Securin in immature Stage 1-12 egg chambers and mature Stage 14 oocytes from $cort^{RH65}$/ + or $cort^{RH65}/cort^{QW55}$ females. A doublet of PIM protein has been noted previously in some strain backgrounds [86]. Blot was probed with anti-α-Tubulin as a loading control. All lanes are from the same blot.

(C) Cyclin A, Cyclin B, Cyclin B3, and PIM/Securin levels are elevated in eggs laid by $cort^{RH65}/cort^{QW55}$ females. By ImageJ analysis, band intensities are greater by the following approximate folds in $cort^{RH65}/cort^{QW55}$ compared to heterozygous controls: Cyclin A, 1.7, Cyclin B, 2.4, Cyclin B3, 2.3, and PIM, 1.7. Western blot was probed with anti-α-Tubulin as a loading control. Non-consecutive lanes from the same blot are shown.

Figure 2-2


A
cortRH65/ +
cortRH65
cortRH65/ +
cortRH65
CycA
TUB
St. 1-11
St. 14
B
cort/ +
cortRH65/ cortQW55
cort/ +
cortRH65/ cortQW55
CycB
CycB3
PIM
TUB
St. 1-12
St. 14
C
cort/ +
cortRH65/ cortQW55
CycA
CycB
CycB3
PIM
TUB
eggs

completed regardless of whether the oocyte is fertilized. Thus, unfertilized eggs represent a time point just after the completion of meiosis. We examined Cyclin levels in eggs laid by *cort* mutant females, which do not complete meiosis (Fig. 2-2C). As a control we used heterozygous unfertilized eggs. We found that all three mitotic Cyclins as well as PIM are elevated in *cort* mutant eggs, which suggests that APC/C^{CORT} targets all of these substrates for degradation after release of the metaphase I arrest. Complementary results for Cyclin levels have been previously observed (Swan and Schupbach 2007). This degradation may take place at the metaphase I to anaphase I transition, the metaphase II to anaphase II transition, or during both transitions. Cyclin B, at least, is likely degraded at both transitions as expression of a non-degradable form of Cyclin B in the female germline results in both meiosis I and meiosis II arrests (Swan and Schupbach 2007).

The sequential CORT-dependent degradation of Cyclins we observe in *Drosophila* female meiosis is parallel to observations made in mitosis that degradation of Cyclin A begins just after nuclear envelope breakdown in prometaphase while degradation of Cyclin B is initiated at the beginning of metaphase (Whitfield et al. 1990; Clute and Pines 1999; den Elzen and Pines 2001; Geley et al. 2001). Nuclear envelope breakdown occurs in *Drosophila* female meiosis in Stage 13, and just after this stage is when we see an increase of Cyclin A but not Cyclins B or B3 in *cort* mutants compared to heterozygous controls. We see CORT-dependent degradation of all three cyclins in eggs, consistent with degradation of Cyclin B and Cyclin B3 not occurring until the metaphase I to anaphase I transition. To our knowledge, this is the first observation of sequential Cyclin degradation during meiosis in a metazoan.

Developmental regulation of appearance of CORT protein in female meiosis

Given the difference in timing of CORT-dependent degradation of Cyclins, we examined the protein expression pattern of CORT during meiosis to see if differential protein regulation of CORT correlates with differential cyclin degradation. Eggs laid by *cort* mutant mothers arrest in metaphase II and never complete meiosis (Page and Orr-Weaver 1996). This strong arrest phenotype indicates a critical role for *cort* specifically in meiosis. However, *cort* mRNA is present throughout oogenesis and early embryogenesis, suggesting a much broader developmental role (Chu et al. 2001). We determined the timing of CORT protein expression to define better the scope of its activity.

To investigate the developmental control of CORT protein expression, we made a polyclonal antibody against a GST-tagged N-terminal fragment of CORT. Anti-CORT serum recognizes a band of approximately 47 kDa in wild-type oocyte extracts (Fig. 2-3A). To test for antibody specificity, we probed oocyte extracts from $cort^{RH65}$ mutants that contain a *cort* allele with a premature stop codon (Chu et al. 2001). The serum does not recognize a band of the same size in these mutants. We also did not detect an N-terminal fragment in this mutant extract. In addition, we probed extracts from *grauzone* (*grau*) mutant oocytes. *grau* encodes a transcription factor that activates expression of *cort* (Chen et al. 2000). The CORT band of 47 kDa is reduced in *grau* mutants, confirming the specificity of our antibody.

CORT expression is specific to oogenesis, as we detected a CORT band in whole female fly extracts but not in female fly extracts from which the ovaries were removed (Fig. 2-3A). We also did not detect CORT in whole male fly extracts. We performed

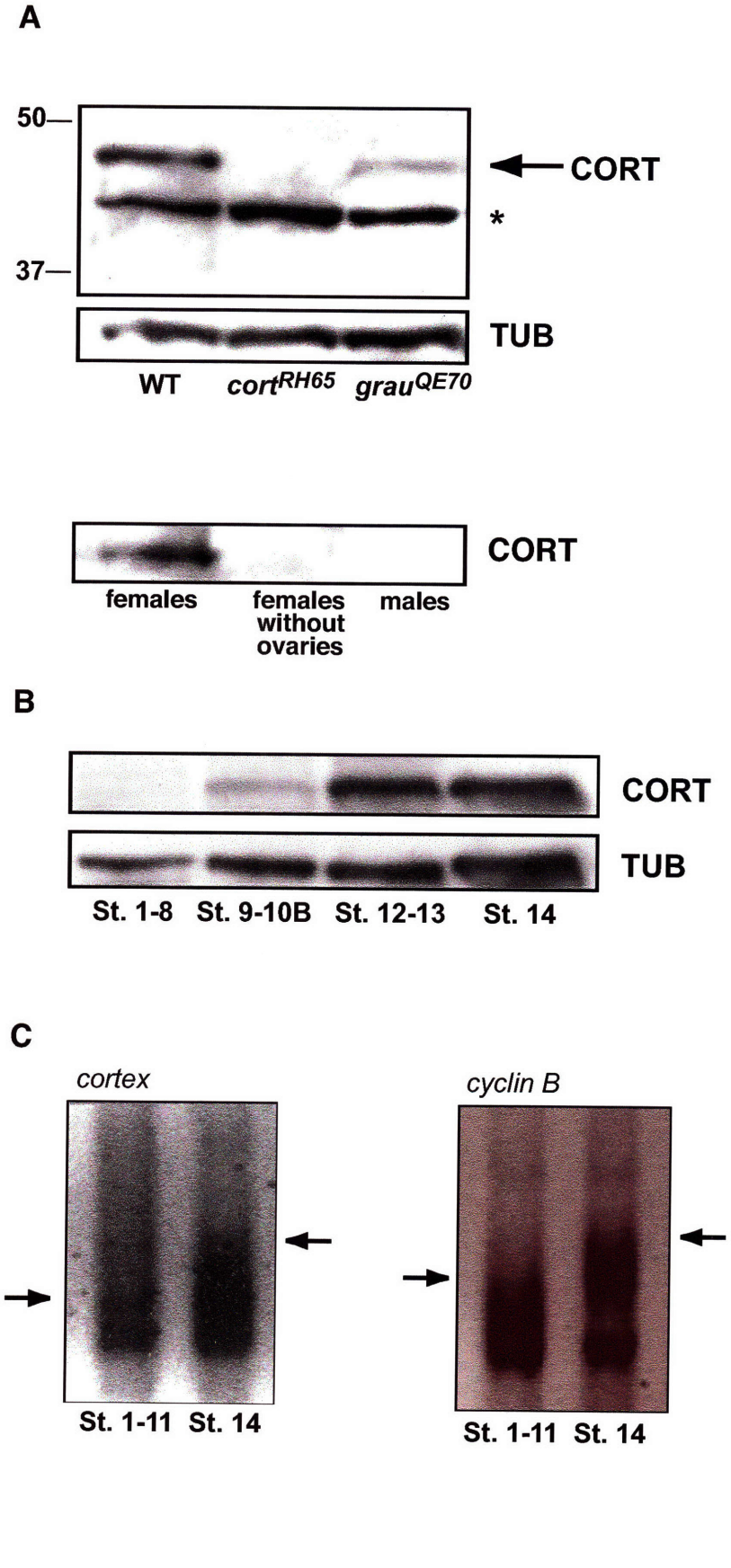
Figure 2-3. The appearance of CORT protein in mature oocytes correlates with polyadenylation of *cort* mRNA.

(A) Top panel: Characterization of CORT antibody. Western blot showing that anti-CORT serum recognizes a band of approximately 47 kDa in wild-type oocyte extracts. The band is absent in $cort^{RH65}$ mutant oocyte extracts and reduced in $grau^{QE70}$ mutant oocyte extracts. The blot was probed with anti-α-Tubulin as a loading control. Asterisk marks a non-specific protein that reacts with anti-CORT serum. Size markers in kilodaltons are shown on left side of blot. Bottom panel: CORT expression is specific to oogenesis. Western blot shows presence of CORT in whole female fly extracts but absence from whole female extracts from which the ovaries were removed or from whole male extracts.

(B) Developmental Western blot of CORT in developing egg chambers. Endogenous CORT levels are low to undetectable in immature ovaries (Stages 1-10B), but strongly increase in mature oocytes (Stages 13-14). Extracts were made from hand-dissected ovaries. By ImageJ analysis, band intensity of CORT in Stages 12-13 is 3.6-fold greater and in Stage 14 is 3.5-fold greater than that of CORT in Stage 9-10B. The blot was probed with anti-α-Tubulin as a loading control.

(C) PAT assays showing elongation of poly(A) tail lengths of *cortex* and *cyclin B* mRNA upon oocyte maturation in wild-type ovaries (transition from immature Stage 1-11 egg chambers to mature Stage 14 oocytes). Arrows indicate the top of each poly(A) smear.

Figure 2-3


A
50
37
CORT
*
TUB
WT
cort^RH65
grau^QE70
CORT
females
females without ovaries
males
B
CORT
TUB
St. 1-8
St. 9-10B
St. 12-13
St. 14
C
cortex
cyclin B
St. 1-11
St. 14
St. 1-11
St. 14

developmental Western analysis on different stages of oogenesis to determine specifically when CORT protein is expressed (Fig. 2-3B). CORT is undetectable in early Stage 1-8 egg chambers, and very low levels are detectable in Stages 9-10B egg chambers. CORT levels increase dramatically in Stage 12-13 egg chambers and remain high in mature Stage 14 oocytes. The appearance of CORT protein occurs at the same time that Cyclin A degradation is triggered (Fig. 2-2A), indicating that APC/C^{CORT} targets Cyclin A as soon as CORT protein is expressed while simultaneously being prevented from targeting Cyclins B and B3 and PIM until after release of the metaphase I arrest.

The timing of appearance of CORT protein correlates with timing of the unmasking of maternal mRNA by cytoplasmic polyadenylation. Many organisms utilize cytoplasmic polyadenylation as a strategy to turn on the translation of specific transcripts at specific developmental timepoints (Tadros and Lipshitz 2005). Elongation of the poly(A) tail of these transcripts is thought to allow for a stable closed-loop conformation of the translational machinery and thus to activate translation. This process occurs during oocyte maturation when oocytes are released from prophase I arrest to reenter the meiotic cell cycle (Kishimoto 2003). In *Drosophila*, oocyte maturation takes place in Stage 13 of oogenesis (King 1970). Given the correlation of the appearance of CORT protein with the timing of oocyte maturation, we investigated the lengths of *cort* poly(A) tails at different developmental timepoints.

We conducted ligase-mediated poly(A) tail (PAT) assays on immature egg chambers and mature Stage 14 oocytes to determine if the poly(A) tail length of *cort* changes upon oocyte maturation (Salles and Strickland 1999). We observed an

elongation of the poly(A) tail in Stage 14 oocytes compared with Stage 1-11 egg chambers (Fig. 2-3C). Poly(A) tails are approximately 20 As in immature stages and elongate to approximately 70 As in mature oocytes. As a positive control, we measured the poly(A) tail length of *cyclin B* in these stages, because *cyclin B* is known to be polyadenylated upon oocyte maturation (Fig. 2-3C) (Benoit et al. 2005). We observed a similar increase in *cyclin B* poly(A) tail lengths as has been previously shown. The appearance of CORT protein in Stage 13 of oogenesis when oocyte maturation occurs together with the elongation of *cort*'s poly(A) tail in mature oocytes suggests that *cort* translation is developmentally regulated by cytoplasmic polyadenylation.

cort mRNA becomes deadenylated in early embryogenesis

If APC/C^{CORT} activity is necessary for meiosis but dispensable for mitosis, Cort may be inactivated in the early embryo to prevent its association with a mitotic APC/C complex. Early *Drosophila* embryos are transcriptionally quiescent, so posttranscriptional control is essential for regulating the activity of maternal gene products. In many organisms, egg activation triggers destabilization of a subset of maternal transcripts (Tadros and Lipshitz 2005). As deadenylation is often the rate-limiting step in mRNA decay, we investigated the polyadenylation status of *cort* mRNA after the completion of meiosis.

We performed PAT assays to measure the poly(A) tail length of *cort* mRNA in mature Stage 14 oocytes and 0-1 hour embryos. We found that *cort* mRNA is deadenylated in early embryos compared to mature oocytes (Fig. 2-4A). The tail decreases from a length of approximately 70 As to 20 As. We used *cyclin B* mRNA as a

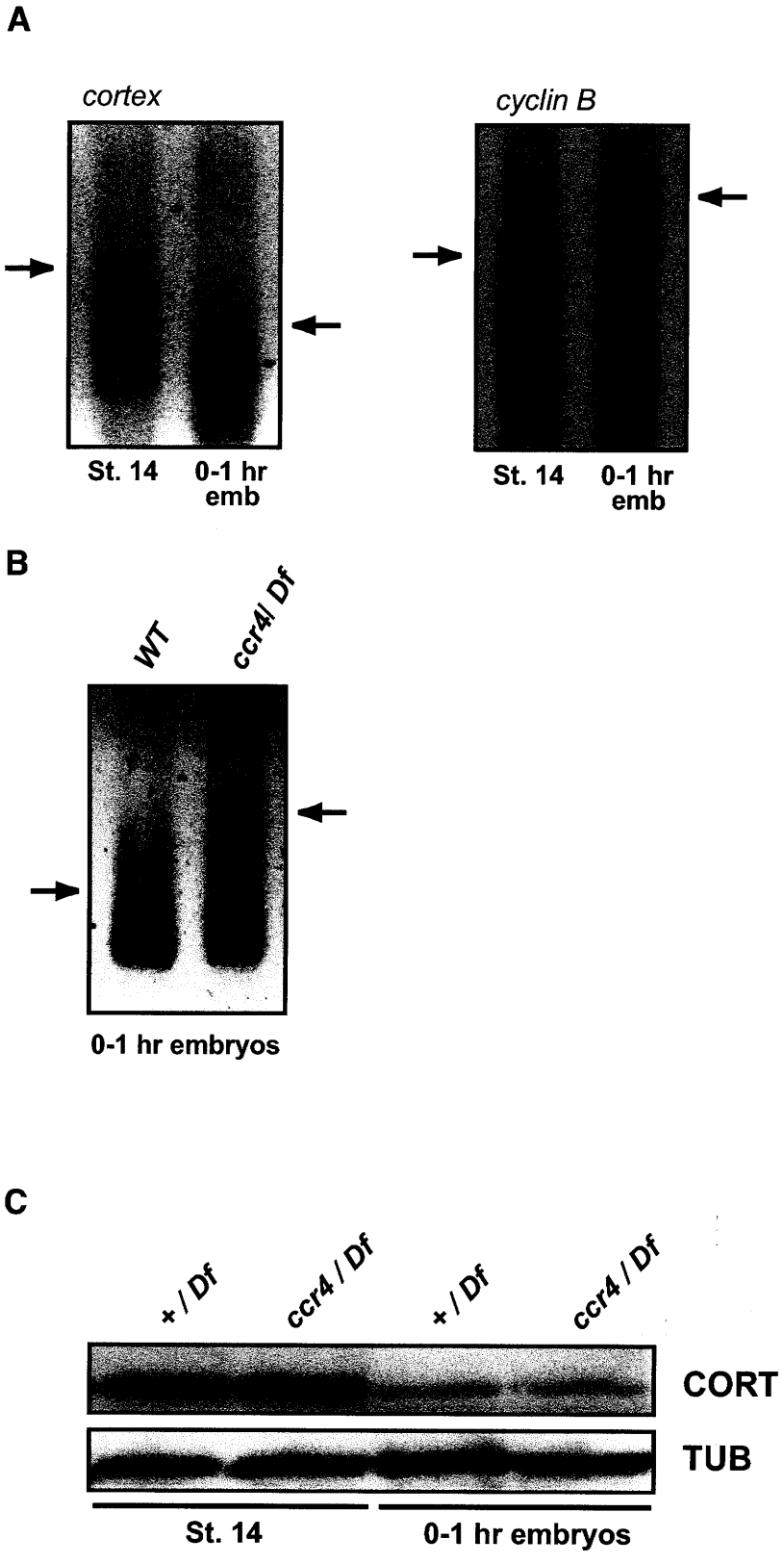
Figure 2-4. *cort* mRNA is deadenylated in early embryos.

(A) PAT assays showing shortening of poly(A) tail length of *cortex* mRNA and elongation of poly(A) tail length of *cyclin B* mRNA in wild-type Stage 14 oocytes and 0-1 hour embryos. Arrows indicate the top of each poly(A) smear.

(B) Deadenylation of *cort* mRNA requires *ccr4*. PAT assay showing elongation of poly(A) tail length of *cortex* mRNA in 0-1 hour embryos from *ccr4/Df* mutant mothers compared to wild-type control embryos. Arrows indicate the top of each poly(A) smear.

(C) *ccr4* is not required to lower CORT protein levels in early embryos. Western blot showing levels of CORT in *ccr4/Df* and +/*Df* sibling control Stage 14 oocytes and 0-1 hour embryos. Blots were probed with anti-α-Tubulin as a loading control.

Figure 2-4


A
cortex
cyclin B
St. 14
0-1 hr emb
St. 14
0-1 hr emb
B
WT
ccr4/ Df
0-1 hr embryos
C
+ / Df
ccr4 / Df
+ / Df
ccr4 / Df
CORT
TUB
St. 14
0-1 hr embryos

positive control that, in contrast, is further polyadenylated upon egg activation (Benoit et al. 2005). CCR4 is the main catalytic subunit of the Ccr4-Pop2-Not deadenylase complex in *Saccharomyces cerevisiae* (Tucker et al. 2002). A CCR4 homolog exists in *Drosophila* and has deadenylase activity (Temme et al. 2004). We measured the poly(A) tail length of *cort* mRNA in embryos from *ccr4* mutant mothers and found that *cort*'s poly(A) tail length is elongated in the mutant (Fig. 2-4B). Thus, deadenylation of *cort* in the early embryo is dependent on the conserved CCR4-NOT deadenylase complex.

To determine whether deadenylation of *cort* mRNA is important for regulating its protein levels, we looked at CORT protein levels in *ccr4* mutants by Western blot (Fig. 2-4C). We found that CORT protein levels are unchanged in both *ccr4/Df* Stage 14 oocytes and 0-1 hour embryos when compared to heterozygous sibling controls. This result suggests that although *ccr4* is required for *cort* deadenylation, it is not required for a subsequent decrease in protein levels. It is likely that deadenylation serves as a backup mechanism to block future synthesis of CORT after fertilization.

CORT protein becomes a target of the APC/C after the completion of meiosis

We performed Western analysis on CORT after the completion of meiosis to determine when CORT protein is expressed in the early embryo (Fig. 2-5A). Surprisingly, we found that CORT protein levels drop dramatically by the time meiosis is completed in unfertilized eggs. We detect CORT at very low levels for up to 1.5 hours of embryogenesis before it disappears. Given the rapid timing of CORT degradation by the end of the meiotic divisions, we hypothesized that CORT itself may be a target of the APC/C.

Figure 2-5. CORT protein becomes a target of the APC/C after the completion of meiosis.

(A) Developmental Western blot showing rapid drop in CORT levels in wild-type unfertilized eggs compared to Stage 14 oocytes. Staged embryo extracts show decreasing levels of CORT as embryogenesis proceeds. Blot was probed with anti-α-Tubulin as a control.

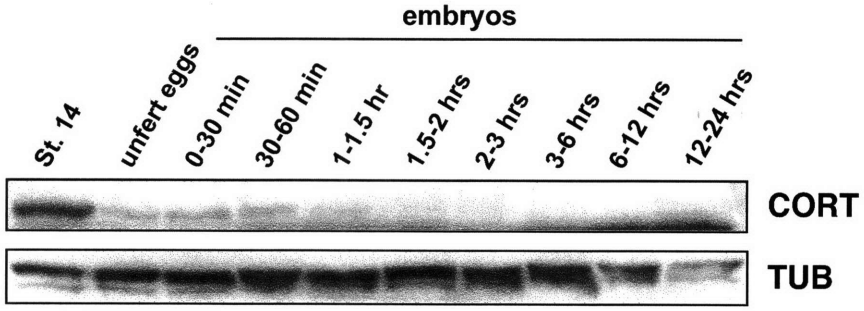
(B) CORT levels increase in *mr* mutants after the completion of meiosis. Western blot showing CORT levels in mr^2/ + and mr^2/ mr^1 ovaries and unfertilized eggs. By ImageJ analysis, band intensity of CORT in mr^2/ + and mr^2/ mr^1 ovaries is approximately identical. Band intensity of CORT in mr^2/ mr^1 eggs is approximately 3.4-fold greater than CORT in mr^2/ + eggs. Blots were probed with anti-α-Tubulin as a loading control.

(C) Putative D-box sequence in C-terminus of CORT (residues 424-432). Conserved residues are underlined.

(D) D-box sequence in CORT is required for its degradation in early embryos. All three conserved residues were mutated to alanine in D-box mutant CORT. 0-30 minute embryos were injected with *myc-cort* wild-type (*WT*) or D-box mutant (*dbox*) RNA or buffer alone (no RNA). Western blot showing levels of wild-type or D-box mutant MYC-CORT in injected embryos. D-box mutant CORT is stabilized compared to wild-type CORT. By ImageJ analysis, band intensity of D-box mutant MYC-CORT is approximately 2.7-fold greater than wild-type MYC-CORT. This blot is representative of 5 independent experiments, which all gave similar results. Blot was probed with anti-α-Tubulin as a control.

Figure 2-5

A



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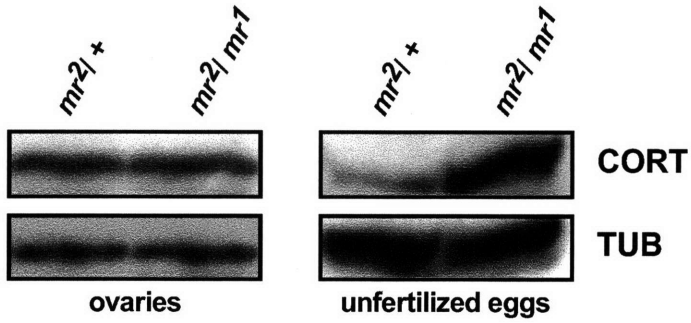
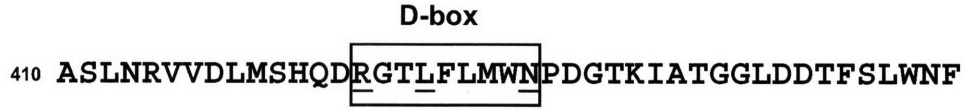
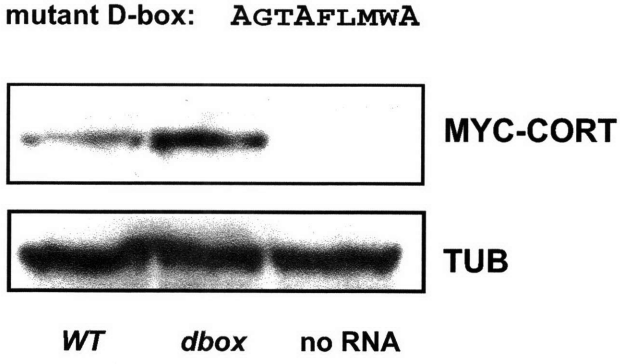


C



D



To test whether the APC/C plays a role in CORT degradation, we looked at CORT levels in *mr*/APC2 mutants (Fig. 2-5B). We found that CORT levels are unaffected in *mr* mutant ovaries, but CORT levels increase strongly in eggs from *mr* mutant females. As a positive control, we probed for Cyclin B in these samples and found that it is also elevated in *mr* mutant eggs (data not shown). These results parallel the timing of changes in CORT protein levels in a wild-type background; CORT levels normally drop by the time that meiosis is completed, and, similarly, the dependence of CORT degradation on *mr* is only apparent in unfertilized eggs, in which meiosis has been completed. These results strongly suggest that CORT is targeted for degradation by the APC/C at some point after the release of the metaphase I arrest and by the time that the meiotic divisions are completed. The specific timing of CORT degradation suggests that it is critical for development of the embryo that CORT protein levels be greatly reduced by the time meiosis is completed.

The APC/C targets proteins for degradation through recognition of specific motifs in its substrates. The two most common motifs are D-boxes (R-X-X-L-X-X-X-X-N) and KEN boxes (K-E-N-X-X-X-E/D/N), although additional motifs have been identified (Glotzer et al. 1991; Pfleger and Kirschner 2000; Thornton and Toczyski 2006). We identified a putative D-box in the C-terminus of CORT (residues 424-432) but no KEN box (Fig. 2-5C).

To determine whether the putative D-box in CORT is functional, we constructed a D-box mutant form and asked whether protein stability is affected in an embryo injection experiment. We mutated all of the conserved residues in CORT's D-box to alanine (Fig.

2-5B) and tagged it with MYC to distinguish the protein from endogenous CORT. We know that a MYC-tagged form of CORT is regulated in a similar way to endogenous CORT, because transgenic MYC-tagged CORT is degraded with similar developmental timing in embryos to endogenous CORT *in vivo* (Supplemental Fig. 1). MYC-tagged D-box mutant *cort* and MYC-tagged wild-type *cort* were transcribed *in vitro*. The RNA was microinjected into 0-30 minute post-deposition wild-type embryos. After incubating the embryos to allow for translation of the RNA and post-translational modifications of the proteins, extracts were made and analyzed by Western blot (Fig. 2-5D). We found that D-box mutant CORT is stabilized compared to wild-type CORT. Thus, the D-box motif in CORT is required for its degradation in early embryos.

CORT is likely to be targeted for degradation by APC/C^{FZY}

Given the dependence of CORT degradation on *mr* and an intact D-box, we wanted to determine which APC/C activator is responsible for CORT's destruction. FZR protein is undetectable in 0-2 hour embryos and, thus, is not a good candidate (Raff et al. 2002). FZY, however, is present in 0-2 hour embryos and is the most likely activator of APC/C-mediated degradation of CORT (Raff et al. 2002).

To test this hypothesis, we first looked at CORT levels in eggs laid by *fzy* mutant females. We were unable to detect an increase of CORT protein in these embryos, but these alleles are hypomorphic and may not show an effect on CORT (data not shown). Next, we looked for genetic interactions between *cort* and *fzy*. If CORT is a substrate of APC/C^{FZY} in the early embryo, we would expect them to antagonize each other in a genetic pathway. Reducing the level of *cort* expression in *fzy* mutants should suppress

the *fzy* phenotype, and increasing the amount of *cort* expression in *fzy* mutants should enhance the *fzy* phenotype.

We carried out these genetic tests using *fzy* female-sterile mutants in which embryos arrest in metaphase after a few mitotic divisions (Dawson et al. 1993). Reducing the gene copy number of *cort* by one in a *fzy* mutant background causes a modest suppression of the *fzy* phenotype. Over 75% of embryos laid by these double mutant females arrest with 3 or more mitotic spindles, whereas only 33% of embryos from *fzy* single mutants develop this far (Fig. 2-6A). Conversely, overexpressing *cort* in the germline of *fzy* mutant females slightly enhances the *fzy* phenotype. In this case, *fzy* embryos containing excess *cort* arrest with fewer mitotic spindles compared to *fzy* alone (Fig. 2-6B). The results of these genetic interaction tests are consistent with CORT being a substrate of APC/C^{FZY}, and suggest that the arrest phenotype of *fzy* embryos is due in part to the presence of excess CORT protein.

DISCUSSION

In this study, we investigated the function of *cort* and its developmental regulation in *Drosophila* female meiosis. We found that CORT protein physically associates with the APC/C *in vivo* and confirmed its function as an APC/C activator. We looked at levels of mitotic APC/C substrates in *cort* mutants and found that Cyclin A is targeted for destruction by APC/C^{CORT} in mature metaphase I arrested oocytes while Cyclin B, Cyclin B3, and PIMPLES/Securin are not targeted until egg activation. Developmental analysis of CORT protein showed that it is translated at oocyte maturation, and appearance of the protein correlates with polyadenylation of *cort* mRNA.

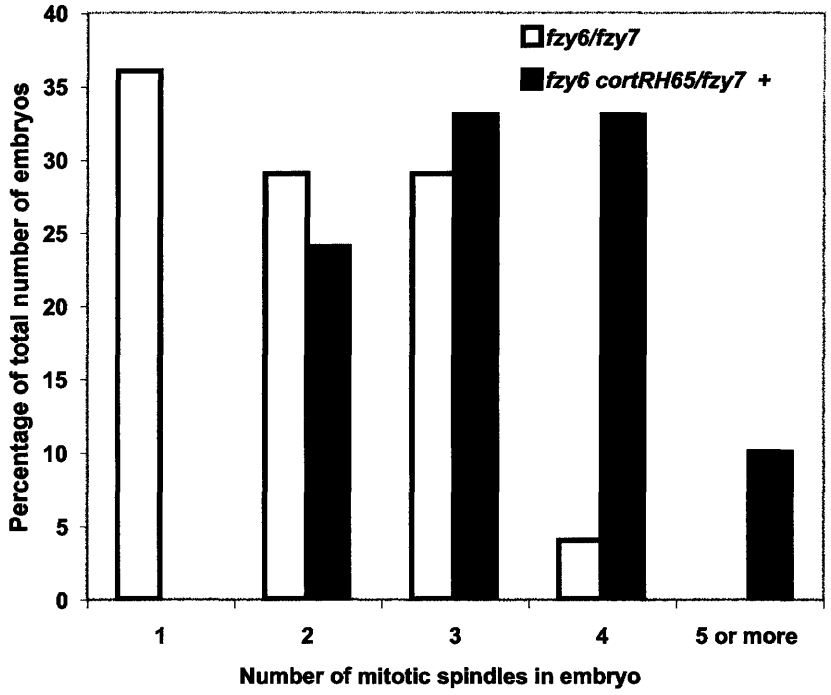
Figure 2-6. *cort* and *fzy* genetically interact in early embryos.

(A) *cort* dominantly suppresses the *fzy* female sterile phenotype. Bar graph showing the percentage of 0-3 hour embryos arresting with 1, 2, 3, 4, and 5 or more mitotic spindles. For fzy^{6}/fzy^{7}, $n = 28$. For $fzy^{6}\ cort^{RH65}/fzy^{7}$ +, $n = 21$.

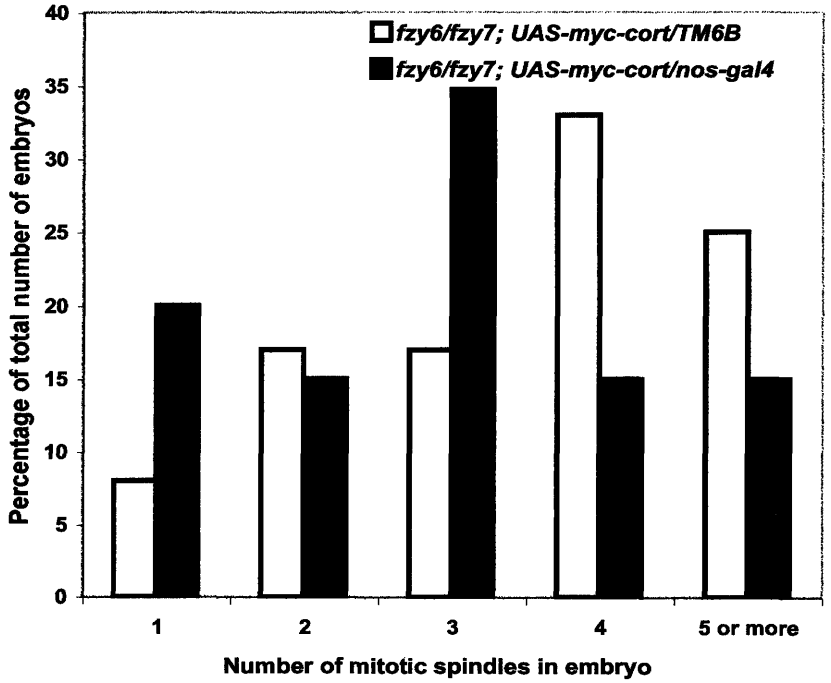
(B) Overexpression of *cort* in the female germline enhances the *fzy* female sterile phenotype. Bar graph showing the percentage of 2-4 hour embryos arresting with 1, 2, 3, 4, and 5 or more mitotic spindles. For fzy^{6}/fzy^{7}; *UAS-myc-cort/ TM6B*, $n = 12$. For fzy^{6}/fzy^{7}; *UAS-myc-cort/ nos-gal4*, $n = 20$.

Figure 2-6

A


fzy6/fzy7
fzy6 cortRH65/fzy7 +
Percentage of total number of embryos
0
5
10
15
20
25
30
35
40
1
2
3
4
5 or more
Number of mitotic spindles in embryo

B


fzy6/fzy7; UAS-myc-cort/TM6B
fzy6/fzy7; UAS-myc-cort/nos-gal4
Percentage of total number of embryos
0
5
10
15
20
25
30
35
40
1
2
3
4
5 or more
Number of mitotic spindles in embryo

Finally, we found that *cort* is regulated post-transcriptionally and post-translationally after the completion of meiosis. *cort* mRNA is deadenylated in early embryos, and CORT protein is degraded after egg activation in an APC/C-dependent manner. CORT contains a conserved D-box motif that is required for the efficiency of its degradation. Our results shed light on the mechanism for the regulation of a meiosis-specific APC/C activator, which results in restriction of its expression to a specific developmental time point.

CORT is an activator of the APC/C

In this study, our demonstration of a physical interaction between CORT and the APC/C strengthens and confirms previous suggestions that *cort* encodes a functional meiosis-specific APC/C activator. A strong metaphase II arrest phenotype in *cort* mutant eggs and distant homology to the Cdc20/ FZY protein family initially suggested that CORT might function as an APC/C activator (Page and Orr-Weaver 1996; Chu et al. 2001). More recently, *cort* was shown to negatively regulate levels of mitotic cyclin proteins, which is consistent with a role for CORT in activating the APC/C (Swan and Schupbach 2007). However, biochemical evidence linking CORT to the APC/C *in vivo* is crucial for this argument. We have shown that CORT physically associates with core subunits of the APC/C in ovaries, strongly supporting CORT's role as an APC/C activator.

Developmental control of CORT levels

Coordination of the meiotic divisions with oogenesis and the transition from meiosis to restart of the mitotic cell cycle in embryogenesis present unique regulatory challenges for the organism. Our studies of *cortex* in *Drosophila* suggest that developmental control of levels of a meiosis-specific APC/C activator is one way in which meiosis is developmentally regulated that has not been previously observed in a multicellular organism. This strategy exploits ongoing regulatory mechanisms occurring during meiosis and embryogenesis: cytoplasmic polyadenylation during oocyte maturation, deadenylation after egg activation, and APC/C-dependent degradation in the early embryo.

Cytoplasmic polyadenylation upon oocyte maturation has been shown to translationally activate maternal transcripts of genes that are required for meiotic entry, transition between meiosis I and meiosis II, and metaphase II arrest in vertebrates (Tadros and Lipshitz 2005). We have shown that *cort* mRNA is polyadenylated at oocyte maturation, which adds an APC/C subunit to this group of transcripts which are translationally unmasked for entry into the meiotic divisions. What is the signal for polyadenylation of *cort*? Masked transcripts contain a *cis*-acting cytoplasmic polyadenylation element (CPE) to which CPE binding protein (CPEB) is bound. Phosphorylation of CPEB upon oocyte maturation triggers elongation of the poly(A) tail and activation of translation (Mendez and Richter 2001). We have not yet identified a CPE in the 3' UTR of *cort*, although CPE sequences are quite variable. In addition, we have tested but have not observed a dependence of *cort* polyadenylation on *orb*, the CPEB homolog in *Drosophila* (data not shown). Because the *orb* alleles we used are

hypomorphic, we cannot fully rule out the possibility that polyadenylation of *cort* is *orb*/CPEB-dependent.

Egg activation triggers maternal transcript destabilization in several organisms, some of which occurs through *ccr4*-dependent deadenylation, and this is likely to be important for localization of maternal transcripts in the embryo and proper zygotic development (Tadros and Lipshitz 2005). We showed in this study that *cort* mRNA is deadenylated in the early embryo in a *ccr4*-dependent manner, but this deadenylation is not required for lowering CORT protein levels. However, we may not be able to detect a difference in protein levels due to the rapid APC/C-dependent degradation of CORT protein that occurs after release of the metaphase I arrest. Deadenylation could serve as a back-up mechanism to ensure that CORT protein levels remain low in the early embryo by destabilizing *cort* mRNA.

The APC/C drives degradation of Cyclin B and other substrates during the rapid syncytial mitotic divisions of early embryogenesis in *Drosophila* (Dawson et al. 1993; Dawson et al. 1995; Huang and Raff 1999; Raff et al. 2002). We found that CORT is targeted for APC/C-dependent degradation by the completion of meiosis in the early embryo. The targeting of an APC/C activator for degradation by another form of APC/C is not unprecedented, as APC/C^{Cdh1} targets Cdc20 for degradation in G1 (Prinz et al. 1998; Shirayama et al. 1998; Huang et al. 2001).

Our data support the conclusion that CORT is targeted by APC/C^{FZY}. First, FZY is thought to be the only activator present in early embryos [68]. Second, we show here that *cort* and *fzy* interact genetically in a way that is consistent with *cort* being a negative downstream target of *fzy* in embryos. Third, in our embryo injection experiments, we

showed that exogenous MYC-CORT is degraded in a D-box-dependent manner in injected embryos. Because the only APC/C activator in early embryos is FZY, degradation of MYC-CORT is likely to occur through APC/C^{FZY} in this assay.

It is also possible the APC/C^{CORT} regulates itself in a negative feedback loop by targeting CORT for degradation when levels of CORT reach a certain threshold at the end of meiosis. To address this possibility, we looked at the degradation of CORT in a homozygous $cort^{QW55}$ background in which there is no functional CORT protein. $CORT^{QW55}$ mutant protein is not degraded at the transition from mature Stage 14 oocytes to unfertilized eggs, unlike in a heterozygous control background (Supplemental Fig. 2). These results suggest that CORT could be targeted by itself, but it remains a possibility that the lesion in the $cort^{QW55}$ allele prevents an interaction between $CORT^{QW55}$ mutant protein and the APC/C machinery. The lesion does not disrupt the D-box, but it could affect proper folding and structure of the protein. In summary, we conclude that CORT is targeted for degradation by the APC/C. It is most likely that FZY is the participating APC/C activator, but CORT may also contribute to targeting itself for degradation.

Role of meiosis-specific activators in Drosophila

Recent work has shown that both *cort* and *fzy* are required for the meiotic divisions in *Drosophila* female meiosis. Mutant analysis suggests that *cort* and *fzy* act redundantly to control the metaphase I to anaphase I transition, whereas they seem to act with different temporal and spatial specificity in targeting Cyclin B for destruction along the meiosis II spindles (Swan and Schupbach 2007). We showed in this study that *cort* cannot functionally substitute for *fzy* in the early embryo, suggesting that they target non-

redundant sets of substrates. Furthermore, homozygous *cort* mutants alone exhibit a strong metaphase II arrest, indicating that the wild-type levels of *fzy* in this background are not able to act in place of *cort* to control passage through metaphase II (Page and Orr-Weaver 1996). Finally, we have observed that FZY is expressed at a uniform level during oogenesis and embryogenesis (data not shown and Supplemental Fig. 3), which is in contrast to our results in this study showing that CORT expression is specifically upregulated during the meiotic divisions. Based on all of these observations, we think it is likely that in addition to the mitotic cyclins, APC/C^{CORT} targets a unique set of substrates in meiosis that are not recognized by APC/C^{FZY}. The identification of these meiotic substrates will be crucial for understanding how the meiotic divisions are controlled in the oocyte.

The study of meiotic control of the APC/C is especially intriguing in *Drosophila* because in addition to *cort*, a female meiosis-specific activator, the genome contains *fizzy-related 2* (*fzr2*), another member of the Cdc20/FZY family. *fzr2* is expressed exclusively in testes and may act as a male meiosis-specific activator (Jacobs et al. 2002). Further study of both *cort* and *fzr2* will be important for understanding differential developmental regulation of the APC/C during meiosis in females versus males.

Sequential degradation of cyclins in meiosis

In mitosis, cyclins are targeted sequentially for destruction by the APC/C. Degradation of Cyclin A begins just after nuclear envelope breakdown in prometaphase, while degradation of Cyclin B does not occur until the metaphase to anaphase transition (Whitfield et al. 1990; Clute and Pines 1999; den Elzen and Pines 2001; Geley et al.

2001). Sequential degradation of Cyclin A, Cyclin B, and, finally, Cyclin B3 in *Drosophila* triggers a series of distinct events leading to exit from mitosis (Sigrist et al. 1995; Parry and O'Farrell 2001). We have found that a similar situation exists in *Drosophila* female meiosis, in which degradation of Cyclin A by APC/C^{CORT} initiates upon nuclear envelope breakdown, but degradation of Cyclin B and Cyclin B3 does not occur until after the metaphase I to anaphase I transition.

The difference in timing of Cyclin A and Cyclin B degradation in mitosis is due to regulation of the APC/C by the spindle assembly checkpoint. The spindle assembly checkpoint inhibits APC/C^{Cdc20} from initiating anaphase until all chromosomes are bi-oriented on the spindle, in part through direct binding of Cdc20 to Mad2 and BubR1 (Musacchio and Salmon 2007). Spindle assembly checkpoint proteins specifically inhibit APC/C-dependent ubiquitination of Cyclin B but not of Cyclin A (Clute and Pines 1999; den Elzen and Pines 2001; Geley et al. 2001). APC/C^{CORT} may be regulated in a similar manner during meiosis I. Indeed, the spindle assembly checkpoint is likely to function during meiosis I in *Drosophila*, as the conserved spindle checkpoint kinase Mps1 is required for delaying entry into anaphase I to allow for proper segregation of achiasmate homologs and maintenance of chiasmate homolog connections in *Drosophila* oocytes (Gilliland et al. 2005; Gilliland et al. 2007). Furthermore, a functional Mad2-dependent checkpoint exists during meiosis I in mouse oocytes, and spindle checkpoint components have been shown to regulate the APC/C during meiosis I in *C. elegans* (Wassmann et al. 2003; Homer et al. 2005; Stein et al. 2007).

To determine whether APC/C^{CORT} is regulated by the spindle checkpoint, we asked if BubR1 or Mad2 physically associate with CORT in Stage 14-enriched ovaries.

We were unable to detect an association with BubR1 or Mad2 (data not shown). Although this negative result does not rule out the possibility of regulation of APC/C^{CORT} by the spindle checkpoint, it suggests that APC/C^{CORT} may be subject to other types of regulation that inhibit it from targeting Cyclin B and Cyclin B3 for degradation until after the metaphase I arrest.

In conclusion, through the investigation of *cortex*, a meiosis-specific APC/C activator, we have found one way in which the meiotic cell cycle may be developmentally controlled during oogenesis. *cort* is developmentally regulated by existing post-transcriptional and post-translational mechanisms, resulting in expression of CORT protein being restricted to the meiotic divisions. Further study of APC/C^{CORT} will continue to elucidate the ways in which developmental control of the APC/C contributes to proper female meiosis in a metazoan.

MATERIALS AND METHODS

Fly stocks

Crosses were performed, and flies were maintained between 22°C and 25°C using standard techniques (Greenspan 1997). The wild-type stocks used were *Oregon R* and *yw*. The $cort^{RH65}$ and $cort^{QW55}$ alleles have been described (Schupbach and Wieschaus 1989; Page and Orr-Weaver 1996; Chu et al. 2001). To obtain *ccr4* mutant flies, $ccr4^{KG877}$, a *ccr4* allele generated by the Berkeley *Drosophila* Genome Project, was placed in trans to *Df(3R)crb-F89-4*, a large deficiency that deletes the *ccr4* locus (Temme et al. 2004). Female-sterile alleles of *morula*, mr^1 and mr^2, were originally isolated from

natural populations and have been described (Lindsley and Zimm 1992; Reed and Orr-Weaver 1997; Kashevsky et al. 2002). Female sterile alleles of *fizzy*, fzy^6 and fzy^7, have been described (Dawson et al. 1993). UASp *myc-cort* was made by PCR amplification of *cort* cDNA (LD43270) and subcloning into pUASp with a 6xMYC tag at the N-terminus. The LD43270 clone is missing coding sequence for nine amino acids on the 5' end that we added during PCR amplification. Expression of *6xmyc-cort* was driven in the female germline with the *nanos-Gal4-VP16* driver (Rorth 1998). The *plu-myc* transgenic line has been described (Fenger et al. 2000). To generate unfertilized eggs, we crossed virgin females to sterile males, which do not produce sperm but are able to stimulate females to lay eggs. The sterile males are from strain $T(Y;2)\#11cn\ bw^D\ mr^2/b\ cn\ mr^1\ bs^2/\ SM6A$, a gift from B. Reed.

Immunoprecipitations

To prepare ovary extracts for immunoprecipitations, whole ovaries were dissected in Grace's insect medium (Gibco) from 32 females fattened 3 days on wet yeast at 25°C. Ovaries were homogenized in 3x volume homogenization buffer (25 mM HEPES, pH 7.5, 0.4 M NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM PMSF, 10% glycerol, complete mini EDTA-free protease inhibitors, 1 tablet/ 10 ml (Roche)), snap frozen in liquid nitrogen, and stored at -80°C. 30 µl Protein A Sepharose beads (Amersham) were pre-coupled to antibodies for 1 hr at 4°C. For pre-coupling, antibodies were as follows, 2 µl mouse IgG (Sigma I5381), 12 µl mouse monoclonal anti-myc, 9E10 (Covance), 2 µl rabbit IgG (Sigma I5006), or 10 µl affinity-purified rabbit anti-Cdc27 (Huang and Raff 1999). After removing an aliquot for input, 70 µl ovary extract was added to antibody-

bound beads and incubated for 2-4 hrs at 4°C. Beads were washed once in ice-cold IP buffer (25 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM EGTA, 0.1% Triton X-100, 10% glycerol, complete mini EDTA-free protease inhibitors, 1 tablet/ 10 ml (Roche)), once in IP buffer plus 0.5 M NaCl, and 4 times in IP buffer. Inputs, immunocomplexes, and supernatants were resolved by SDS-PAGE and analyzed by immunoblot as described below.

Polyclonal antibodies against CORT

A fusion between GST and 152 amino acids from the N-terminus of CORT was used to produce antibodies in guinea pigs. The construct encoding GST-CORT_N was made by PCR amplification of *cort* cDNA (LD43270) as described above, followed by subcloning into pGEX-4T-1 expression vector (Pharmacia). GST-CORT_N was expressed in TOP10 *E. coli* cells by IPTG induction. The majority of GST-CORT_N was insoluble so it was gel-purified from the insoluble material after cell lysis. Crude lysate was clarified, and the insoluble pellet resuspended in 5x Sample Buffer (60 mM 1 M Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol, 0.1% bromophenol blue). Sample was resolved by SDS-PAGE on a preparative 10% 150:1 (30% acrylamide/ 2% bis-acrylamide) gel. Vertical strips from either side of the gel were stained with GelCode Blue (Pierce) and used as a guide to cut out the unstained band of GST-CORT_N. Gel slice was pulverized with cold 1X SDS Electrophoresis Buffer (25 mM Tris base, 192 mM glycine, 0.1% SDS) through a 10 ml syringe and gently rocked for 30 minutes at 4° to elute protein. Gel slice mixture was filtered through 125 μm nylon mesh (Tetko), and the eluate concentrated in Amicon Centricon YM-10

(Millipore). Concentrated protein was injected into guinea pigs for antibody production (Covance). The anti-CORT antibody recognizes a band of approximately 47 kDa that is the CORT protein.

Protein Extracts and Immunoblotting

Protein extracts were made by homogenizing staged egg chambers, whole ovaries, unfertilized eggs, or embryos in 3:1 Urea Sample Buffer (8 M urea, 2% SDS, 100 mM Tris, pH 7.5, 5% Ficoll)/embryo (vol./vol.). Unfertilized eggs were collected for 0-2 hours. Whole fly extracts were made by homogenizing flies directly in 5x Sample Buffer. Protein extracts were resolved by SDS-PAGE and transferred to Immobilon-P membranes (Millipore). 10.5-14% acrylamide gels were used for immunoprecipitations (Fig. 2-1) and substrate blots (Fig. 2-2). 10% acrylamide gels were used for all CORT blots and RNA injection assays (Fig. 2-3 and 2-5). Equal amounts of protein were loaded per lane and confirmed by anti-α-Tubulin blotting. Blots were probed with the following antibodies: mouse monoclonal anti-MYC, 9E10 (1:1000, Covance), affinity-purified rabbit anti-Cdc27 (1:500 (Huang and Raff 1999)), affinity-purified rabbit anti-MR (1:200 (van Roessel et al. 2004)), guinea pig anti-CORT serum (1:2000), rat monoclonal anti-α-tubulin, YL1/2 and YOL1/34 (1:200, Harlan Sera-lab), mouse monoclonal anti-Cyclin A, A19 (1:50, gift of Pat O'Farrell), mouse monoclonal anti-Cyclin B, F2F4 (1:200 (Lehner and O'Farrell 1990)), rabbit anti-Cyclin B3 serum (1:4000 (Sigrist et al. 1995)), rabbit anti-PIM serum (1:10,000 (Stratmann and Lehner 1996)), and affinity-purified rabbit anti-FZY (1:1000 (Raff et al. 2002)). Alkaline phosphatase- or horseradish peroxidase-

conjugated secondary antibodies were used to detect bound primary antibodies. Protein was detected using ECL Plus (Amersham).

PAT Assays

Ovary or embryo mRNA was isolated using the PolyATtract System 1000 (Promega). LM-PAT assays were performed using 100 ng mRNA as described (Salles and Strickland 1999). cDNA was made using the Reverse Transcription System (Promega). PCRs were performed with message-specific primers, and a fraction of the PCR product was tested on a gel to permit approximately equal loading of the PCR product for the experiment. PCR products were separated on a 2% MetaPhore agarose gel and stained with ethidium bromide.

Mutagenesis and RNA Injection Assays

Point mutations were introduced into *cort* cDNA using the Phusion Site-Directed Mutagenesis Kit (Finnzymes). Wild-type or mutated D-box *cort* cDNA was subcloned into pCS2 containing a 6xMYC tag at the N-terminus. Capped mRNAs were synthesized from these vectors using the SP6 mMessage mMachine Kit (Ambion). mRNA was purified using the MEGAclear Kit (Ambion). 0-30 minute *yw* embryos were dechorionated and prepared for injection. Samples were prepared containing 250 ng/μl wild-type or mutant *cort* RNA in injection buffer (5 mM KCl, 0.1 M K_2HPO_4, pH 7.8). A no RNA control contained injection buffer alone. Each sample was injected into 150 embryos. After 40 minutes at room temperature, the embryos were harvested in heptane, washed 2 times in embryo wash (0.4% NaCl, 0.03% Triton X-100), and homogenized in

20 µl USB. Extracts were resolved by SDS-PAGE and analyzed by immunoblotting as described above. The experiment was repeated 5 independent times to confirm results.

Egg Fixation, Staining, and Microscopy

Eggs were collected for 0-3 hours for Fig. 2-6A and for 2-4 hours for Fig. 2-6B, dechorionated in 50% bleach, devitellinized in methanol and heptane, and fixed in methanol for 3 hours at room temperature or overnight at 4°C. Eggs were stained for DNA with Propidium Iodide and for Tubulin with rat monoclonal anti-α-Tubulin, YL 1/2 and YOL 1/34 (1:20, Harlan Sera-lab). Antibodies were detected using fluorescent secondary antibodies (Jackson Immunoresearch). Imaging was performed using a Zeiss Axioskop.

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Supplemental Table. *Rescue of cort mutants by tagged transgenes*

Genotype	Percentage of mitotically dividing embryos (*n*)
$cort^{RH65}$/ $cort^{RH65}$; *UAS-myc-cort*^{3C}/ *TM6B*	0 (34)
$cort^{RH65}$/ $cort^{RH65}$; *UAS-myc-cort*^{3C}/ *nos-gal4*	64 (44)
$cort^{RH65}$/ $cort^{RH65}$; *UAS-myc-cort*^{3A}/ *nos-gal4*	26 (211)

Females of the indicated genotypes were fattened on wet yeast for 2 days before embryo collection. 2-4 hour embryos were dechorionated, methanol fixed, and stained with DAPI. Two independent transgenic lines are shown.

Figure S1. MYC-CORT is degraded in early embryos.

MYC-CORT expression was driven in the female germline with *nos-gal4* in a WT background. Western blot showing levels of MYC-CORT in Stage 14 oocytes, unfertilized eggs, and developing embryos. MYC-CORT was detected by probing with anti-MYC. Control sibling females contain *nos-gal4* but not *UAS-myc-cort*. Blot was probed with anti-α-Tubulin as a loading control.

Figure S2. $CORT^{QW55}$ mutant protein is not degraded at egg after egg activation.

Western blot showing levels of CORT in St. 14 oocytes and unfertilized eggs in $cort^{QW55}$/ *CyO* and $cort^{QW55}$ backgrounds. CORT is degraded at this transition in the heterozygous background but not in the homozygous mutant background. Blot was probed with anti-α-Tubulin as a loading control.

Figure S3. FZY is expressed at uniform levels during embryogenesis

Developmental Western blot showing levels of FZY in Stage 14 oocytes, unfertilized eggs, and developing embryos. Blot was probed with anti-α-Tubulin as a loading control.

Figure S1

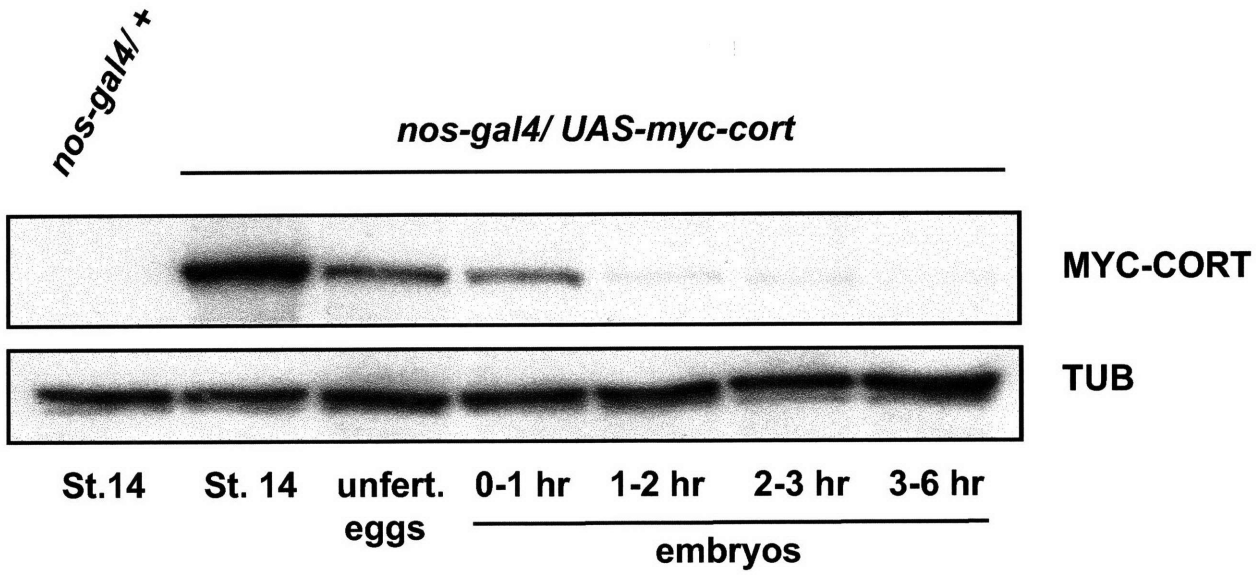

nos-gal4/ +
nos-gal4/ UAS-myc-cort
MYC-CORT
TUB
St.14
St. 14
unfert. eggs
0-1 hr
1-2 hr
2-3 hr
3-6 hr
embryos

Figure S2

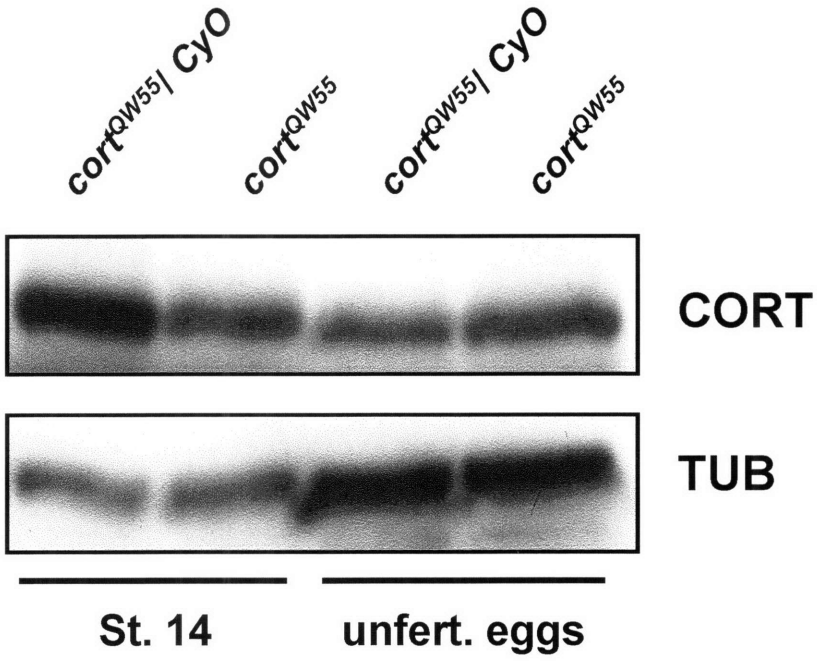
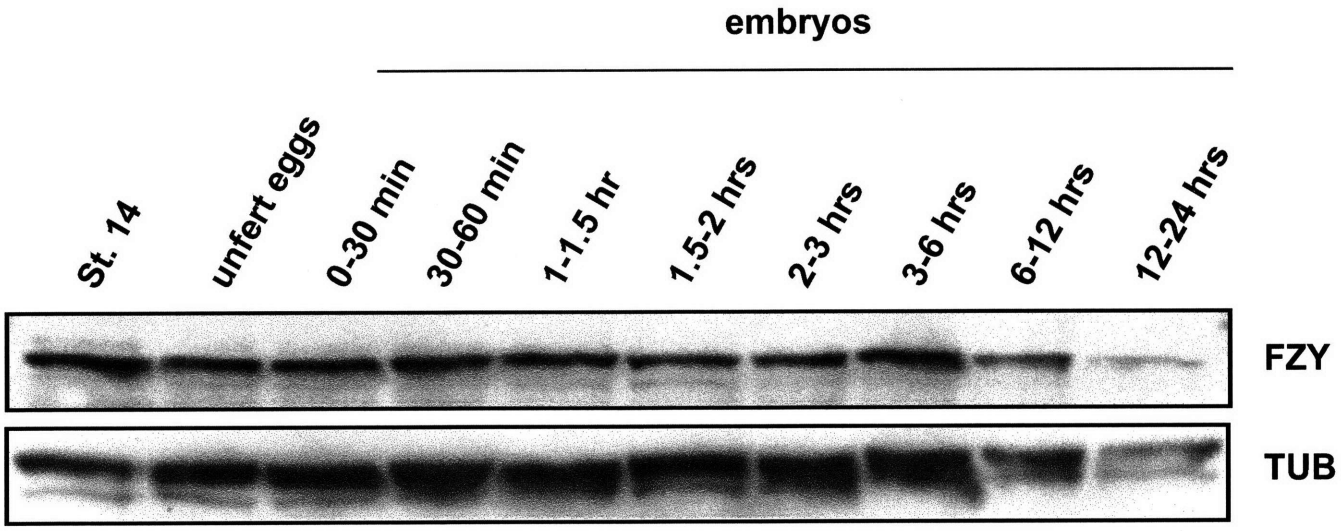

cort^QW55| CyO
cort^QW55
cort^QW55| CyO
cort^QW55
CORT
TUB
St. 14
unfert. eggs

Figure S3


embryos
St. 14
unfert eggs
0-30 min
30-60 min
1-1.5 hr
1.5-2 hrs
2-3 hrs
3-6 hrs
6-12 hrs
12-24 hrs
FZY
TUB

Chapter Three

A Genetic Screen for Substrates of the Anaphase-Promoting Complex in Meiosis

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* J.P. conducted genetic interaction tests, phenotypic analysis, and quantification of *cort*; *cycB3*/+ and *cort pim*/*cort* + double mutants, performed MEI-S332 immunofluorescence in *cort*; *cycB3*/+ embryos, constructed the dominantly marked starting strain for the deficiency screen, screened 15 out of 58 completed deficiencies, quantified the phenotypes in the original suppression test with *Df(3R)p-XT103*, quantified the phenotypes in the large-scale suppression tests with the three Exelixis deficiencies, and performed the BEL Western blot.

ABSTRACT

APC/C-mediated proteolysis is crucial for cell cycle transitions during mitosis. Many mitotic APC/C substrates have been studied, but little is known about APC/C substrates in meiosis. Drosophila utilizes a female meiosis-specific APC/C activator, *cortex*, which may target a unique set of APC/C substrates. We tested candidate APC/C^{CORT} substrates for their ability to genetically suppress *cort*. Neither *pimples* nor *cycB3* suppressed *cort*, although reducing the gene copy number of *cycB3* caused an increase in microspindles containing DNA with localized MEI-S332. We initiated a dominant suppressor screen to identify substrates of APC/C^{CORT} using mutants in *grauzone*, a gene encoding a transcriptional activator of *cort*, to reduce but not eliminate the levels of CORT protein. We have currently screened 45% of the third chromosome deficiency kit and identified at least one deficiency, *Df(3R)p-XT103*, that suppresses *grau*. Protein levels of BEL, one candidate gene within *Df(3R)p-XT103*, are elevated in *cort* mutants, consistent with its identity as an APC/C^{CORT} substrate. We hope to better understand the role of the APC/C in meiosis by identifying meiosis-specific APC/C substrates in this screen.

INTRODUCTION

Regulated proteolysis of specific proteins is crucial for proper cell cycle transitions. The ubiquitin-proteasome system controls this degradation through the transfer of ubiquitin to substrate proteins, tagging them with multi-ubiquitin chains. The 26S proteasome recognizes these chains and degrades the protein (for review, see Pickart 2001). The Anaphase-Promoting Complex (APC/C) is a large multisubunit E3 ubiquitin ligase that catalyzes the addition of ubiquitin chains onto substrates in mitosis and G1 (for review, see Peters 2002). Substrate specificity of the APC/C is conferred by its association with two main activator proteins, Cdc20/Fizzy (Fzy) and Cdh1/Fizzy-related (Fzr), which are not only required for robust E3 ligase activity, but also for the targeting of two subsets of substrates with minimal overlap. Generally, Cdc20 activates the APC/C in prometaphase and at the metaphase-anaphase transition, while Cdh1 becomes active in anaphase and G1 (Dawson et al. 1995; Schwab et al. 1997; Visintin et al. 1997; Sigrist and Lehner 1997).

The temporally regulated degradation of various substrates involved in different aspects of cell division controls each transition in mitosis. In prometaphase, degradation of Cyclin A, a mitotic cyclin, is required for subsequent sister chromatid separation in anaphase (Parry and O'Farrell 2001). Occurring at the same time, degradation of Nek2A, a centrosomal kinase, may be required for anaphase onset and the ability of an intercentriolar linkage to be re-established late in mitosis (Hames et al. 2001; Hayes et al. 2006). Sister chromatid separation and anaphase onset is strictly controlled by APC/C-mediated degradation of Securin, an inhibitor of Separase, and Cyclin B, a mitotic cyclin that directly inhibits Separase (Cohen-Fix et al. 1996; Stemmann et al. 2001).

The APC/C targets proteins involved in spindle dynamics as well as mitotic regulators for anaphase progression and mitotic exit. Xkid, a chromokinesin, kinesins Kip1 and Cin8, and spindle-associated protein Ase1 are all degraded in anaphase to allow for chromosome movements toward the spindle poles and proper spindle disassembly (Juang et al. 1997; Funabiki and Murray 2000; Gordon and Roof 2001; Hildebrandt and Hoyt 2001). Cyclin B continues to be degraded through anaphase and G1 to inhibit mitotic activities and, as a result, promote events associated with mitotic exit such as chromosome decondensation, spindle disassembly, and cytokinesis (Murray and Kirschner 1989; Luca et al. 1991; Gallant and Nigg 1992; Holloway et al. 1993; Surana et al. 1993; Clute and Pines 1999). Finally, Plk1/Polo kinase, a protein with multiple roles in mitosis, must be degraded for mitotic exit to occur, and degradation of Cdc20 allows the switch from APC/C^{Cdc20} to APC/C^{Cdh1} in G1 (Charles et al. 1998; Prinz et al. 1998; Shirayama et al. 1998). In addition, APC/C^{Cdh1} mediates the degradation of replication proteins Geminin, Orc1, and Cdc6 in G1 to prevent inappropriate DNA replication prior to S-phase (McGarry and Kirschner 1998; Petersen et al. 2000; Araki et al. 2003).

As evidenced by the above examples, our understanding of the function of degradation of specific APC/C substrates in mitosis is quite extensive. In contrast, very little is known about specific APC/C substrates in meiosis and how their degradation promotes meiotic cell cycle transitions. APC/C-mediated degradation of cyclins and securin is known to occur in meiosis, and in some organisms has been shown to be required for both meiotic divisions (Salah and Nasmyth 2000; Homer et al. 2005; Oelschlaegel et al. 2005; Swan and Schupbach 2007). Spo13, a meiotic regulator that

promotes monopolar chromosome attachment and protection of centromeric cohesion in *S. cerevisiae*, is the only meiosis-specific APC/C substrate identified thus far, although the degradation of Spo13 is not essential for meiotic progression or spore viability (Katis et al. 2004; Lee et al. 2004; Sullivan and Morgan 2007).

Meiosis-specific APC/C activators, which have been identified in yeast and Drosophila, may provide an opportunity to identify meiosis-specific APC/C substrates. An appealing hypothesis is that these activators serve to target unique proteins in meiosis that Cdc20 and Cdh1 are unable to recognize. In particular, meiosis-specific activators in Drosophila may contribute to our understanding of the role of the APC/C in the developmentally regulated meiotic cell cycle of a multicellular organism.

cortex (*cort*) encodes a distant member of the Cdc20/FZY protein family in Drosophila that is expressed exclusively during oogenesis (Chu et al. 2001). *cort* mutant females lay eggs that do not complete meiosis but arrest terminally in metaphase II (Lieberfarb et al. 1996; Page and Orr-Weaver 1996). CORT physically associates with core APC/C subunits Cdc27 and Cdc16 in oogenesis, strongly suggesting that CORT functions as an activator of the APC/C (Pesin and Orr-Weaver, submitted). Furthermore, mitotic Cyclins and PIMPLES, the securin homolog in Drosophila, are likely to be substrates of APC/C^{CORT} because levels of these proteins are elevated in *cort* mutants (Swan et al. 2005; Swan and Schupbach 2007; Pesin and Orr-Weaver, submitted). It is not known whether failure to degrade these proteins is the cause of the metaphase II arrest in *cort* mutants. No other substrates of APC/C^{CORT} have been identified.

We tested candidate APC/C^{CORT} substrates for their ability to genetically suppress *cort*. Because this approach was not successful, we are screening a collection of

deficiencies for dominant suppressors of *grauzone*, a transcriptional activator of *cort*, in order to identify meiosis-specific substrates of APC/C^{CORT}.

RESULTS

cortex mutants are not suppressed by candidate APC/C^{CORT} substrates

We predicted that *cort* mutants would be suppressed by reducing the copy number of putative substrate genes. Presumably, the metaphase II arrest in *cort* mutants is the result of an accumulation of APC/C^{CORT} substrate proteins in *cort* eggs. Lowering the gene copy number of a substrate in a *cort* background may reduce the amount of substrate protein present in the egg and allow progression through metaphase II, and possibly, into embryonic mitotic divisions. We decided to use this reasoning as the basis for a genetic screen to identify APC/C^{CORT} substrates.

Before beginning the screen, we tested two candidate genes that we expected to be critical substrates of APC/C^{CORT} to confirm that the screen would work. Cyclin B and Securin are critical substrates of the APC/C in mitosis, as their degradation is required for anaphase onset and mitotic exit (Cohen-Fix et al. 1996; Shirayama et al. 1999; Stemmann et al. 2001; Wasch et al. 2002). Although *pimples* (*pim*), the homolog of Securin in Drosophila, has not been shown to be required for meiotic divisions, we have shown that PIM protein is elevated in *cort* mutant eggs (Pesin and Orr-Weaver, submitted). In addition, levels of Cyclin B3, a mitotic cyclin required for female fertility, are elevated in *cort* mutants (Jacobs et al. 1998; Pesin and Orr-Weaver, submitted).

In order to test for dominant suppression of *cort* by these putative substrates, we generated double mutant females homozygous for *cort* and heterozygous for *cycB3* or

pim. We collected 0-30 minute eggs from these females, stained the DNA with DAPI, and analyzed the phenotypes by fluorescence microscopy (Table 3-1). Although neither double mutant produced eggs that completed meiosis or initiated mitotic divisions, we found that *cort*; *cycB3*/+ eggs are more frequently arrested with anaphase II spindles, as opposed to metaphase II spindles, and are more likely to arrest with 1 or 2 major spindles plus 1 or more microspindles, tiny spindles containing single chromosomes or DNA fragments, when compared with a *cort* single mutant control (Figure 3-1A). *cort pim*/ *cort* + double mutants eggs showed a slight increase in the presence of microspindles, but generally showed a similar frequency of phenotypes to the control, and thus, did not suppress *cort*.

The phenotypic classes we observed for *cort* mutants are consistent with what had been seen previously (Page and Orr-Weaver 1996). Both *cort* single mutant and double mutant eggs arrested with a small frequency in metaphase I, suggesting a requirement for *cort* in progression through meiosis I. Aberrant chromosome morphology on metaphase II spindles may be indicative of defects in chromosome segregation in meiosis I. The presence of microspindles may be indicative of defects in meiosis I or meiosis II segregation, such as chromosomes, single chromatids, or DNA fragments becoming dissociated from the major spindles and nucleating microtubules to form tiny spindles (Theurkauf and Hawley 1992; McKim and Hawley 1995).

We hypothesized that the increased number of eggs with microspindles in the *cort*; *cycB3*/+ double mutant is a result of increased occurrences of anaphase II that lead to single chromatids falling off of the major spindles. To test our hypothesis we stained double mutant eggs for MEI-S332 and assessed the localization of MEI-S332 to the DNA

Table 3-1. Suppression Tests for Candidate Genes

pe	1 spindle	2 metaII spindles	Aberrant chromosome morphology on metaII spindles	2 meta/ana or ana II spindles	1 or 2 major spindles plus 1 or more microspindles	Total e exami
cort^{RH65}	3%	75%	11%	3%	8%	63
cycB3/+	1%	60%	3%	11%	25%	97
^{65} *pim*/ ^{5} +	2%	84%	0	1%	12%	96

e collected for 0-30 minutes from females of the designated genotype, stained with DAPI, and analyzed for terminal phen

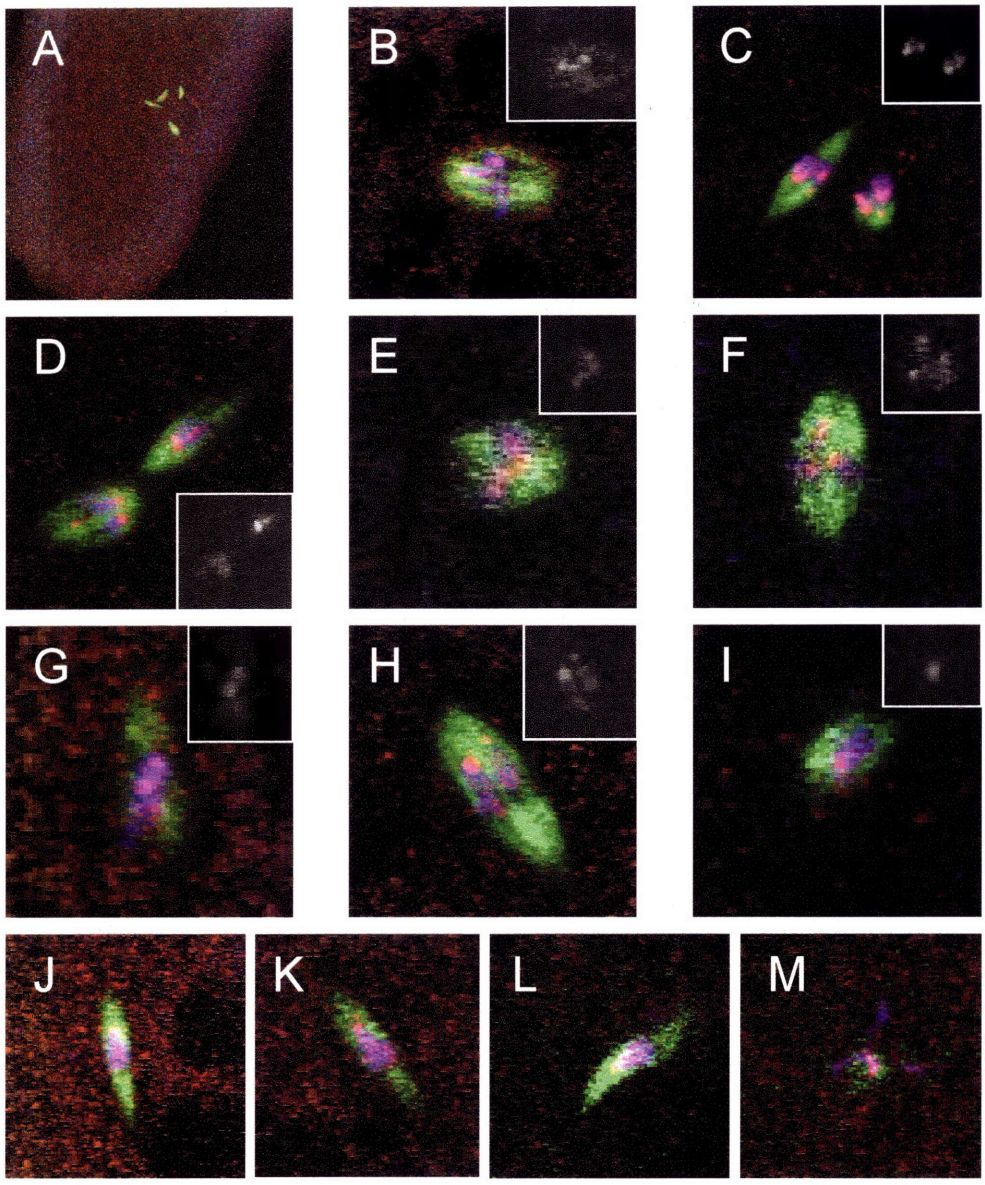


Figure 3-1. MEI-S332 localizes to DNA on all spindles in *cort*; *cycB3*/+ mutants
(A) Example of microspindles in a $cort^{RH65}$; *cycB3*/+ egg stained for DNA (red) and Tubulin (green). (B-M) Mutant eggs with DNA stained in blue, Tubulin in green, and MEI-S332 in red. Insets show the MEI-S332 channel alone. (B) Metaphase I arrest in $cort^{RH65}$, (C) Metaphase II arrest in $cort^{RH65}$, (D-F) metaphase-anaphase II in $cort^{RH65}$, (E) and (F) are from one egg, (G-I) Spindles from one metaphase-anaphase II $cort^{RH65}$; *cycB3*/ + egg, (I) is a microspindle, (J-K) Spindles from one metaphase-anaphase II $cort^{RH65}$; *cycB3*/ + egg

on microspindles. MEI-S332, the Drosophila member of the Shugosin protein family, localizes to meiotic centromeres in oocytes in prometaphase I and is delocalized from chromosomes in anaphase II (Moore et al. 1998). The absence of MEI-S332 localization on DNA forming microspindles would indicate that this DNA is single sister chromatids and suggest that anaphase II occurred in these eggs.

In single *cort* mutants, MEI-S332 localized to chromosomes in a metaphase I arrested egg, in a metaphase II arrested egg, and in an egg that arrested at an intermediate point between metaphase II and anaphase II (Figure 3-1B-F). In *cort*; *cycB3*/+ double mutants, MEI-S332 also localized to DNA on metaphase II/anaphase II spindles, as well as to DNA on microspindles (Figure 3-1G-M). The presence of MEI-S332 on microspindle DNA prevents us from concluding the identity of this DNA. It could be univalents or single sisters, and these eggs may be in an early stage of anaphase at which MEI-S332 is normally still localized to chromosomes. Additionally, *cort* itself may be required for delocalization of MEI-S332, perhaps through a role in activating the separase pathway (Lee et al. 2004), and in this case, localization of MEI-S332 to microspindles is not informative. In summary, we are unable to conclude whether the phenotypic differences between *cort* and *cort*; *cycB3*/+ in female meiosis are indicative of dominant suppression of *cort* by *cycB3*.

A screen for dominant suppressors of *grauzone*

The failure of the candidate genetic tests with *cort* may be explained by the fact that the *cort* alleles we used were nulls (Chu et al. 2001). Reducing the amount of

substrate accumulated in a metaphase II arrested egg may have no effect when no CORT protein is present to then promote onset of anaphase II and meiotic exit. Therefore, we decided to use *grauzone* mutants as the basis for a dominant suppression screen.

grauzone (*grau*) encodes a zinc finger transcription factor that is required for activating *cort* during female meiosis (Chen et al. 2000; Harms et al. 2000). *grau* mutant eggs exhibit identical phenotypes to *cort*, and the only critical function of *grau* during female meiosis is to promote *cort* function, as the addition of one extra copy of *cort* rescues the female meiosis defect of *grau* eggs (Lieberfarb et al. 1996; Page and Orr-Weaver 1996; Harms et al. 2000). Hypomorphic *grau* alleles exist, and a small percentage of eggs laid by these females complete meiosis and initiate mitotic divisions (Chen et al. 2000; Harms et al. 2000). We reasoned that a transheterozygous combination of these alleles would be better suited for a genetic interaction screen because a low level of *grau* and *cort* activity is present in these eggs and is likely to be sensitive to small changes in levels of APC/C substrates.

We initiated a screen to identify dominant suppressors of a weak *grau* transheterozygote using the third chromosome deficiency collection available from the Bloomington Stock Center (Figure 3-2). We analyzed eggs laid by females transheterozygous for *grau* and heterozygous for third chromosome deficiencies by staining the DNA with DAPI. As a control, for each set of deficiencies screened, we screened eggs laid by sibling females homozygous for *grau* and heterozygous for the dominant marker *Ki* instead of a deficiency. We expected most deficiencies to have no effect on the *grau* phenotype; these eggs should arrest in metaphase II or anaphase II like the *grau*; *Ki*/+ control (Figure 3-3A,B). For deficiencies that suppress *grau*, we expected

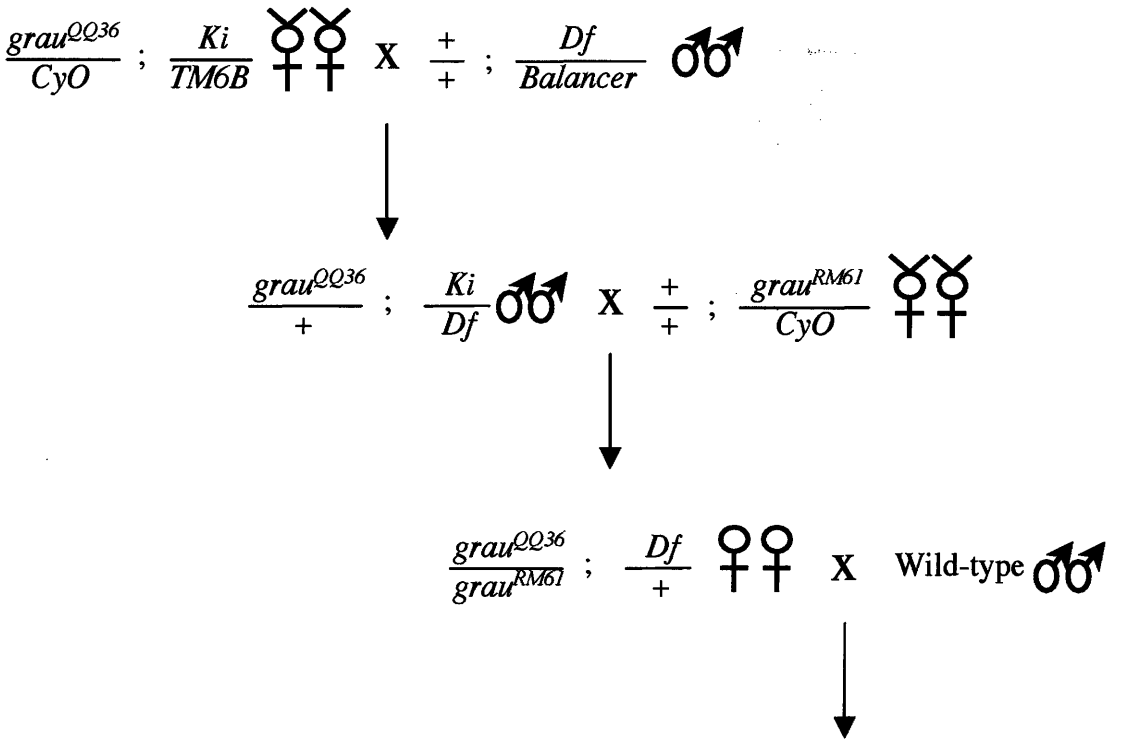


Figure 3-2. Screen for deficiencies that dominantly suppress *grau*
Males carrying a balanced deficiency (*Df*) on the third chromosome were mated to females heterozygous for $grau^{QQ36}$ on the second chromosome and for *Kinked (Ki)*, a dominant bristle marker, on the third chromosome. Male progeny resulting from this cross carrying $grau^{QQ36}$ on the second chromosome and *Ki* and the *Df* on the third were mated to females heterozygous for $grau^{RM61}$. Female progeny transheterozygous for *grau* and heterozygous for the *Df* resulting from this cross were mated to wild-type males, and eggs were collected for analysis as described above and in Materials and Methods.

to observe mitotic divisions in the embryos, indicating completion of meiosis and initiation of embryonic development (Figure 3-3C).

The third chromosome deficiency kit covers approximately 93% of the third chromosome. We have currently screened through 58 of 131 total lines in the kit (45%). The deficiencies we have screened are listed in the Materials and Methods section. We detected strong suppression by *Df(3R)p-XT103* (discussed below). We also detected weak suppression by *Df(3R)H-B79*, but this result remains to be confirmed by repeating the suppression test on a larger scale.

Df(3R)p-XT103 dominantly suppresses *grauzone*

We identified one deficiency, *Df(3R)p-XT103*, that dominantly suppresses *grau*. Whereas all other deficiencies screened gave no eggs containing mitotically dividing cells, approximately 19% (n=26) of *grau*; *Df(3R)p-XT103*/+ eggs showed clear mitotic divisions (Figure 3-3D). In addition, approximately 23% of these eggs contained a rosette-like structure that may indicate the presence of polar bodies and suggests that meiosis was completed. We repeated the suppression test for *Df(3R)p-XT103* on a larger scale to confirm our results. *grau* was again dominantly suppressed in this test. 12% (n=300) of these embryos were mitotically dividing in a syncyntial blastoderm stage, and 6% had developed to a gastrulation stage. In a separate collection from these females, we did not fix and stain the eggs but allowed them to develop. 2% (n=600) of these eggs developed to larval stages, and 1% developed to adulthood. We conclude that a gene within *Df(3R)p-XT103* dominantly suppresses *grau*.

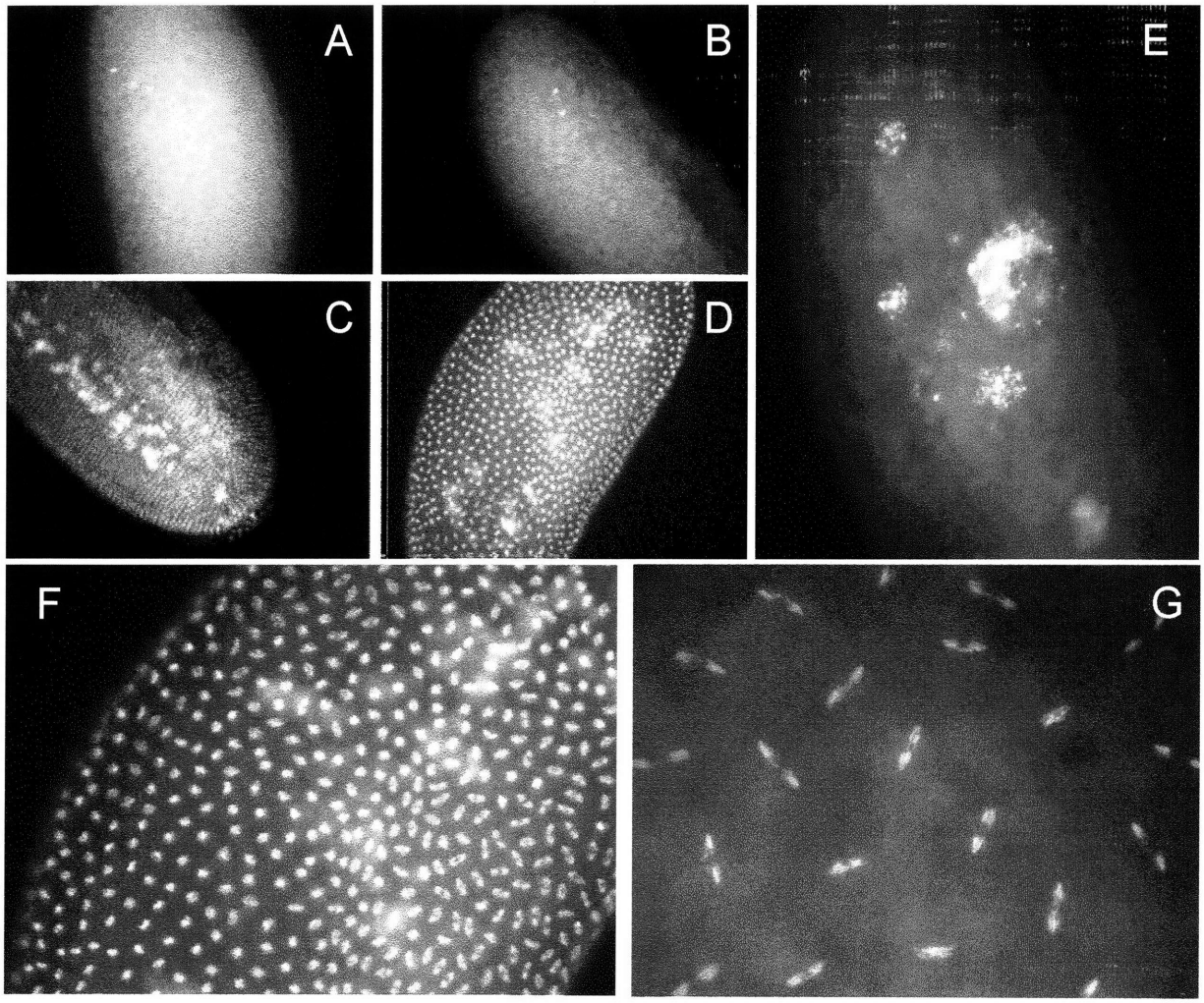


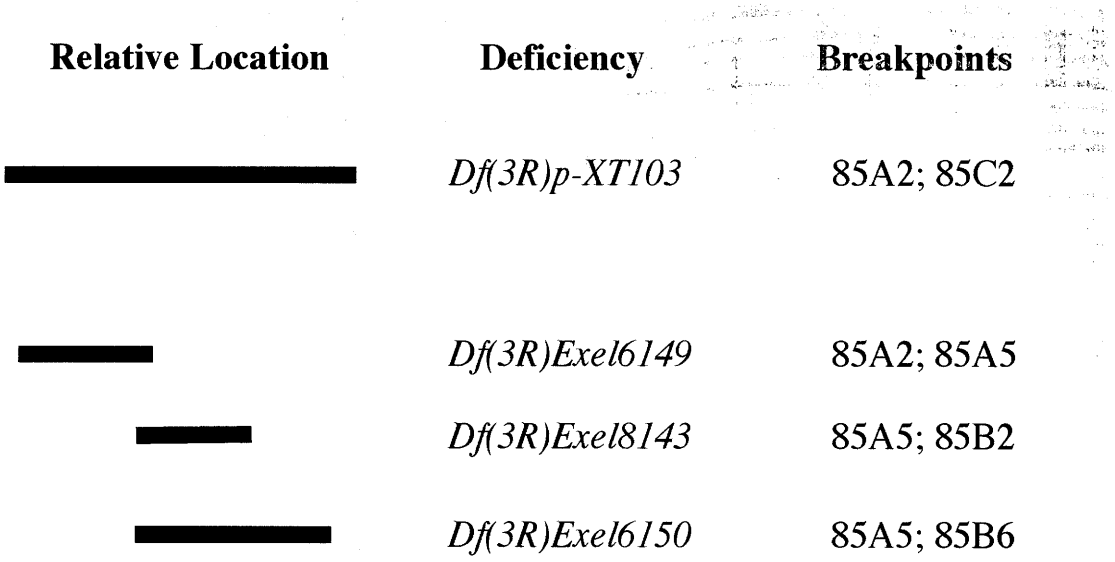
Figure 3-3. *Df(3R)p-XT103* dominantly suppresses *grauzone*
2-8 hour embryos from females of the specified are stained for DNA. (A) $cort^{RH65}$ egg arrests in metaphase II. This phenotype is identical to that of a *grau* egg. (B) Metaphase II arrest from a double mutant that is not suppressed, $grau^{QQ36}$/ $grau^{RM61}$; *Df(3R)ry614*/+, (C) Wild-type embryo that has undergone several mitotic cycles, (D) Suppressed double mutant embryo, $grau^{QQ36}$/ $grau^{RM61}$; *Df(3R)p-XT103*/+, that has progressed to a similar stage as the wild-type embryo, (E-F) Suppressed embryos exhibit some aberrant DNA and spindle morphology. All embryos are from $grau^{QQ36}$/ $grau^{RM61}$; *Df(3R)p-XT103*/+ females. (E) Large clusters of DNA that appears to be fragmented, (F) Asynchronous cycling of nuclei, (G) Chromosome bridges and lagging chromosomes on anaphase spindles.

Close inspection of *grau*; *Df(3R)p-XT103*/+ eggs revealed mitotic defects in those eggs that began to mitotically divide. In some embryos we observed large masses of DNA that appeared to be fragmented or degrading (Figure 3-3E). We also observed asynchronous cycling of nuclei with nuclei in different stages of mitosis (Figure 3-3F). In syncytial embryos with nuclei in anaphase, anaphase figures often contained lagging chromosomes or chromatin bridges between the two dividing masses of DNA (Figure 3-3G). These defects are not unexpected because we do not predict that reducing levels of one APC/C^{CORT} target will be sufficient to suppress fully the *grau* meiotic arrest. A likely scenario is that reduction in levels of one substrate is enough to allow completion of meiosis and initiation of mitotic divisions. Eventually, accumulation of other APC/C^{CORT} substrates prevents proper mitotic divisions to result in the observed defects.

Testing of smaller deficiencies within the *Df(3R)p-XT103* region

We used smaller molecularly defined deficiencies from the Exelixis collection at Harvard Medical School to narrow down the region of the interacting gene within *Df(3R)p-XT103*. These deficiencies cover most of *Df(3R)p-XT103* and divide it into three regions, two of which are partly overlapping (Figure 3-4A). We performed large-scale genetic suppression tests with these deficiencies and *grau* as described above and quantified the different classes of phenotypes in the eggs (Figure 3-4B). *Df(3R)Exel8143* and *Df(3R)Exel6150* showed a significant suppression of *grau* compared to the control, whereas *grau*; *Df(3R)Exel 6149*/+ eggs are only weakly suppressed.

A



B

Genotype	2 metaphase/ anaphase II spindles	Presence of polar bodies	Large masses of fragmented DNA	Syncytial divisions	Gastrulating	*n*
$grau^{QQ36}/grau^{RM61}$; *Df(3R)p-XT103/+*	ND	ND	ND	12%	6%	300
$grau^{QQ36}/grau^{RM61}$; *Df(3R)Exel6149/+*	95%	4.5%	-	-	-	155
$grau^{QQ36}/grau^{RM61}$; *Df(3R)Exel8143/+*	84%	11%	2.7%	1.3%	1.0%	223
$grau^{QQ36}/grau^{RM61}$; *Df(3R)Exel6150/+*	89%	8.9%	1.7%	-	-	179
$grau^{QQ36}/grau^{RM61}$; *Ki/+*	100%	-	-	-	-	121

Figure 3-4. Mapping location of suppressor with smaller deficiencies
(A) Diagram of relative location of smaller Exelixis deficiencies to original suppressing deficiency, (B) Phenotypes of double mutants of *grau* and smaller deficiencies, last row is a control not containing a deficiency

To determine whether the suppression caused by *Df(3R)Exel8143* and *Df(3R)Exel6150* is significantly different from that caused by the original larger *Df(3R)p-XT103*, we performed pairwise comparisons with Fisher's Exact Test. By this test, *Df(3R)Exel8143* and *Df(3R)Exel6150* are suppressing *grau* to a significantly lesser degree than *Df(3R)p-XT103*. The amount of suppression between *Df(3R)Exel8143* and *Df(3R)Exel6150* is not significantly different, but the level of their suppression is significantly greater than that of *Df(3R)Exel6149*. These results suggest that two genes within *Df(3R)p-XT103* are suppressing *grau*, one within the overlapping region of *Df(3R)Exel8143* and *Df(3R)Exel6150*, and one within *Df(3R)Exel6149*. When they are both deleted together, the amount of suppression is stronger than if each is deleted on its own. This interpretation makes sense if the suppressing genes are APC/C^{CORT} substrates, because cell cycle transitions are usually triggered by APC/C-mediated degradation of more then one protein.

Candidate substrates within *Df(3R)p-XT103*

We looked for genes within the region deleted by *Df(3R)p-XT103* that seemed like possible candidate substrates of APC/C^{CORT} and found *belle* (*bel*), *Cenp-C*, *cks85A*, and *pif-1A*. All of these genes have roles relevant to mitosis and are expressed during oogenesis or early embryogenesis. *bel and Cenp-C* are located within the smaller deficiency *Df(3R)Exel6149*, and *cks85A* and *pif-1A* are located within the overlapping region between *Df(3R)Exel8143* and *Df(3R)Exel6150* (Figure 3-4).

bel encodes a DEAD-box protein that is most closely related to the Ded1p subfamily of proteins which have roles in translation initiation, remodeling RNPs, and RNA processing (Johnstone et al. 2005). *bel* is required for male and female fertility, and ovaries from *bel* mutant females usually arrest around Stage 8 or 9. Mature eggs are occasionally produced, but they contain defective dorsal appendages (Johnstone et al. 2005).

Cenp-C encodes a centromere protein that was identified in Drosophila in a screen for modifiers of *pim/securin* overexpression (Heeger et al. 2005). CENP-C is the ortholog to the vertebrate kinetochore protein that localizes to the centromere and is required for normal bipolar kinetochore attachments. *cks-85A* encodes one of two Cks/Suc1 homologs in Drosophila. These proteins interact with Cyclin-dependent kinases and are thought to stimulate Cdk-dependent phosphorylation of mitotic proteins (Pines 1996). Cks85A protein co-immunoprecipitates with Cdk1, Cdk2, and mitotic cyclins in embryos (Swan et al. 2005). Finally, little is known about *pif-1A*, but it has weak homology to microtubule-binding proteins Drosophila D-CLIP-190 and vertebrate Dynactins (Rascle et al. 2003).

To determine if any of these genes was the suppressing gene in the region, we tested whether strong alleles of *bel* or *cks85A* could dominantly suppress *grau*. Neither *bel* nor *cks85A* suppressed *grau* (data not shown). This negative result does not rule out the possibility that one of these genes is an APC/C^{CORT} substrate, although it does suggest that absence of one of these genes alone cannot account for the strong suppression seen by *Df(3R)p-XT103*.

One prediction for an APC/C^{CORT} substrate is that levels of the protein will be elevated in *cort* mutant eggs, as has been shown for the mitotic cyclins and PIM (Swan et al. 2005; Swan and Schupbach 2007; Pesin and Orr-Weaver, submitted). We analyzed BEL protein levels in *cort* mutant ovaries and eggs by Western blot (Figure 3-5). BEL is only slightly elevated in $cort^{RH65}/cort^{QW55}$ ovaries, but more significantly elevated in $cort^{RH65}/cort^{QW55}$ eggs. These results are consistent with BEL being a substrate of APC/C^{CORT} during the meiotic divisions, and, together with the failure of *bel* alone to suppress *grau*, suggest that *bel* may be one gene in this region that strongly suppresses *grau* in combination with another interacting gene.

DISCUSSION

In this study, we made progress towards identifying APC/C^{CORT} substrates in meiosis. First, we found that mutations in candidate substrates do not strongly genetically suppress *cort*, but *cort; cycB3*/+ double mutant embryos do exhibit an increase in the number of microspindles containing chromatin with localized MEI-S332. The limitation of these initial tests was the likely absence of CORT function in the available alleles. In order to identify APC/C^{CORT} substrates, we are currently conducting a dominant interaction screen with *grau* mutants, a gene encoding a transcriptional activator of *cort*. These alleles retain residual CORT that could be suppressed by reducing substrate levels. We have screened just under half of the third chromosome deficiency kit from Bloomington Stock Center and identified one strongly suppressing deficiency and one possible weakly suppressing deficiency. *Df(3R)p-XT103* dominantly suppresses *grau*, and a small percentage of these double mutant embryos progress to

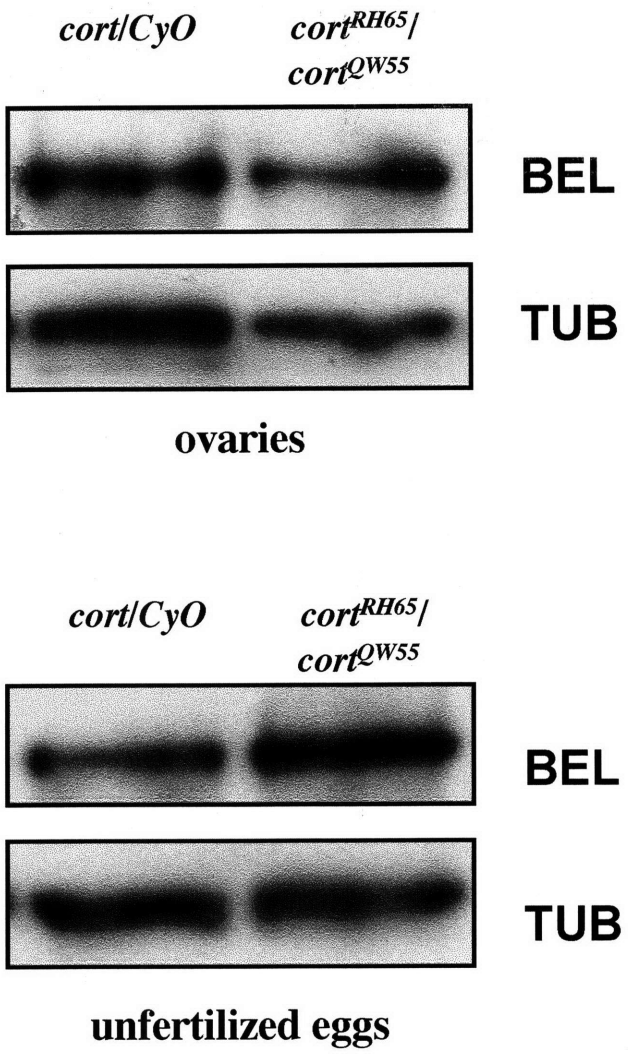


Figure 3-5. BEL protein levels are elevated in *cort* mutant eggs
Western blot showing levels of BEL in whole ovaries and unfertilized eggs of the indicated genotypes. Blot was probed with anti-α-Tubulin as a loading control. By ImageJ analysis, BEL levels are approximately 1.2-fold greater in $cort^{RH65}/cort^{QW55}$ ovaries compared to *cort/CyO* ovaries. BEL levels are approximately 1.6-fold greater in $cort^{RH65}/cort^{QW55}$ unfertilized eggs compared to *cort/CyO* unfertilized eggs.

mitotic divisions in blastoderm and gastrula stages. We identified candidate interacting genes within this region and found that the protein levels of one, BEL, are elevated in *cort* mutant eggs.

After observing that a strong *cort* allele is not dominantly suppressed by candidate substrate genes, *cycB3* and *pim*, we undertook a dominant suppression screen to identify APC/C^{CORT} substrates using *grau* transheterozygotes. We expected this genotype to be more sensitive to interacting genes, because the alleles are hypomorphic, and mutant eggs contain low levels of *cort* function. This approach is proving to be successful, as we have identified at least one suppressing deficiency. We will not know whether this screen achieves our goal of identifying APC/C^{CORT} substrates until we have identified an interacting gene within an interacting deficiency that is an APC/C substrate.

A positive control for the screen would be useful in evaluating our results. Among the critical mitotic APC/C substrates of mitotic cyclins and securin, the only candidate APC/C^{CORT} substrates that are located on the third chromosome are *cycB3* and *cycA*. Levels of these proteins are elevated in *cort* mutants and are good candidates for substrates (Swan et al. 2005; Swan and Schupbach 2007; Pesin and Orr-Weaver, submitted). We have tested the deficiency in the kit that uncovers *cycB3*, *Df(3R)XTA1*, but this deficiency did not suppress *grau*. *cycA* is located within two deficiencies in the kit, *Df(3L)vin5* and *Df(3L)vin7*, but we have not yet tested these deficiencies. Although we would expect *cycB3* to suppress *grau*, this negative result does not rule out the possibility that it is a substrate, but it does suggest that eliminating one genetic copy of one substrate may not always be enough to suppress *grau*'s meiotic defect.

In addition to APC/C^{CORT} substrates, we may also identify APC/C^{CORT} inhibitors in this screen. Reducing inhibition of APC/C^{CORT} could increase APC/C^{CORT} activity just enough to progress through metaphase II, complete meiosis, and start mitotic divisions. One candidate inhibitor of APC/C^{CORT} is *rca1*, the Drosophila homolog of Emi1, an inhibitor of APC/C^{Cdc20} in mitosis (Reimann et al. 2001). *rca1* is located on the second chromosome and is not testable in this screen.

Other candidate inhibitors of APC/C^{CORT} are components of the spindle checkpoint pathway. This pathway inhibits APC/C^{FZY} in metaphase of mitosis in response to spindle damage or unattached kinetochores and is also thought to inhibit APC/C during meiosis I (for review, see Musacchio and Salmon 2007; Baker et al. 2004; Homer et al. 2005; Yin et al. 2006; Stein et al. 2007; Gilliland et al. 2007). Among the spindle checkpoint genes in Drosophila, *mad2* and *ald/mps1* are located on the third chromosome. *mad2* is located within *Df(3L)ZN47*, which we have not yet tested. *ald/mps1* is located within *Df(3R)DG2*, which we have tested but did not observe any suppression of *grau*. It is not known whether *ald/mps1* directly inhibits the APC/C during meiosis, and therefore, its failure to suppress *grau* in the screen is not informative.

Finally, in addition to a substrate or inhibitor of APC/C^{CORT}, we may identify negative upstream regulators of *cort*. It would be useful to measure *cort* transcript and protein levels to test this possibility.

A weakness of our screen is the use of *grau* alleles instead of *cort* alleles. Even though *cort* seems to be the only critical target of *grau* during female meiosis, we may still uncover interactors that are negative regulators of *grau* that are unrelated to *cort*

function. Because little is known about *grau* outside of its role in activating *cort*, we may not know how to interpret positive hits from the screen that are unrelated to APC/C^{CORT}.

In our screen we identified a strongly suppressing deficiency, *Df(3R)p-XT103*. A small percentage of the embryos transheterozygous for *grau* and heterozygous for *Df(3R)p-XT103* completed meiosis and progressed into embryogenesis to the developmental stages of syncytial blastoderm or gastrulation. Although the meiotic defect was suppressed in these embryos, they often displayed defects in the subsequent mitotic divisions such as asynchrony of diving nuclei and lagging chromosomes or bridges in anaphase figures during syncytial divisions. An additional defect we observed was large fragmented or degrading nuclei during preblastoderm stages.

What is the cause of these mitotic defects? We hypothesize that *Df(3R)p-XT103* contains a gene(s) that encodes a substrate of APC/C^{CORT}. When levels of this substrate are reduced, *grau* embryos are able to complete meiosis and start mitotic divisions. Even though levels of one substrate are reduced, the embryo still contains elevated levels of all other APC/C^{CORT} substrates. These proteins may interfere with the mitotic machinery, especially if they are not recognized and targeted for degradation by APC/C^{FZY}, which is the main APC/C present in early embryos (Raff et al. 2002).

We have shown that the bulk of CORT is rapidly degraded after egg activation and is undetectable after 1.5 hours of embryogenesis (Pesin and Orr-Weaver, submitted). Thus, any APC/C^{CORT} specific substrates still present in these embryos will not be targeted for degradation because CORT is not synthesized again during development. Some studies suggest that APC/C substrates bind to core APC/C in addition to binding APC/C activators (Yamano et al. 2004; Eytan et al. 2006). In these suppressed embryos,

excess APC/C^{CORT} substrates may be binding to core APC/C and blocking access of APC/C^{FZY} substrates. Thus, the mitotic defects in these embryos could be the result of interferences with normal APC/C^{FZY} function.

We used smaller Exelixis deficiencies to narrow down the region within *Df(3R)p-XT103* that contains the suppressing gene. This approach revealed the possibility that more than one gene within this region is responsible for the strong suppression of *Df(3R)p-XT103*. We will evaluate future interacting deficiencies from the screen with this possibility in mind.

We have identified four candidate APC/C^{CORT} substrates within the *Df(3R)p-XT103* region. An antibody is available for BEL, and we showed by Western blot analysis that levels of this protein are elevated in *cort* mutant eggs. This result is consistent with BEL being a substrate of APC/C^{CORT} but is not proof. BEL could be elevated in any egg that arrests before mitotic divisions begin. We have identified a potential D-box sequence in BEL, but, ideally, we need an *in vitro* ubiquitination or degradation assay to test BEL's identity as a substrate.

Without a working *in vitro* system to test whether proteins are APC/C^{CORT} substrates, a couple of strategies will help to narrow down the set of possible interacting genes in this deficiency and in other deficiencies that we may identify in the future. First, a systematic search for D-boxes and KEN boxes will tell us which proteins contain putative APC/C recognition motifs. Second, looking in online expression databases such as www.flyatlas.org will tell us which genes are expressed in ovaries.

In conclusion, in this study we have initiated a dominant suppression screen to identify substrates of APC/C^{CORT} in meiosis. Identification of these substrates will greatly enhance our understanding of how APC/C-mediated proteolysis triggers meiotic events.

MATERIALS AND METHODS

Fly Stocks

Crosses were performed, and flies were maintained between 22°C and 25°C using standard techniques (Greenspan 1997). The *cort*^{RH65} and *cort*^{QQ36} alleles and the *grau*^{QQ36} and *grau*^{RM61} alleles were generated in an EMS screen for female-sterile loci on the second chromosome (Schupbach and Wieschaus 1989). These alleles have been described (Page and Orr-Weaver 1996; Chen et al. 2000; Chu et al. 2001). The *cycB3*^{2} allele has been described (Jacobs et al. 1998). The *pim*^{IL} allele has been described (Stratmann and Lehner 1996). All deficiencies from the third chromosome deficiency kit as well as the Exelixis deficiencies were obtained from the Bloomington Drosophila Stock Center.

Deficiency Screen

The strategy used for the screen is outlined in Figure 3-2. The following deficiencies on the third chromosome were tested in the screen:

Chromosomal arm 3L: Df(3L)emc-E12; Df(3L)R-G5; Df(3L)Aprt-1; Df(3L)Aprt-32; Df(3L)R-G7; Df(3L)BSC23; Df(3L)HR119; Df(3L)GN24; Df(3L)XDI98; Df(3L)pbl-X1; Df(3L)66C-G28; Df(3L)BSC13; Df(3L)29A6; Df(3L)66C-I65; Df(3L)BSC14; Df(3L)eyg[C1]; Df(3L)Ly; Df(3L)fz-D21; Df(3L)fz-M21; Df(3L)st8P; Df(3L)BSC8;

Df(3L)W10; Df(3L)W4; Df(3L)H99; Df(3L)fz2; Df(3L)kto2; Df(3L)XS533; Df(3L)ri-XT1; Df(3L)ME107; Df(3L)Pc-2q

Chromosomal arm 3R: Df(3R)ME15; Df(3R)3-4; Df(3R)e1025-14; Df(3R)Tpl10; Df(3R)Scr; Df(3R)p-XT103; Df(3R)BSC24; Df(3R)6-7; Df(3R)T-32; Df(3R)ry81; Df(3R)kar-Sz11; Df(3R)ry614; Df(3R)red1; Df(3R)ea; Df(3R)C4; Df(3R)DG2; Df(3R)Cha7; Df(3R)H-B79; Df(3R)e-BS2; Df(3R)mbc-30; Df(3R)mbc-R1; Df(3R)crb87-4; Df(3R)crb87-5; Df(3R)XTA1; Df(3R)Espl3; Df(3R)Tl-P; Df(3R)D605; Df(3R)3450.

Egg Fixation, Staining, and Microscopy

Eggs for Figure 3-1 were collected for 0-30 minutes, dechorionated in 50% bleach, devitellinized in methanol and heptane, and fixed in methanol for 3 hours at room temperature or overnight at 4°C. Eggs were stained for DNA with TOTO-3 (Molecular Probes) or 4',6-diamidino-2-phenylindole (DAPI), with rat monoclonal anti-α-Tubulin, YL 1/2 and YOL 1/34 (1:20, Harlan Sera-lab), and with guinea pig anti-MEI-S332 serum (1:1000, Tang et al. 1998). Antibodies were detected using fluorescent secondary antibodies (Jackson Immunoresearch). Imaging was performed using a Zeiss microscope with LSM510 confocal imaging software (Keck Imaging Facility).

Embryos for the screen were collected for 2-8 hours, dechorionated in 50% bleach, devitellinized in methanol and heptane, and fixed in methanol overnight at 4°C. Embryos were stained for DNA with DAPI, and imaging was performed using a Zeiss Axioskop with an AxioCam Hrm camera and AxioVision AC software. All images were processed using Adobe Photoshop.

Protein Extracts and Immunoblotting

Protein extracts were made by homogenizing whole ovaries or unfertilized eggs in 3:1 Urea Sample Buffer (8 M urea, 2% SDS, 100 mM Tris, pH 7.5, 5% Ficoll)/ tissue (vol./vol.). Protein extracts were resolved by SDS-PAGE and transferred to Immobilon-P membranes (Millipore). Equal amounts of protein were loaded per lane and confirmed by anti-Tubulin blotting. Blots were probed with rabbit anti-BEL serum (1:2500, Johnstone et al. 2005) and with rat monoclonal anti-α-Tubulin, YL 1/2 and YOL 1/34 (1:200, Harlan Sera-lab). Alkaline phosphatase- or horseradish peroxidase-conjugated secondary antibodies were used to detect bound primary antibodies. Protein was detected using ECL Plus (Amersham).

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Chapter Four

Conclusions and Perspectives

In this thesis we have investigated the problems of meiotic cell cycle control in a multicellular organism. Unlike mitosis, the molecular components of meiotic cell division and their regulation, particularly in metazoans, are not well understood. We have used meiosis in female Drosophila as a model for understanding the role of the Anaphase-Promoting Complex/ Cyclosome (APC/C) and its regulation in this non-canonical cell cycle.

The molecular control of meiosis in female organisms is an interesting problem for two reasons. First, meiosis is achieved through the use of both general mitotic regulators and meiosis-specific proteins (Marston and Amon 2004). In this thesis we looked at the APC/C, a general mitotic regulator, which in Drosophila is modified in meiosis with the addition of a meiosis-specific activator subunit. Understanding how mitotic and meiotic proteins act together in meiosis will provide insight into differences and similarities between the two types of cell division. Second, female meiosis takes place in a unique developmental context. During oogenesis, meiosis is arrested twice to coordinate growth and development of the oocyte with meiotic division and fertilization. This additional complexity of female meiosis provides an opportunity to study how developmental signals are coupled with cell cycle controls.

Below we discuss our major findings in this thesis and address the questions that remain.

I. Function of Putative Meiosis-specific APC/C Activators in Drosophila

Our work and the work of Swan and Schupbach have demonstrated that *cortex* (*cort*) encodes a protein that very likely functions as a female meiosis-specific activator of the APC/C. It is required for and can trigger the degradation of mitotic cyclins and

associates biochemically with the core APC/C (Swan and Schupbach 2007; Pesin and Orr-Weaver, submitted). More definitive proof of CORT's function would require an *in vitro* ubiquitination assay demonstrating the ability of CORT to cause APC/C-dependent ubiquitination of substrates.

We initially set out to perform a heterologous ubiquitination assay using Xenopus APC/C, recombinant E1 and E2, radiolabeled substrates, and tagged recombinant CORT, but our attempts at purifying recombinant CORT were unsuccessful (Appendix One). CORT is most closely related to Cdc20, and, for several groups, generation of active recombinant *S. cerevisiae* and human Cdc20 has been technically difficult, so our results are not unexpected (Kraft et al. 2006). In spite of these difficulties, Araki et al. successfully generated active Drosophila FZY and FZR by coupled *in vitro* transcription and translation methods (Araki et al. 2003). It may be worth revisiting this technical challenge, not only to provide further evidence that CORT is a functional APC/C activator, but also to use as an assay for evaluating putative APC/C^{CORT} substrates (discussed below).

Intriguingly, the Drosophila genome contains another member of the Cdc20/FZY family in addition to *fzy*, *fzr*, and *cort* that is expressed exclusively in testes. This gene, *fizzy-related 2* (*fzr2*), encodes a protein that is more similar to FZR than to FZY, unlike CORT, which is more closely related to FZY (Chu et al. 2001; Jacobs et al. 2002). Indeed, misexpression of *fzr2* suppresses a *fzr* phenotype but not one of *fzy* mutants (Jacobs et al. 2002). *fzr2* transcripts are detected by *in situ* hybridization in premeiotic spermatocytes. Perhaps APC/C^{FZR2} is a unique form of the APC/C that is specifically required during the extended G2/meiotic prophase during male meiosis. In mouse

oocytes APC/C^{Cdh1} is required specifically during meiotic prophase to prevent premature entry into the meiotic divisions (Reis et al. 2006; Marangos et al. 2007). No mutant alleles exist for *fzr2*, but P element insertions are located both just upstream and downstream of the gene and could be useful in generating a mutant. Mutant analysis of *fzr2* will be crucial for determining the role of this putative male-meiosis specific APC/C activator.

Meiosis-specific APC/C activators have only been identified in yeast and flies. However, the existence of multiple Cdh1 homologs with different developmental expression patterns in chicken suggests that meiosis-specific activators in vertebrates may have yet to be identified (Wan and Kirschner 2001).

II. Meiosis-specific APC/C Substrates

Why are meiosis-specific APC/C activators necessary? We hypothesize that meiosis-specific activators are crucial because they target the degradation of a unique set of substrates in meiosis. These substrates could be proteins expressed only in meiosis or mitotic proteins that must be degraded with different dynamics in meiosis. *S. cerevisiae* Spo13, which is expressed exclusively in meiosis, is the only meiosis-specific APC/C substrate to be identified so far, but it seems to be targeted by APC/C^{Cdc20} rather then APC/C^{Ama1} (Sullivan and Morgan 2007). However, the multiple Cdh1 homologs in chicken have different substrate specificities *in vitro*, suggesting that one function of these different putative activators is to target different sets of substrates (Wan and Kirschner 2001).

We have initiated a genetic screen to identify APC/C^{CORT} substrates. Although the screen and mapping of interacting genes are not yet complete, we have identified a

possible limitation with our approach. Ultimately, to identify a new APC/C substrate with confidence, we must be able to show that this substrate is ubiquitinated and/or degraded in an APC/C^{CORT}-dependent manner in an *in vitro* system. As discussed above, previous difficulties with producing recombinant CORT prevented us from setting up such an *in vitro* assay system, but this effort should be revisited.

III. Developmental Regulation of CORT Protein Levels

A major finding of our work is that CORT protein levels are developmentally regulated in order to restrict its expression to the meiotic divisions. This pattern of expression is in striking contrast to that of FZY, which is expressed at uniform levels during oogenesis and embryogenesis (Pesin and Orr-Weaver, submitted).

First, polyadenylation of *cort* mRNA is tightly correlated with the appearance of elevated CORT protein levels in mature oocytes (Pesin and Orr-Weaver, submitted). In order to demonstrate a causal relationship between these events, future experiments should identify elements in the 3' UTR of *cort* that are required for its polyadenylation and show that this polyadenylation is required for the translational activation of *cort*.

Perhaps a more important problem is determining the biological significance of this translational activation. Is it critical that CORT protein not be present until the meiotic divisions, or is the organism simply taking advantage of an existing developmental mechanism to turn on *cort* translation along with other meiotic regulators? We utilized our transgenic lines to address this question. In these lines, we used the *UAS-gal4* system to target expression of a transgene containing *cort* cDNA flanked by non-endogenous UTRs in the female germline (Pesin and Orr-Weaver, submitted). Translation of *cort* in this system should be independent of cytoplasmic polyadenylation

and should occur earlier in oogenesis than that of endogenous *cort*. We examined the cytology of ovaries in which *cort* is overexpressed by staining the DNA with DAPI (data not shown). We observed no gross defects in whole ovary morphology, and the karyosome structure of the oocyte nucleus in meiotic prophase appeared normal. Furthermore, these females are fertile, so overexpression of *cort* does not affect the production of viable embryos. Therefore, at least with this level of overexpression of *cort* in the early ovary, premature presence of CORT protein does not appear to be detrimental to the process of oogenesis.

We showed that the bulk of CORT protein is rapidly degraded by the end of meiosis in an APC/C-dependent manner, and degradation of exogenous CORT in the embryo is dependent on the presence of a D-box (Pesin and Orr-Weaver, submitted). APC/C-mediated degradation of an APC/C activator is not a novel mode of regulation. Cdc20 is targeted for degradation by APC/C^{Cdh1} during mitotic exit and G1 (Prinz et al 1998). Additionally, Cdh1 may be subject to autoubiquitination in G1 (Listovsky et al. 2004).

The biological significance of this rapid degradation of CORT after egg activation remains a mystery. We hypothesize that CORT must be degraded at this time to prevent it from interfering with the rapid syncytial divisions in the early embryos that are driven in part by localized degradation of Cyclin B on the spindle (Huang and Raff 1999; Wakefield et al. 2000; Raff et al. 2002). This interference could occur in two ways. First, APC/C^{CORT} could continue to degrade substrates, those that are present in the syncytial embryo, in a way that prevents or hinders normal mitotic divisions. Second,

excess CORT in the embryo could bind to and saturate APC/C complexes and block FZY from binding, thus preventing APC/C^{FZY} from functioning.

To address this question, we looked for defects in embryos from flies overexpressing *cort* in our *cort* transgenic lines but observed no phenotype. These lines may not be useful in assessing the consequences of excess CORT in embryos because this transgenic protein was also subject to degradation after egg activation (Pesin and Orr-Weaver, submitted, Fig. S1). To address this question in a different way, we are making flies carrying a *cort* D-box mutant transgene under control of the *UAS* response element. We will drive expression of this gene in the female germline and assess the phenotype in early embryos from these transgenic females. This experiment should tell us whether it is crucial for the normal development of the embryo that CORT be degraded by the end of the meiotic divisions.

IV. Inhibitors of APC/C^{CORT}

The APC/C is subject to regulation by several inhibitors in both mitosis and meiosis (for review, see introduction of this thesis). We have been unable to identify an inhibitor of APC/C^{CORT}, but several experiments can still be done to address this issue.

rca1 is the Drosophila homolog of Emi1, an important inhibitor of APC/C^{Cdc20} in prometaphase and APC/C^{Cdh1} at the G1 to S transition (Reimann et al. 2001; Hsu et al. 2002; Margottin-Goguet et al. 2003). In Drosophila, *rca1* is thought to inhibit APC/C^{FZR} in G2 but not APC/C^{FZY}, but it is not known if *rca1* plays a role in inhibiting APC/C during meiosis. Furthermore, a homolog for Emi2, a related meiosis-specific APC/C inhibitor, has not been identified in Drosophila.

Emi1 has been shown to bind directly to the APC/C, and this interaction may occur through the activator subunit or a core subunit or both (Reimann et al. 2001; Miller et al. 2006). Therefore, we predict that RCA1 will co-immunoprecipitate with CORT in ovaries if it is an inhibitor of APC/C^{CORT}. We are currently testing this hypothesis with anti-RCA1 serum from Frank Sprenger (Grosskortenhaus and Sprenger 2002).

Emi1 is thought to inhibit APC/C^{Cdc20} from targeting Cyclin A for degradation in prophase (Margottin-Goguet et al. 2003). In Drosophila ovaries, Cyclin A appears at high levels in Stage 12 of oogenesis, when the oocyte in still in prophase, but begins to be degraded in Stage 13, when nuclear envelope breakdown occurs and the oocyte enters the meiotic divisions (Vardy, Pesin, and Orr-Weaver, unpubl.). We believe that this degradation of Cyclin A is mediated by APC/C^{CORT}. The analysis of Cyclin A protein levels in *rca1* mutant ovaries and *rca1*; *cort* double mutants will allow us to test a role for RCA1 in preventing premature APC/C^{CORT}-dependent degradation of Cyclin A in Stage 12. *cort* translation may not be fully activated at Stage 12, but low levels are CORT are present beginning in Stage 10 (Pesin and Orr-Weaver, submitted).

The spindle checkpoint inhibits APC/C^{Cdc20} in metaphase of mitosis (for review, see Musacchio and Salmon 2007). In meiosis I of metazoans, the spindle checkpoint is required for normal chromosome segregation (for review, see introduction of this thesis). This requirement for spindle checkpoint in meiosis I appears to be true for Drosophila, and, therefore, we might expect APC/C^{CORT} to be inhibited by components of this checkpoint (Gilliland et al. 2005; Gilliland et al. 2007; Malmanche et al. 2007). The fact that CORT-dependent degradation of Cyclin A occurs before CORT-dependent degradation of Cyclin B and Cyclin B3 supports this idea, as it mirrors the specific

inhibition of Cyclin B degradation but not Cyclin A degradation by the spindle checkpoint in mitosis (Geley et al. 2001; Pesin and Orr-Weaver, submitted).

To test whether CORT is subject to regulation by the spindle checkpoint, we tested for co-immunoprecipitation of MAD2 or BUBR1, two spindle checkpoint proteins that have been shown to bind directly to Cdc20, but did not detect an association with CORT (Appendix Two) (Fang et al. 1998; Tang et al. 2001). Cdc20 is also regulated during spindle checkpoint arrest by phosphorylation by Bub1 kinase (Tang et al. 2004). We tested whether CORT is phosphorylated in ovaries by treating Stage 14-enriched ovary extracts with lambda phosphatase. We did not detect a mobility shift of CORT in a polyacrylamide gel after treatment with phosphatase (Appendix Two). These results suggest that CORT may not be regulated by the same mechanisms that inhibit Cdc20 in mitosis. It is possible that the developmental restriction of CORT protein levels specifically to the meiotic divisions reduces the need for other modes of APC/C^{CORT} inhibition.

V. Localization of CORT in Meiosis

An important remaining question concerning the function and regulation of CORT is determining its localization in meiosis. We have attempted immunofluorescence with our anti-CORT serum in mature oocytes but have not yet found the ideal conditions for this antibody to work *in situ*.

The localization of CORT during the meiotic divisions is important for understanding its role in the spatiotemporal degradation of Cyclin B. The dynamics of Cyclin B accumulation and degradation differ between embryonic syncytial mitoses and meiosis in Drosophila. In mitosis Cyclin B accumulates throughout the spindle and is

degraded in a wave starting at the spindle poles and spreading to the spindle equator (Huang and Raff 1999). In contrast, Cyclin B first accumulates in meiosis on the spindle mid-zone. By immunofluorescence, Cyclin B is not degraded along the spindle in anaphase I, but in meiosis II is degraded first from the spindle mid-zone, and next along the entire spindle (Swan and Schupbach 2007). *cort* is required for Cyclin B degradation from the meiosis II spindle midzone, while *fzy* is required for its degradation along the entire spindle (Swan and Schupbach 2007). Therefore, we hypothesize that CORT localizes to the spindle mid-zone in meiosis II. It will be interesting to see where CORT localizes in metaphase I-arrested oocytes because Cyclin B does not appear to be locally degraded at that time. In Drosophila syncytial mitoses, FZY localizes to spindles and both FZY and FZR can bind to microtubules *in vitro*, suggesting that CORT is also likely to associate with the spindle (Raff et al. 2002).

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Appendix One:

Attempts to Generate Recombinant CORT Protein

An *in vitro* assay system is a useful tool for studying the biochemical properties and functions of enzymes. We proposed to generate recombinant CORTEX (CORT) protein for use in an *in vitro* ubiquitination assay for studying CORT function and identifying substrates of APC/C^{CORT}. We used the Bac-to-Bac Baculovirus Expression System (GIBCO-BRL) to express CORT in *Sf9* insect cell culture. We chose this system for its advantage of being a eukaryotic system.

We made recombinant baculovirus encoding full-length MBP-CORT, infected *Sf9* cells for 39 hours, lysed cells, and incubated cleared lysates with an amylose resin. Although we saw expression of MBP-CORT by Western blot, most of MBP-CORT did not bind to the beads and remained in the flow-through sample (Figure 1A). We hypothesized that this failure to bind could be due to a saturation of the beads. To address this issue, we tried incubating the same amount of cell lysate with twice as many beads but did not observe any increase in the amount of MBP-CORT binding to the beads (data not shown). We also tried incubating the flow-through with amylose beads but still observed very little MBP-CORT binding (Figure 1B).

CCT chaperonin is required for the generation of functional Cdc20 in *S. cerevisiae* (Camasses et al. 2003). Because CORT is a member of the Cdc20/FZY protein family, it may also need a chaperonin to fold. CCT chaperonin is expressed in *Sf9* cells, but it may not be expressed at high enough levels to fold all recombinant CORT being expressed (Vassilev et al. 1995). Alternatively, the N-terminal MBP tag may be preventing folding of CORT by the chaperonin. The fraction of MBP-CORT that does not bind to the amylose beads could be the unfolded fraction.

We decided to focus on the fraction of MBP-CORT that did bind to the beads. We eluted MBP-CORT from the beads by incubation with maltose. Very little MBP-CORT eluted with the bulk of the protein remaining on the beads (Figure 1C). We conclude that most of MBP-CORT is precipitating on the beads and unable to be eluted. Theoretically, scaling up of our purification may yield enough protein to use in assays. We concentrated the eluate with Amicon Centricon YM-10 (Millipore) but were unable to obtain enough protein by this method.

We tried similar methods to express and purify MYC-CORT in this system but were never able to detect expression (data not shown). We also tried using cleared lysate of cells containing MBP-CORT or cold *in vitro* transcribed and translated (IVT) CORT in *in vitro* ubiquitination assays with APC/C purified from Xenopus interphase extracts (with the help of Danny Ooi, Kirschner Lab, Harvard Medical School). We were unable to detect any CORT-dependent ubiquitination of ^{35}S-labeled Cyclin A, Cyclin B, Cyclin B3, or PIMPLES/Securin (data not shown).

Our difficulties in generating pure and functional recombinant CORT protein are not unexpected, as other groups had difficulty with the production of recombinant *S. cerevisiae* and human Cdc20 in both bacteria and *Sf9* cells and by IVT (Kraft et al. 2006). Araki et al. were successful in generating active Drosophila Fzy and FZR by IVT, and this approach is a direction worth pursuing. Some other options to consider are trying different tags on CORT in the baculovirus-*Sf9* system, as well as the co-infection of *Sf9* cells with baculovirus encoding tagged CORT and baculovirus encoding CCT chaperonin.

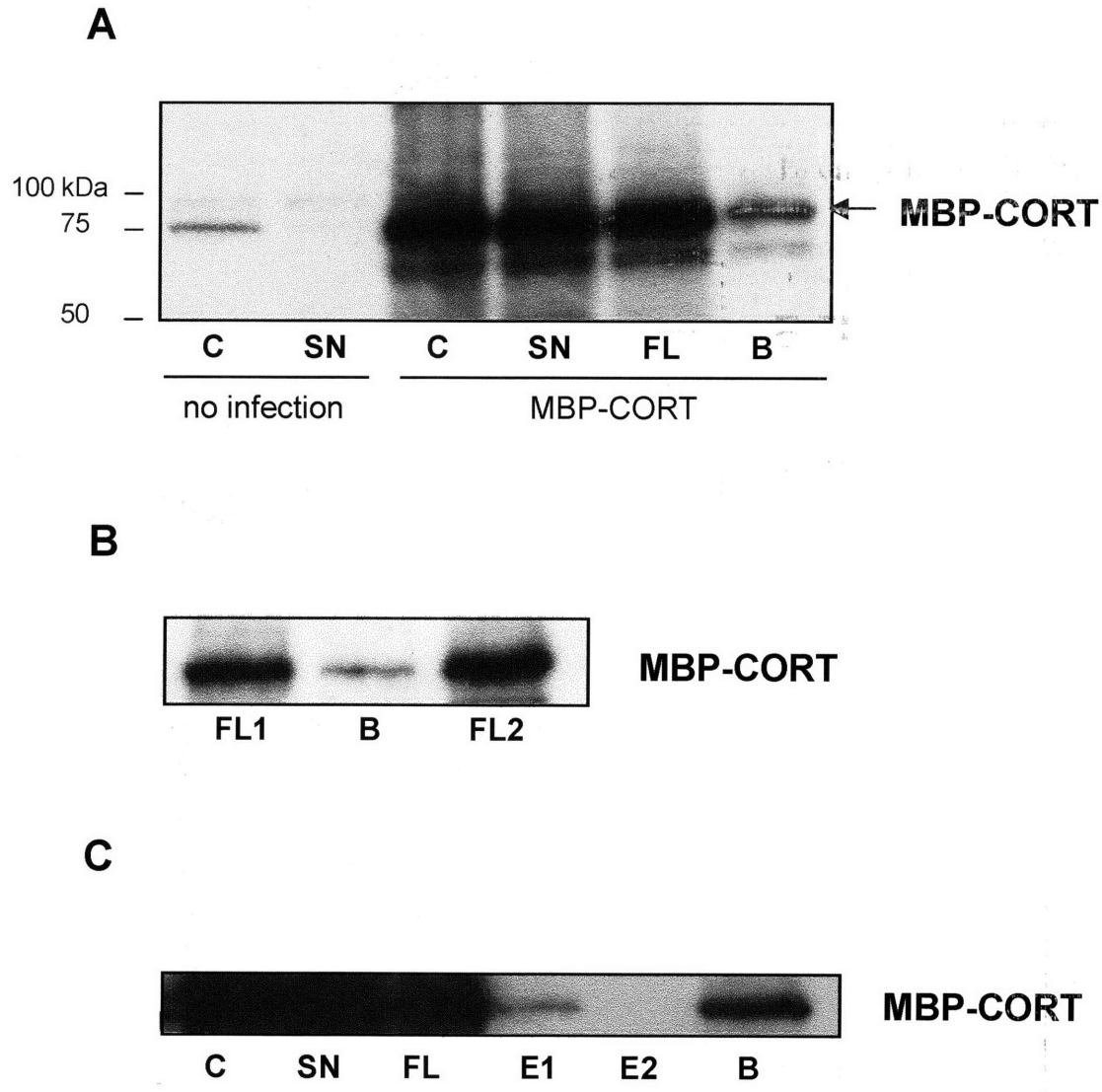

Figure 1. Expression and Purification of MBP-CORT in Baculovirus-infected *Sf9* Cells

Lanes are labeled as C, crude lysate, SN, cleared lysate, FL, flow-through, B, beads, E, eluate. All membranes were probed with anti-MBP. A. Infection with baculovirus encoding MBP-CORT produced a band of approximately 73 kilodaltons, which is the predicted size for MBP-CORT. The majority of MBP-CORT does not bind to the beads and remains in the flow-through sample. Beads lane shows beads sample post-incubation. B. Flow-through sample (FL1) from an identical experiment to (A) was incubated with amylose beads. Flow-through (FL2) from this second incubation still contained most of the MBP-CORT. Beads lane shows beads sample after the second incubation. C. Very little MBP-CORT elutes after a 3.5 hour incubation with maltose (E1). No additional MBP-CORT elutes after a 24 hour elution (E2). Beads lane shows beads sample after elution. C, SN, and FL lanes were overexposed in order to see band in eluate and bead lanes.

MATERIALS AND METHODS

Sf9 cells were routinely grown in suspension at 27°C. cDNA encoding full-length *cort* was subcloned into a derivative of pFastBac1 containing an N-terminal maltose binding protein (MBP) tag. Recombinant baculovirus was generated following protocols from the manufacturer (GIBCO-BRL). Infections were performed by addition of amplified baculovirus stock (at 1:100 (v/v) virus/cells) to growing *Sf9* cells (1×10^6 cells/ml).

Frozen *Sf9* cell pellets (from 25-ml infected cultures incubated for 39 hours) were resuspended in 300 µl of ice-cold lysis buffer (50 mM HEPES pH 7.6, 500 mM NaCl, 1 mM EGTA, 5 mM $MgCl_2$, 5 mM DTT, 10% glycerol, protease inhibitors), briefly sonicated, and cleared by microcentrifugation at 4°C for 15 minutes. Cleared lysates were added to 200 µl amylose resin (New England BioLabs), and samples were rotated at 4°C for 3 hours to allow binding. The beads were washed 3 times at follows: ice-cold lysis buffer, lysis buffer with 800 mM NaCl, lysis buffer (300 µl each). To elute, 500 µl lysis buffer containing 10 mM maltose was added to washed beads and incubated at 4°C for 3.5 or 24 hours.

Samples were resolved by SDS-PAGE and transferred to Immobilon-P membranes (Millipore). Blots were probed with rabbit anti-MBP serum (1:10,000, New England BioLabs). Horseradish peroxidase-conjugated secondary antibodies were used to detect bound anti-MBP. Protein was detected using ECL Plus (Amersham).

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Appendix Two:

Role of the Spindle Checkpoint and Phosphorylation in Regulation of CORT

The spindle checkpoint inhibits APC/C^{Cdc20} during metaphase of mitosis and in meiosis I (for review, see introduction of this thesis). We predicted that APC/C^{CORT} may also be under control of the spindle checkpoint in meiosis I, because it targets mitotic cyclins for degradation sequentially, suggesting that the complex is inhibited from targeting Cyclin B, Cyclin B3, and PIMPLES for degradation until release of the metaphase I arrest (Pesin and Orr-Weaver, submitted). The spindle checkpoint is thought to inhibit Cdc20 in part through direct binding of BubR1 and Mad2 (Fang et al. 1998; Tang et al. 2001).

We tested whether BubR1 or Mad2 co-immunoprecipitate with CORT in ovary extracts enriched for mature Stage 14 oocytes. We did not observe co-immunoprecipitation of BubR1, although BubR1 is a large protein and did not completely transfer to the membrane during semi-dry transfer during Western blot analysis (data not shown). We also did not observe any co-immunoprecipitation of Mad2 with MYC-tagged CORT (Figure 1). These results suggest that CORT may not be regulated by the spindle checkpoint. However, spindle checkpoint inhibition of the APC/C takes place at or near kinetochores (Musacchio and Salmon 2007). It is possible we may not detect a localized association with co-immunoprecipitation from a whole ovary extract. A better approach may be to look for co-localization by immunofluorescence in oocytes.

Another mode of inhibition of APC/C^{Cdc20} by the spindle checkpoint is direct phosphorylation of Cdc20 by Bub1 (Tang et al. 2004). We looked for evidence of phosphorylation of CORT in ovary extracts enriched with mature St. 14 oocytes. We treated extracts with lambda phosphatase but did not observe any mobility shift of the

CORT band by Western blot (Figure 2). This assay would have benefited from a positive control to be sure that the phosphatase was active. However, we repeated this assay twice and observed the same results.

cks30A encodes one Drosophila homolog of the Cks proteins, small conserved proteins that are thought to stimulate Cdk-dependent phosphorylation of mitotic proteins (Pines 1996; Swan et al. 2005). Swan et al. proposed that *cks30A* mediates Cyclin A degradation through direct phosphorylation and activation of CORT (Swan et al. 2005). This assertion was based solely on the similarity of *cort* and *cks30A* phenotypes because both mutant ovaries contain elevated levels of Cyclin A. A subsequent study showed that the phenotype of misexpressing *cort* in the wing is suppressed in a $cks30A^{KO}$ background (Swan and Schupbach 2007).

We tested whether *cks30A* has an effect on the phosphorylation state of CORT. We looked for a mobility shift of CORT protein by Western blot in $cks30A^{KO}$ ovaries but did not observe any change compared to a heterozygous control (Figure 2). These results suggest that Cks30A does not directly regulate CORT through phosphorylation. Another possibility is that Cks30A promotes mitotic phosphorylation of core APC/C subunits, and this phosphorylation enhances CORT binding to the APC/C. This possibility may be more likely, since data from Xenopus support this model (Patra and Dunphy 1998; Fang et al. 1998; Kramer et al. 2000).

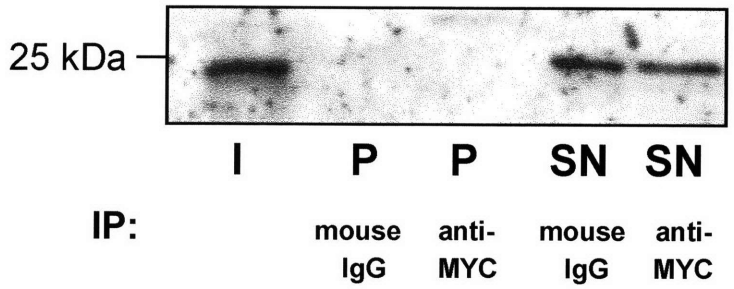

Figure 1. Mad2 does not co-immunoprecipitate with CORT
MYC-CORT was immunoprecipitated from whole ovary extracts from *UAS-myc-cort/ nos-gal4* females with anti-MYC or control mouse IgG, and immunoprecipitates were examined for the presence of Mad2. I, input, P, pellet, SN, supernatant.

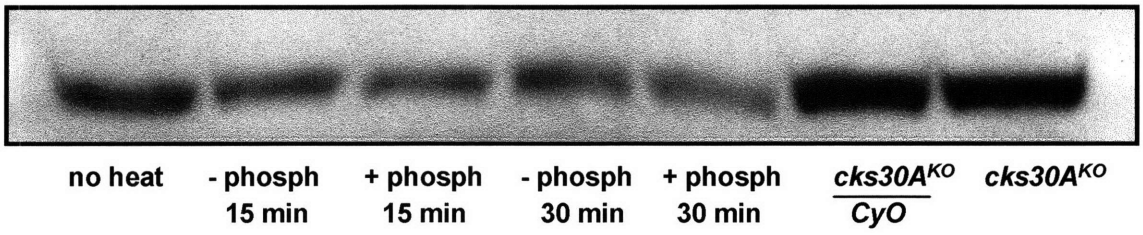

Figure 2. CORT is not phosphorylated in ovaries
Western blot showing mobility of CORT under different conditions. In first five lanes, wild-type *OrR* ovary extracts were treated with lambda phosphatase for 15 or 30 minutes at 30°C. One sample was not treated with phosphatase or heat as a negative control. Blot was probed with anti-CORT serum. In the last two lanes ovary extracts from *cks30A*^{KO} mutants or heterozygous controls were probed for CORT.

MATERIALS AND METHODS

Co-immunoprecipitations and Western blotting were carried out as described in Chapter Two of this thesis. In Figure 2, 10% acrylamide gels were used. For detection of Mad2, affinity-purified anti-Mad2 antiserum (Rb1223) was used at 1:3500 (Logarinho et al. 2004).

For phosphatase experiments, whole ovaries were dissected from wild-type *OregonR* females, and 30 µl whole ovary volume was homogenized in 140 µl phosphate buffer and treated as described (Clarke et al. 2005).

cks30A^{KO} alleles have been described (Swan et al. 2005). *cks30A*^{KO} and heterozygous control extracts were made from whole ovaries as described in Chapter Two of this thesis.

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