A Meiosis-Specific Form of the Anaphase Promoting Complex/Cyclosome Regulates the Oocyte-to-Embryo Transition in Drosophila

by

Zachary James Whitfield

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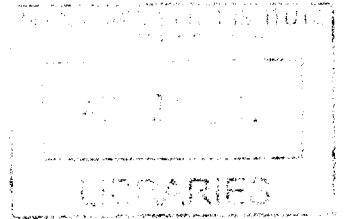in Partial Fulfillment of the Requirements for the Degree of

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Signature of Author...
Department of Biology
August 8th, 2013

Certified by...
Terry L. Orr-Weaver
Professor of Biology
Thesis Supervisor

Accepted by.........................
Stephen P. Bell
Chair, Committee on Graduate Students
Department of Biology

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Abstract

Cell cycle transitions during mitosis and meiosis must proceed in an irreversible manner. At the heart of this is the Anaphase Promoting Complex/Cyclosome (APC/C), an E3 ubiquitin ligase. The APC/C targets its substrates for degradation, and thus progresses the cell cycle irreversibly forward. Many substrates of the APC/C have been identified in mitosis, but how the APC/C regulates meiosis is less well understood. The Drosophila gene *cortex* encodes a female, meiosis-specific activator of the APC/C. We set out to identify specific substrates of APC^{Cort} both genetically and biochemically. A genetic screen identified five deficiencies that suppress an arrest caused by low APC^{Cort} activity. In some cases, these deficiencies could be narrowed to regions containing only a few genes. IP/mass-spectrometry was also performed to identify interactors of Cortex. One hit was Matrimony, a potent inhibitor of Polo kinase during prophase I. Cort and Mtrm can interact directly *in vitro*, while a mitotic APC/C activator, *fzy/cdc20*, cannot. Mtrm proteins levels are drastically reduced upon completion of meiosis, and this reduction is dependent on *cort*. When expressed in cell culture, Cort causes a proteasome dependent decrease in Matrimony protein levels. Cort and Mtrm also interact genetically, and overexpression of Mtrm in the early embryo causes developmental defects in a subset of embryos. This work contributes to our understanding of the meiotic cell cycle and the specific regulation that distinguishes it from mitosis.

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

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I first would like to thank my advisor, Terry Orr-Weaver, for her guidance, support, and advice through the years. Terry was always willing to take time out of her day to discuss anything from the latest experiments to career advice. She fostered a wonderful lab environment in which I really learned to be a scientist. Perhaps I'm most grateful for Terry's ability to keep a level head, ensuring that even when things were not going as well as I would have liked, there was always a way forward.

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Table of Contents

Chapter One

Introduction

I. The role of the Anaphase Promoting Complex/Cyclosome in Cell Division

Mitosis is the incredibly intricate and beautiful process by which a cell divides to form two daughter cells. During mitosis, duplicated sister chromosomes are captured by the microtubules of the mitotic spindle and aligned along the metaphase plate. Once all sisters are attached and aligned properly, anaphase is rapidly induced and chromosomes are segregated toward opposite ends of the cell. Cytokinesis then serves to divide the cell in half, creating two cells with identical genomic content. Importantly, mitosis (and the entire cell cycle for that matter) proceeds only in a forward, irreversible direction. At the heart of this irreversibility is active protein degradation.

Degradation of cell cycle regulators at the appropriate time during mitosis ensures irreversible mitotic progression. Entry into mitosis and maintenance of the mitotic state is, in part, regulated by the mitotic Cyclins (Cyclin A and Cyclin B) together with their Cyclin dependent kinase (Cdk) partner. A host of substrates are phosphorylated by these complexes to drive entry and progression through mitosis (Errico et al., 2010). Chromosome condensation, an early event in mitosis, is driven initially by Cyclin A/Cdk1 and later by Cyclin B/Cdk1 by phosphorylation of condensin subunits (Kimura et al., 1998; Morgan, 2007). Upon entry into the nucleus, Cyclin B/Cdk1 phosphorylates nuclear lamins of the nuclear envelop to promote one of the hallmarks of mitotic entry, nuclear envelope breakdown (NEB) (Heald and McKeon, 1990; Peter et al., 1990). Cyclin B also promotes centrosome separation and spindle assembly through phosphoregulation of kinesin motors (Blangy et al., 1997). Cyclin/Cdk activity eventually leads to replicated sister chromatids aligned along the metaphase plate by the mitotic spindle. High Cyclin/Cdk activity maintains cells in this

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mitotic state, and so there must be efficient mechanisms to shut off Cyclin/Cdk activity upon anaphase onset and mitotic exit.

The Anaphase Promoting Complex/Cyclosome (APC/C) is responsible for ubiquitylating many cell cycle regulators (including the mitotic cyclins), marking them for degradation by the 26S proteasome and thus progressing the cell cycle forward. Degradation of Cyclins lowers Cdk activity so that mitotic exit can proceed normally, but not before Cyclin/Cdks themselves activate the APC/C (Hershko et al., 1994; Rudner and Murray, 2000). Phosphorylation by Cyclin/Cdk plays a role in activating the APC/C as well as regulating co-activator binding (Schteinberg et al. 1999). These co-activators (discussed below), bind the APC/C and confer to it substrate specificity. The APC/C also takes a more direct role in promoting anaphase onset by ubiquitylating the separase inhibitor securin (Nasmyth, 2002). Once securin is degraded, separase cleaves the cohesin complex which normally encircles replicated sister chromatids. Cleavage of cohesin allows sisters to physically separate at anaphase and segregate away from each other (Gruber et al., 2003; Haering et al., 2008; Haering et al., 2002). These are just a few examples of a myriad of known APC/C substrates that must be recognized and ubiquitylated at a precise time during the cell cycle. Recent structural studies of the APC/C have given insight into APC/C substrate recognition and regulation.

II.   Structure of the APC/C

The Anaphase Promoting Complex is a large, multi-subunit E3 ubiquitin ligase. It is composed of around 15 subunits (Pines, 2011) (Table 1-1), which together form an

overall triangular shape containing a central cavity (Schreiber et al., 2011)

(Figure 1-1A). The individual subunits of the APC/C act in protein binding/recruitment,

catalysis, and structural support. The tetratricopeptide repeat (TRP) containing subunits

primarily serve as scaffolding, with Apc3/Cdc27 and Apc8/Cdc23 also helping to bind

Apc10/Doc1 and/or the APC/C co-activators (to be discussed below) (Matyskiela and

Morgan, 2009; Vodermaier et al., 2003; Wendt et al., 2001). The TPR subunits

(Apc3/Cdc27, Apc6/Cdc16, Apc8/Cdc23) exist as dimers (Schreiber et al., 2011), with

the N-termini of Cdc16, Cdc27, and Cdc23 directing homodimerization (Zhang et al.,

2013; Zhang et al., 2010a; Zhang et al., 2010b). These three dimers stack on top of

one another in the APC/C, forming a left-handed suprahelix (Zhang et al., 2013). This

suprahelix of TPR subunits forms the backbone and roof of the central cavity (often

called the arc lamp) (Figure 1-1A).

Another section of the APC/C consists of the Cullin and RING subunits Apc2 and

Apc11 respectively. Apc2 binds Apc11, which in turn activates the E2 ubiquitin

conjugating enzyme (Tang et al., 2001). Between these two domains is the PC repeat

containing Apc1, which serves to bridge the catalytic (APC2 and 11) and structural

(TPR subunits) halves (Pines, 2011; Thornton et al., 2006; Vodermaier et al., 2003).

Recombinant protein complexes of portions of the *S. cerevisiae* APC/C form stable

structures without needing to be within in the context of the entire APC/C (Schreiber et

al., 2011). This supports the APC/C having distinct regions of activity within the holo-

enzyme. These subunits make up the majority of the APC/C, but additional core and

non-core subunits are needed for substrate recruitment (Figure 1-1A). Known

interactions between all APC/C subunits are listed in Table 1-1.

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Table 1

APC/C Subunit	Interactors	Reference(s)
Apc1	Apc8/Cdc23	(Schreiber et al., 2011)
	Apc4	(Schreiber et al., 2011)
	Apc2	(Schreiber et al., 2011)
	Apc10/Doc1	(Buschhorn et al., 2011)
Apc2	Apc1	(Schreiber et al., 2011)
	Apc11	(Thornton et al., 2006)
	Apc10/Doc1	(Thornton et al., 2006)
	Cdh1	(Schreiber et al., 2011)
	Cdc20	(Schreiber et al., 2011)
Apc3/Cdc27	Apc3/Cdc27	(Zhang et al., 2010b)
	Apc6/Cdc16	(Thornton et al., 2006)
	Apc9*	(Schreiber et al., 2011; Thornton et al., 2006).
	Apc10/Doc1	(Buschhorn et al., 2011)
	Cdc20	(Izawa and Pines, 2011; Matyskiela and Morgan, 2009)
	Cdh1	(Izawa and Pines, 2011; Matyskiela and Morgan, 2009)
	Apc7	(Yu et al., 1998)
Apc4	Apc5	(Schreiber et al., 2011)
	Apc1	(Schreiber et al., 2011)
	Apc15/Mnd2	(Hall et al., 2003; Uzunova et al., 2012)
Apc5	Apc4	(Schreiber et al., 2011)
	Apc8/Cdc23	(Schreiber et al., 2011)
	Apc15/Mnd2	(Hall et al., 2003; Uzunova et al., 2012)
Apc6/Cdc16	Cdc16	(Zhang et al., 2010a)
	Cdc27	(Thornton et al., 2006)
	Cdc23	(Thornton et al., 2006)
	Cdc26	(Zhang et al., 2010a)
	Apc13/Swm1	(Schreiber et al., 2011)
	Apc10/Doc1	(Buschhorn et al., 2011)
Apc7 *	Cdc20	(Vodermaier et al., 2003)
	Cdh1	(Vodermaier et al., 2003)
	Apc3/Cdc27	(Yu et al., 1998)

Table 1-1 (continued on next page)

Apc8/Cdc23	Cdc23	Zhang 2013
	Cdc16	(Thornton et al., 2006)
	Apc13/Swm1	(Hall et al., 2003; Schreiber et al., 2011)
	Apc1	(Schreiber et al., 2011)
	Apc15/Mnd2	(Hall et al., 2003; Schreiber et al., 2011)
	Cdc20	(Izawa and Pines, 2011; Matyskiela and Morgan, 2009)
	Cdh1	(Izawa and Pines, 2011; Matyskiela and Morgan, 2009)
	Apc5	(Schreiber et al., 2011)
Apc9**	Apc3/Cdc27	(Schreiber et al., 2011; Thornton et al., 2006)
Apc10/Doc1	Cdc16	(Buschhorn et al., 2011)
	Apc1	(Buschhorn et al., 2011)
	Cdc27	(Buschhorn et al., 2011)
	Apc2	(Thornton et al., 2006)
Apc11	Apc2	(Thornton et al., 2006)
Cdc26	Cdc16	(Zhang et al., 2010a)
Apc13/Swm1	Apc8/Cdc23	(Hall et al., 2003; Schreiber et al., 2011)
	Apc6/Cdc16	(Schreiber et al., 2011)
Apc15/Mnd2	Apc8/Cdc23	(Hall et al., 2003; Schreiber et al., 2011)
	Apc4	(Hall et al., 2003; Uzunova et al., 2012)
	Apc5	(Hall et al., 2003; Uzunova et al., 2012)
Apc16	None Reported	
Cdc20	Apc2	(Schreiber et al., 2011)
	Cdc23	(Izawa and Pines, 2011; Matyskiela and Morgan, 2009)
	Cdc27	(Izawa and Pines, 2011; Matyskiela and Morgan, 2009)
	Apc7	(Vodermaier et al., 2003)
Cdh1	Apc2	(Schreiber et al., 2011)
	Cdc23	(Izawa and Pines, 2011; Matyskiela and Morgan, 2009)
	Cdc27	(Izawa and Pines, 2011; Matyskiela and Morgan, 2009)
	Apc 7	(Vodermaier et al., 2003)

Table 1-1. List of Interactions Between Subunits of the APC/C
A list of known interactions between subunits of the APC/C, including activators.
* Apc7 is found only in metazoans.
**Apc9 seems to be specific to *S. cerevisiae*

A

B
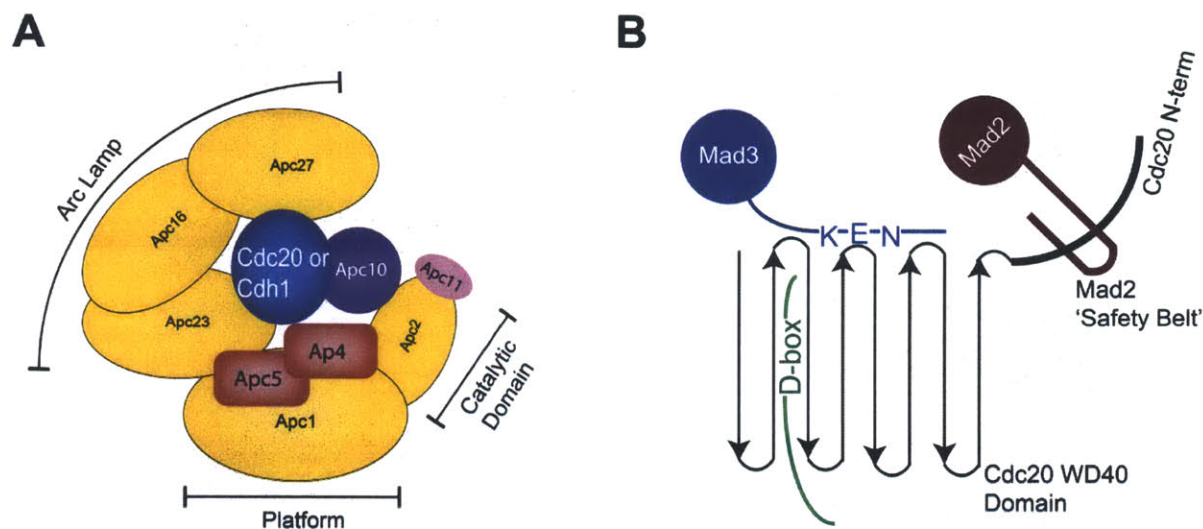
Figure 1-1. Layout of the APC/C and MCC-Cdc20

A) A 2D schematic of select subunits found within the APC/C. Cdc23, Cdc16, and Cdc27 exist as homodimers. Substrates are bound between the activator (Cdc20 or Cdh1) and Apc10. The substrate can then be ubiquitylated by an E2 ubiquitin conjugating enzyme recruited by Apc11.

B) A diagram representing highlights of the MCC's interaction with Cdc20. Cdc20's WD40 repeats (thin black lines/arrows) are viewed in 2D from the side. The KEN box binding site of Mad3 (and likely substrates themselves) lies on top of the propeller structure. The proposed D-box receptor lies between two separate blades of the propeller. Cdc20's N-terminus (with the Mad2-binding motif) is sequestered by the Mad2 'safety belt.' Based on structures in Chao et al. 2012 and Tian et al. 2012.

III. Recognition of APC/C substrates

a. Role of APC/C activators and Apc10/Doc1

The APC/C makes use of co-activators, subunits that are not part of the core APC/C enzyme but instead bind at precise times during the cell cycle to aid in substrate recruitment. In mitosis, these activators are Cdc20 and Cdh1 (Visintin et al., 1997). There are common features shared by all APC/C activators. The most notable is the presence of a WD40 domain, which forms a bladed propeller structure typically used to mediate protein interactions (Smith et al., 1999). The WD40 domain of APC/C activators is thought to mediate substrate binding (see below). Additionally, APC/C activators contain a C-box and an IR-tail that stabilizes activator binding to the APC/C (Schwab et al., 2001; Vodermaier et al., 2003). Despite these common motifs, the substrates targeted by APC^{Cdc20} and APC^{Cdh1} do not completely overlap.

Mutational analysis and crosslinking studies showed that the WD40 domain of Cdh1 binds directly to its substrates, specifically the destruction box of Cyclin B (Kraft et al., 2005). The destruction box is one of many motifs recognized by APC co-activators, and is discussed in more detail below. Other studies also implicated components of the APC/C itself, specifically Apc10/Doc1, in APC/C substrate recognition (Carroll and Morgan, 2002; Passmore et al., 2003). APC/C lacking Apc10/Doc1 is significantly reduced in its ubiquitylation processivity, and has a decreased affinity for substrates but not components of the APC/C itself (Carroll and Morgan, 2002; Passmore et al., 2003). Different residues within Apc10/Doc1 were also identified that affected either its association with the APC/C or the APC/C's ability to bind substrates (Carroll et al., 2005). More recently though, our understanding of the precise way in which the APC/C

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and it activator recognize substrates has been greatly facilitated by structural analysis of the APC/C.

b. Structural insight into binding of APC/C substrates

Two studies confirmed structurally that Cdh1 and Apc10/Doc1 form the destruction box (discussed below) receptor in budding yeast and/or vertebrates. Buschhorn and colleagues (Buschhorn et al., 2011) performed single particle electron microscopy (EM), along with crosslinking and 3D reconstruction to gain a better picture of Apc10/Doc1's location within the APC/C. Using *in vitro* translated Apc10/Doc1 containing specific crosslinkable residues, Apc10/Doc1 was found to contact/interact with Cdc27, Cdc16, and Apc1. Cryo-negative EM was then used to determine the precise location of Apc10/Doc1 within the APC/C itself (both human and yeast). Comparing structures of APC/C^{WT}, APC/C$^{\Delta APC10/Doc1}$, or APC/C where Apc10/Doc1 was N-terminally tagged, Apc10/Doc1 was located at the surface of the cavity where ubiquitylation is believed to occur.

Next, substrate binding to human APC/C with Cdh1 as the co-activator was analyzed. By cryo-EM, the APC/C substrate Hsl1 was found to bind stably between Cdh1 and Apc10/Doc1. Interestingly, this caused a structural shift of Apc2 toward Cdh1 near its substrate binding site (Buschhorn et al., 2011). A similar study was also performed by da Fonseca and colleagues primarily using yeast APC/C (da Fonseca et al., 2011). They too found Apc10/Doc1 and the activator Cdh1 situated close to each other in the central cavity of the APC/C. Interestingly, the density of Cdh1 decreased in APC/C$^{\Delta\,Apc10/Doc1}$, hinting at a possibility for structural stabilization between Apc10/Doc1 and APC/C co-activators (at least Cdh1). Consistent with the results of Buschhorn et al.,

a fragment of Hsl1 also was found to intercalate between Apc10/Doc1 and Cdh1. Furthermore, the presence of Hsl1 causes a shift of Cdh1 toward Apc10/Doc1, and additionally results in new connections between the two proteins.

c. Role of specific motifs in APC/C mediated recognition

The APC/C relies on the presence of particular motifs within its substrates for recognition. The destruction box (D-box) (Glotzer et al., 1991) (RxxLxx(I/V/x)xN) and the KEN box (Pfleger and Kirschner, 2000) (KEN) are the two most well-known and well-characterized APC/C recognition motifs. The above structural studies addressed the role these motifs played in the APC/C's ability to recognize the Hsl1 substrate. Because Hsl1 contains both a KEN and D-box, deFonseca et al. examined synthetic peptides containing either a D-box or a KEN box. The D-box peptide behaved similarly to Hsl1, while the synthetic KEN box peptide caused a shift of Cdh1 toward Doc1/Apc10 but without forming new connections. Lastly, a direct interaction between Doc1/Apc10 and a Dbox containing peptide was shown by HSQC NMR. This interaction is dependent on the presence of a Dbox and does not happen with a KEN box containing peptide (da Fonseca et al., 2011).

The above study indicates the KEN box is not recognized as well by the Cdh1-Apc10 receptor. Specific studies focusing on the binding of KEN box substrates to the APC/C are needed to understand alternative substrate recognition mechanisms employed by the APC/C. Studies of the structure of the Mitotic Checkpoint Complex, a cellular machine used to inhibit the APC/C, have begun to address binding of the KEN box by the APC/C (Chao et al., 2012) (see below).

In addition to the D-box and KEN box, other varied recognition motifs have also been identified (Pines, 2011). The Xenopus chromokinesin Xkid is required for chromosome congression, but it is degraded by the APC/C at anaphase onset (Funabiki and Murray, 2000). This degradation is dependent on a non-canonical recognition motif GxEN (Castro et al., 2003). When this motif is mutated, Xkid is stabilized both in vivo and in vitro. Interestingly, peptides containing the GxEN motif are able to inhibit degradation of Cyclin B by competition, suggesting that the GxEN motif functions in APC/C substrate recognition (Castro et al., 2003). Another non-canonical APC/C motif was found in the meiosis-specific yeast protein Spo13 (Katis et al., 2004; Lee et al., 2002; Shonn et al., 2002; Sullivan and Morgan, 2007). The motif LxExxxN is necessary for Spo13 degradation by the APC/C at anaphase I. Mutation of this motif stabilizes the protein both in vitro and in vivo. Interestingly, stabilized Spo13 had little effect on progression through meiosis, but negatively affected mitosis (Sullivan and Morgan, 2007). Whether these motifs are recognized by the APC/C in similar ways to the D-box is yet to be determined, but should provide interesting insights into how the APC/C is able to recognize so many substrates at different times during the cell cycle.

IV. Spindle Assembly Checkpoint mediated inhibition of the APC/C

a. SAC inhibition of Cdc20 through the Mitotic Checkpoint Complex

Given the irreversible consequences of APC/C activity, there must be a mechanism to inhibit the APC/C until chromosome segregation is ready to occur. The spindle assembly checkpoint (SAC) exists for just this reason (Murray, 2011). Many core components of the SAC were initially identified genetically in screens for mutants that failed to arrest in the presence of microtubule poisons (Hoyt et al., 1991; Li and

Murray, 1991). The SAC functions by inhibiting the activity of Cdc20, the APC/C

activator responsible for triggering the onset of anaphase (Fang et al., 1998; Hwang et

al., 1998; Kim et al., 1998). Unattached kinetochores are bound by the SAC component

Mad1, which in turn recruits Mad2. Mad2 can exist in two states, open and closed. It is

thought that binding of Mad2 to Mad1 at unattached kinetochores induces a change

from the open to closed confirmation. Closed Mad2 (c-Mad2) not bound to Mad1 is able

to diffuse and inhibit Cdc20 (De Antoni et al., 2005; Kulukian et al., 2009; Morgan,

2007). Mad2 bound to Cdc20 (via Cdc20's Mad2 binding motif and the 'safety belt' of

Mad2) can help stabilize the formation of the mitotic checkpoint complex (MCC) (Chao

et al., 2012; Luo et al., 2002; Sironi et al., 2002). The MCC consists of Mad2,

Mad3/BubR1, Bub3, and Cdc20 (Murray, 2011). Mad3 contains a KEN box that is

essential to MCC inhibition of APC^{Cdc20}, likely by acting as a pseudo-substrate (Burton

and Solomon, 2007; King et al., 2007; Sczaniecka et al., 2008). Mechanistic insights

into the MCC's function, however, were lacking until recently.

b. Structural insights into MCC mediated inhibition of the APC/C

Structural studies have again elicited a deeper understanding of how the MCC

inhibits Cdc20 function. Chao et al. solved the structure of the *Schizosaccharomyces*

pombe MCC, consisting of Mad2, Mad3, and Cdc20 (Bub3 was left out due it its non-

essentiality in fission yeast) (Chao et al., 2012). As expected, Mad2 bound to Cdc20's

Mad2 binding motif using its 'safety belt.' Interestingly, the structure of the MCC reveals

both a KEN and D-box binding site on Cdc20. The KEN box of Mad3 sits atop the

WD40 propeller structure of Cdc20 (Figure 1-1B). The residues within Cdc20 that

contact the KEN box are conserved within Cdh1, hinting at a conserved mode of binding

to KEN boxes in substrates. Importantly, mutation of these KEN box contacting residues inhibits Cdc20's ability to activate the APC/C. This suggests two things. One, that these residues likely bind the KEN box in actual substrates, and two, that Mad3 functions as a pseudo-substrate inhibitor of the APC.

These structural MCC studies also shed light on specific residues involved in D-box recognition. Somewhat fortuitously, MCC crystallization caused the C-terminus of Mad3 to interact with the WD40 domain of a neighboring crystallized Cdc20 (Chao et al., 2012). This interaction fell between blades 1 and 7 of the Cdc20 WD40 repeat, and the authors hypothesized that the C-terminus of Mad3 was simulating a D-box peptide (Figure 1-1B). They went on to show that mutation of residues within these blades inhibited APC^{Cdc20} ubiquitylation of Securin. Again, this suggests that the space between blades 1 and 7 of the WD40 propeller may be the endogenous D-box receptor (Chao et al., 2012). Very similar KEN and D-box binding sites were found for human Cdc20, further suggesting these modes of substrate recognition are conserved evolutionarily (Tian et al., 2012) (Figure 1-1B).

c. APC/C mediated degradation while the SAC is active

The rigorous inhibition of the APC/C by the SAC creates a paradox with respect to how some of the APC/C's earlier substrates (namely Cyclin A) are still degraded normally while the SAC is active. Cyclin A binds to Cdc20 during prometaphase (SAC is active) and Cyclin A's N-terminus is required for its degradation (den Elzen and Pines, 2001; Geley et al., 2001; Jacobs et al., 2001; Wolthuis et al., 2008). Di Fiore and Pines found a precise region of Cyclin A's N-terminus that can bind to Cdc20 both *in vivo* and *in vitro* (Di Fiore and Pines, 2010). Furthermore, this fragment of Cyclin A is able to

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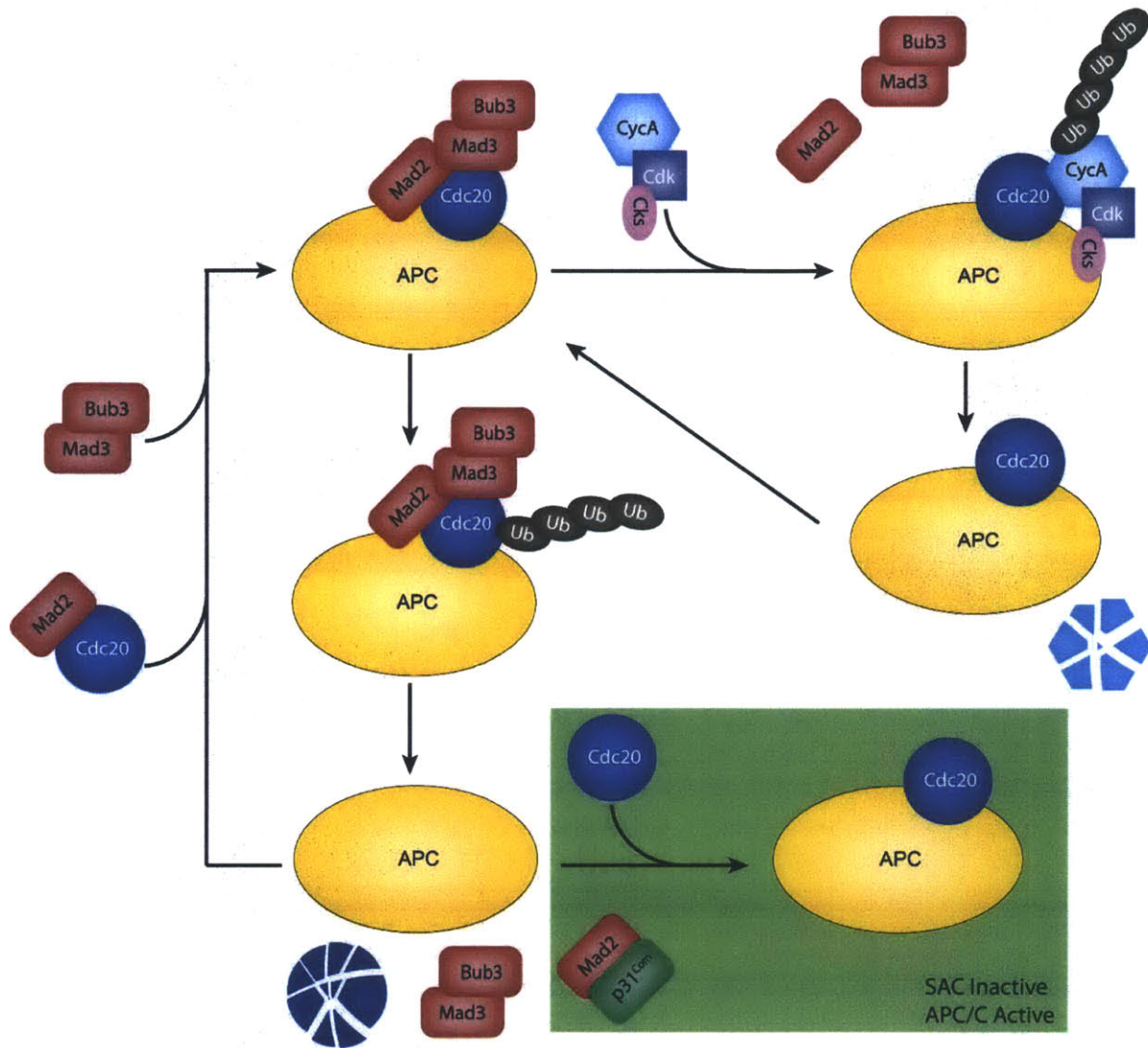
Figure 1-2. Inhibition and Release of Cdc20 by the MCC

Cdc20 is inhibited by the MCC (upper left). Constant turnover of MCC-Cdc20 by Cdc20 autoubiquitylation/degradation and new protein synthesis allows rapid APC/C activation upon fulfillment of the SAC and Mad2 inhibition by p31[comet] (bottom right). During prometaphase, Cyclin A is still ubiquitylated and degraded because it can bind Cdc20 and compete it away from the MCC (upper right). After Cyclin A degradation, free APC[Cdc20] is quickly inhibited by the MCC.

compete with the SAC (specifically BubR1) for binding of Cdc20. This sets up a model whereby Cyclin A displaces the SAC complex from Cdc20, and together with Cdk1 and its small subunit Cks1 (which is required for degradation of Cyclin A) (Swan and Schüpbach, 2007; Wolthuis et al., 2008), bind to the APC/C and trigger Cyclin A ubiquitylation (Di Fiore and Pines, 2010) (Figure 1-2, top half). Other APC/C substrates are degraded also while the SAC is active, such as Nek2A. Nek2A binds to the APC/C directly as a dimer, but this binding is unable to complete away the SAC (Sedgwick et al., 2013). It is therefore proposed that Nek2A binds to a pool of APC/C without MCC bound, and then is ubiquitylated by a small fraction of Cdc20 that has escaped the MCC during prometaphase (Sedgwick et al., 2013).

d. Release of APC^{Cdc20} inhibition by the SAC

There are multiple mechanisms by which Cdc20 inhibition is relieved once the SAC is fulfilled. Numerous studies have implicated the ubiquitylation and/or degradation of Cdc20 itself by the APC/C during prometaphase as a means to escape from MCCs (Nilsson et al., 2008; Reddy et al., 2007; Visconti et al., 2010). This creates a cyclical model where Cdc20 is inhibited by the MCC, degraded (thus disassembling MCCs), and then resynthesized (to again be inhibited by the MCC). Once the SAC is fulfilled, the MCC is inhibited, and resynthesized Cdc20 can rapidly activate the APC/C and initiate anaphase onset (Figure 1-2). Recently, a number of groups have reported on the role of Apc15/Mnd2 in promoting Cdc20 turnover for inactivation of the SAC (Foster and Morgan, 2012; Mansfeld et al., 2011; Uzunova et al., 2012). Apc15 was shown to be a core component of the APC/C, and knockdown of Apc15 lengthens the time from nuclear envelope breakdown to anaphase (Mansfeld et al., 2011; Uzunova et al., 2012).

Lack of Apc15 also results in stabilization of the levels of Cyclin B1 and Cdc20 itself, but not Cyclin A, indicative of a role in the SAC. Inhibition of the SAC alleviated the delay in Apc15 knockdown cells, and it was further shown that Apc15 regulates the APC/C specifically through the SAC (Mansfeld et al., 2011; Uzunova et al., 2012). Without Apc15, components of the MCC remain bound to the APC/C, hinting at its role in MCC turnover. Apc15/Mnd2 is required for efficient APC^{MCC} driven auto-ubiquitylation of Cdc20 (Foster and Morgan, 2012; Uzunova et al., 2012), which directly leads to increased turnover of Cdc20 (degradation and release from MCCs) (Uzunova et al., 2012). Constant turnover of Cdc20 allows it to rapidly activate the APC/C after fulfillment of the SAC. Without turnover, Cdc20 remains bound to MCCs, and cannot properly trigger release from the SAC (Foster and Morgan, 2012; Mansfeld et al., 2011; Uzunova et al., 2012).

In addition to autoubiquitylation/degradation of Cdc20, other mechanisms exist to help relieve SAC inhibition. p31^{comet} is known to structurally mimic the open conformation of Mad2, thus binding c-Mad2 and relieving the closed form's inhibition of Cdc20 (Habu et al., 2002; Mapelli et al., 2006; Xia et al., 2004; Yang et al., 2007) (Figure 1-2). The protein Cuedc2 was also found to play a part in SAC inactivation. After being phosphorylated by Cdk1, Cuedc2 is able to bind to Cdc20 and promote its release from the MCC (Mad2 specifically) (Gao et al., 2011).

V.   Role of the APC/C outside of mitosis

a. The APC/C in regulation of the endocycle and other differentiated cell types

The APC/C has also been shown to play roles outside of the canonical mitotic cell cycle (Eguren et al., 2011). In Drosophila, the gene *morula* encodes Apc2, part of

the catalytic domain of the APC/C (Kashevsky et al., 2002b; Reed and Orr-Weaver, 1997). Female-sterile alleles of *morula* revealed defects in endoreduplication of the polyploid nurse cells in female ovaries. Without Morula/Apc2, endocycling nurse cells aberrantly reenter the mitotic state (Kashevsky et al., 2002b; Reed and Orr-Weaver, 1997). Furthermore, mutants of Fzr/Cdh1 are completely unable to enter the endocycle in Drosophila larval salivary glands (Sigrist and Lehner, 1997). Similarly, mice mutant for Cdh1 have placentas with underdeveloped endocycling trophoblast giant (Garcia-Higuera et al., 2008; Li et al., 2008). Cdh1 has been further implicated in even more developmental processes, including muscle differentiation and neuron development (Eguren et al., 2011; Gieffers et al., 1999; Li et al., 2007). In Drosophila, Cdh1acts within neuromuscular synapses to regulate both growth and transmission (van Roessel et al., 2004). Furthermore, Cdh1 regulates glial migration in Drosophila neurons, through regulation of the adhesion molecule Fas2 (Silies and Klambt, 2010). More recently, Cdc20 has also been shown to play a role in neurogenesis, regulating both dendrite morphogenesis and presynaptic differentiation (Kim et al., 2009; Yang et al., 2009).

b. The APC/C in meiosis

Perhaps the most well-known non-mitotic role of the APC/C is its regulation of meiosis (Pesin and Orr-Weaver, 2008).In contrast to mitosis, meiosis serves to create cells with half of their normal genetic content. In higher organisms, these cells are the sperm and egg, which will eventually fuse to once again produce a diploid organism. Meiosis is composed of two consecutive cell divisions without an intervening S-phase. Before meiosis, sister chromatids are replicated during pre-meiotic S-phase, similar to

S-phase in mitosis. The first division of meiosis, known as meiosis I, begins with the

recently replicated homologous chromosome pairs undergoing meiotic recombination.

These recombination events serve to physically link the homolog pairs together,

ensuring they align opposite each other at the metaphase plate of meiosis I and

properly segregate away from each other at anaphase I. Meiotic recombination is aided

by formation of the synaptonemal complex, a meshwork of proteins that forms a

transient, zipper-like structure between homologous chromosome pairs and stabilizes

them while meiotic recombination takes place (Fraune et al., 2012).

Pre-meiotic S-phase, as well as meiotic recombination, occurs within the nucleus

of the cell. After completion of recombination, nuclear envelope breakdown (also known

as germinal vesicle breakdown (GVBD) in oocytes) occurs and the meiotic divisions

begin. With newfound access to the DNA, the meiotic spindle takes shape and aligns

homologous chromosome pairs along the metaphase plate in meiosis I. In contrast to

mitosis, meiosis I requires segregation of homologs, and thus kinetochores of

duplicated sister chromatids must orient toward the same pole (known as mono-

orientation). Anaphase I onset at the hands of the APC/C (see below) triggers

homologs to segregate away from each other. During meiosis II, a more mitotic like

division occurs in which sister chromatids segregate from each other. Because no S-

phase takes place between meiosis I and II, the genetic content of cells completing

meiosis is halved.

The APC/C is known to play key roles in entry, maintenance, and progression

through meiosis. APC^{Cdc20} regulates meiotic entry in budding yeast by degrading Ume6

(Mallory et al., 2007). Ume6 is a transcriptional inhibitor that shuts down early meiotic

genes during vegetative growth (Strich et al., 1994). Cdc20-mediated degradation of Ume6 is required to induce the proper meiotic transcriptional program, whereas non-degradable, D-box mutant forms of Ume6 block meiotic induction (Mallory et al., 2007). Recently, complete degradation of Ume6 by APC^{Cdc20} was found to occur as a two-step process (Mallory et al., 2012). Ume6 is first degraded upon a switch from glucose to acetate-containing media, and this degradation is dependent on its acetylation by Gcn5. The remaining Ume6 is degraded upon entry into meiosis (nitrogen deprivation) and its association with Ime1 (Mallory et al., 2007; Mallory et al., 2012). Other early steps in meiosis, such as meiotic recombination, are also regulated by the APC/C (Okaz et al., 2012; Trickey et al., 2008).

During oogenesis in higher organisms, progression of meiosis must be coordinated with development. Female meiosis typically arrests at two distinct points: first during prophase I and second at metaphase I or II (discussed below). Maintenance of the prophase I arrest in mouse by the APC/C seems to rely primarily on the activity of APC^{Cdh1}. Mice with Cdh1 specifically knocked out in the oocyte show precocious resumption of meiosis from prophase I (Holt et al., 2011). Additionally, these oocytes contain increased levels of Cyclin B1, but not Securin or Cdc25B. These data are consistent with Cdh1 being necessary for maintaining low levels of Cyclin B1 to prevent premature meiotic resumption. However, knockdown of Cyclin B1 in Cdh1 knockout oocytes did not completely restore proper prophase I arrest, and so there are likely other APC^{Cdh1} substrates that need to be kept at low levels (Holt et al., 2011). It also has been proposed that competition between endogenous APC/C substrates (securin and Cyclin B) plays a role in managing the time of prophase I release (and cell cycle

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transitions in general) (Marangos and Carroll, 2008). Overexpression of securin in prophase I arrested mouse oocytes stabilized Cyclin B1 and increased the rate of germinal vesicle breakdown/prophase I release. Conversely, knockdown of securin resulted in decreased Cyclin B1 levels and delayed prophase I release. Marangos and Carroll suggest the presence of other endogenous APC/C substrates (such as Securin) competes the APC/C away from Cyclin B, thus helping to finely tune its levels (Marangos and Carroll, 2008).

c. Meiosis-specific activators of the APC/C

The APC/C also functions during the remainder of meiosis, where it targets some of the same substrates for degradation as it does in mitosis (Pesin and Orr-Weaver, 2008). Although much of the APC/C's function during meiosis is regulated by Cdc20 and Cdh1, meiosis-specific APC/C activators have been found in a number of organisms (Table 1-2). *S. cerevisiae* contains Ama1 (Cooper et al., 2000), *Schizosaccharomyces pombe* has Mfr1(Blanco et al., 2001), and Fzr2 (Jacobs et al., 2002) and Cortex (Chu et al., 2001; Page and Orr-Weaver, 1996) are found in *Drosophila melanogaster* (further discussed below). Study of these proteins has shed light on some of the meiosis-specific roles of the APC/C.

The budding yeast APC/C activator Ama1 initially was characterized as having a role later in meiosis during spore formation (Cooper et al., 2000). It capable of degrading Clb1 (Cooper et al., 2000), Pds1/Securin (Oelschlaegel et al., 2005; Penkner et al., 2005), and Cdc20 itself at the end of meiosis (Tan et al., 2011). However, more recently Ama1 has been shown to play a role in prophase I of meiosis as well (Okaz et al., 2012). *ama1Δ* cells progress through prophase I and begin assembly of the meiosis

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I spindle much faster than wild-type cells. Normally, prophase I is prolonged to permit homolog pairing and recombination. Release from prophase I is usually dependent on the activity of the transcription factor Ndt80 (Chu and Herskowitz, 1998). In *ndt80Δ ama1Δ* double mutants, however, cells exit prophase I and assemble a meiotic spindle. Additionally, these cells accumulate the mitotic Cyclins as well as Cdc5/Polo kinase, whose expression normally requires Ndt80. These data indicate deletion of *ama1* bypasses the requirement of Ndt80. Okaz et al. found that APC^{Ama1} normally suppresses the levels of the mitosis-inducing Ndd1 protein during early prophase I, thus delaying induction of mitotic proteins (M-phase Cyclins and Cdc5). Ama1 was also found to trigger proteolysis of the M-phase Cyclins and Cdc5 directly. Additionally, Ama1 plays a role in proper formation of the synaptonemal complex (through suppression of Cdc5), proper segregation of homologs at meiosis I (through its ability to promote SC formation and proper crossing over), and for activation of the Recombination Checkpoint in response to double-strand breaks (Okaz et al., 2012).

Mnd2 is an inhibitor of APC^{Ama1}, whose activity is crucial to regulate meiotic events. Ama1 protein appears in pre-meiotic S-phase, but its activity must be partially repressed to prevent premature ubiquitylation and degradation of securin (Oelschlaegel et al., 2005; Penkner et al., 2005). It is currently unknown how Ama1 can be inhibited by Mnd2, but still carry out its role in prophase I. It is possible the situation mirrors that of Cdc20, which still targets Cyclin A and Nek2A for degradation during prometaphase despite being inhibited by the SAC (Di Fiore and Pines, 2010; Sedgwick et al., 2013) (discussed above). Nevertheless, Mnd2 binds and inhibits APC^{Ama1} during prophase I,

Table 2

Activator	Species	Substrates	Timing of Degradation	Reference(s)
Ama1	*S. cerevisiae*	Clb1	Prophase I	(Okaz et al., 2012)
		Clb4	Prophase I	(Okaz et al., 2012)
		Cdc20	Anaphase II/Meiotic Exit	(Tan et al., 2011)
		Pds1/Securin	Prophase I (normally inhibited by Mnd2)	(Oelschlaegel et al., 2005; Penkner et al., 2005)
		Ndd1	Prophase I	(Okaz et al., 2012)
		Cdc5/Polo	Prophase I	(Okaz et al., 2012)
		Ssp1	Cytokinesis/meiotic exit	(Diamond et al., 2009)
Mfr1	*S. pombe*	Cdc13/Cyclin B	Mainly Anaphase II (possibly anaphase I also)	(Blanco et al., 2001), (Kimata et al., 2008)
Cortex	*D. melanogaster* (female-specific)	Cyclin A	Oocyte Maturation (prometaphase I)	(Pesin and Orr-Weaver, 2007; Swan and Schüpbach, 2007)
		Cyclin B	Egg activation (likely anaphase of MI and MII)	(Pesin and Orr-Weaver, 2007; Swan and Schüpbach, 2007)
		Cyclin B3	Egg activation (likely anaphase of MI and MII)	(Pesin and Orr-Weaver, 2007; Swan and Schüpbach, 2007)
		Pimples/Securin	Egg activation (likely anaphase of MI and MII)	(Pesin and Orr-Weaver, 2007)
Fizzy-related 2	*D. melanogaster* (male-specific)	Unknown	N/A	N/A

Table 1-2. Meiosis-Specific APC/C activators and Their Substrates

A list of currently known meiosis-specific APC/C activators, their known substrates, and time of action.

but the protein is lost from meiotic cells at anaphase II (Oelschlaegel et al., 2005; Penkner et al., 2005). This loss of Mnd2 likely allows Ama1 to control spore formation and degradation of Cdc20 (Oelschlaegel et al., 2005; Penkner et al., 2005; Tan et al., 2011) later in meiosis. Further work is necessary to explore how Mnd2 prevents APC^{Ama1} from targeting substrates like Pds1/Securin, but allows it to target its prophase I substrates (Ndd1, Cdc5, and Clb4).

Another known meiosis-specific APC/C activator exists in *Schizosaccharomyces pombe*. Mfr1 (sometimes referred to as Fzr1) mRNA and protein appear only during late meiosis I and into meiosis II. It is required for the degradation of Cdc13/Cyclin B at anaphase II, whose downregulation as a mitotic Cyclin is necessary for meiotic exit and spore formation (Blanco et al., 2001). Mfr1 is regulated by the specific APC/C inhibitor, Mes1 (Izawa et al., 2005; Kimata et al., 2011). Based on double mutant studies of Mes1 and Cdc20/Slp1or Mes1 and Fzr1/Mfr1, Kimata et al. determined that Mes1's primary function in meiosis I is to inhibit Fzr1/Mfr1. *In vitro* however, Mes1 can equally inhibit APC/C ubiquitylation mediated by either Slp1 or Fzr1/Mfr1. However, APCSlp1 can uniquely mediate the degradation of Mes1, thus overcoming its inhibition. Therefore, it is proposed that Mes1 functions primarily as an inhibitor of Mfr1/Fzr1 until meiosis II. Upon Mes1's complete degradation during meiosis II, APC$^{Mfr1/Fzr1}$ is freed to mediate onset of anaphase II.(Kimata et al., 2011).

A remarkable situation is found in Drosophila, where not only do two meiosis-specific activators exist, but they also show sex specific expression. While the male meiosis specific activator *fizzy-related 2 (fzr2)* is not well characterized (Jacobs et al., 2002), the female, meiosis-specific regulator *cortex (cort)* has been better studied.

Cortex was shown to be an activator of the APC/C (Chu et al., 2001; Pesin and Orr-Weaver, 2007; Swan and Schüpbach, 2007), and is required for proper progression through meiosis in female Drosophila (Page and Orr-Weaver, 1996; Swan and Schüpbach, 2007). *cort* mutant females are viable but completely sterile. In contrast to wild-type oocytes that normally arrest at metaphase I and then complete meiosis after egg activation (Horner and Wolfner, 2008b), *cort* mutant eggs arrest indefinitely after activation at metaphase II (Page and Orr-Weaver, 1996). Cdc20/Fizzy also is active during Drosophila oogenesis, and leaky/hypomorphic alleles of *fizzy* cause arrest at anaphase II (Swan and Schüpbach, 2007). Only mutation of both *cort* and *fzy* causes significant arrest in meiosis I. This led to the hypothesis that Cort and Fzy serve functionally redundant roles in meiosis I, but have non-overlapping roles during meiosis II (Swan and Schüpbach, 2007). The requirement of Cortex specifically during meiosis means it likely plays a unique role in promoting the oocyte-to-embryo transition.

VI.   The Oocyte-to-Embryo Transition

a. Overview

During the oocyte-to-embryo transition, an egg ultimately becomes capable of supporting the rapid mitotic divisions of early embryogenesis. Early embryos are typically transcriptionally silent, and so their proper development relies on 'stockpiles' of nutrients, mRNAs, and proteins synthesized during oogenesis. This stockpiling of molecules occurs concurrently with meiotic progression, and so must be coordinated with development. Two major developmental transitions contribute to the oocyte-to-embryo transition (in most sexual organisms): oocyte maturation and egg activation. Degradation and synthesis of both mRNA and protein are actively controlled during

these processes to ensure the correct molecules are loaded into the embryo. Numerous regulatory networks exist to coordinate meiosis with development of the oocyte.

b. Prophase I and Meiotic Maturation

During prophase I, an oocyte begins to prepare itself for progression through the remainder of meiosis. DNA recombination between homologous chromosome pairs sets the oocyte up for proper chromosome segregation during meiosis I (Kurahashi et al., 2012). After completing recombination, oocytes typically arrest in diplotene of prophase I (Lesch and Page, 2012). During this time an oocyte generates tremendous maternal stockpiles of mRNAs and proteins, reflected by a period of physical growth.

The growth of an oocyte is coordinated by cross-talk between the oocyte itself and its surrounding somatic cells (together termed a follicle) (Von Stetina and Orr-Weaver, 2011). In rodents, many factors have been implicated in the initiation of oocyte growth including Kit Ligand and Leukemia Inhibitory Factor, as well as numerous factors from the TGF-Beta family (Knight and Glister, 2006). Without proper soma-germline crosstalk and oocyte growth, defects in meiotic progression and chromosome segregation occur. LHβCTP mutant mice exhibit an abnormal endocrine signaling environment surrounding their follicles. This directly leads to disrupted follicular growth. (Hodges et al., 2002; Hunt et al., 2003).

To ensure proper coordination of meiotic progression with development, and to allow adequate time for growth, the prophase I arrest must be properly maintained. This arrest can last from days in Drosophila to decades in humans (Von Stetina and Orr-Weaver, 2011), and so a robust mechanism is required to prevent premature prophase I

exit. Originally deemed Maturation Promoting Factor, Cyclin B/Cdk1's activity must be kept off to prevent re-entry into meiosis (Nurse, 1990; Sagata, 1996). The activity of the Anaphase Promoting Complex/Cyclosome (APC/C) acts to keep Cyclin levels (and thus Cdk activity) low (Holt et al., 2011) (discussed above). Additionally, mammalian oocytes must maintain high levels of Cyclic AMP (cAMP). cAMP activates Protein Kinase A (PKA), which in turn activates an inhibitor of Cdk1 (Wee1) and inhibits an activator (Cdc25) (Verlhac et al., 2010; Von Stetina and Orr-Weaver, 2011).

Drosophila oocytes also contain a variety of mechanisms to properly maintain the prophase I arrest. The Cdk inhibitor Dacapo is differentially regulated between the oocyte itself and its supporting nurse cells. Within the oocyte Dacapo is maintained at high levels to inhibit CycE/Cdk2, thus preventing re-replication (Hong et al., 2003). Additionally, the mitotic Cyclins are kept at low levels in the oocyte through the action of the translation repressor Bruno. In the absence of Bruno, oocytes exit meiosis, accumulate mitotic Cyclins, and begin to divide mitotically. It is hypothesized that Bruno acts together with the APC/C to downregulate Cyclin levels in developing ovarian cysts (Kashevsky et al., 2002a; Sugimura and Lilly, 2006). Mutants of the gene Matrimony (Mtrm) prematurely undergo Germinal Vesicle Breakdown (GVBD) and exit the prophase I arrest (Xiang et al., 2007). *Mtrm* mutants were initially discovered as being haplo-insufficient for achiasmate (non-recombinant) chromosome segregation, and Mtrm was later characterized to function as an inhibitor of Polo kinase (Harris et al., 2003; Xiang et al., 2007). In the absence of Matrimony, Polo is prematurely activated and likely activates Cdc25, thus activating CycB/Cdk1 and triggering GVBD.

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The signal to exit prophase I properly and resume meiosis differs between organisms. In mouse, a cyclical hormonal surge of luteinizing hormone (LH) triggers the resumption of meiosis. This pathway is activated initially in the somatic cells of the follicle, and the message is transmitted through gap junctions to the oocyte (Von Stetina and Orr-Weaver, 2011). This triggers an intricate, multifaceted cascade ultimately leading to reduced levels of cAMP, and thus increased Cdk activity (Norris et al., 2009; Zhang et al., 2008).

In Drosophila, the initial signal that triggers meiotic maturation remains unknown. However, many of the players involved in exit from prophase I are beginning to be elucidated (Figure 1-3A). Similar to mammals, the Cdk1 activating phosphatase Cdc25/Twine is at the center of this pathway. Twine mutants are greatly delayed in exiting prophase I (Von Stetina et al., 2008; Xiang et al., 2007). The protein kinase Greatwall (Yu et al., 2004) directly phosphorylates and activates the small phosphoprotein alpha-Endosulphine, which in turn antagonizes the protein phosphatase PP2A (Gharbi-Ayachi et al., 2010; Mochida et al., 2010; Rangone et al., 2011). Inhibition of PP2A allows for activated Cdc25 to build up, thus activating Cdk1 and leading to prophase I exit (Glover, 2012).

c. Establishment and maintenance of the secondary meiotic arrest

After release of the initial prophase I arrest, most organisms go on to arrest at a secondary point in meiosis. In most vertebrates, this secondary arrest occurs in metaphase of meiosis II. Insects, however, come to their secondary arrest at metaphase I (Von Stetina and Orr-Weaver, 2011). No matter the point in meiosis the
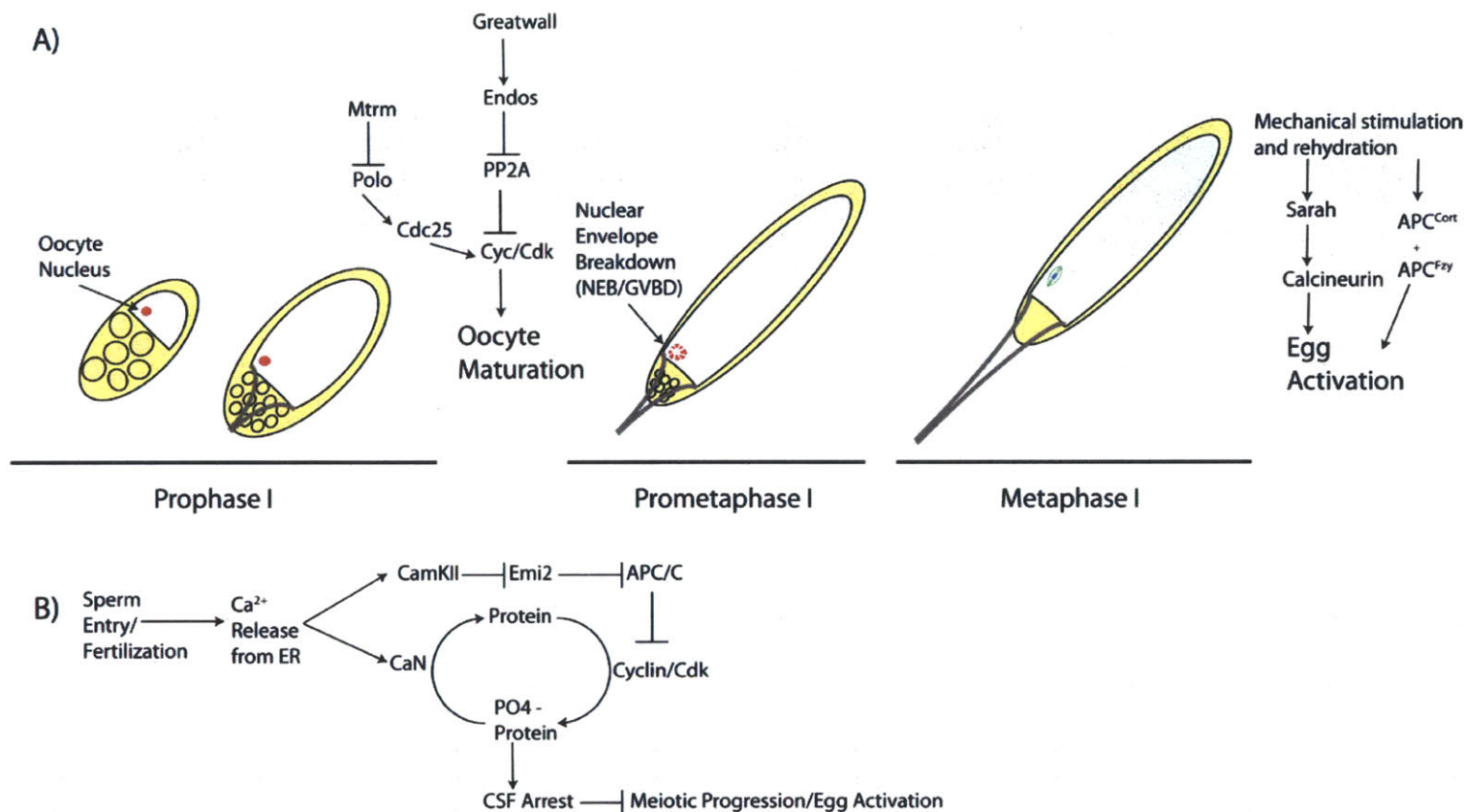
Figure 1-3. The Oocyte-to-Embryo Transition in Drosophila and Vertebrates
A) Depiction of meiotic maturation and activation in *Drosophila melanogaster*. Meiosis initially arrests in prophase I, and then comes to its secondary arrest at metaphase I. The nuclear envelope (red) breaks down upon release from prophase I. The metaphase I spindle is shown with microtubules (green) and DNA (blue).

B) Outline of egg activation in vertebrates. CSF arrest is maintained through the action of Cyclin/Cdk complexes. Phosphorylation of a host of proteins, including Mos kinase and the nuclear lamins, maintains the meiotic arrest. Fertilization triggers release of calcium and activation of CamKII and CaN. These proteins lead to the inhibition of Cdk activity and a reversal of the egg's global phosphorylation state.

secondary arrest occurs, it must be properly maintained to retain coordination between development and meiotic progression. The existence of a 'cytostatic factor' responsible for maintaining the arrest of frog oocytes was originally hypothesized by Masui and Markert (Masui and Markert, 1971). The cytostatic factor was shown to involve the Mos/MapK cascade many years later (Sagata et al., 1989). Since then, many more factors have been found to play a role in the CSF arrest of various organisms, including the APC/C inhibitor Emi2, the spindle assembly checkpoint (SAC), and Cdk2 (Gabrielli et al., 1993; Grimison et al., 2006; Schmidt et al., 2005). In short, Mos kinase triggers a phosphorylation cascade leading to the activation of the $p90^{RSK}$ kinase. $p90R^{RSK}$ inhibits APC/C activity through activation of the SAC as well as Emi2 (Horner and Wolfner, 2008b). More recently in mouse oocytes, the role of maintaining proper steady state phosphorylation of Cdk1 (independent of Cyclin degradation) has also been implicated in properly maintaining the CSF arrest (Oh et al., 2013).

In Drosophila, maintenance of the metaphase I arrest also is reliant on some of these mechanisms. Mutants for a component of the spindle assembly checkpoint, Mps1, do not arrest in metaphase I and proceed directly to anaphase I (Gilliland et al., 2007). Additionally, other components of the SAC show defects in female meiosis (Malmanche et al., 2007), though whether these effects are by inhibition of the APC/C has been called into question (Batiha and Swan, 2012). Physical forces must also be maintained between the chromosomes at the metaphase I plate to ensure proper arrest in Drosophila oocytes. Mutants in two genes involved in meiotic recombination (*mei-9* and *mei-218*) show reduced frequency of crossover formation, and these mutants eventually bypass the secondary metaphase I arrest (McKim et al., 1993). Without the

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tension generated at bi-oriented kinetochores with proper chiasmata, the metaphase I arrest is not maintained (Jang et al., 1995).

d. Egg Activation

Egg activation is the process by which an oocyte at its secondary arrest re-initiates and completes meiosis (Horner and Wolfner, 2008b). This process requires fertilization in vertebrates but is independent of fertilization in insects. In vertebrates, fertilization sets off a cascade of events to trigger the resumption and completion of meiosis (Figure 1-3B). In mouse, sperm is thought to bring in an activated phospholipase that eventually leads to the release of Ca^{2+} from the ER (Saunders et al., 2002; Swann and Lai, 2013). Calcium triggers relief of the CSF-mediated metaphase II arrest by activating calmodulin kinase II (CamKII) (Markoulaki et al., 2004; Tatone et al., 2002) and the phosphatase calcineurin (CaN) (Mochida and Hunt, 2007; Nishiyama et al., 2007). Active CamKII phosphorylates the APC/C inhibitor Emi2, leading to further phosphorylation by Polo kinase and eventual SCF-mediated degradation (Liu and Maller, 2005; Rauh et al., 2005). CaN likely acts by 'reversing' the phosphorylations helping to maintain the CSF arrest. In addition to dephosphorylating Fzy/Cdc20 and the APC itself, the levels of nearly all reactive anti-MPM2 and anti-phosphoSer-Pro species were increased when an inhibitor of Calcineurin was added to Ca^{2+} activated Xenopus egg extracts (Mochida and Hunt, 2007). These data indicate Calcineurin acts 'globally' to undo the mitotic phosphorylation state of the activated egg.

In Drosophila, exit from the secondary arrest at metaphase I is not dependent on fertilization. Rather, mechanical stimulation and osmotic pressure trigger resumption and completion of meiosis. Although the molecular mechanism linking mechanical

stimulation and meiotic resumption is not known (Horner and Wolfner, 2008b), some of the downstream players have been identified (Figure 1-3A). As discussed above, proper progression past the metaphase I arrest relies on the activity of both APC$^{Cdc20/Fzy}$ and APC^{Cort} (Page and Orr-Weaver, 1996; Swan and Schüpbach, 2007). Despite playing a pivotal role in vertebrate activation, no direct ties to calcium signaling have been made in Drosophila. However, mutants of the Calcineurin regulator *sarah* (*sra*) or germline clones missing components of Calcineurin itself (*canB2*, or both *canA* subunits) arrest at anaphase I after egg activation (Horner et al., 2006; Takeo et al., 2010; Takeo et al., 2006). These genetic data support Sra acting as an activator of Calcineurin. *In vivo*, Sarah is phosphorylated by glycogen synthase kinase 3β (*gs3kβ/shaggy*), which serves to activate Sra at egg activation. Presumably, the activation signal (mechanical stimulation and/or swelling of the egg) feeds into activation of *shaggy* through a yet unknown mechanism (Takeo et al., 2012). Calcium was also shown to play a more direct role in activation of Drosophila oocytes *in vitro*. Oocytes can be activated by application of hydrostatic or osmotic pressure (Horner and Wolfner, 2008a; Mahowald et al., 1983; Page and Orr-Weaver, 1997). Horner and Wolfner (Horner and Wolfner, 2008a) found that complete depletion of Ca^{2+} from the activating buffers severely inhibited activation, as judged by membrane cross-linking and protein translation. These data support a direct role for calcium signaling in Drosophila egg activation.

e. Post-transcriptional control of protein activity during egg activation

Given the key role phosphorylation plays in the regulation of egg activation, Krauchunas et al. investigated the phosphorylation changes that take place during egg activation in Drosophila (Krauchunas et al., 2012). Using 2D-gel analysis combined with

mass spectrometry, a number of proteins were identified whose phosphorylation state either increase or decrease during egg activation. Many of these proteins were determined to be necessary for proper oogenesis and/or embryogenesis (Krauchunas et al., 2012). Whereas protein phosphorylation is a key regulator of the oocyte-to-embryo transition, many other post-transcriptional and post-translational changes are known to occur also. These changes are particularly important given the transcriptional silence of oocytes and the early embryo.

At the mRNA level, poly-adenylation of transcripts' poly-A tails promotes their translation (Kronja and Orr-Weaver, 2011). The Cytoplasmic Polyadenlyation Element Binding protein (CPEB) binds a CPE in the 3'UTR of an mRNA, and recruits Symplekin and CPSF. Additionally, both a polyA polymerase and deadenylase are recruited prior to maturation, though deadenylase activity wins out and tail length is kept short. At oocyte maturation, a phosphorylation driven removal of the deadenylase allows poly-adenylation to occur (Barnard et al., 2004; Richter, 2007). This method of regulation allows specific proteins to increase upon meiotic maturation. The Drosophila Gld2-type polyA polymerase Wispy is required for proper progression through meiosis and early embryogenesis (Benoit et al., 2008; Brent et al., 2000; Cui et al., 2008). This is likely due to its role in promoting the polyadenylation of a number of meiotic and maternal genes such as *bicoid* (Benoit et al., 2008), *dmos* (Cui et al., 2008), and *cortex* (Benoit et al., 2008). Poly-A tails of sufficient length help promote efficient translation. Translation initiation and elongation are controlled by a wide variety of both inhibitors and activators (Kronja and Orr-Weaver, 2011). In addition to Bruno (mentioned above), Drosophila also utilizes the Pan Gu (PNG) kinase complex and the translational repressor Pumilio

(Pum) to regulate translation of messages (Gerber et al., 2006; Vardy and Orr-Weaver, 2007). Pum is known to associate with hundreds of mRNAs, with much focus on its regulation of Cyclin A and B translation (Gerber et al., 2006; Vardy and Orr-Weaver, 2007; Vardy et al., 2009). PNG enhances translation of Cyclin A during oogenesis and early embryogenesis by affecting poly-A tail length, likely by counteracting the activity of Pum (Vardy et al., 2009). In a similar manner, PNG also regulates translation of Cyclin B during egg activation, though this occurs by both poly-A dependent and independent mechanisms (Vardy and Orr-Weaver, 2007). Polyadenylation followed by translation directly leads to the appearance of proteins, but degrading proteins is also crucial in regulating the oocyte-to-embryo transition.

Protein degradation plays a key role during the oocyte-to-embryo transition in a large number of organisms (DeRenzo and Seydoux, 2004). In addition to advancing the meiotic cell cycle, as previously discussed, it is also thought that degradation of maternal proteins helps to set the stage for proper embryogenesis. In *C. elegans*, the microtubule severing proteins MEI-1 and MEI-2 act in complex to regulate meiotic spindle formation (Srayko et al., 2000), but are degraded upon the completion of meiosis (Clark-Maguire and Mains, 1994; Kurz et al., 2002). This degradation is necessary for robust mitotic spindles to form (in contrast to the shorter spindles of *C. elegans* meiosis) (Dow and Mains, 1998; Pellettieri et al., 2003). The proteins OMA-1 and OMA-2 function redundantly to promote oocyte maturation in *C. elegans* (Detwiler et al., 2001). OMA-1 and -2 continue to function at the 1-cell embryo stage, but are then rapidly degraded (Nishi and Lin, 2005; Pellettieri et al., 2003). Delayed degradation of OMA-1 results in embryonic lethality and cell fate specification problems between the C-

and EMS-blastomeres (Lin, 2003). It is clear that certain meiotic proteins must be cleared away for the proper embryogenesis to proceed.

VII.   Summary of Thesis

The existence of sex and meiosis-specific activators of the Anaphase Promoting Complex in Drosophila suggests a specific role for the APC/C during meiotic progression. I focused on Cortex, the female, meiosis-specific activator of the APC/C in *Drosophila melanogaster.* Given the key role of active protein degradation in meiotic progression, and Cortex's narrow window of developmental expression, identifying substrates of APC^{Cort} should give unique insight into meiosis-specific regulation of cell division.

In this thesis, I set out to find substrates of the Cortex form of the APC/C. First, I followed up on a genetic screen to identify substrates of APC^{Cort}. Next, mass spectrometry was used to identify Cortex interacting proteins. I then focused on one of the hits, Matrimony, and further characterize its relationship to Cortex. I found that degradation of Matrimony during the oocyte-to-embryo transition by APC^{Cort} is important for proper embryogenesis.

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Chapter Two

A genetic screen to identify substrates of the Cortex form of the Anaphase Promoting Complex/Cyclosome

Zachary J. Whitfield, Jillian A. Pesin, Chin Chin Yao, and Terry L. Orr-Weaver

[1]Whitehead Institute, Department of Biology, Massachusetts Institute of Technology, Nine Cambridge Center, Cambridge, MA, 02142

Abstract

The APC/C targets specific proteins for degradation to progress the cell cycle forward irreversibly. Identification of APC/C substrates sheds light on the specific regulatory mechanisms controlling the cell cycle. A deficiency screen was carried out to identify substrates of a female, meiosis-specific form of the APC/C (APC^{Cortex}). Five deficiencies were identified that suppressed the arrest in eggs with low APC^{Cort} activity. These large deficiencies were narrowed down to smaller regions, and finally individual genes were tested for their ability to suppress. Despite narrowing some regions down to only a few candidate genes, no single gene tested so far suppressed consistently. With the growing resources available to the Drosophila community, testing mutants of the remaining genes within these regions may soon be possible.

Introduction

The APC/C's role in mitosis has been and continues to be extensively characterized, but its role in meiosis is only just starting to be elucidated. Much of the APC/C's role in meiosis has stemmed from the study of meiosis-specific activators and their unique role in meiotic progression (Blanco et al., 2001; Okaz et al., 2012; Pesin and Orr-Weaver, 2007, 2008; Swan and Schüpbach, 2007). There are many aspects of meiosis that distinguish it from mitosis (particularly in oogenesis), and these unique aspects likely require meiosis-specific regulation. Here we highlight examples of regulators that may be subject to meiosis-specific degradation.

Whereas the job of mitosis is to segregate sister chromatids, meiosis must first segregate homologous chromosome pairs in meiosis I, and then segregate sister chromatids in meiosis II. The 'reductional' division of meiosis I is unique in that it requires both kinetochores of a single pair of sisters to align toward the same spindle pole. In *S. cerevisiae*, this is accomplished by the monopolin complex (Rabitsch et al., 2003; Toth et al., 2000). One hypothesis is that Monopolin serves as a sort of 'clamp,' crosslinking microtubule binding sites of sister kinetochores together to ensure they maintain the same orientation (Corbett et al., 2010; Watanabe, 2012). The meiosis-specific Spo13 protein is required for proper localization of the Monopolin complex (Katis et al., 2004; Lee et al., 2004). Similar sister kinetochore mono-orientation also occurs in higher eukaryotes.

Although monopolin has not been identified in higher eukaryotes, other proteins have been shown to facilitate sister kinetochore co-orientation. In some higher eukaryotes, the meiosis-specific cohesin subunit Rec8 is necessary for sister-chromatid

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co-orientation (Chelysheva et al., 2005; Severson et al., 2009). In Drosophila, the proteins Solo and Ord are known to be required for sister-chromatid cohesion and sister kinetochore co-orientation (Goldstein, 1980; Miyazaki and Orr-Weaver, 1992; Yan et al., 2010). The requirement for sister-chromatid co-orientation is a unique aspect of meiosis, and thus these components may need to be degraded after meiosis. Supporting this hypothesis, Spo13 is targeted for APC/C mediated degradation at anaphase I (though not by Ama1, the meosis-specific APC/C activator in budding yeast) (Sullivan and Morgan, 2007). Rec 8 is cleaved in meiosis, but it is not clear whether Solo or Ord are subjected to regulated degradation.

In addition to differences between mitosis and meiosis, there are also key differences between meiosis in oocytes and spermatocytes. Meiotic progression in oocytes contains distinct arrest points (in prophase I and metaphase I or II) that allow for the coordination of meiotic progression and development (Von Stetina and Orr-Weaver, 2011). The maintenance and release of these arrests is directly tied to the APC/C activity (Pesin and Orr-Weaver, 2008).

Another major difference between male and female meiosis is the structure of the meiotic spindle. In spermatocytes, a centriole-containing microtubule organizing center (MTOC) nucleates and organizes the spindle, as in mitosis. Female meiotic spindles are acentriolar, however, and assemble their spindles without the aid of complete centrosomes (Manandhar et al., 2005; Schatten, 1994). Depending on the organism, meiotic spindles in the female are nucleated and organized in varying ways. The ability of chromatin itself to direct spindle assembly was demonstrated in Xenopus egg extracts (Heald et al., 1996). In mouse oocytes, numerous MTOCs cluster together and

self-organize to form a bipolar spindle surrounding the DNA (Schuh and Ellenberg, 2007). These MTOCs are acentriolar, but do contain some components of the pericentriolar material (Carabatsos et al., 2000; Gueth-Hallonet et al., 1993; Palacios et al., 1993). In Drosophila, microtubule asters along with the motor protein Nonclaret disjunctional (Ncd) associate with the germinal vesicle and activate microtubule nucleation (Skold et al., 2005) , which eventually forms the arrested metaphase I spindle in Drosophila oocytes (Matthies et al., 1996; Theurkauf and Hawley, 1992). Protein degradation is known to play a role in centrjole maintenance during mitosis, where APC/C and SCF mediated degradation prevent centriole over-duplication (Pagan and Pagano, 2011). Given the need to rid the oocyte of centrioles during oogenesis, proteins responsible for centriole biogenesis could also be regulated by degradation in a meiosis-specific manner.

Two molecular pathways are known to play a role in triggering microtubule nucleation in female meiotic spindles. When GTP bound, the small GTPase Ran is thought to locally release spindle assembly factors (SAFs) to help trigger microtubule nucleation. The presence of Ran's GEF, RCC1, on chromatin ensures a gradient of high Ran-GTP near the DNA, and thus SAFs are released close to chromatin (Clarke and Zhang, 2008). Inhibition of the Ran gradient disrupts spindle assembly in a variety of organisms (Cesario and McKim, 2011; Dumont and Desai, 2012; Kalab et al., 2006; Ohba et al., 1999). In addition to the Ran gradient, the chromosomal passenger complex (CPC) also regulates microtubule nucleation. Its components help to localize the kinase Aurora B to the centromere and microtubules (Sampath et al., 2004; Tseng

et al., 2010), where it phosphorylates and inhibits inhibitors of spindle assembly (Budde et al., 2001; Kelly et al., 2007; Rivera et al., 2012).

In addition to these differences in spindle organization, the mechanisms of homolog segregation in meiosis I are dramatically different between Drosophila females and males. In oogenesis, as in most other examples of meiosis, homologs pair and then synapse via the synaptonemal complex (SC), followed by recombination between homologs (Szekvolgyi and Nicolas, 2010). In contrast, Drosophila males do not undergo meiotic recombination and instead pair at specific pairing sites independent of the SC (McKee et al., 2012). Such stark differences between recombination and pairing mechanisms in male and females could hint at a need for both sex- and meiosis-specific regulation.

The discovery of sex-specific, meiosis-specific APC/C activators in Drosophila (Chu et al., 2001; Jacobs et al., 2002; Page and Orr-Weaver, 1996) provides a unique opportunity to truly tease apart specific regulation that occurs not just during meiosis, but during female and male meiosis. As highlighted above, male and female meiosis differ in many regards, any of which has the potential to be regulated by the meiotic APC/C. There is a predicted Drosophila male meiosis activator of the APC/C, Fzr2, but its substrates have not been identified. Cortex, the female, meiosis-specific activator of the APC/C in Drosophila, is known to target Cyclin A, B, B3, and Pimples/Secruin for degradation during meiosis (Pesin and Orr-Weaver, 2007; Swan and Schüpbach, 2007). However, these substrates are also regulated by Fizzy/Cdc20 (Swan and Schüpbach, 2007) and thus it is likely that Cortex has other unique substrates to justify its incredibly specific expression pattern.

A genetic screen was carried out to find deficiencies on the third chromosome that suppress the meiotic arrest caused by *low* APC^{Cort} activity. This screen has the potential to identify substrates and negative regulators of Cortex whose loci reside on chromosome three.

Results

I.   Rational for suppression screen

The screen was carried not in a *cort* mutant background, but rather in eggs laid by females mutant for the gene *grauzone*. Grauzone serves as Cortex's dedicated transcription factor (Harms et al., 2000). Cortex seems to be Grauzone's only essential target, because the *grau* phenotype can be rescued by inserting additional copies of the *cortex* gene into the genome (Harms et al., 2000). *grau* mutant eggs arrest at metaphase II, phenocopying *cort* mutant eggs, further illustrating that Grauzone's only crucial target is *cort* (Page and Orr-Weaver, 1996). Importantly, *grau* eggs contain low levels of residual Cortex protein (Pesin and Orr-Weaver, 2007), and about 3% of *grau* eggs go on to develop (Harms et al., 2000), suggesting that these eggs are just on the cusp of having enough APC^{Cort} activity to properly develop. Therefore, *grau* serves as a sensitized background ideal for a genetic screen.

We hypothesized this screen would uncover substrates and negative regulators of APC^{Cort} activity. *grau* eggs contain some, but not enough, Cort protein to progress properly through meiosis. In this sensitized background, if a deficiency removes half of the genomic copies encoding an APC^{Cort} substrate, the reduction in substrate levels may 'free up' the little APC^{Cort} present to properly degrade its remaining substrates. Alternatively, if one substrate must be degraded to permit exit from meiosis, reduction of

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the levels of this protein could permit adequate degradation of the remainder for completion of meiosis. In either of these cases, the metaphase II arrest of *grau* mutants would be relieved and meiosis completed. A distinct mechanism could involve a repressor of Cort function. If a deficiency uncovers such an inhibitor of Cortex, the resulting increase in APC^{Cort} activity could allow for sufficient substrate degradation to occur and meiosis to be completed. A key aspect of this screen is that it requires the presence of residual APC^{Cort} activity, preventing the use of known *cort* alleles, which are nulls (Page and Orr-Weaver, 1996).

The screen was performed using a collection of deficiencies spanning the 3rd chromosome (representing 40% of the total genome). Suppression was scored by DAPI staining embryos and scoring for development past metaphase II (i.e. suppression of the *grauzone* arrest).

II. *Df(3R)p-XT103* identified as first hit in grauzone suppression screen

The deficiency screen covered 93% of the 3rd chromosome and required dominant suppression (Figure 2-1). This approach initially identified one strongly suppressing deficiency: *Df(3R)p-XT103* (Figure 2-2). Because the goal of this screen was to identify single genes responsible for the suppression, smaller deficiencies of the region eliminated by *p-XT103* were used to narrow down the suppressing region (Figure 2-3C). However, all three deficiencies spanning the region also suppressed, indicating that more than a single gene was responsible for suppression of *grau* by *p-XT103* (Pesin, 2007).
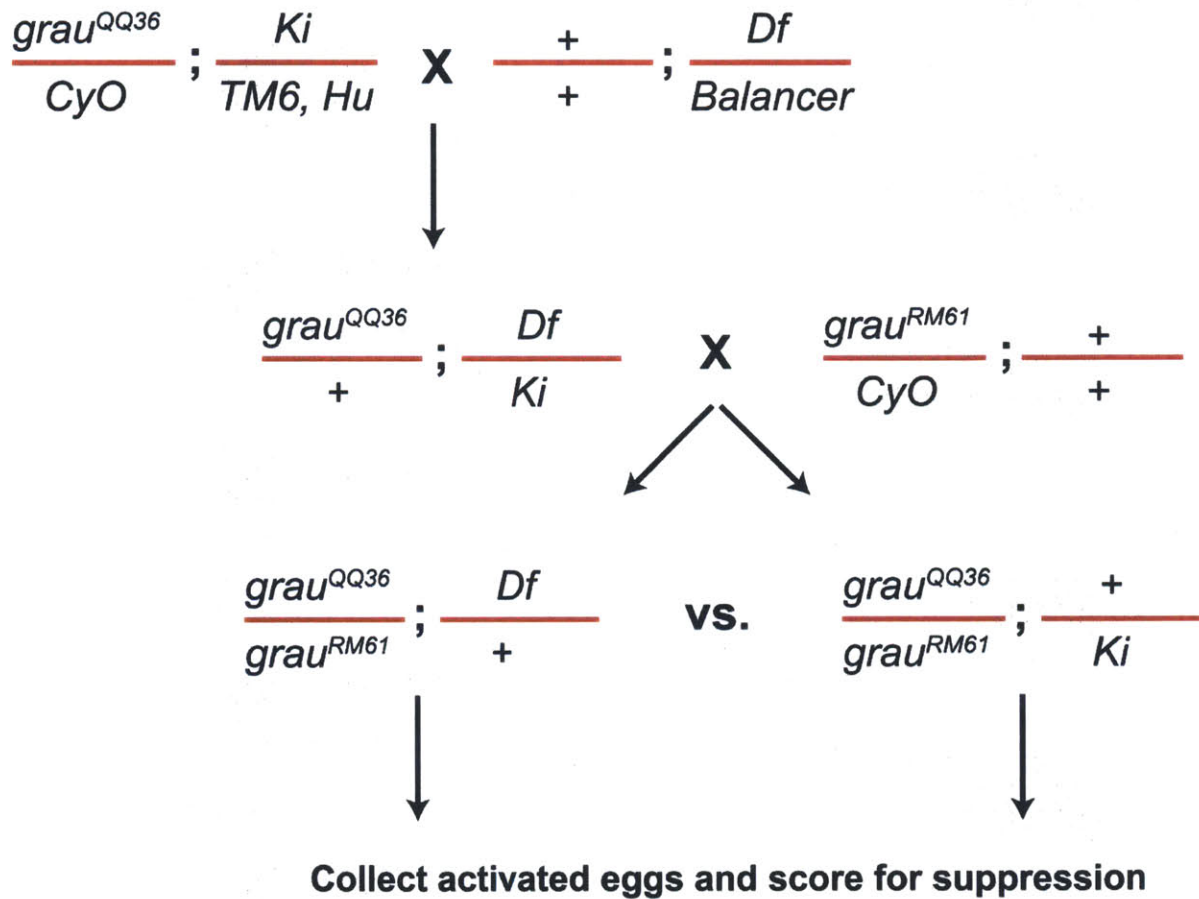
$$\frac{grau^{QQ36}}{CyO} \; ; \; \frac{Ki}{TM6, \, Hu} \quad \mathbf{X} \quad \frac{+}{+} \; ; \; \frac{Df}{Balancer}$$

$$\frac{grau^{QQ36}}{+} \; ; \; \frac{Df}{Ki} \quad \mathbf{X} \quad \frac{grau^{RM61}}{CyO} \; ; \; \frac{+}{+}$$

$$\frac{grau^{QQ36}}{grau^{RM61}} \; ; \; \frac{Df}{+} \quad \mathbf{vs.} \quad \frac{grau^{QQ36}}{grau^{RM61}} \; ; \; \frac{+}{Ki}$$

Collect activated eggs and score for suppression

Figure 2-1. Overview of Deficiency Screen Crosses

Schematic for testing a given deficiency's ability to dominantly suppress the metaphase II arrest in *grau* mutant eggs. Adult $grau^{QQ36/RM61}$ females were collected and sorted based on presence of the deficiency or the dominant marker *Kinked (Ki)* (known as the sibling control). Development of eggs laid by these females was monitored by immunofluorescence.

III. Verification of other potential hits identified in the screen

During the initial screen, other possible suppressing deficiencies were identified:

Df(3L)XG5, Df(3R)Exel6144, Df(3R)Exel9012, Df(3R)BSC137, Df(3R)H-B79, and

Df(3L)fz-GF3b. Of these, *Df(3L)XG5, Df(3R)Exel6144, Df(3R)Exel9012,* and

Df(3R)BSC137 were confirmed to suppress the *grauzone* arrest to varying degrees.

Figure 2-2 summarizes the locations of the five confirmed suppressing deficiencies. As

with *p-XT103*, it was necessary to narrow down the regions within these deficiencies

responsible for suppression. Given the large collections of stocks bearing specific

chromosomal deletions available to the Drosophila community (Cook et al., 2012; Roote

and Russell, 2012), smaller deficiencies were obtained that together spanned much of

the initial larger deficiencies (with the exception of *BSC137*, which has not been

followed up yet) (Figure 2-3).

IV. Narrowing down regions of the suppressing deficiencies

In all cases tested, the suppressing regions could be narrowed down to a smaller

region (Table 2-1, Figure 2-3). *Df(3L)XG5* was narrowed down to a region containing

about 34 genes. Interestingly, the smaller deficiency (*Df(3L)BSC575*) suppressed better

than the 'parental' *Df(3L)XG5* (Table 2-1). One possible explanation for this is that a

second gene within *XG5* (but not *BSC575*) normally has a positive role on Cortex

activity or protein levels. By reducing its copy number in *XG5*, Cort activity is even

further reduced and thereby suppression of *grau* is reduced as well. This is supported

by a specific increase in Cort protein levels in *grau$^{QQ36/RM61}$; BSC575/+* eggs (Figure 2-

4A). Cort protein levels remained low in *grau$^{QQ36/RM61}$; XG5/+* eggs (Figure 2-4B). Given

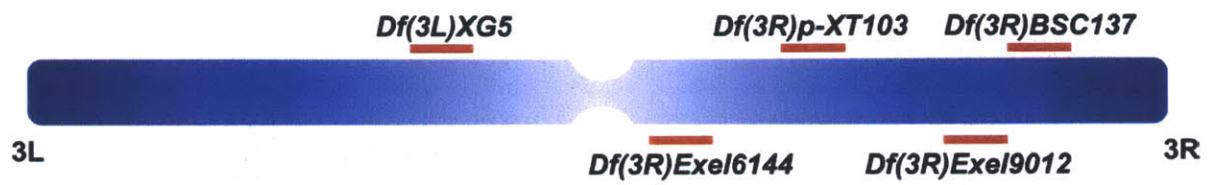these data, it would follow that the gene within both *XG5* and

Figure 2-2. Location of the Five Hits from the Deficiency Screen

The 3rd chromosome is indicated by the blue bar. Relative locations of the five

deficiencies are indicated by red lines (not to scale).

BSC575 is a negative regulator of Cortex protein levels (i.e. not likely to be a substrate).

Next, *Df(3R)Exel6144* was narrowed down to an interval containing only seven genes (*Df(3R)ED5177*). The levels of suppression by *Exel6144* and *ED5177* varied from experiment to experiment, but were usually comparable to each other. Finally, *Df(3R)Exel9012* was narrowed down to a genomic region containing only one gene. It is worth noting, however, that *Exel9012* was narrowed down by a *lack* of suppression in neighboring deficiencies, rather than finding a smaller deficiency that did suppress (as in the previous cases).

V.   Testing of individual mutants within the smaller suppressing regions

With the suppressing deficiencies narrowed down to more manageable sizes, it was possible to begin testing mutants of single genes within the deficiencies. Given that surrounding deficiencies did not show suppression of *grau*, *Exel9012's* region of suppression was narrowed down to the cytological location 94E11-94E13, a span of about 50kb (another deficiency, *Df(3R)BSC619*, contains the entirety of *Exel9012* and also suppresses) (Figure 2-3). Only one gene, *pointed* (*pnt*), is annotated within this region. *pnt* is a transcription factor involved in both eye and glial differentiation (Brunner et al., 1994; Klambt, 1993; O'Neill et al., 1994). A null allele of *pnt*, *pnt$^{\Delta 88}$* (Scholz et al., 1993), was tested for its ability to suppress the *grau* arrest. Unfortunately, *pnt$^{\Delta 88}$* was unable to suppress the metaphase II arrest in *grau* mutants (Table 2-2). Given that *pnt$^{\Delta 88}$* is a null allele, the lack of suppression should not be due to not using a strong enough allele. It is possible that the suppression observed with the deficiency is
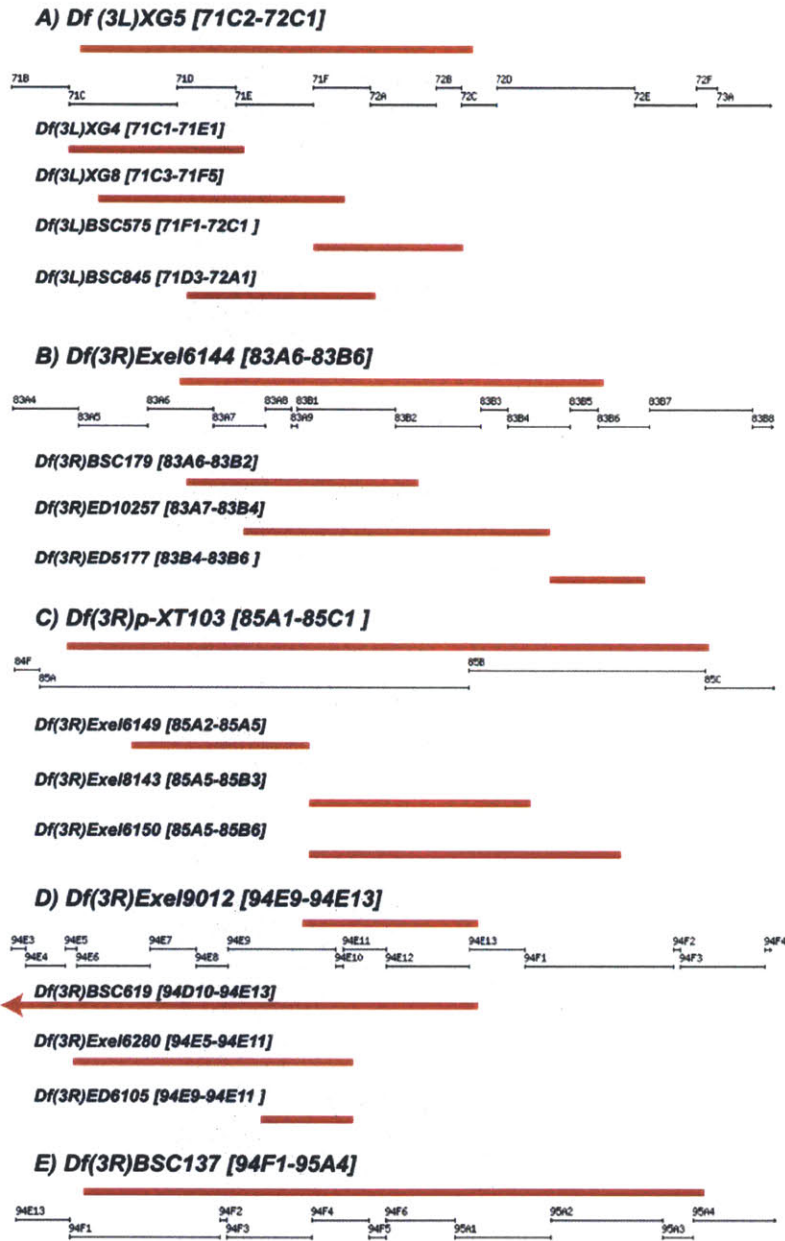
73

Figure 2-3. Narrowing Down of the Suppressing Deficiencies

The 'parental' deficiency is indicated at the top of each panel in red. Cytological location along the 3rd chromosome is shown beneath (courtesy of FlyBase). The relative locations of the smaller deficiencies used to narrow down the 'parental' deficiency are shown underneath.

due to removal of a regulatory region(s) of a gene whose coding sequence lies outside the suppressing region. The $pnt^{\Delta 88}$ allele may not affect this regulatory element.

About seven genes are uncovered by the suppressing deficiency *Df(3R) ED5177*, spanning a region of 24kb. Of these genes, two stood out as good candidates to be responsible for the suppression. The *asterless* (*asl*) gene is required for proper centriole assembly, and so could require specific regulation during the acentriolar meiotic divisions in females. The gene *krasavietz/extra bases* (*kra/exba*) is also uncovered by *ED5177*. Kra is a translational inhibitor involved in directing proper movement of CNS axons. Interestingly, females containing germline clones mutant for Kra stall in oogenesis, illustrating a role for Kra during oogenesis as well (Lee et al., 2007). Null (or very strong hypomorphic) alleles for both of these genes (*asl*^{MecD} and *kra*^{1}) (Blachon et al., 2008; Lee et al., 2007) were tested for their ability to suppress *grauzone*. However, neither of these two alleles could overcome the *grauzone* arrest (Table 2-2). Other genes also fall within the suppressing region of *ED5177*. The gene *Regena* (*Rga*) is a homolog of the transcriptional regulator Cdc36/Not2 in yeast, and is necessary for proper expression levels of numerous genes, including *white* (Frolov et al., 1998). A P-element insertion allele of *Rga*, *Rga*^{03834}, did not suppress the *grauzone* phenotype (Table 2-2). This allele is thought to be a null (Frolov et al., 1998; Temme et al., 2004), and so *Rga* is not likely to have been responsible for suppression in *ED5177*. *Another transcription unit* (*Atu*) also is found in this region, and this gene has been implicated in hemocyte proliferation through its genetic interaction with AML1-ETO (Sinenko et al., 2010). To date, no alleles of *Atu* have been tested for their ability to suppress *grauzone*.
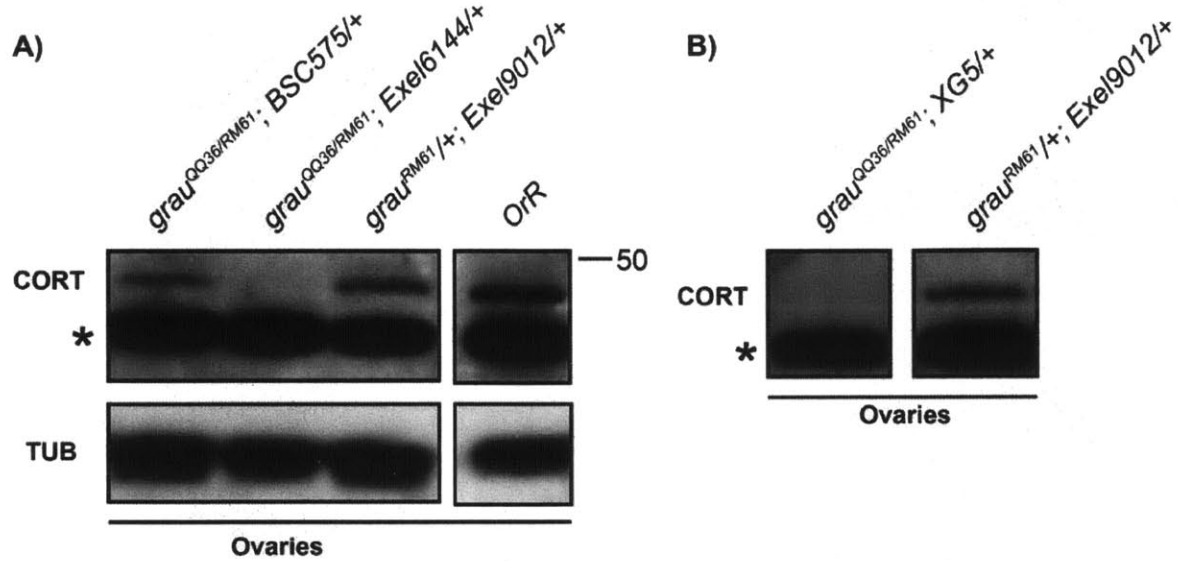
Figure 2-4. Cortex Protein Levels are Partially Restored by *BSC575* but not *XG5*

A) Western blot showing Cortex protein levels in the indicated genetic background. *grau*^{RM61}/+ and *OrR* are used as controls for normal Cortex protein levels. Tubulin serves as a loading control. * indicates a non-specific band.

B) Western blot showing Cortex protein levels are not restored by the presence of *Df (3L)XG5*. The *grau*^{RM61}/+ background is used as a control and the non-specific band is used as a loading control.

Lastly, *Df(3L)BSC575* is a strongly suppressing deficiency that uncovers about 34 genes. The best candidate within this region is *early girl* (*elgi*), a putative E3 ubiquitin ligase identified by yeast two-hybrid as an interactor with *α-endosulfine* (*endos*) (Von Stetina et al., 2008). *elgi* mutants undergo premature nuclear envelop breakdown during oogenesis and progress quickly into metaphase I. Given its role in meiotic maturation, two alleles of *early girl* were tested for suppression of *grauzone*. Both *elgi^1* and *elgi^2* were previously generated by imprecise excision of a P-element inserted upstream of the *elgi* coding sequence (Von Stetina et al., 2008). *elgi^1* is presumably a null and results in no *elgi* transcript, whereas the *elgi^2* locus still generates a small amount of transcript encoding a truncated form of the protein. *elgi^1* did not produce significant suppression of *grau*. *elgi^2*, however, did exhibit suppression (though much lower than the BSC575) (Table 2-2). Given that *elgi^1* is a stronger allele than *elgi^2*, it is difficult to explain the discrepancy. The truncated protein generated by *elgi^2* is thought to act in a dominant-negative fashion (Von Stetina et al., 2008), and so could be a possible reason for the difference between the two alleles. The lack of suppression by *elgi^1*, however, indicated that *elgi* was unlikely to be the gene responsible for suppression in *BSC575*.

Discussion

The initial deficiency screen identified numerous putative hits in addition to *p-XT103*. Of the six additional hits, four consistently suppressed the *grau* phenotype. To identify the specific gene(s) responsible for suppression, smaller deficiencies were used to narrow down the suppressing region. The suppressing region was successfully delineated in three cases (with the fourth still to be studied). In these cases, mutants of single genes were tested for suppression activity.

Genotype	Df Tested	Total	Suppressed	Percent Suppression
grau$^{RM61/QQ36}$; Df/+	Df (3L)XG5	130	20	15.4
grau$^{RM61/QQ36}$; Df/+	Df(3L)XG4	175	1	0.6
grau$^{RM61/QQ36}$; Df/+	Df(3L)XG8	35	0	0.0
grau$^{RM61/QQ36}$; Df/+	Df(3L)BSC575	303	151	49.8
grau$^{RM61/QQ36}$; Df/+	Df(3L)BSC845	361	15	4.2
grau$^{RM61/QQ36}$; Df/+	Df(3R)Exel6144	132	30	22.7
grau$^{RM61/QQ36}$; Df/+	Df(3R)BSC179	100	3	3.0
grau$^{RM61/QQ36}$; Df/+	Df(3R)ED10257	40	0	0.0
grau$^{RM61/QQ36}$; Df/+	Df(3R)ED5177	253	95	37.5
grau$^{RM61/QQ36}$; Df/+	Df(3R)Exel9012	241	58	24.1
grau$^{RM61/QQ36}$; Df/+	Df(3R)Exel6280	90	1	1.1
grau$^{RM61/QQ36}$; Df/+	Df(3R)ED6105	100	5	5.0
grau$^{RM61/QQ36}$; Df/+	Df(3R)BSC619	23	14	60.9
grau$^{RM61/QQ36}$; Df/+	Df(3R)p-XT103	89	25	28.1
grau$^{RM61/QQ36}$; +/+*	Df(3R)p-XT104 Sibling Control	39	3	7.7
grau$^{RM61/QQ36}$; +/+*	Df(3L)BSC575 Sibling Control	212	0	0.0
grau$^{RM61/QQ36}$; +/+*	Df(3R)ED5177 Sibling Control	52	0	0.0

Table 2-1. Summary of Deficiency Screen Results

'Total' indicates total number of embryos scored, while 'Suppressed' indicates the number that exhibited development (normal or abnormal) past metaphase II. Cells highlighted in yellow indicate the positive control (*p-XT103*) and selected negative (sibling) controls.

* +/+ indicates either *TM6,Hu/+* or *Ki/+*.

Genotype	Allele Tested	Total	Suppresed	Percent Suppression
$grau^{RM61/QQ36}$; Gene/+	$elgi^1$	330	10	3.0
$grau^{RM61/QQ36}$; +/+	$elgi^1$ Sibling Control	239	1	0.4
$grau^{RM61/QQ36}$; Gene/+	$elgi^2$	211	37	17.5
$grau^{RM61/QQ36}$; +/+	$elgi^2$ Sibling Control	229	0	0.0
$grau^{RM61/QQ36}$; Gene/+	Asl^{MecD}	382	1	0.3
$grau^{RM61/QQ36}$; +/+	Asl^{MecD} Sibling Control	154	0	0.0
$grau^{RM61/QQ36}$; Gene/+	Kra^1	52	2	3.8
$grau^{RM61/QQ36}$; +/+	Kra^1 Sibling Control	94	2	2.1
$grau^{RM61/QQ36}$; Gene/+	$pnt^{\Delta 88}$	137	2	1.5
$grau^{RM61/QQ36}$; Gene/+	Rga^{03834}	56	0	0.0

Table 2-2. Summary of Single Mutant Screen Results

'Total' indicates total number of embryos scored, while 'Suppressed' indicates the number that exhibited development (normal or abnormal) past metaphase II. *grau* mutant siblings without the indicated mutation served as negative controls.

* +/+ indicates either *TM6,Hu*/+ or *Ki*/+.

Alleles of *pnt, asl, kra, Rga,* and *elgi* were tested, but did not consistently

suppress the *grau* metaphase II arrest. Most of these alleles are characterized as nulls,

and so should have sufficed in acting as a 'deficiency' for its respective gene. One

explanation for the lack of suppression by all of these genes is simply they are not the

genes responsible for suppression in the narrowed down deficiencies. It is not always

possible to test all individual genes in a given region due to lack of available mutant

alleles. In these cases, it might be beneficial to make use of the Transgenic RNAi

Project (TRiP) (http://www.flyrnai.org/TRiP-HOME.html) lines now widely available to

the Drosophila community. With efficient germline expression now possible, genes

without appropriate alleles could be knocked down and tested for their ability to

suppress *grau*. This comes with its own caveats however, as complete knockdown

(compared to heterozygous mutation) of a gene could adversely affect oogenesis in

numerous ways.

It is also possible, that in some cases, the deficiencies affect the regulatory

region of a nearby gene (that is itself outside the deficiency). Genes such as *Abd-B* are

known to be controlled by distant regulatory regions (Sipos and Gyurkovics, 2005), and

so this is not out of the realm of possibility. If a deficiency removes the regulatory

region of a gene outside of the deficiency, it would prove difficult to identify, because it

could be 50Kb or more away. However, it might still be found if a deficiency uncovering

the gene itself also exhibited suppression.

In this screen, suppression was scored as any development (normal or

otherwise) past the metaphase II arrest, as judged by DAPI staining. Suppression

ranged from embryos with large, abnormal masses of DNA to those that completed

meiosis and developed normally (Figure 2-5). This range of phenotypes was observed

for any single deficiency. We propose that a given embryo's chance to develop directly

correlates to APC^{Cort}'s ability to degrade its substrates. A *grau* embryo that developed

normally likely degraded most Cort substrates. On the other hand, an embryo showing

suppression with large DNA masses likely degraded enough APC^{Cort} substrates to

complete meiosis, but 'residual' substrates then interfered with proper embryogenesis. If

this hypothesis is correct, a more quantitative look at the distribution of phenotypes for a

given deficiency would prove useful. Deficiencies that show a large number of normally

developing embryos must be better at promoting degradation of many APC^{Cort}

substrates. Deficiencies that suppress, but then lead to abnormal development, may be

better at promoting degradation of only a few key substrates (whose degradation is

required for *meiotic* progression).

One way to more easily identify candidate APC/C substrates is by searching for

the presence of known APC/C destruction motifs. Most canonical are the destruction

box and KEN box (RxxLxxxxN and KEN respectively). Together with the Bioinformatics

and Research Computing (BaRC) group at the Whitehead, the presence of these motifs

was searched for in the Drosophila proteome. In addition to the above motifs, we also

searched for KxxLxxxxN (a modified D-box) (Pines, 2006), or proteins that contained

both a shorter D-box motif as well as a KEN box (KxxL/KEN or RxxL/KEN). In total,

3,653 genes were identified that contained one or more of the above motifs in at least

one protein isoform. This represents about 25% of the Drosophila genome, and

because it is unlikely that this number of proteins is controlled by the APC/C, additional

regulation besides simply the presence of a destruction motif must dictate APC/C

$$\frac{grau^{QQ36}}{grau^{RM61}} ; \frac{+}{Ki} \quad \textbf{vs.} \quad \frac{grau^{QQ36}}{grau^{RM61}} ; \frac{Exel9012}{+}$$

Figure 2-5. Representative Image of Suppression by a Deficiency

DAPI stained embryos of the indicated genotype. While the *grau* mutant alone remains arrested in meiosis, presence of the *Exel9012* deficiency allows normal development in a subset of embryos.

targets. It is also possible that a novel or less well characterized motif will be recognized by APC^{Cort}, given our hypothesis that some substrates will be uniquely ubiquitylated by APC^{Cort}.

In summary, a screen to find dominant suppressing deficiencies of the *grau* phenotype was carried out. Five deficiencies in total were found, and this work describes the classification of three of them. The three suppressing deficiencies were narrowed down to a single suppressing region, but attempts at finding a single gene responsible for suppression was unsuccessful.

We decided that a more biochemical approach to identifying substrates of APC^{Cort} might prove useful, as detailed in the next chapter.

Materials and Methods

Fly Stocks

The *grau*^{RM61}, *grau*^{QQ36} (Page and Orr-Weaver, 1996; Schupbach and Wieschaus, 1989), *asl*^{MecD} (Blachon et al., 2008), *kra*^{1} (Lee et al., 2007), *pnt*$^{\Delta 88}$ (Scholz et al., 1993), *elgi*^{1}, and *elgi*^{2} (Von Stetina et al., 2008) have been described previously. The following deficiency stocks were used in this study: BL 6551 (*Df (3L)XG5*), BL 6550(*Df(3L)XG4*), BL 6554 (*Df(3L)XG8*), BL 27587 (*Df(3L)BSC575*), BL 27888 (*Df(3L)BSC845*), BL 7623 (*Df(3R)Exel6144*), BL 9159 (*Df(3R)BSC179*), BL 23146 (*Df(3R)ED10257*), BL 8103 (*Df(3R)ED5177*), BL 7990 (*Df(3R)Exel9012*), BL 7746 (*Df(3R)Exel6280*), Kyoto 150336 (*Df(3R)ED6105*), BL 25694 (*Df(3R)BSC619*), BL 1962 (*Df(3R)p-XT103*), BL 4962 (*Df(3R)H-B79*), and BL 3124 *Df(3L)fz-GF3b*.

Embryo Collection and Immunofluorescence

Females were collected for 1-2hrs and aged 2-3hrs. Eggs were then dechorionated in 50% bleach, fixed in methanol, and prepared for immunofluorescence as described (Pesin and Orr-Weaver, 2007). DAPI (1ug/ml) was used to visualize DNA.

Western blotting

Whole ovaries were hand dissected from fattened females and homogenized in NP-40 lysis buffer (150mM NaCl, 50mM Tris, pH 8.0, 2.5mM EDTA, 2.5mM EGTA, 1%NP-40, 1mM PMSF, complete mini EDTA-free protease inhibitors, 1 tablet/10 ml [Roche]). Protein lysates were spun at 14,000 RPMs for 15 minutes at 4C, and supernatant was used as protein sample. Equal protein amount was loaded on 10% SDS-PAGE gels as determined with Bradford reagent [BioRad]. Protein was transferred to Immobilon-P membranes (Millipore).

Antibodies used were guinea pig anit-Cortex (1:2000)(Pesin and Orr-Weaver, 2007) and rat anti-tubulin (yol1/34 and yl1/2) (1:400-1:1000)(Novus Biologicals). Secondary antibodies used were Peroxidase-conjugated anti-guinea pig and Alkaline Phosphatase-conjugated anti-rat (1:5,000; Jackson ImmunoResearch).

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Chapter Three

A Meiosis-Specific Form of the APC/C Promotes the Oocyte-to-Embryo Transition by Decreasing Levels of the Polo Kinase Inhibitor Matrimony

Zachary J. Whitfield[1], Jennifer Chisholm[2], R. Scott Hawley[2,3], and Terry L. Orr-Weaver[1*]

[1]Whitehead Institute, Department of Biology, Massachusetts Institute of Technology, Nine Cambridge Center, Cambridge, MA, 02142

[2]Stowers Institute for Medical Research, 1000 East 50th Street, Kansas City, MO 64110

[3]Department of Molecular and Integrative Physiology, University of Kansas Medical Center, Kansas City, Kansas 66160

*Corresponding Author: Terry L. Orr-Weaver (weaver@wi.mit.edu)

Abstract

Oocytes are stockpiled with proteins and mRNA that are required to drive the initial mitotic divisions of embryogenesis. But are there proteins specific to meiosis whose levels must be decreased to begin embryogenesis properly? The Drosophila protein Cortex (Cort) is a female, meiosis-specific activator of the Anaphase Promoting Complex/Cyclosome (APC/C), an E3 ubiquitin ligase. We performed immunoprecipitation of Cortex followed by mass spectrometry, and identified the Polo kinase inhibitor Matrimony (Mtrm) as a potential interactor with Cort. *In vitro* binding assays showed Mtrm and Cort can bind directly. We found Mtrm protein levels are reduced dramatically during the oocyte-to-embryo transition, and that this downregulation did not take place in *cort* mutant eggs, consistent with Mtrm being a substrate of APC^{Cort}. We showed that Mtrm is subject to APCCort mediated proteasomal degradation and have identified a putative APC/C recognition motif in Mtrm that when mutated partially stabilized the protein in the embryo. Furthermore, overexpression of Mtrm in the early embryo caused aberrant nuclear divisions and developmental defects, and these were enhanced by decreasing levels of active Polo. These data indicate APC^{Cort} ubiquitylates Mtrm at the oocyte-to-embryo transition, thus preventing excessive inhibition of Polo kinase activity due to Mtrm's presence.

Introduction

The oocyte-to-embryo transition is the developmental course by which an oocyte not only switches from a meiotic to a mitotic program, but becomes fully competent to support early embryogenesis. Initially, fertilization introduces the haploid genomic content of the sperm into the egg. Egg activation, triggered by fertilization in vertebrates and independent of fertilization in insects, signals the resumption and completion of meiosis in the egg (Horner and Wolfner, 2008; Von Stetina and Orr-Weaver, 2011). Following successful completion of meiosis, pronuclear fusion creates a single diploid nucleus from the individual haploid sperm and egg nuclei. The single diploid nucleus must then transition to a mitotic cell cycle within the same cytoplasm in which the meiotic divisions took place.

The oocyte-to-embryo transition can proceed normally only if the preceding events of meiosis are completed successfully. During *Drosophila melanogaster* oogenesis, an oocyte enters prophase I following completion of pre-meiotic S-phase. After homologous chromosome pairs synapse and recombine, the oocyte enters a prolonged prophase I arrest. Oocyte maturation then releases this primary arrest, allowing the oocyte to continue meiosis until its secondary arrest at metaphase I, in what is known as a stage 14 oocyte. Lastly, egg activation triggers resumption and completion of meiosis concordantly with the oocyte-to-embryo transition itself (Horner and Wolfner, 2008; Von Stetina and Orr-Weaver, 2011).

The switch from meiosis to mitosis is controlled by cellular proteins and structures produced during gametogenesis, with both the sperm and egg making unique contributions. The centrosome, necessary for proper spindle formation during mitotic

divisions, is brought into the acentrosomal egg by the sperm (Schatten, 1994). The initial rapid divisions of a developing embryo are driven by the maternal stockpile of nutrients, mRNA, and translational machinery that are 'packed' into the egg during oocyte differentiation (Von Stetina and Orr-Weaver, 2011). Additionally, the egg also contains numerous meiosis-specific proteins. These meiosis-specific proteins are crucial for proper meiotic progression, but are not necessarily needed after the switch to mitosis.

There are known examples of proteins uniquely employed in meiosis that need to be removed prior to mitosis (DeRenzo and Seydoux, 2004). In *C. elegans*, the MBK-2 kinase promotes the oocyte-to-embryo transition. One target is the katanin subunit MEI-1 (Quintin et al., 2003), and phosphorylation of MEI-1 by MBK-2 marks it for degradation before the completion of meiosis (Stitzel et al., 2006). A gain-of-function MEI-1 protein that persists into embryogenesis often leads to a short, mispositioned mitotic spindle (Dow and Mains, 1998). The *Saccharomyces cerevisiae* meiosis-specific protein Spo13 prevents the biorientation of sister chromatids at meiosis I, ensuring homologs segregate together (Katis et al., 2004; Lee et al., 2004). Spo13 is actively targeted for degradation during anaphase I by the Cdc20 form of the Anaphase Promoting Complex/Cyclosome (APC/C) (Sullivan and Morgan, 2007). Interestingly, a non-degradable form of Spo13 does not result in a significant meiotic phenotype; however, overexpression of Spo13 leads to mitotic cell cycle defects (Lee et al., 2002; Shonn et al., 2002; Sullivan and Morgan, 2007). This demonstrates the necessity of degrading a meiosis-specific protein not for proper meiotic progression, but subsequent mitotic progression.

The unique mechanisms of meiosis such as segregation of homologs in meiosis I, absence of DNA replication between divisions, and the meiotic arrests during oogenesis require either unique regulators or altered control of factors that also are used in mitosis. For example, during mitosis the mitotic cyclins are completely degraded as the cell progresses through the metaphase to anaphase transition and exits from mitosis. In contrast, the mitotic cyclins are left at an intermediate level after the metaphase to anaphase transition of meiosis I; low enough to exit from meiosis I, but high enough to prevent re-replication (Furuno et al., 1994; Iwabuchi et al., 2000). This altered control of mitotic regulators may need to be removed upon the start of embryogenesis. The APC/C inhibitor Emi2 is responsible for maintaining Cyclin B1 levels after meiosis I in mouse oocytes, but it is quickly degraded to allow for meiotic exit (though it has been shown to reestablish its levels in early embryogenesis in *Xenopus*) (Inoue et al., 2007; Madgwick et al., 2006; Nishiyama et al., 2007; Tischer et al., 2012). This illustrates how normal mitotic cell cycle regulation can be altered through the use of unique meiotic proteins.

Regulated degradation of proteins, particularly by the APC/C, plays an indispensable role in progression through the mitotic and meiotic divisions (Pesin and Orr-Weaver, 2008; Pines, 2011). The APC/C ubiquitylates numerous proteins during mitosis, targeting them for degradation and promoting mitotic progression and exit. Similarly, during oogenesis proper cell cycle regulation by the APC/C is crucial in maintaining coordination between meiosis and development. The APC/C must use activator proteins (Cdc20/Fizzy and Cdh1/Fizzy-related in mitosis) to recognize its substrates. Interestingly, meiosis-specific activators of the APC/C are known to exist in

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both budding (Cooper et al., 2000) and fission yeast (Blanco et al., 2001) in addition to sex and meiosis-specific APC/C activators in Drosophila (Chu et al., 2001; Pesin and Orr-Weaver, 2007, 2008). Elucidating the function and targets of these meiosis-specific APC/C activators will give valuable insights into meiotic regulation and the transition from meiosis into mitosis.

The Drosophila protein Cort is a female, meiosis-specific activator of the APC/C (Chu et al., 2001; Pesin and Orr-Weaver, 2007; Swan and Schüpbach, 2007). It is expressed exclusively during oogenesis, and is itself targeted for degradation by the APC/C soon after meiotic completion (Pesin and Orr-Weaver, 2007). Cort is dispensable for viability, but absolutely essential for fertility. Eggs laid by *cort* mutant mothers arrest in metaphase II (Page and Orr-Weaver, 1996). During Drosophila female meiosis, Cort and Fzy/Cdc20 both contribute to meiotic progression, whereas Fizzy-related/Cdh1 is not believed to play a role. Cort cooperates with Fizzy/Cdc20 during meiosis to degrade the Cyclins (Pesin and Orr-Weaver, 2007; Swan and Schüpbach, 2007), but whether it also has other substrates is unknown. Identifying additional substrates of APC[Cort] will give further insight into the differential regulation of meiosis and mitosis, as well as the necessary steps to transition from oocyte to embryo.

Here we show that degradation of the female, meiosis-specific protein Mtrm during meiotic completion is dependent on the activity of Cort. Furthermore, we show that this downregulation of Mtrm is crucial to the proper onset of embryogenesis.

Results

Identification of Mtrm as a candidate APC^{Cort} Substrate

To identify substrates and regulators of APC^{Cort}, a dual mass spectrometry

approach was taken. First, functional myc-tagged Cort (Pesin and Orr-Weaver, 2007)

was immunoprecipitated from whole ovaries, and co-immunoprecipitated proteins were

identified by mass spectrometry. In addition to isolating multiple components of the

APC/C as expected (Pesin and Orr-Weaver, 2007), the Polo inhibitor Mtrm was

recovered as a potential substrate or interacting protein (Table 1). Mtrm also was

identified in our second set of mass spectrometry experiments, using quantitative mass

spectrometry to identify proteins whose levels are increased in *cort* eggs vs. unfertilized

controls. Unfertilized eggs have completed meiosis, but have not initiated

embryogenesis, and therefore provide the best control for *cort* mutant eggs. Multiplex,

dimethyl labeling was used to label protein extracts from eggs laid by *cort* and wild-type

females. Given the difference in mass of the two dimethyl labels used, quantitative

mass spectrometry could identify proteins enriched in *cort* eggs compared to wild-type.

One of the top hits in both replicates was Mtrm (Table 2). Together, these data indicate

Mtrm can physically associate with Cortex, and that its levels are increased when Cort

function is absent.

Mtrm was identified initially in a genetic screen for dominant effects on

achiasmate chromosome segregation in Drosophila oocytes (Harris et al., 2003), and it

was later shown to function as a direct inhibitor of Polo kinase during meiosis I (Xiang et

al., 2007). Given Mtrm's essential role during female meiosis, we sought to explore

further its relationship to Cort.

Cort binds to the Polo inhibitor Mtrm *in vitro*

To confirm the physical interaction between Cort and Mtrm identified by IP-mass

spec, *in vitro* binding assays were performed. GST tagged Mtrm and GST alone were

expressed and purified from bacteria (Figure 3-1B), and then incubated with *in vitro*

translated 6xMyc-Cort produced in rabbit reticulocyte lysate. Cort strongly bound to

GST-Mtrm beads, but not to GST-only beads or beads alone, consistent with the

physical interaction between these two proteins being direct (Figure 3-1A). Moreover, *in*

vitro translated Cortex lacking its C-terminus binds GST-Mtrm much less efficiently

(Figure 3-2). The C-terminus of Cortex is made up mainly of its WD40 repeats (Chu et

al., 2001), which are known to mediate substrate binding in other APC/C activators(Kraft

et al., 2005). These data are consistent with Cortex binding Matrimony directly through

its WD40 propeller.

Cort and Fzy/Cdc20 are both required for degradation of the mitotic cyclins

during female meiosis (Pesin and Orr-Weaver, 2007; Swan and Schüpbach, 2007), and

therefore share at least a subset of their substrates. We also tested whether the

interaction between Cort and Mtrm was specific, or whether Mtrm might be a target of

all forms of the APC/C (or an APC/C regulator). In contrast to 6xMycCort, little to no *in*

vitro translated 6xMycFzy/Cdc20 bound to GST-Mtrm (Figure 3-1A). Importantly, *in vitro*

translated Fzy/Cdc20 could bind Cyclin A, a known substrate (Di Fiore and Pines, 2010;

Wolthuis et al., 2008). Full length Cortex also bound Cyclin A, albeit to a lesser extent

than it binds Matrimony (Figure 3-2). Thus, the interaction between Cort and Mtrm is

specific, suggesting regulation between these two female, meiosis-specific proteins.

	Experiment 1*	Experiment 2*	Experiment 3*
Cortex	98(0)	118(2)	102(0)
Cdc16	9(0)	8(0)	4(0)
Cdc27	0(0)	4(0)	5(0)
Cdc23	3 (0)	4 (0)	5 (0)
Shattered/Apc1	10(0)	7(0)	5(0)
Matrimony	2 (0)	2(0)	0 (0)

Table 1. Immunoprecipitation of Cortex identifies APC/C components and Matrimony.

Data summarizing three independent IP/mass spec experiments are shown. The number of total spectra identified that immunoprecipitated/Co-IP'd with Cortex is indicated. The number of peptides identified in the negative control is shown in parentheses. In experiments 1 and 2, random mouse IgG was used as a negative control. Experiment 3 used anti-myc antibody in a strain not expressing 6xMyc-Cortex (*OrR*) as a control. * Number of spectra indicated were searched for in MASCOT and analyzed by Scaffold (see Materials and Methods for more details).

	H/L	H/L Normalized	Peptides	Intensity H	Intensity L
Biological Replicate 1	3.658	4.516	5	25.241	21.145
Biological Replicate 2	-4.985	-4.286	5	20.076	25.270

Table 2. Identification of Mtrm by quantitative mass spectrometry.

Only data pertaining to Mtrm peptides is shown. In replicate 1, $cort^{RH65}$ eggs were labeled with heavy (H) dimethyl isotope, while wild-type (*OrR*), unfertilized eggs were labeled with the light (L) dimethyl isotope. In replicate 2, the labeling was switched (hence the difference in signs). Both experiments show enrichment for Mtrm protein (at significance level B, as determined by MaxQuant and Perseus (Cox and Mann, 2008)) when Cort is mutant compared to the wild-type control.
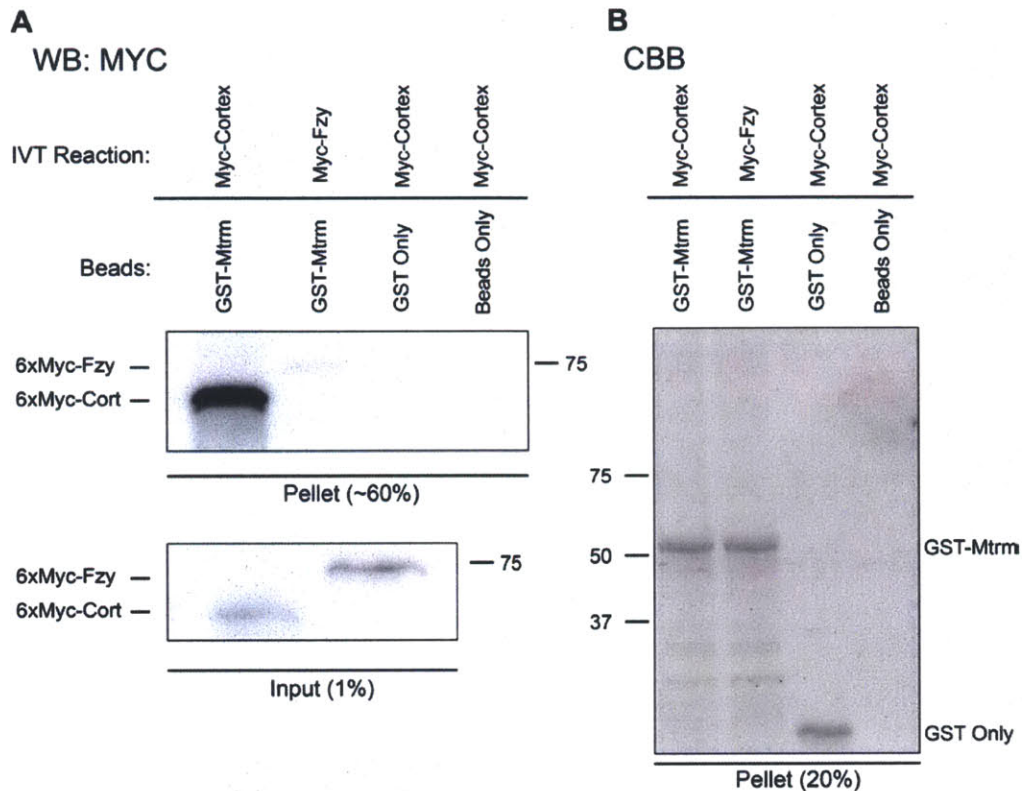
Figure 3-1. Cort physically interacts with Mtrm in vitro.

A) Western blot showing in vitro translated Myc-tagged Cort stably binds to GST-Mtrm, but not to GST only or beads only. In vitro translated Myc-tagged Fzy/Cdc20 is unable to bind GST-Mtrm. About 60% of each pellet sample was subjected SDS-PAGE followed by Western blotting (remaining pellet sample was used for B). Lower panel shows 1% of total input of *in vitro* translated 6xMycCort and 6xMycFzy/Cdc20. Panels were probed with anti-Myc (9E10) antibody. Molecular weight markers are indicated to the side of the blot. B) Coomassie stain of purified proteins used in binding assay. 25% of the final washed pellet was subjected to SDS-PAGE followed by coomassie staining. Molecular weight markers are indicated to the side of the gel.

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Decreased Mtrm protein levels after meiosis are Cort dependent

Mtrm protein levels increase throughout meiosis I (Von Stetina et al., 2011). Interestingly, its levels are drastically reduced by the time meiosis is completed (Figure 3-3A; compare heterozygous stg. 14 oocyte to heterozygous activated egg). This pattern of expression mimics that of Cort, which itself is a substrate of the APC/C (Pesin and Orr-Weaver, 2007). As with Cort, such a sharp transition in Matrimony protein levels suggests active degradation, potentially through the action of APC[Cort].

Our quantitative mass spectrometry experiments (see above), indicated Mtrm is enriched in *cort* mutant eggs. To confirm this, western blots were performed to compare Mtrm protein levels in *cort* mutant eggs to heterozygous control unfertilized eggs. In contrast to heterozygous unfertilized eggs, activated eggs laid by homozygous *cort* females retained high levels of Mtrm protein, consistent with it being a substrate of APC[Cort] (Figure 3-3A,B). Moreover, unfertilized eggs laid by females mutant for *morula/APC2*, a component of the APC/C itself, also showed elevated levels of Mtrm. This shows APC/C function is necessary to trigger the decrease in Mtrm protein (Figure 3-3B). Importantly, *fzy/cdc20* mutant unfertilized eggs did not show elevated Mtrm levels, again illustrating Mtrm is not a general APC/C substrate (Figure 3-3B). Together these data demonstrate the decrease in Mtrm protein upon meiotic completion (or during meiosis II) is dependent specifically on APC[Cort] function. We hypothesized the relatively large pool of Mtrm present in the ovary is necessary for proper progression through meiosis, but such high levels may be detrimental in early embryogenesis.
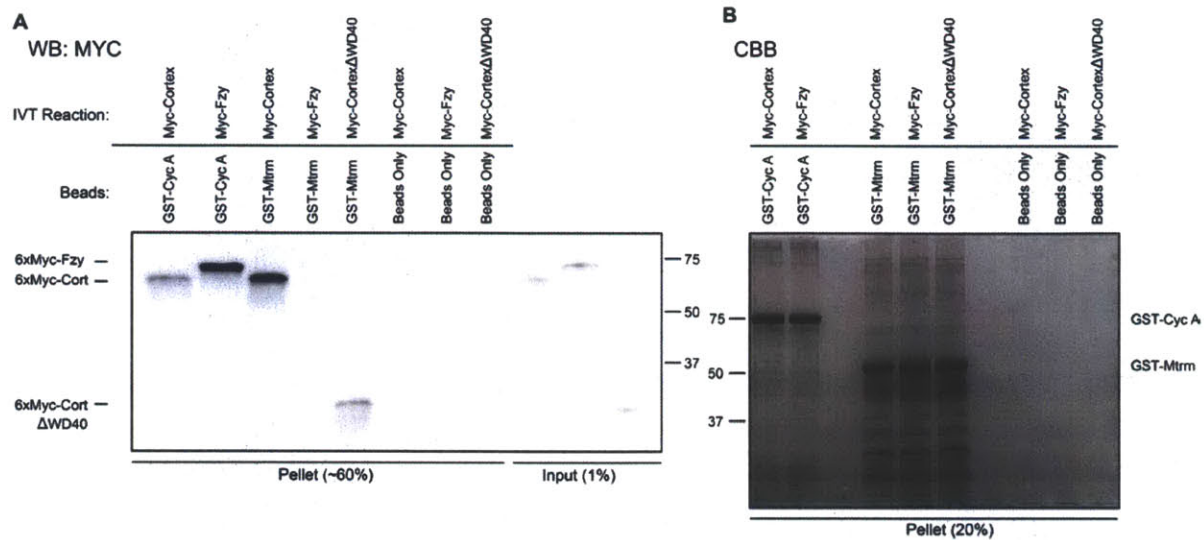
Figure 3-2. *in vitro* binding assays with cyclin A and CortΔWD40.

A) Western blot showing *in vitro* translated Myc-tagged Fzy/Cdc20 stably binds to GST-CycA. Myc-Cortex also binds, but somewhat less efficiently. Myc-CortexΔWD40 (AA 1-148 of Cortex) is impaired in its ability to bind Gst-Mtrm. Glutathione beads alone serve as a negative control. Quantification indicates Myc-Fzy binds to GST-CycA 155x better than to GST-Mtrm. 6xMyc-Cortex (full length) binds GST-Mtrm 5.8x better than 6xMyc-CorttΔWD40. About 60% of each pellet sample was subjected SDS-PAGE followed by Western blotting (remaining pellet sample was used for B). Right side of panel shows 1% of total input of *in vitro* translated 6xMycCort, 6xMycFzy/Cdc20, and 6xMyc-CortΔWD40. Blot was probed with anti-Myc (9E10) antibody. Molecular weight markers are indicated to the side of the blot. B) Coomassie stain of purified proteins used in binding assay. 20% of the final washed pellet was subjected to SDS-PAGE followed by coomassie staining. Molecular weight markers are indicated to the side of the gel.
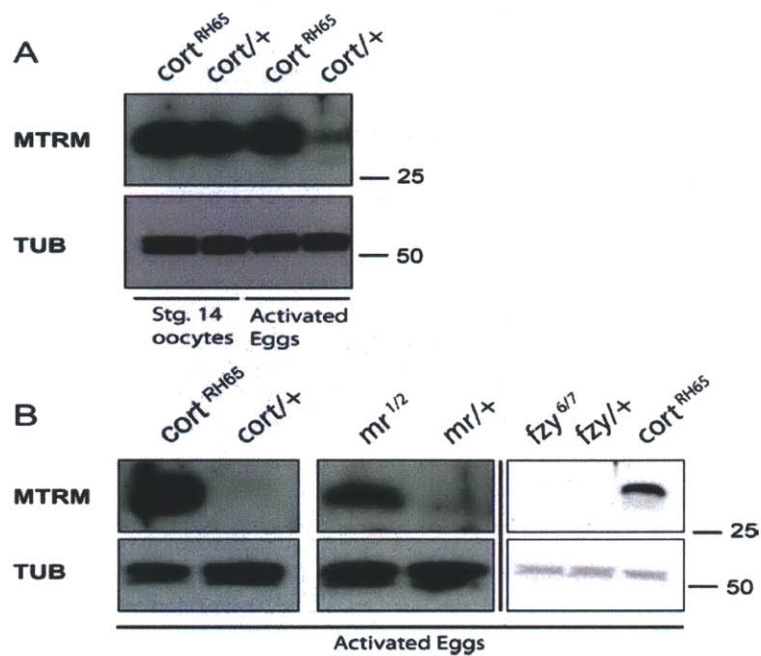
Figure 3-3. Cort activity is required for Mtrm destabilization.

A) Western blots showing *cort* homozygous and heterozygous mutant female oocytes have equal amounts of Mtrm protein at metaphase I (stage 14 oocytes). Activated (fertilized) eggs from *cort* mutant females have increased Mtrm levels compared to control heterozygous unfertilized, activated eggs (*cort/+*). Molecular weight markers are indicated to the side of the blot. B) Mtrm protein levels also are increased in unfertilized eggs from *morula* transheterozygous females (mr^1/mr^2) compared to unfertilized controls (*mr/+*). However, *fzy/cdc20* transheterozygous unfertilized eggs (fzy^6/fzy^7) do not show elevated Mtrm levels compared to control heterozygous unfertilized eggs (*fzy/+*). All panels show western blots probed with the indicated antibody. Alpha-tubulin was used to confirm equal loading. '+' indicates the presence of the *CyO* balancer chromosome.. Molecular weight markers are indicated to the side of the blot.

Requirement for APC motif in Mtrm for APC^{Cort} dependent destabilization

We exploited Drosophila cell culture to study the effects of Cort on Mtrm stability, as it permits the expression of proteins in an easily manipulated system. Neither Cort nor Mtrm is expressed endogenously in Drosophila Kc167 cell culture cells, but both can be expressed transiently through transfection (Figure 3-4A). In a stable cell line expressing Cort, Cyclin A protein levels were decreased markedly and Cyclin B levels marginally (Figure 3-5A), indicating functional APC^{Cort}. The changes in mitotic Cyclin protein levels did not detectably affect cell cycle progression, however, as measured by the mitotic index (Table 3) and FACS analysis (Table 4).

If Mtrm is targeted for degradation by APC^{Cort}, levels of Mtrm protein should be reduced in the presence of Cort. Indeed, levels of a Myc-tagged Mtrm were reduced when functional Cort was expressed (Figure 3-4A). Moreover, expression of functionally null alleles of Cort, Cort^{QW55} (a missense mutation) or Cort^{RH65} (a nonsense mutation) (Chu et al., 2001; Page and Orr-Weaver, 1996; Schupbach and Wieschaus, 1989), failed to decrease Mtrm protein levels. Therefore, wild-type Cort function is required to bring about the observed decrease in Mtrm protein. Consistent with APC^{Cort} affecting Mtrm levels through degradation, these cells contained similar amounts of *mtrm* transcript, illustrating the effect is post-transcriptional (Figure 3-4B and C). Additionally, reduction of Mtrm levels was not observed when a 6xMyc-tagged Fzy/Cdc20 was expressed, again showing the selectivity of APC^{Cort} for Mtrm (Figure 3-5B).
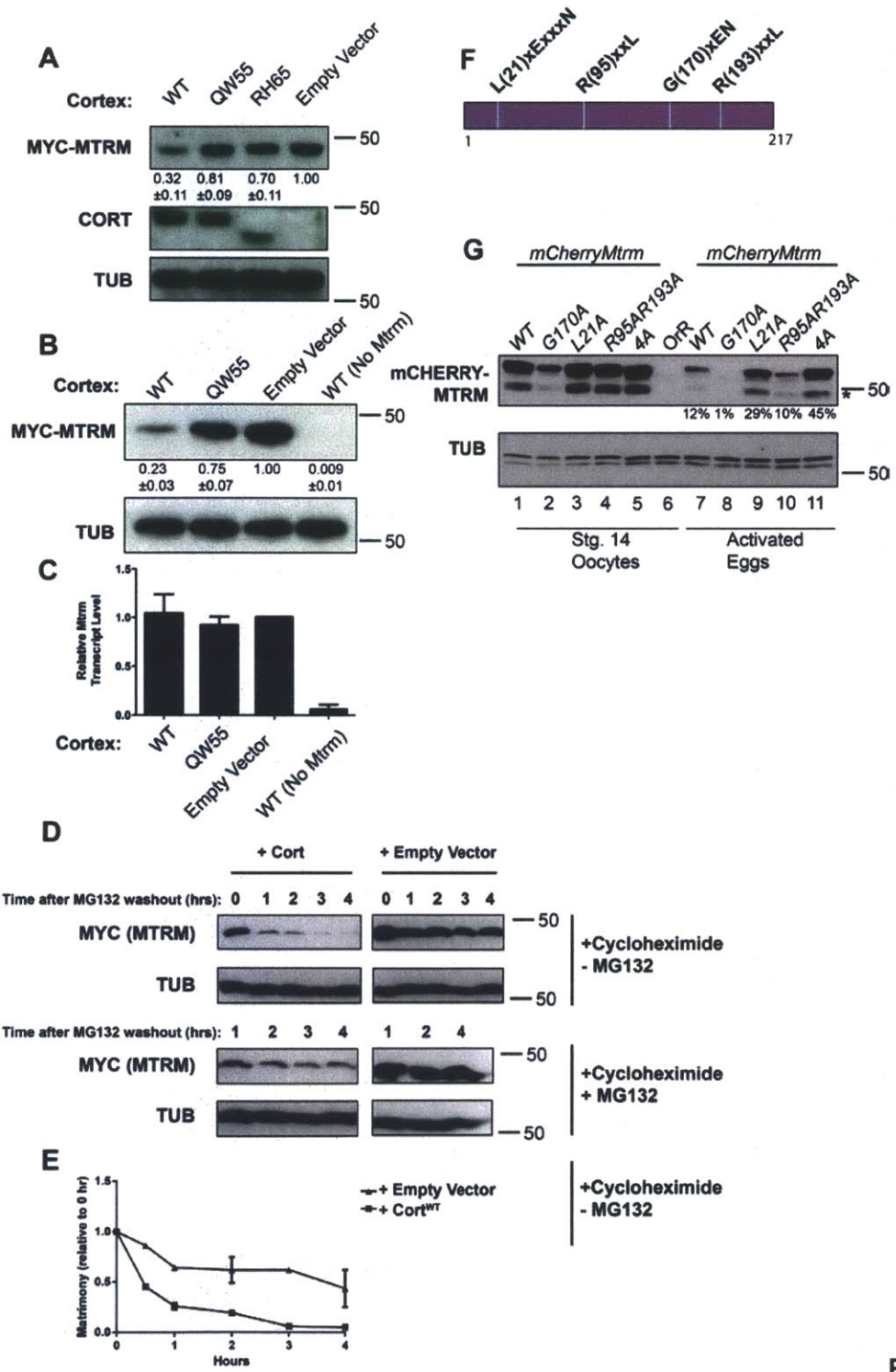
Figure 3-4

Figure 3-4. Cort expression leads to proteasome-mediated degradation of Mtrm in cell culture.

A) Western blots showing levels of Mtrm and Cort in transfected Kc167 cells. *pMT-cort* and *pMT-6xmyc-mtrm* were transfected into Kc167 cells. The form of transfected Cort is indicated above each lane. Only wild-type Cort leads to decreased levels of tagged Mtrm protein. The *RH65* mutation results in a premature stop codon in Cort. Myc-Mtrm band intensity (and SEM from two experiments) is quantified below the Myc-Mtrm panel. Band intensity is normalized to tubulin and is expressed relative to empty vector. B/C) Cells transfected with *pMT-mtrm* (lanes 1-3; lane 4 transfected with *pMT-empty*) and the indicated form of Cort were split and subjected to both western blot (B) and quantitative PCR (C). Myc-Mtrm band intensity is quantified as in A). For qPCR, *mtrm* transcript levels are normalized to Actin5c and shown relative to empty vector. D) Western blot showing Mtrm protein levels over time. Time indicates hours post MG132 washout. Rate of Matrimony degradation is faster in the presence of Cortex versus empty vector. The rate of degradation is slowed in continued presence of MG132. E) Quantification of – MG132 blot in D) The 1, 2, and 4 hour time-points are averages of two experiments. Mtrm amount is normalized to tubulin and shown relative to amount at 0 hr time point. F) Illustration of candidate APC/C recognition motifs. G) The L21A mutation stabilizes mCherry-Mtrm in embryos. Western blots of stage 14 oocytes and fertilized eggs (1hr collection) are shown. The 4A mutant consists of L21A, R95A, R193A, and H94A (a mutation in a possible APC/C initiation motif (Williamson et al., 2011)). Percentage below mCherry egg lanes indicates remaining protein left, normalized to tubulin and relative to amount at stage 14. * denotes cleavage product due to hydrolysis of acylimine linkage in the mCherry tag (Gross et al., 2000). Myc-Mtrm was detected using anti-Myc antibody (A,B,D) and mCherry-Mtrm was detected using anti-RFP (G).

	Total # Cells	# Cells PH3 +	% PH3 +
Cortex Stable Line (Induced)	811	24	2.96
Cortex Stable Line (Uninduced)	1452	45	3.10

Table 3. Quantification of mitotic index in the Cortex stable line.

Cells were induced (or not) with 0.5mM CuSO4. Total cells were counted using DAPI and mitotic cells were counted using anti-phospho histone H3.

Cell Type	CuSO4?	%G1	%S	%G2
Cortex Stable Line	0.5 mM CuSO4 (1 day)	13.3	6.15	67.4
Kc167	0.5 mM CuSO4 (1 day)	11	7.18	67.7
Cortex Stable Line	No CuSO4 (1 day)	15.7	11.9	56.4
Kc167	No CuSO4 (1 day)	17.8	13.7	51.7
Cortex Stable Line	0.5 mM CuSO4 (2 day)	16.6	9.12	61.7
Kc167	0.5 mM CuSO4 (2 day)	14.1	9.7	62.7
Kc167	0.5 mM CuSO4 (1 day) + MG132 (25uM; 8hrs)	3.78	0	88.7

Table 4 Analysis of Cortex stable line by FACS.

The stable Cortex cell line or Kc167 cells were incubated with or without $CuSO_4$ and cell cycle progression was analyzed by FACs (after 1 or 2 days of treatment). Cells are predominantly in G2, as is typical of Kc cells (Joyce et al., 2012). No significant cell cycle arrest is induced by ectopic expression of Cortex. A significant arrest in G2 was detected when MG132 was added to the medium for 8hrs.

We next used this cell culture-based system to determine whether APC^{Cort}'s

effect on Mtrm was truly the result of degradation. Mtrm protein was accumulated

during arrest with the proteasome inhibitor MG132, upon release of the arrest

translation was inhibited with cycloheximide and Mtrm protein levels examined over

time in the presence or absence of Cortex (Figure 3-4D,E). Mtrm protein levels

decreased rapidly,by 30 minutes, in the presence of Cort. Importantly, this decrease

was abolished in the continued presence of MG132 to inactivate the proteosome. Mtrm

levels remained higher when an empty vector was transfected in place of Cort. These

data establish that APC^{Cort} affects Mtrm levels through proteasome-mediated

degradation. Given the decrease in Mtrm is mediated through degradation, we

searched Mtrm's primary amino acid sequence for APC/C recognition motifs that could

influence its stability during the oocyte-to-embryo transition. Four motifs previously

implicated in APC/C mediated degradation (Pines, 2011) are present within Mtrm's 217

amino acid sequence (Figure 3-4F). To examine the role these motifs play in Mtrm

protein stability at the oocyte-to-embryo transition, transgenic flies expressing mCherry-

Mtrm under the control of *mtrm's* endogenous promoter were created. mCherry-Mtrm

protein levels decreased at the oocyte-to-embryo transition as expected (Figure 3-4G,

lanes 1 and 7). Point mutants in the four candidate APC motifs were also examined for

their effect on (mCherry-) Mtrm protein stability. Whereas the G170A mutation and the

double R95A/R193A mutations did not stabilize mCherry-Mtrm in activated eggs (Figure

3-4G, compare lanes 2 and 8; 4 and 10 respectively), mutation of leucine 21 exhibited

partial stabilization (Figure 3-4G, lanes 3 and 9). A quadruple mutant of mCherry-Mtrm

that also contains the L21A mutation is partially stabilized as well (Figure 3-4G; lanes 5
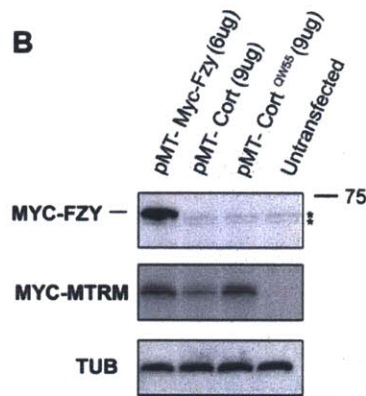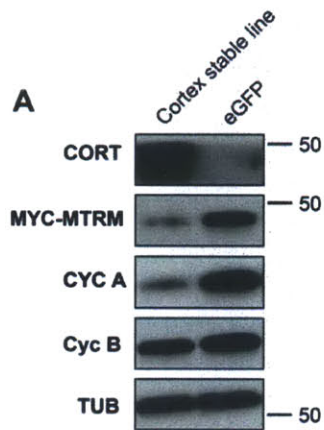
Figure 3-5. Levels of cell cycle proteins in cell culture system.

A) A cell line with a stable *cort* gene shows decreased Cyclin protein levels. Western blots comparing levels of indicated proteins in a *cort* stable line and cells transfected with *pMT-eGFP* instead. Both populations were also transfected with *pMT-6xmyc-mtrm*. Molecular weight markers are indicated to the side of the blot . B) Expression of myc-tagged Fizzy/Cdc20 does not decrease myc-tagged Mtrm levels. Amount of plasmid used to transfect cells is indicated above each lane. Cells were also transfected with equal amounts of *pMT-6xmyc-mtrm* (except last lane). The *s indicate non-specific bands. Both Myc-Fzy and Myc-Mtrm were detected using anti-myc antibodies. Molecular weight markers are indicated to the side of the blot.

and 11). Importantly, both Mtrm-L21A and Mtrm-4A are functional, as judged by their ability to rescue $mtrm^{+/-}$ induced nondisjunction (Table 5). Given mCherry-Mtrm-L21A is still partially degraded at the oocyte-to-embryo transition, L21 is not likely to be the only residue responsible for Matrimony degradation. It is intriguing to note, however, that L21 is part of the related LxExxxN APC/C destruction motif found in Spo13, another meiosis-specific substrate of the APC/C (Sullivan and Morgan, 2007).

Genetic interactions reveal an antagonistic relationship between Cort and Mtrm

We next investigated the genetic relationship between Cort and Mtrm, specifically in the background of a mutant with low APC^{Cort} activity. Mutants with low APC^{Cort} activity arrest without completing meiosis, presumably due to a failure to degrade key substrates. If Mtrm were such a substrate, we hypothesized that decreasing its levels could lead to suppression of the reduced APC^{Cort} phenotype. All alleles of *cort* are null (Page and Orr-Weaver, 1996), however mutation of Cort's dedicated transcription factor *grauzone* results in decreased levels of *cort* transcript (Harms et al., 2000) and protein (Pesin and Orr-Weaver, 2007). Activated eggs laid by *grauzone* mutant females also arrest in meiosis II (just as *cort* eggs do) (Page and Orr-Weaver, 1996), thus illustrating that such low levels of APC^{Cort} cannot efficiently cause degradation of key substrates. Decreasing levels of the Mtrm substrate may be sufficient to permit progression past the meiotic arrest. Alternatively, the reduced levels of one key substrate may afford low APC^{Cort} enough opportunity to target its remaining substrates for degradation. Thus we used this sensitized background to test whether decreased *mtrm* permitted progression past the *grauzone* metaphase II arrest.

Genotype	Adjusted Total**	%X NDJ	% $4th$ NDJ
FM7w/yw; spa^{pol}	1117	2.1	0.6
No rescue construct*	1572	35.8	20.2
mtrm^{WT*}	1574	1.4	2.6
mtrm^{L21A*}	2193	1.1	2.6
mtrm^{4A*}	1540	1	3.9

Table 5. Mtrm-4A and L21A are competent to rescue chromosome non-disjunction in *mtrm/+* heterozygotes.

Both mCherry-Mtrm-L21A and 4A can rescue non-disjunction caused by heterozygous deletion of *mtrm*.

* Full genetic background is *FM7w/yw; nanos-GAL4:VP16/mtrm$^{Df(3L)66C-T2-10}$; spa^{pol}*

**Adjusted totals were calculated as in Hawley et al (Hawley et al., 1992).

Strikingly, when one copy of the *mtrm* gene was removed, we observed partial suppression of the *grau* phenotype. *grau* eggs typically arrest with two spindles at metaphase II, but *grau* eggs also mutant for one copy of *mtrm* contained, on average, an increased number of spindles (Figure 3-6). The increase in spindle number indicates some form of progression past the typical metaphase II arrest of *grau* eggs. These spindles appear acentriolar, and thus are likely not mitotic. Supporting this, no gamma-tubulin (a common component of centrosomes) is present at the spindle poles (Figure 3-7). These likely arise from completion of meiosis followed by all meiotic products (including polar bodies) forming bipolar spindles and possibly dividing, reminiscent of the effect of *polo* mutation on meiosis II (Riparbelli et al., 2000) Importantly, the observed increase in spindle number is not due to a restoration of Cort protein levels (Figure 3-8). Thus the *mtrm* mutation partially suppresses the *grau* phenotype, allowing further progression through the oocyte-to-embryo transition.

Increased Mtrm levels in the embryo lead to developmental defects

Proteins and mRNA deposited into the oocyte during oogenesis control the early embryonic divisions, but it is possible some of these proteins function in meiosis and then need to be removed. We hypothesized degradation of Mtrm at the oocyte-to-embryo transition by APC[Cort] is a crucial step necessary to ensure proper development of the syncytial embryo. To test this hypothesis, we overexpressed a transgenic *mtrm* using the *UAS-GAL4* system. 3xFLAG-Mtrm was overexpressed in the ovary using the maternal alpha tubulin driver, resulting in excess Mtrm being present in the early embryo (Figure 3-9A/B).

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This surplus of Mtrm caused a variety of defects in early embryogenesis, which we categorized into three phenotypes (Figure 3-10A-C). We observed some embryos undergoing nuclear fallout (Figure 3-10A). During nuclear fallout, nuclei at the surface of an embryo that have detached from their centrosomes fall back into the middle of the embryo (Takada et al., 2003). We also found embryos that exhibited complete mitotic catastrophe (Figure 3-10B), showing only scattered DNA with no real spindle organization. DNA masses seemed to contain varying chromosomal content, and were usually associated with tubulin. These embryos were found with variable amounts of total DNA, some containing DNA over the entire expanse of the embryo (late arrest), while others only contained DNA in a particular section of the embryo (early arrest). Lastly, some embryos showed scattered DNA/tubulin over a portion of the embryo, whereas the rest of the embryo appeared to reach the blastoderm stage (Figure 3-10C). These embryos seemingly underwent an abortive/abnormal development up to the blastoderm stage. Given the centrosome's crucial role in spindle organization and the requirement for Polo kinase for proper centrosome attachment in the early embryo (Archambault et al., 2007), there are many ways these phenotypes could be obtained. In summary, these data illustrate that the downregulation of Mtrm protein following meiosis is biologically significant to early embryonic development.

The defects observed from *mtrm* overexpression likely result from low Polo kinase activity, given Mtrm's known function as its inhibitor. If true, mutating *polo* should further exacerbate the *mtrm* overexpression phenotype. Indeed, overexpression of Mtrm in conjunction with heterozygous *polo^11* results in a substantially higher proportion of defective embryos (Figure 3-10H). Additionally, the observed defects are often more
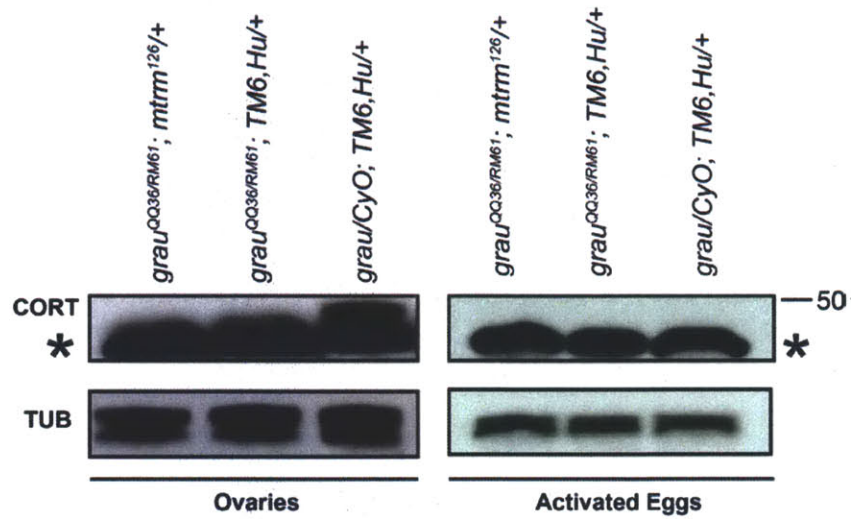
Figure 3-8. Cort is not restored in $grau^{QQ36/RM61}$; $mtrm^{126}$/+ mutants

The partial suppression of the *grau* phenotype in $grau^{QQ36/RM61}$; $mtrm^{126}$/+ activated

eggs is not due to restoration of Cort protein. Western blot showing presence of Cort in

grau/CyO ovaries but not *grau* or *grau; mtrm^{126}*/+ ovaries. Cortex levels are also not

restored in $grau^{QQ36/RM61}$; $mtrm^{126}$/+ fertilized eggs. * indicates a non-specific band.

Ovary and fertilized eggs panel are from two separate blots. Molecular weight markers

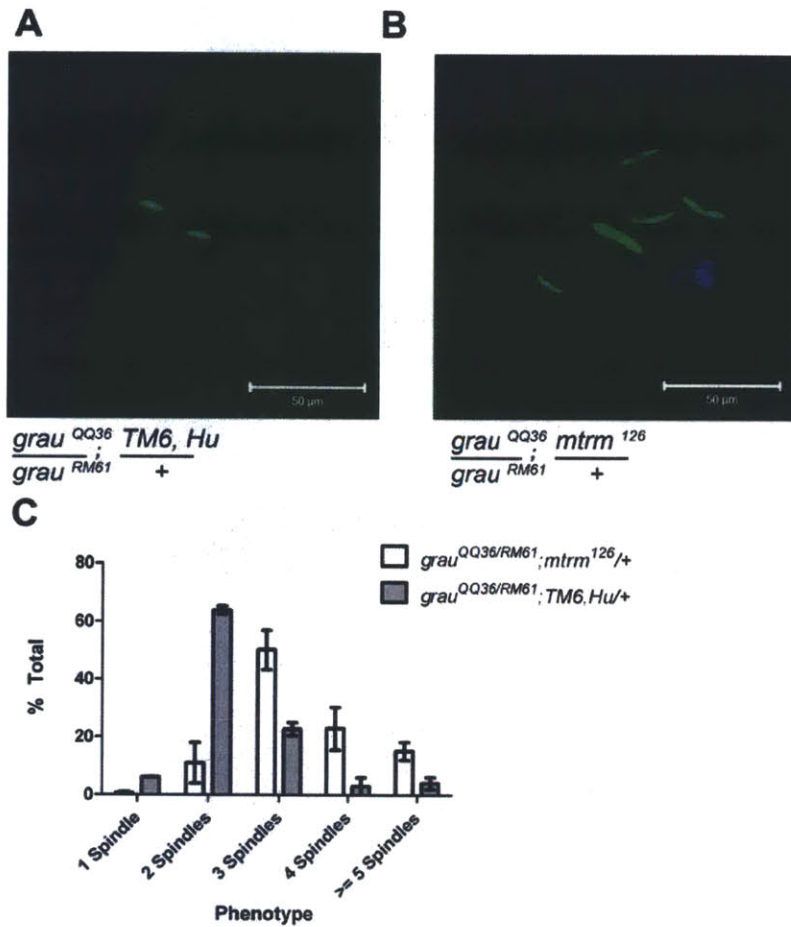are indicated to the side of the blot.

A

B

$\dfrac{grau^{QQ36}}{grau^{RM61}} ; \dfrac{TM6, Hu}{+}$

$\dfrac{grau^{QQ36}}{grau^{RM61}} ; \dfrac{mtrm^{126}}{+}$

C

$grau^{QQ36/RM61};mtrm^{126}/+$

$grau^{QQ36/RM61};TM6,Hu/+$

Figure 3-6. *cort* and *mtrm* show an antagonistic relationship in vivo.

A/B) Fertilized eggs from females of the indicated genotypes are shown. When *mtrm* is mutated in conjunction with *grauzone*, an increased number of spindles is observed. Even mutation of a single copy of the *mtrm* gene dominantly suppresses the *grauzone* phenotype. Tubulin is shown in green and DNA in blue. Scale bar indicates 50um.

C) Quantification of eggs from A and B. The TM6 balancer siblings served as the wild-type control for *mtrm*. n=167 for $grau^{QQ36}/^{RM61};mtrm^{126}/+$ and n=67 for $grau^{QQ36}/^{RM61};TM6/+$.
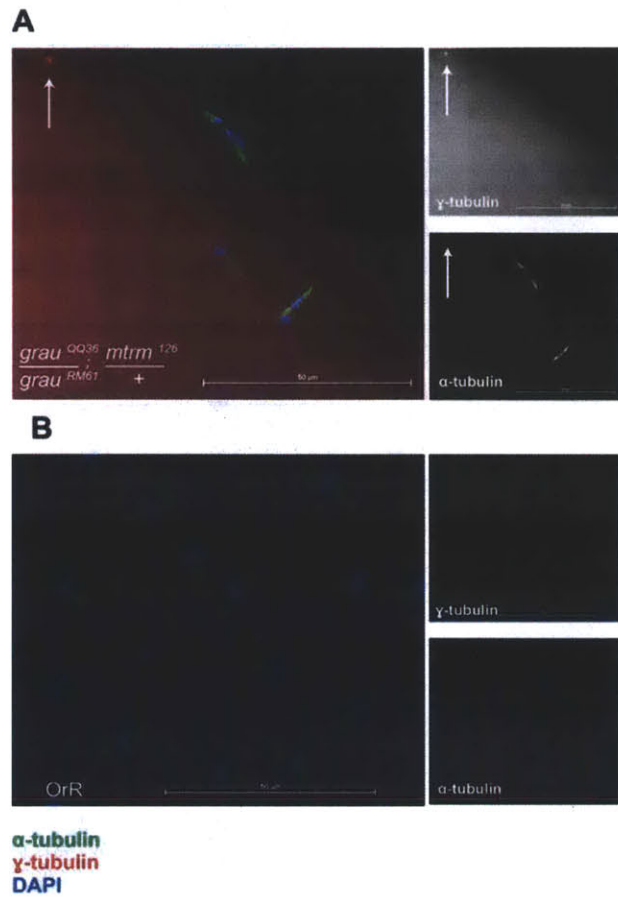
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A

$$\frac{grau^{QQ36}}{grau^{RM61}}; \frac{mtrm^{126}}{+}$$

γ-tubulin

α-tubulin

B

OrR

γ-tubulin

α-tubulin

α-tubulin
γ-tubulin
DAPI

Figure 3-7. *grau;mtrm/+* spindles are meiotic in structure

A) An egg laid by a *grau*$^{QQ36/RM61}$;*mtrm*^{126}/+ female is shown. A free centrosome (presumably deposited by the sperm) is indicated by the arrow. Although the free centrosome shows the presence of both alpha-and gamma-tubulin, the spindles contained in the egg are not enriched for gamma-tubulin at their poles. Scale bars represent 50um.

B) Mitotically dividing embryo from an *OrR* female. Centrosomes are readily detected by the presence of gamma-tubulin at the spindle poles. Scale bars represent 50um.

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severe, with DNA completely fragmented and tubulin in almost random configurations (Figure 3-10G). In our hands the heterozygous $polo^{11}$ mutation alone also exhibited defects similar to *mtrm* overexpression alone, but these fell primarily into one phenotypic category (Figure 3-10H). These data are consistent with increased Mtrm in the early embryo causing developmental defects due to excessive inhibition of Polo kinase activity (and potentially other, unknown targets).

To address the possibility that Matrimony affects proteins other than Polo, we expressed a mutant form of Matrimony deficient in Polo binding. Mtrm-T40A is unable to bind Polo, and cannot rescue chromosome non-disjunction in *mtrm/+* heterozygotes (Bonner et al., 2013; Xiang et al., 2007). In contrast to wild-type Matrimony, expression of Mtrm-T40A did not cause any developmental defects (Figure 3-10H). Importantly, expression of both the WT and T40A transgenes is similar using the maternal alpha tubulin driver (Figure 3-9C). Thus, high levels of Matrimony in the early embryo cause developmental defects due to inhibition of Polo kinase activity.

Discussion

Despite its pivotal role in development, regulation of the oocyte-to-embryo transition is poorly understood. Given the maternal stockpiles in the oocyte, mechanistic differences between meiosis and mitosis, and meiosis-specific forms of the APC/C, it is crucial to determine which proteins need to be degraded to switch correctly from meiosis to mitosis. The meiosis-specific activator Cort is essential for the transition from oocyte to embryo despite Fzy/Cdc20's presence. Cortex's existence raised the possibility that degradation of particular meiosis-specific proteins may be necessary for the onset of embryogenesis. Here we show this to be the case: the Cort form of the
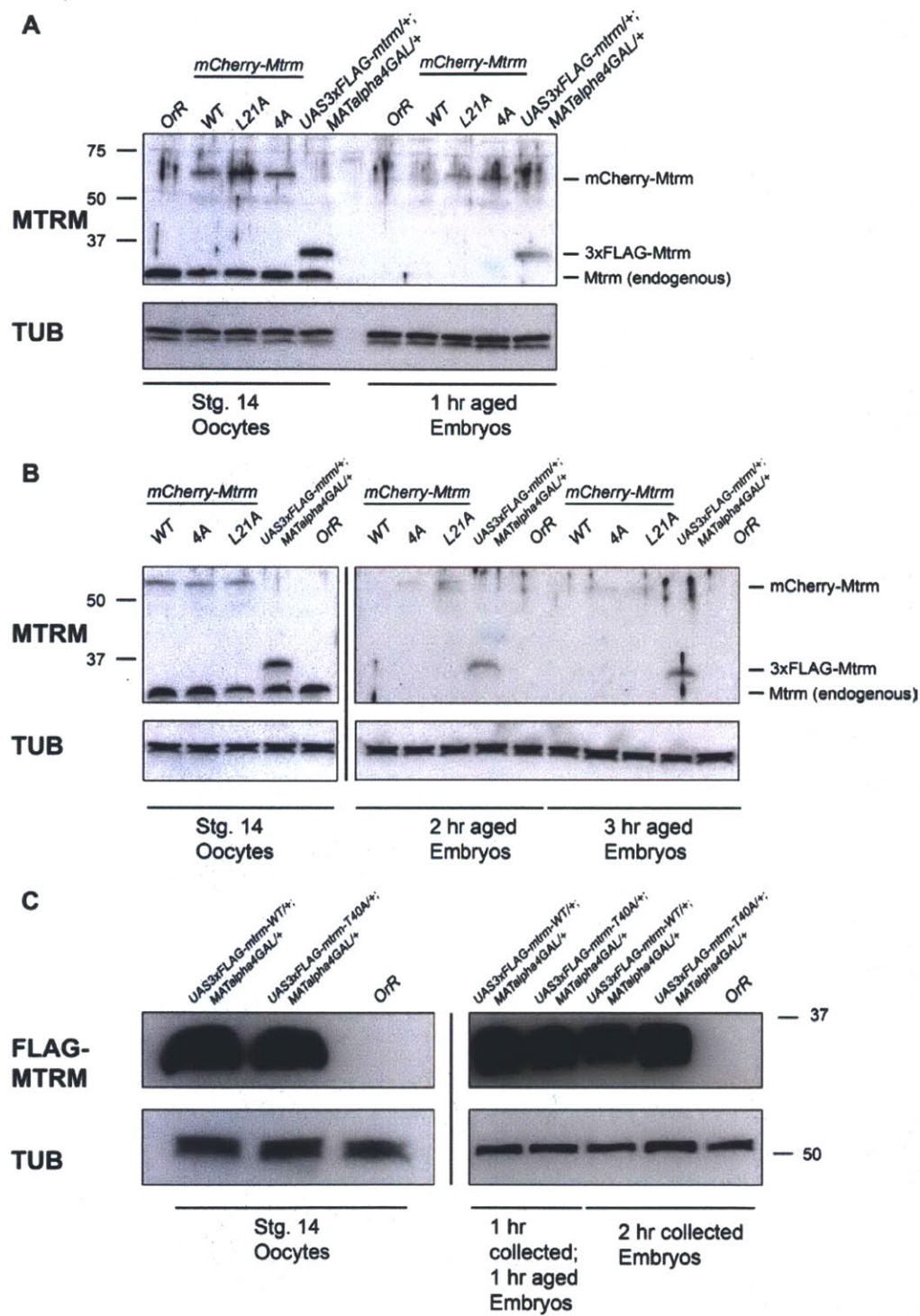
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Figure 3-9.

Figure 3-9. Comparison of Mtrm protein levels from various transgenic lines.

A) Western blot showing protein amounts from the indicated genotypes. (UAS) 3xFLAG-

Mtrm is seen at higher levels than stabilized mCherry-Mtrm (expressed from the

endogenous *mtrm* promoter) in both stage 14 oocytes and activated, fertilized eggs

(collected for 1 hour and left to develop for 1 hour in A. Molecular weight markers are

indicated to the side of the blot.

B) Activated eggs were collected for 30 minutes and left to develop for 2 or 3 hrs.

Molecular weight markers are indicated to the side of the blot.

C) Activated eggs were collected/aged as indicated. Molecular weight markers are

indicated at the side of the blot. Stg. 14s and activated eggs are from two different blots.

APC/C is required for Mtrm's destruction at the oocyte-to-embryo transition.

Furthermore, reduced levels of Mtrm heading into embryogenesis are necessary for

proper development, indicative of requirements for differential levels of the protein in

meiosis and mitosis.

A requirement for reduction in levels of Mtrm is illustrated by the deleterious

effects of overexpression of the protein in the embryo. A crucial role for Mtrm

degradation in the transition from oocyte to embryo is supported by the observation that

reduction in levels of the protein can suppress the developmental block caused by low

activity of Cort. In the grau mutants, levels of Cort are reduced, and the mutant oocytes

arrest in meiosis. By mutating a single copy of the mtrm gene this arrest was

overcome, the eggs progressed and several nuclear divisions occurred.

Mtrm provides key insights into how protein degradation can be regulated at the

oocyte-to-embryo transition. Mtrm is not completely removed from the embryo,

illustrating that its protein levels are important and degradation does not have to be an

all-or-none process. In this case, APC^{Cort} acts as a rheostat, allowing for high levels of

Mtrm in meiosis and low levels in mitosis. Consistent with this, it is interesting that

stabilized forms of Mtrm (Figure 3-4G) present at lower levels than the overexpressed

wild-type form (Figure 3-9A/B) did not exhibit an embryonic phenotype (only 2/69

mCherry-L21A-mtrm embryos exhibited abnormal development). mCherry-Mtrm also is

present at levels lower than endogenous Mtrm in stage 14 oocytes, and therefore may

never reach high enough levels to be able to cause the more substantial developmental

defects seen with the overexpressed form of Mtrm. This offers evidence for a specific

threshold of Mtrm that can be tolerated in the early embryo.

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Polo kinase is a critical regulator of both mitosis and meiosis, and is conserved

from yeast to humans. *polo* (and its orthologs) help regulate mitotic/meiotic entry,

chromosome segregation, centrosome dynamics, and cytokinesis (Archambault and

Glover, 2009). With such diverse roles during mitosis and meiosis, Polo function must

be carefully regulated. Upregulation of human Polo-like kinase (Plk1) is prevalent in

many human cancers, and identifying potent inhibitors of Plk1 is the focus of much

research (Strebhardt and Ullrich, 2006). In Drosophila, without inhibition by Mtrm during

prophase of meiosis I, Polo prematurely triggers nuclear envelope breakdown (through

activation of the Cdc25 phosphatase) and eventually leads to chromosome

nondisjunction (Xiang et al., 2007). Mutation of *polo* has direct consequences on female

meiotic progression as well. During Drosophila embryogenesis, expression of Scant, a

hyperactive form of the Polo antagonist Greatwall kinase, leads to dissociated

centrosomes from prophase nuclei (Archambault et al., 2007). Embryos homozygous

for *polo*[1] show a wide array of defects, including irregular DNA masses with

disorganized spindles (Riparbelli et al., 2000), reminiscent of our *mtrm* overexpression

phenotype (Figure 3-10). These data illustrate the importance of Polo kinase in both

mitosis and meiosis, and that improper regulation of its activity can have disastrous

consequences on cell division.

Current evidence suggests that Mtrm regulates Polo activity during both meiosis

and mitosis (Archambault et al., 2007; Von Stetina et al., 2011; Xiang et al., 2007). Our

results shed light on how the oocyte/embryo might use the same protein to regulate

Polo during such drastically different cell divisions. Our data indicate meiosis requires

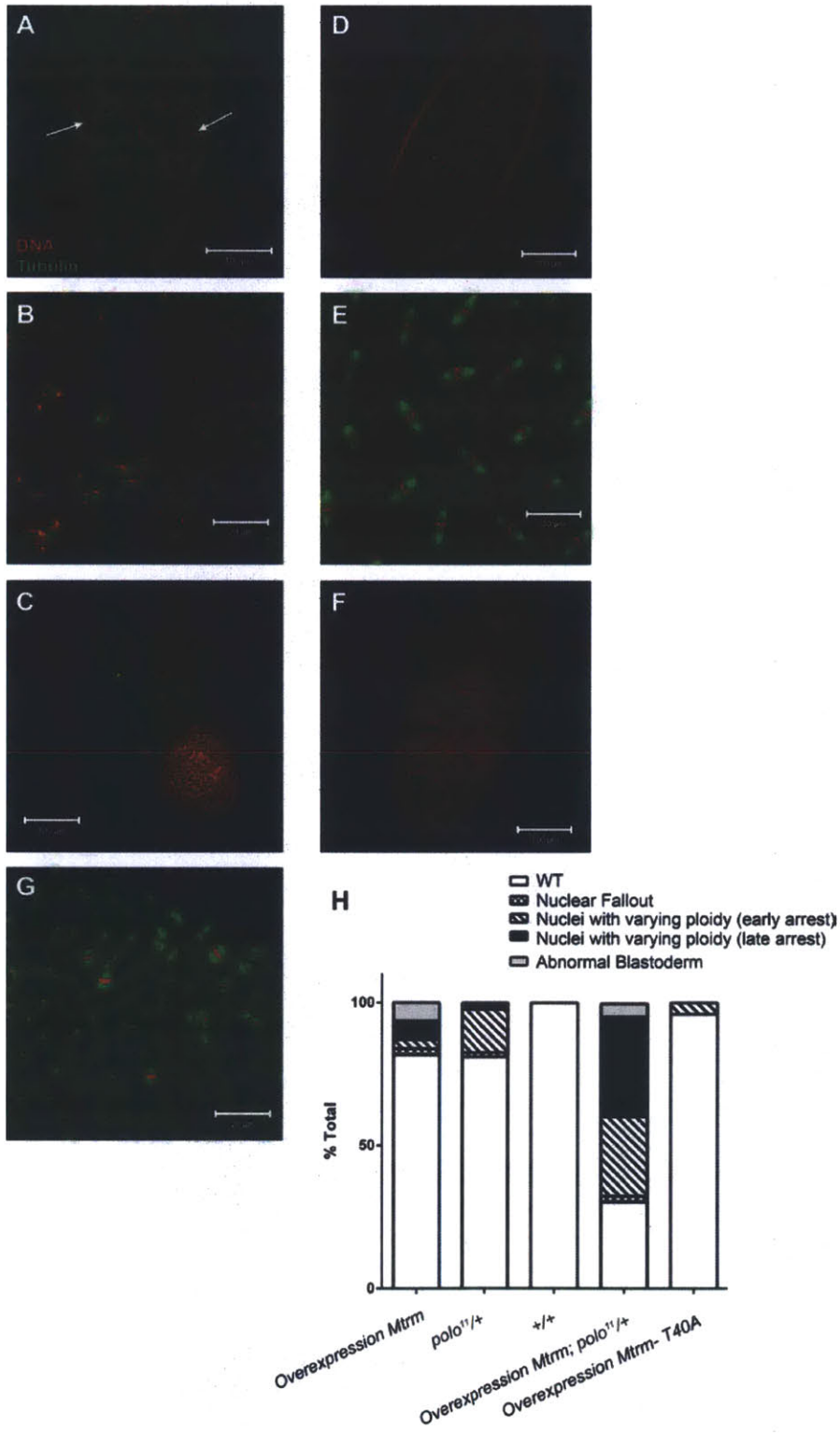high levels of Mtrm protein/Polo inhibition, while low levels of Mtrm are needed for early

Figure 3-10

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Figure 3-10. Developmental defects result from increased Mtrm expression.

A,B,C) Representative images of fertilized eggs laid by females overexpressing

3xFLAG-Mtrm using the *MATalpha4-GAL-VP16* driver. A) Embryo undergoing 'nuclear

fallout.' Nuclei can be seen having fallen below the surface of the embryo (white

arrows). B) An embryo showing scattered DNA with disorganized tubulin. C) An embryo

that underwent uneven development across its length, showing abnormal development

up to the blastoderm stage.

D,E,F) Control fertilized eggs showing proper development at comparable stages to

those in A-C). Scale bar indicates 100um in A, C, D, and F. It indicates 20um in B, E,

and G. G) A fertilized egg laid by females overexpressing 3xFLAG-Mtrm and

heterozygous for *polo^{11}*. These embryos predominantly had scattered DNA and

disorganized tubulin. H) Quantification of embryos shown in A-G. The genotype for

overexpression Mtrm is *UAS3xFLAGmtrm/+; P{matα4-GAL-VP16}V37/+* (n=93), the

genotype for *polo^{11}/+* is *polo^{11}/ P{matα4-GAL-VP16}V37* (n=137), +/+ is the control for

driver alone and is *TM6,Sb/ P{matα4-GAL-VP16}V37* (n=109), the genotype for

overexpression Mtrm;*polo^{11}/+* is *UAS3xFLAGmtrm/+; polo^{11}/P{matα4-GAL-VP16}V37*

(n=86), and the genotype for Overexpression Mtrm-T40A is *UAS3xFLAGmtrm-T40A/+;*

P{matα4-GAL-VP16}V37/+ (n=45).

embryogenesis. This is likely a mechanism to allow for fine tuning of Polo activity during the rapid divisions of the syncytial embryo.

The results here provide an interesting biological counterpoint to a recent study on the *S. cerevisiae* meiosis-specific APC/C activator Ama1. Previously, Ama1 had been known to act later in meiosis, regulating spore formation and Cdc20 degradation at meiosis II (Cooper et al., 2000; Tan et al., 2011). Okaz et al. showed APC^{Ama1} also acts earlier in meiosis to clear out mitotic regulators (including Polo/Cdc5) during the extended meiotic prophase I. Consequently, cells lacking Ama1 exit prematurely from prophase I (Okaz et al., 2012). It is interesting that two meiosis-specific APC/C activators have now been tied to regulation of Polo kinase. Ama1 has a direct, inhibitory effect early in meiosis, whereas Cort seemingly activates Polo indirectly through degradation of Mtrm late in meiosis.

Mtrm is not likely to be the only specific substrate of Cort, and it will be exciting to search for more APC^{Cort} substrates in the future. It will also be interesting to examine whether Cort targets continue to follow a graded versus all-or-none pattern of degradation during the oocyte-to-embryo transition. Further study of meiosis-specific APC/C activators will give valuable insight into the distinctions between meiotic and mitotic regulation and the control of the onset of embryogenesis.

Materials and Methods

Fly stocks

The *grau^{RM61}*, *grau^{QQ36}*, *cort^{RH65}*, *cort^{QW55}* (Chu et al., 2001; Page and Orr-Weaver, 1996; Schupbach and Wieschaus, 1989), *mtrm^{126}* (Xiang et al., 2007), *mr^1,mr^2*

(Kashevsky et al., 2002; Reed and Orr-Weaver, 1997) $twine^{HB5}$ (Courtot et al., 1992; Schupbach and Wieschaus, 1989), $polo^{11}$ (Archambault et al., 2007), $polo^{9}$ [Donaldson et al., 2001], and fzy^{6}, fzy^{7} (Dawson et al., 1993) alleles have all been described. The UASp *myc-cort* transgenic and lines were generated previously (Pesin and Orr-Weaver, 2007) and was driven by *w-;nanos-GAL4:VP16 (Van Doren et al., 1998)*. The *UASp-3xFLAG-mtrm^{WT}, UASp-3xFLAG-mtrm^{T40A}* , and *mCherry-mtrm^{WT}* (driven by its genomic promoter) were generated previously (Bonner et al., 2013; Xiang et al., 2007). *mCherry-mtrm^{4A}*, *mCherry-mtrm^{L21A}*, *mCherry-mtrm^{G170A}*, and *mCherry-mtrm$^{R95/R193A}$* were generated for this study (see below). w˙; P{matα4-GAL-VP16}V37 was obtained from Bloomington Stock Center (BL 7063). *Oregon R* was used as a wild-type control. Flies were maintained at 22 or 25 °C (Greenspan, 2004).

Transgenic Lines

To construct the *mtrm^{FL}* constructs driven by the genomic *mtrm* promoter, the following fragments were generated by PCR from a wild-type *mtrm* construct and *pFPV-mCherry* (a gift from the Susan Abmayr lab) and ligated into *pBluescriptSKII+*: *BamHI-mtrm* 5'UTR-*AvrII*, *AvrII-mCherry-PacI*, *PacI-mtrm* + 3'UTR-*XhoI*. The Stowers Molecular Biology facility deleted the *AvrII* and *PacI* sites using the Stratagene QuikChange II XL Site-Directed Mutagenesis Kit. The Stowers Molecular Biology facility made the point mutations using the Stratagene QuikChange II XL Site-Directed Mutagenesis Kit. The insert was digested and ligated into *pCasPeR4-attB*, and the sequence verified. The *pCasPeR4-attB-mtrm* constructs were injected into *y,w; attP40* embryos and integrations into the attP40 site were recovered.

IP-Mass Spec

Whole ovaries were dissected from 100 to 200 fattened females containing the *UASp-myc-cort* transgene being driven by *nanos-GAL4*. Ovary protein extracts were made by homogenizing in 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]). 110 µl Protein G magnetic bead slurry was coupled (and/or crosslinked using dimethylpimelimidate [Sigma]) to 27.5 µl anti-Myc [9e10] antibody or mouse random IgG. Whole ovary extract was split evenly and incubated with the anti-Myc or random IgG beads for 3hrs at 4 °C. Beads were then washed in 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, IP buffer + 0.5M NaCl once, then washed in IP buffer four more times. Bound proteins were eluted in sample buffer. Immunoprecipitated proteins were resolved by SDS-PAGE and silver stained. Bands were cut from the silver stained gel and reduced, alkylated and digested with trypsin. The resulting peptides were extracted and the volume reduced to 15 µl. The digestion extracts were analyzed by HPLC/ tandem mass spectrometry using a Waters NanoAcquity UPLC system and a ThermoFisher LTQ linear ion trap mass spectrometer operated in a data dependent manner. Tandem mass spectra were extracted by Extract_MSn. Charge state deconvolution and deisotoping were not performed. All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.4.0). Mascot was set up to search the refseq_fly_lc2_042413 database (27878 entries) assuming the digestion enzyme trypsin. Mascot was searched with a fragment ion mass tolerance of 1.00 Da and a parent ion tolerance of 3.0 Da. Iodoacetamide derivative of cysteine was specified in

Mascot as a fixed modification. Oxidation of methionine was specified in Mascot as a variable modification. Scaffold (version Scaffold_4.0.5, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Peptide Prophet algorithm (Keller et al., 2002). Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al., 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

Quantitative Mass Spectrometry

Eggs were collected from $cort^{RH65}$ females (kept with OrR males) and OrR virgins (mated to $twine^{HB5}$ males). Collections lasted for two hours, after which eggs were placed at 4°C until sample processing could take place. Eggs of each genotype were pooled together and homogenized in 10 volumes of SDT buffer (4% w/v SDS, 100mM Tris-HCl pH 7.6 and 0.1M DTT), boiled at 95°C for 5 minutes, sonicated, spun at 13,000 RPMs for 5 minutes at 4°C, and the supernatant was frozen in liquid nitrogen and kept at
-80°C. Protein concentration was determined by comparison to egg lysates of known concentrations on a Gel Code Blue (Pierce) stained SDS-PAGE gel followed by quantification using ImageJ.

Samples were submitted to the proteomics core facility at EMBL (Heidelberg, Germany) for quantitative mass spectrometry analysis. In brief, protein digestion was done as

previously described (Wisniewski et al., 2009), as was dimethyl labeling (Boersema et al., 2009) and LC-MS/MS (Hansson et al., 2012). Analysis was performed using MaxQuant software. Statistical significance was determined as in Cox and Mann (2008).

Westerns/Immunoblots

Whole ovaries and staged egg chambers were hand dissected from fattened females and homogenized in NP-40 lysis buffer (150mM NaCl, 50mM Tris, pH 8.0, 2.5mM EDTA, 2.5mM EGTA, 1%NP-40, 1mM PMSF, complete mini EDTA-free protease inhibitors, 1 tablet/10 ml [Roche]). Unfertilized eggs were obtained by mating virgin females of the indicated genotype to sterile $twine^{HB5}$ males and collecting for 2hrs (or O/N in the case of mr females). The eggs were then dechorionated in 50% bleach, and homogenized in NP-40 lysis buffer. Protein lysates were spun at 14,000 RPMs for 15 minutes at 4°C, and supernatant was used as protein sample. Equal protein amount was loaded on 10% SDS-PAGE gels as determined with Bradford reagent [BioRad]. Protein was transferred to Immobilon-P membranes (Millipore).

Antibodies used in this study were guinea pig anti-Mtrm (1:1000) (Xiang et al., 2007), mouse anti-CycA (1:50) [Developmental Studies Hybridoma Bank], mouse anti-CycB (1:50) [Developmental Studies Hybridoma Bank], rat anti-tubulin (yol1/34 and yl1/2) (1:400-1:1000) [Novus Biologicals], guinea pig anti-Cort (1:2000) (Pesin and Orr-Weaver, 2007), mouse anti-Myc 9E10 (1:400-1:1000) [Covance]. Mouse anti-RFP 3F5 (Chromotek) (1:500) was used to detect mCherry. Secondary antibodies used were Peroxidase-conjugated anti-mouse, Peroxidase-conjugated anti-guinea pig, and Alkaline Phosphatase-conjugated anti-rat (1:10,000; Jackson ImmunoResearch).

In vitro binding assays

In vitro binding assays using purified GST-Mtrm were done essentially as described

(Gutierrez et al., 2010), with some adjustments. *mtrm* cDNA (LD47919) was cloned into

pGEX6p-1 (GE Healthcare) for expression of GST-Mtrm. *6xmyc-cort, 6xmyc-cort*

ΔWD40 and *6xmyc-fzy/cdc20* cDNAs were cloned into pOT2. *cortΔWD40* encodes the

first 444 nucleotides of the *cort* ORF, followed by a stop codon (TGA). *In vitro*

transcription/translation was done using the TnT T7 Coupled Reticulocyte Lysate

System (Promega) according to manufacturer's instructions. 5 µl of the *in vitro*

translation reaction was added to beads in 500 µl IP buffer and rotated for 2hrs at 4 °C.

Beads were washed 3x in IP buffer and bound proteins were eluted with 40 µl 2x

sample buffer. 10 µl was analyzed by Coomassie to check levels of GST tagged

proteins, and 25 µl was analyzed by SDS-PAGE/ western blotting.

Cell culture, Transfection, qPCR, and FACS analysis

Kc167 Drosophila cell culture cells were maintained at 25 °C in Schneider's serum

media (Invitrogen) supplemented with 10% FBS (Sigma) and 50ug/ml Pen/Strep.

pMT-6xmyc-mtrm and *pMT-cort* were generated by cloning the respective constructs

into pMT-puro under control of the metallothionein promoter. Kc167 cells were

transfected with the indicated constructs using Cellfectin II (Invitrogen) according to

manufacturer's instructions. 48hrs after transfection, protein expression was induced

with 0.5mM $CuSO_4$ for 1-3 days. After induction, protein lysate was prepared by

homogenizing cells in NP-40 lysis buffer for SDS-PAGE/Western as described.

The Cort stable line was generated by transfecting Kc167 cells with pMT-Cort as above, and selecting for stable transfectants with puromycin (5ug/ml) over multiple passages for ~3 weeks.

For quantitative PCR, transfected Kc cells from a T25 (5ml) flask were resuspended in 2mls of 1xPBS. 1.2 mls was used to make protein extract as described and subjected to immuno blotting. 800 μl was used to isolate total RNA for absolute qPCR. Primers against *mtrm* were used to measure transgene expression, and primers against *act5C* were used for normalization. Quantitative PCR was performed using PerfeCTa SYBR Green FastMix (Quanta BioSciences) and analyzed on 7300 qPCR system software (Applied Biosystems).

For FACS analysis, the Cortex stable line or Kc cells alone were grown with or without $CuSO_4$ for one or two days. For proteasome inhibition, MG132 was added to 25uM 8hrs before cells were to be fixed. Kc cells were first washed in 5mls 1xPBS, and resuspended in 500ul PBS. Cells were then transferred into 4.5mls ice cold 70% ethanol and rotated for 2hrs at 4°C. Fixed cells were kept at -20°C until used for cell cycle analysis. Cells were pelleted at 2000 RPMs for 5 minutes and washed 2x in 5mls PBS (once in PBS, spun 2000 RPMs for 10 minutes). Cells were resuspended in 500ul PBS containing 50ug/ml propidium iodide, 0.15% Trition X-100, and 100ug/ml RNAse A, and rotated O/N at 4°C. Cells were then filtered and run on cell analyzer for cell cycle analysis.

Mtrm *in vivo* Degradation Timecourse

Kc167 cells in T75 flasks were transfected as above with *pMT6xmyc-mtrm* and either *pMT-cort^{WT}* or *pMT-empty* vector. 48hrs after transfection, $CuSO_4$ was added to the

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medium at a final concentration of 0.5mM. At the same time, MG132 (EMD Chemicals) was added to the media to 25uM. After eight hours of induction/treatment, cells were washed twice with serum media to remove MG132 and $CuSO_4$ and then resuspended in 7mls serum media. 700ul of resuspended cells were added to 5mls fresh media in T25 flasks containing 100 uM cycloheximide (Sigma-Aldrich) with or without MG132 (25uM). Cells were allowed to grow for the indicated amounts of time, and then were harvested for protein extraction/western blotting as above.

Non-disjunction Assays

Non-disjunction assays were carried out as in Bonner et al (Bonner et al., 2013).

Embryo Collection and Immunofluorescence

Females were allowed to lay eggs for 2hrs (Figure 3-6) or 1-2hrs with 3hr aging (Figure 3-10). Eggs were prepared for immunofluorescence as described (Pesin and Orr-Weaver, 2007). Propidium iodide (Figure 3-10) or DAPI (Figure 3-6) was used to stain DNA and anti-alpha tubulin [DM1A]-FITC (1:250) or anti-alpha tubulin (yol 1/34) 1:500 was used to visualize microtubules. Anti-gamma-tubulin (GTU-88; Sigma-Aldrich) was used at 1:500 to visualize gamma-tubulin on centrosomes. Anti-phospho-H3 (Rabbit polyclonal; Upstate/Millipore) was used at 1:300. When appropriate, secondary antibodies used were Alexa-488 anti-rat and Alexa-568 anti-mouse (1:1000; Life Technologies).

Acknowledgements

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Chapter Four

Discussion and Future Perspectives

I.   Introduction

Mitosis and meiosis exist to complete two very different tasks. Whereas mitotic divisions maintain diploid DNA content from mother to daughter cells, meiosis halves the genomic content of a germ cell. This reduction ensures restoration of the diploid state at fertilization and pronuclear fusion. To reduce the genomic content successfully, a number of meiosis-specific processes must occur. Meiotic recombination, segregation of homologs at meiosis I, and segregation of sister chromatids at meiosis II all require distinct meiotic regulation. The discovery and characterization of meiosis-specific APC/C activators have shed light on the differential regulation surrounding meiosis.

Both of the meiotic APC/C activators in yeast are required for proper meiotic progression. Ama1 (*S. cerevisiae*) and Mfr1 (*S. pombe*) play roles late in meiosis and are necessary for proper spore formation (Blanco et al., 2001; Cooper et al., 2000). Additionally, Ama1 regulates the lengthened prophase of meiosis I, ensuring proper timing of metaphase onset (Okaz et al., 2012). In Drosophila, sex-specific meiotic activators exist, and an exciting area of research is to find out why these sex-specific activators evolved and what function they serve in meiotic progression. In all cases, a mitotic APC/C activator is expressed alongside the meiosis-specific one (Pesin and Orr-Weaver, 2008), indicating specific roles for both types of activator in meiosis.

During oogenesis, both *cort* and *fzy/cdc20* are expressed and necessary for meiotic completion (Page and Orr-Weaver, 1996; Swan and Schüpbach, 2007). Some evidence indicates they may regulate substrate degradation at the spatial level. During mitosis of syncytial embryos, Cyclin B associated with the mitotic spindle is degraded at

anaphase onset (Raff et al., 2002). The same holds true for the meiotic spindles of meiosis II (though with slightly different timing). *cort* and *cdc20/fzy* mutants both show elevated levels of Cyclin B on metaphase II spindles, but in different patterns (Swan and Schüpbach, 2007). Whereas spindles in *cort* eggs accumulate Cyclin B at the spindle midzone, *fzy/cdc20* mutants accumulate Cyclin B along the length of the entire spindle. These data suggest Cort and Fzy/Cdc20 play unique roles in degradation of substrates at distinct spatial locations.

Might these activators also target a unique set of substrates distinct from the mitotic APC/C activators also present during meiosis? The current study supports this hypothesis, showing the female, meiosis specific APC/C activator Cortex specifically targeting the female-specific protein Matrimony (Mtrm) for degradation at the oocyte-to-embryo transition (Whitfield et al, submitted, Chapter 3).

II.   A Search for Meiosis-Specific Substrates of APC^{Cort} (Genetic Approach)

A deficiency screen to identify substrates of Cortex was undertaken and identified numerous hits (at the deficiency level). The screen was designed to require dominant suppression by any interacting deficiencies and should therefore identify only the strongest genetic interactors of *cort* (by proxy of *grauzone*). Although the screen identified numerous suppressing regions, isolation of single genes within them proved unsuccessful. Given that suppressing regions could be narrowed down with smaller deficiencies (typically from different deficiency collections), secondary background mutations on the deficiency chromosomes are an unlikely cause of genetic suppression. In the cases described in this study, it is likely that the actual genes responsible for

suppression were simply not tested. This bottleneck stems from a lack of available alleles to properly test all genes within a suppressing region. In the case of *Exel9012*, where only one annotated gene is found within the suppressing region, it is possible the deficiency uncovers the regulatory region of a nearby gene, thus lowering its expression. This possibility is bolstered by the ability of a nearby deficiency to also suppress (*BSC137*; data not shown). It is also possible that a protein coding gene within the region has not been annotated or that a noncoding RNA is responsible.

III. A Search for Meiosis-Specific Substrates of APC^{Cort} (Biochemical Approach)

This study identified Matrimony as a substrate of APC^{Cort}. *In vivo,* Mtrm protein levels were found to decrease rapidly upon the oocyte-to-embryo transition, and this decrease relied on *cort* and *morula/apc2* activity (Chapter 3). Moreover, this degradation is dependent on the leucine 21 of Mtrm's primary amino acid sequence. Mutation of this motif to alanine resulted in partially stabilized protein after the oocyte-to-embryo transition. Strikingly, L21 is found within the sequence LxExxxN, a motif found to be necessary for APC/C mediated degradation of the meiosis-specific yeast protein Spo13 (Sullivan and Morgan, 2007). While not targeted for degradation by the meiosis-specific APC/C activator Ama1, Spo13 is one of the only known meiosis-specific substrates of the APC/C.

We wondered whether the LxExxxN motif might be common among meiotic proteins. With the help of the BaRC bioinformatics group, a search for Drosophila proteins harboring the LxExxxN motif was performed. 1,882 genes unique genes were found to contain this motif. A careful evaluation of the proteins identified still needs to

be performed, but it is interesting to note that the genes identified were enriched for GO categories such as cell cycle, cell cycle regulation, and meiosis I.

Cortex also was shown to mediate Mtrm degradation in a secondary biological context. Although not endogenously expressed in Kc167 Drosophila cultured cells, plasmids encoding both Cortex and Mtrm were transiently transfected. It was found that expression of a wild-type form of Cortex specifically decreased Mtrm protein levels. This decrease in protein levels was found to be post-transcriptional and dependent on proteasome activity. In the absence of a functional *in vitro* ubiquitination assay, this system can still illustrate the effect Cort exerts on its substrates.

Given that Matrimony protein levels are severely down regulated at the oocyte-to-embryo transition, we hypothesized that excess Mtrm protein would be detrimental to embryonic development. Overexpression of Mtrm does lead to severe developmental defects, but only in a small fraction of embryos. The overexpressed Mtrm is still subject to proteolysis, and so only a small fraction of what is initially overexpressed makes it into the early embryo. It would be useful to create transgenic flies capable of overexpressing the stabilized L21A mutant form of Matrimony. In addition to being initially overexpressed, Mtrm-L21A would persist at higher levels longer into embryogenesis, thus likely affecting a larger number of embryos.

IV.   Implications for Human Meiosis

Faithful meiotic chromosome segregation seems to be a specific problem for humans. The risk of mis-segregating a chromosome during meiosis in *S. cerevisiae* is estimated to be 1 in 10,000 and about 1 in 2,000-6,000 in Drosophila. Rates are considerably higher in mammals. Mice are estimated to experience non-disjunction at a

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rate of around 1% (Hassold and Hunt, 2001; Hunt et al., 2003; Koehler et al., 1996; Sears et al., 1992). The error rate in humans however, is even higher. 1-2% of sperm are estimated to be aneuploid, while a striking 20% of eggs are thought to contain incorrect chromosomal content (Delhanty, 1997; Hassold and Hunt, 2001; Volarcik et al., 1998). In Drosophila, oocytes lacking Cortex exhibit signs of chromosome mis-segregation during meiosis I (Page and Orr-Weaver, 1996). The exact cause of this mis-segregation is unknown, but identifying the reason(s) should shed light on the general processes that can go wrong during meiosis. This high rate of error in the human egg is partially attributed to the long prophase I arrest an egg must tolerate during a female's reproductive lifetime. It is thought that abnormal spindle morphology and loss of proper chromosome cohesion over time contributes to increased incidence of aneuploidy in older females (Kurahashi et al., 2012; Revenkova et al., 2010; Volarcik et al., 1998). These data illustrate the importance of understanding the prophase I arrest and how it is properly regulated for such a long period of time in humans. Study of factors directly involved in maintenance and regulation of the prophase I arrest will aid in this endeavor. Mtrm is absolutely essential for maintenance of the prophase I arrest during Drosophila oogenesis and its heterozygous mutation can lead to chromosome non-disjunction (Xiang et al., 2007). It is exciting to speculate the existence of a Mtrm-like protein in humans, particularly given the attention paid to regulation of the prophase I arrest. Conversely, there may have been an evolutionary loss of Mtrm-like proteins in mammals, resulting in a less well-regulated prophase I and a higher rate of meiotic non-disjunction.

In addition to proper regulation of the prophase I arrest, recombination between homologous chromosome pairs must be carefully controlled to ensure accurate chromosome segregation at meiosis I (Champion and Hawley, 2002; Hassold et al., 2007). As discussed above, mutants of the meiosis-specific APC/C activator Ama1 do not properly form cross overs between homologs and exhibit meiotic non-disjunction, illustrating a direct role for meiosis-specific APC/C activators in regulation of another meiosis-specific process (Okaz et al., 2012).

While meiosis specific activators have yet to be identified in higher eukaryotes, at least some of their functions are likely to be conserved evolutionarily. As discussed above, both Ama1 and Cortex regulate meiosis-specific processes that are conserved throughout evolution (meiotic recombination and meiotic chromosome segregation). These processes are at the heart of what makes meiosis unique, and their regulation is likely conserved (to some degree). For example, Polo kinase plays a key role in prophase I regulation from yeast to mouse (Jordan et al., 2012; Okaz et al., 2012; Xiang et al., 2007). Interestingly, meiosis-specific APC/C activators in yeast and fly have been directly or indirectly tied to regulation of Polo activity (This thesis, Ch. 3, Okaz et al., 2012). Examples such as these illustrate meiosis-specific APC/C activators and their substrates are involved in some of the unique aspects of meiotic regulation. It is enticing to hypothesize that the *mechanism* by which meiosis-specific activators regulate meiosis also is conserved evolutionarily. However, despite the pivotal role they play in yeast and fly meiosis, meiosis-specific APC/C activators have yet to be found in other species. If APC activators truly do not exist in higher eukaryotes, their function may be mediated by other proteins.

The clearest choice for proteins that could have adapted to serve the role of meiotic APC activators would be the *mitotic* APC activators themselves, Cdc20 and Cdh1. Given a typical APC recognition motif consists of only three amino acids, it is conceivable that a substrate of Cortex, for example, could evolve a D-box or a KEN box (converting it to a substrate of Cdc20 or Cdh1in higher eukaryotes). This would allow for the substrate to still be efficiently degraded in an APC/C dependent manner, but without the need for an actual meiosis-specific APC activator. While simply evolving a D-box or KEN box would not convert most proteins into substrates of the APC/C, a protein that was already a target of APC^{Cort} or APC^{Ama1} would be much more readily adapted to ubiquitylation by APC^{Cdc20} or APC^{Cdh1}. So while meiosis-specific APC/C activators have yet to be found outside of yeast and flies, it is very possible their functions are conserved. Further study of meiosis-specific APC/C activators, particularly the sex-specific activators found in Drosophila, will shed light on the unique features of meiotic regulation.

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