Regulation of Anaphase Promoting Complex/Cyclosome to Control M Phase Exit

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Wanli Tang

Department of Pharmacology and Cancer Biology
Duke University

Date:_____

Approved:

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Sally Kornbluth, Supervisor

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Xiao-Fan Wang

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Daniel Lew

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Ann Marie Pendergast

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Gerard Blobe

Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
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ABSTRACT

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Abstract

The Anaphase Promoting Complex/Cyclosome (APC/C) is a RING E3 ligase that plays essential roles both within and outside of the cell cycle. At the onset of anaphase, the APC/C targets cyclin B and securin for degradation, initiating chromosome separation and mitotic exit. Regulation of APC/C activity is critical for a functional cell cycle, and this is largely mediated by cytostatic factor (CSF) activity and the Spindle Assembly Checkpoint (SAC).

Prior to fertilization, vertebrate eggs are arrested in metaphase of meiosis II by CSF activity, a key component of which is the APC/C inhibitor Emi2. Although the roles and regulation of Emi2 in maintaining CSF arrest have been extensively studied, its function during the oocyte maturation process, especially at the meiosis I to meiosis II (MI-MII) transition, was not well understood. Studies presented in this dissertation characterize an Emi2-mediated auto-inhibitory loop of the APC/C that provides the molecular basis of a critical biochemical event during the MI-MII transition—the partial degradation of cyclin B. In brief, phosphorylation of the Emi2 N-terminus by Cdc2/cyclin B targets it for proteasomal degradation in meiosis I (MI). During anaphase of MI, the APC/C triggers its own inactivation by degrading cyclin B, therefore stabilizing its inhibitor, Emi2. The timely inactivation of APC/C activity prevents the

complete inactivation of Cdc2 kinase, which is crucial for prohibiting S phase onset and parthenogenetic activation of the oocytes.

To better understand the regulation of the APC/C, a number of the studies presented here are aimed at identifying the mechanism for Emi2 inhibition of the APC/C. Many APC/C inhibitors have been reported to function as "pseudosubstrates", which inhibit the APC/C by preventing substrate binding. After carefully examining the ubiquitin reactions mediated by the APC/C *in vitro*, we have found that it is the last step in the ubiquitylation process, where ubiquitin is transferred from a charged E2 to the substrate, that is targeted by Emi2. In addition, biochemical studies have also revealed that Emi2 itself has RING-dependent ligase activity and this activity enables it to inhibit the APC/C in a sub-stoichiometrical manner.

Although the ultimate goal for both CSF activity and the SAC signaling pathway is APC/C inhibition, a much more complicated regulatory network is known to control SAC. Previous research in our lab has identified Xnf7 to be an APC/C inhibitor that is required for the SAC pathway in *Xenopus* egg extract. In an effort to characterize the human Xnf7 homolog, we have found that Trim39, a protein that has been implicated in apoptosis regulation, is required for the SAC pathway in RPE cells. Like Emi2, both Xnf7 and Trim39 are RING E3 ligases whose activity is essential for their function. Interestingly, the ligase activity of both proteins appears to be regulated by the checkpoint. While we continue to characterize the roles and regulation of both Trim39

and Xnf7 in the SAC, future investigations into the mechanisms that underlie APC/C inhibition by all the three E3 ligases—Emi2, Xnf7 and Trim39—would be of great interest.

Dedication

To my wonderful husband, Zhizhong Li, for his trust, support and love.

Contents

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List of Abbreviations

APC/C	Anaphase Promoting Complex/Cyclosome
BubR1	Budding uninhibited by benzimidazole
CamKII	Calcium/Calmodulin-dependent kinase II
Caspase	Cysteine-dependent aspartate-directed protease
Cdk	cyclin dependent kinase
CSF	Cytostatic factor
D box	Destruction box
ELB	Egg lysis buffer
FL	Full length
GVBD	Germinal vesicle breakdown
IR	Isoleucine arginine region
IVT	*In vitro* translated
Mad2	Mitotic-arrest deficient homologue 2
MCC	Mitotic checkpoint complex
MI	Meiosis I
MII	Meiosis II
MPF	Maturation promoting factor
MS	Mass spectrometry

ND	Non-degradable
Noc	Nocodazole
OA	Okadaic acid
Plx1	*Xenopus* polo-like kinase 1
PP1	Protein phosphatase
PP2A	Protein phosphatase 2A
Pre-RC	Pre-replication complex
ROS	Roscovitine
SAC	Spindle assembly checkpoint
SCF	Skp1/cullin/F-box protein
RING	Really interesting new gene
TPR	Tetratriopeptide repeat
TRIM	Tripartite motif
UPS	Ubiquitin-proteasome system
Ub/U	Ubiquitin
WT	Wild type
ZBR	Zinc binding region

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First and foremost, I want to thank my thesis advisor, Dr. Sally Kornbluth, for her tremendous support and guidance throughout these years. Her enthusiasm for science has been such a great inspiration to me and has made an incredible imprint on my scientific career. Ever since the day I joined the lab, she has constantly encouraged me to think creatively and critically, which has really made me who I am today. No matter how busy she is, she always has time for her students when they are in need, to which I am deeply grateful. I feel extremely lucky to be her student and I just could not imagine a better mentor.

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I will always be very grateful for the help and training I have had from two previous students, Judy and Jessie, who were more like sisters to me. Jessie worked in

the same bay with me when I first joined the lab. For someone who was always doing five experiments at one time, she was incredibly patient with me and my hundreds of random questions on a daily basis. I have worked closely with Judy on some of the same or related projects since my rotation. As a senior student, she was such an amazing teacher to me who not only taught me all kinds of techniques but also selflessly showed me her way of scientific research. She has contributed significantly to the Emi2 projects, and this dissertation would not be possible without her.

I would also like to thank Chris for his generous help with preparation of figures as well as this dissertation, which has been greatly improved with his critical reading and meticulous editing. A special thanks to Jen, who has also graciously included me in her project and helped me with her knowledge of the cell cycle in my first year; to Chen, for his great input in the Emi2 mechanism project and Trim39 project; to Liguo, for cooperating on the Trim39 project and driving it forward while I was away working on the dissertation; to Reichen, for his help with the Emi2 mechanism project; to Taisuke, for kindly teaching me all tissue culture-related techniques, from cell passaging to FACS analysis; to Manabu, for showing me how to perform RT-PCR, gel filtration and protein expression in Sf9 cells; to Josh, for his help with lentivirus production and related experiments; to Mike, for introducing me to the Gateway system, which greatly improved my cloning efficiency; and to all other former and current members in the

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1. Introduction

1.1 The cell cycle

The cell cycle, or cell division cycle, consists of a series of ordered events that lead to the production of two identical daughter cells. In eukaryotes, the cell cycle can be divided into two separate phases: interphase and M phase (Fig. 1). During interphase, the cell replicates its genetic information and undergoes significant protein synthesis in preparation for division. This phase can be further categorized into three subphases: G1 (Gap 1), S (Synthesis) and G2 (Gap 2) phases. During G1, the cell synthesizes all of the enzymes necessary for DNA replication, which are used during S phase to replicate the entire genome of a cell in order to produce two exact copies of each chromosome. The repair of any damage to the newly replicated DNA occurs during G2 as well as the final preparations for entry into M phase.

Nuclear division takes place during M phase, when the duplicated chromosomes are separated into two identical sets in two daughter cells. M phase can be sub-divided into five distinct stages: prophase, prometaphase, metaphase, anaphase, and telophase. During prophase, chromatin condenses into highly-ordered chromosomes and the mitotic spindle begins to assemble. Prometaphase starts when the nuclear membrane breaks down and the spindle microtubules extend to reach the chromosomes. Metaphase is reached when the spindle structure has properly formed and all of the chromosomes are aligned along an equatorial position equidistant from the two

centrosome poles, often referred to as the metaphase plate. In anaphase, the attached sister-chromatids are separated and pulled to the two opposing poles of the cell by the spindle microtubules. Events that reverse the effects of prophase and prometaphase occur in telophase, when the chromosomes are decondensed and the nuclear membrane reforms, completing nuclear division. After M phase, cells normally undergo the process of cytokinesis, in which the cytoplasm and other cellular contents divide into two daughter cells (Alberts et al., 2002).

1.1.1 Mitosis and meiosis

Mitosis and meiosis are two types of cell division that serve different purposes and yield distinct outcomes (Fig. 2). Mitosis is the process of autosomal cell reproduction through which a cell replicates its genetic information once and divides once, generating two daughter cells containing exactly the same number of chromosomes as the parental cell. In contrast, meiosis results in the production of gametes (i.e., sperm or egg cells). During meiosis, a diploid germ cell undergoes one round of DNA synthesis and two rounds of division (called meiosis I [MI] and meiosis II [MII]) resulting in four cells, each with half of the chromosomes as the parental cell. In MI, pairs of homologous chromosome separate into two cells, reducing the ploidy of the original cell by a factor of two. In MII, the coupled sister-chromatids segregate and four haploid cells are created.

Whereas mitosis serves as the fundamental mechanism for cell self-reproduction, meiosis provides the basis of sexual reproduction. The halving of ploidy in gametes is essential for stable sexual reproduction, as fertilization would otherwise result in a doubling of chromosome copy number with each generation. In addition, paired homologous chromosomes can exchange genetic information during MI, which further promotes the genetic diversity of the species (Alberts et al., 2002).

1.1.2 Regulation of the cell cycle by kinases and phosphatases

The events comprising the cell cycle are highly coordinated, both temporally and spatially. Efficient regulation of these events is not only crucial for ensuring successful cell division, but is also necessary for cell survival. The phosphorylation of proteins play critical roles in essentially every aspect of cell activity, especially during the cell cycle (Johnson, 2009). The regulation of protein activity by phosphorylation, mediated by a class of proteins known as kinases, has been intensively investigated over the past two decades, with a number of kinases identified in multiple model organisms. Recently, the important functions of phosphatases in cell cycle regulation have also been discovered and an increasing number of studies have been directed to their roles and regulation (Bollen et al., 2009; Queralt and Uhlmann, 2008).

1.1.2.1 Roles of cyclin-dependent kinases and other kinases

Cyclin-dependent kinases (Cdks) were the first kinases identified to control cell cycle progression and they are by far the most important (Fig. 3). Cdks need to be

associated with their respective cyclin binding partners in order to be active, and different Cdk/cyclin pairs govern different cell cycle stages. While the protein levels of the Cdks remain relatively stable throughout the cell cycle, the levels of cyclins change, resulting in a fluctuating pattern of Cdk kinase activity. During interphase, external mitogenic factors trigger the expression of cyclin D, which pairs with Cdk4 and Cdk6 and drives G1 phase progression during which cyclin E is expressed. Cdk2/cyclin E promotes the G1/S transition and the synthesis of cyclin A, also known as the S phase cyclin. After S phase entry, cyclin E is quickly degraded and the Cdk2/cyclin A pair predominates and triggers DNA replication (Draetta and Beach, 1988; Draetta et al., 1988; Nigg, 2001).

Cdk1/cyclin B (or Cdc2/cyclin B), the first discovered Cdk complex, is the primary kinase that drives M phase entry. The Cdc2/cyclin B kinase complex was first identified as a cytoplasmic factor capable of driving MI entry in *Xenopus* oocytes in the absence of hormonal stimuli and was described accordingly as "maturation promoting factor" (or MPF) (Doree and Hunt, 2002; Dunphy et al., 1988; Gautier et al., 1988; Jones, 2004; Masui, 2001). As the master kinase of M phase, the activity of Cdc2/cyclin B is under tight regulation by phosphorylation. Before the G2/M transition, kinases Wee1 and Myt1 keep Cdc2/cyclin B inactive by phosphorylating the Thr14 and Tyr15 residues of Cdc2 (Coleman and Dunphy, 1994; Lew and Kornbluth, 1996). Before M phase entry

is initiated, the dual specificity phosphatase Cdc25 mediates the dephosphorylation of both sites, activating the kinase complex.

Besides Cdks, a number of other kinases have been demonstrated to regulate particular events during the cell cycle, including: 1) kinases in the Mos-MAPK pathway that mediate meiosis progression in vertebrate germ cells (Kosako et al., 1994b; Peter et al., 2002); 2) Polo-like kinases (Plk) that contribute to the regulation of Cdk activity and also function in centrosome maturation and mitotic spindle formation (Archambault and Glover, 2009; Nigg, 1998); and, 3) Aurora kinases that coordinate the centrosome cycle and the cell cycle (Lukasiewicz and Lingle, 2009). These and many other kinases compose the highly elaborate and efficient network that controls the flow of molecular events during a cell cycle (Nigg, 2001).

1.1.2.2 Roles of phosphatases in M phase exit

Exit from M phase requires not only inactivation of Cdc2/cyclin B but also dephosphorylation of mitotic phosphoproteins. In yeast, this process is catalyzed by Cdc14, which is mainly regulated by localization. Recent studies in our laboratory have established that protein phosphatase-1(PP1) is required for mitotic exit, but not exit from meiosis, in vertebrate cells (Wu et al., 2009). Other phosphatases, like PP2A and calcineurin, have also been found to be involved in dephosphorylating mitotic substrates. While the specific roles of these phosphatases remain to be determined, it is worth noting that their activities are also under tight control as with the kinases. For

example, the activity of PP1 is regulated by phosphorylation as well as inhibitor association. Together, by controlling the phosphorylation status of substrate proteins, the kinases and phosphatases coordinate and promote the progression of the cell cycle from one phase to the next.

1.1.3 Regulation of the cell cycle by the ubiquitin-proteasome system

Several regulatory features of the cell have evolved to ensure rapid transition between cycle phases as well as cycle unidirectionality. For example, key regulators are often degraded by the ubiquitin-proteasome system (UPS) after their use in driving a particular transition, allowing the cell cycle to move forward in only one direction (Reed, 2003; Sumara et al., 2008). The UPS promotes the covalent attachment of multiple ubiquitin molecules to substrate proteins, leading to the degradation of polyubiquitylated substrates by the 26S proteasome. Formation of polyubiquitin chains on substrates requires the sequential action of three enzymes: a ubiquitin-activating enzyme (E1), a ubiquitin conjugating enzyme (E2) and an E3 ligase (Fig. 4). Specificity of the ubiquitylation is usually achieved at the level of substrate recognition, which is mediated by the E3 ligase (Ciechanover, 1998; Jentsch, 1992a, b; Scheffner et al., 1995).

More than one thousand E3 ligases have been identified, all of which fall into two major categories (HECT domain-containing ligases and RING finger-containing ligases) and utilize different catalytic mechanisms. The HECT E3s typically form a thioester bond with ubiquitin before transferring it to their substrates (Ingham et al.,

2004). On the contrary, RING E3s bind simultaneously with E2s and substrates, promoting the ubiquitin transfer directly from E2 to substrates by bringing them into close proximity (Jackson et al., 2000; Petroski and Deshaies, 2005).

Two structurally related RING E3 ligase complexes, the Anaphase Promoting Complex/Cyclosome (APC/C) and the Skp1/cullin/F-box protein complex (SCF), play essential roles in cell cycle control (Nakayama and Nakayama, 2006). The activities of the two E3s are confined in largely non-overlapping phases during the cell cycle and each of them has a specific set of substrates. Generally, many SCF substrates (e.g., the Cdk inhibitors P21 and P27) are restraining factors that keep the cell cycle on hold until certain criteria are satisfied for a cell to advance into the next stage. Alternatively, most of the activity of the APC/C can be summarized as degradation and inactivation of cell cycle-advancing factors, like cyclin A, cyclin B and Plk. As key factors of cell cycle regulation, the activities of both of these E3s are tightly controlled by phosphorylation. In addition, a great deal of crosstalk occurs between APC/C and SCF. SCF promotes the activation of the APC/C by degrading its inhibitor, Emi1, during interphase, while certain substrate recognition subunits for SCF (also referred as F-box proteins), like Skp2 and Tome1, are substrates for APC/C. Working closely together, these two RING-containing ligases play essential roles in regulating and promoting cell cycle progression (Bashir et al., 2004; Cardozo and Pagano, 2004; Hansen et al., 2004; Peters, 1998; Vodermaier, 2004; Wei et al., 2004) .

1.2 The Anaphase Promoting Complex/Cyclosome

The Anaphase Promoting Complex/Cyclosome (APC/C), discovered 15 years ago by Heshko and Kirschner (King et al., 1995; Sudakin et al., 1995), is a large, evolutionarily conserved protein complex (estimated to be 1.5 MDa by biochemical fractionation and scanning transmission electron microscopy). It is the most complicated molecular machinery that has been identified to catalyze ubiquitylation reactions. Aside from its well-established roles in cell cycle control, recent studies have uncovered a number of unexpected aspects of APC/C function, including roles in controlling cell death and neural activities (Aulia and Tang, 2006; Carroll and Morgan, 2005; Harper et al., 2002; Heilman et al., 2005; Kim and Bonni, 2007; Wasch and Engelbert, 2005). Understanding the mechanism and regulation of the APC/C is of substantial interest for its implications in the process of cancer development as well as neuron degeneration.

1.2.1 The structure and composition of APC/C

The vertebrate APC/C is composed of twelve core subunits that remain associated throughout the cell cycle (Table 1). The stoichiometry of the subunits and their spatial organization has just started to be elucidated (Gieffers et al., 2001; Thornton et al., 2006). The catalytic core of the complex consists of APC2 and APC11, which contain a Cullin domain and a RING finger, respectively. The Cullin domain of APC2 associates with the RING finger on APC11, which is directly responsible for E2

recruitment and is capable of independently mediating ubiquitylation reactions *in vitro* (Gmachl et al., 2000; Leverson et al., 2000; Tang et al., 2001). The Tetratricopeptide Repeat (TRP)-containing subunits (D'Andrea and Regan, 2003), including APC3 (Cdc27), APC6 (Cdc16), APC7 and APC8 (Cdc23), are found to mediate the binding of the APC/C to its activators and they contain most of the phosphorylation sites on the APC/C. APC10 contains a DOC domain and is known to cooperate with APC/C activators to recruit substrates and to enhance the processivity of the ubiquitylation reactions (Carroll et al., 2005; Carroll and Morgan, 2002). APC1, APC4 and APC5 are proposed to act, in part, as a bridge and connect the catalytic core of APC/C to the TRP subunits (Fig. 5). The functions of the other subunits remain obscure (Peters, 2006; Thornton and Toczyski, 2006; van Leuken et al., 2008).

UbcH5 and UbcH10 (also known as UbcX or E2-C in other species) are the two E2s that collaborate with human APC/C (Aristarkhov et al., 1996; Yu et al., 1996) and both can independently support the ligase activity of the APC/C *in vitro*. Researchers have found that UbcH5 mediates the ubiquitylation reactions for several E3s while UbcH10 exclusively supports APC/C. In addition to facilitating ligation reactions, a recent study has shown that the N-terminal extension of UbcH10 plays a regulatory role in restraining APC/C activity. The ubiquitylation reactions supported by UbcH10 with a mutated N-terminal extension were more resistant to APC/C inhibitors. Linking this UbcH10 N-terminal extension to UbcH5 consistently rendered it more limited in

substrate selection (Summers et al., 2008). Recent work in yeast has provided insight into the cooperative mode of action by Ubc4 (homolog of UbcH5a) and Ubc1 (a homolog of vertebrate E2-25K), with mono-ubiquitination at multiple lysines being catalyzed by Ubc4 followed by Ubc1-mediated assembly of polyubiquitin chains (Rodrigo-Brenni and Morgan, 2007). Whether this mechanism is conserved in other model organisms is still unknown.

Activation of the APC/C requires association with its activators, Fizzy/Cdc20 (Fang et al., 1998; Kramer et al., 1998; Lorca et al., 1998) and Fizzy-related/Cdh1 (Kraft et al., 2005; Visintin et al., 1997), which act sequentially to promote APC/C activity at different cell cycle stages. Substrate recognition by the APC/C is known to rely largely upon these activators. Both Cdc20 and Cdh1 contain a C-terminal WD40 domain that recruits substrates (Passmore and Barford, 2005) and two conserved sequence motifs are required for APC/C binding: a C-box located in the N-terminus of the protein and an IR tail at the C-terminus (Schwab et al., 2001; Thornton and Toczyski, 2006; Vodermaier et al., 2003; Vodermaier and Peters, 2004). Recent studies have revealed that these activators also promote the intrinsic ligase activity of the APC/C, probably by triggering a conformational change of the E3 ligase (Dube et al., 2005; Passmore et al., 2005). For example, the C-box of Cdc20 has been demonstrated to be necessary for its role in stimulating the ligase activity of the APC/C (Kimata et al., 2008a).

1.2.2 Roles of the APC/C in the cell cycle

Several key events during the cell cycle require proper APC/C activity, including the initiation of anaphase, exit from M phase, and preparation for the next S phase. The APC/C was originally named after its roles in anaphase initiation, with securin and cyclin B being its main substrates. The segregation of chromosomes requires the degradation of a subunit of the cohesin protein complex that holds together the sister chromatids. The protease separase, which is restrained by securin before anaphase, is responsible for the destruction of cohesin. When all of the chromosomes are aligned at the metaphase plate, Cdc20-associated APC/C (APC/C^{Cdc20}) mediates the ubiquitylation and destruction of securin. Free from securin, the active separase then cleaves cohesin, resulting in the separation of chromosomes. At the same time, the APC/C^{Cdc20}-mediated degradation of cyclin B inactivates the Cdc2 kinase. Once Cdc2 is inactive, the APC/C^{Cdc20} complex disassembles and newly assembled APC/C^{Cdh1} begins to degrade late mitotic substrates, including Plk and Aurora kinases. As the mitotic kinases become either inactivated or degraded, the phosphatases that have been previously inhibited by the Cdc2 kinase become active and facilitate the dephosphorylation of M phase substrates, which enables the disassembly of the spindle structure, reformation of the nuclear membrane and formation of a cytokinetic furrow (Floyd et al., 2008; Lindon, 2008; Lindon and Pines, 2004; Peters, 2002; Thornton and Toczyski, 2006; Wasch and Cross, 2002).

The roles of the APC/C during the cell cycle do not end with the exit of M phase. In G1 phase, the APC/C^{Cdh1} becomes activated and plays at least two critical roles: First, it maintains a stable G1 by causing the destruction of S phase cyclin and stabilizing the Cdk inhibitor P27 before a cell is ready for the S phase entry. As previously described, degradation of P27 is mediated through the E3 ligase SCF^{Skp2}. By promoting the destruction of Skp2, APC/C allows the accumulation of P27 and thus prohibits the unscheduled activation of Cdk2/cyclin A. In addition to preventing a premature S phase entry, the activity of APC/C^{Cdh1} also enables the onset of DNA replication. Formation of pre-replication complexes (pre-RCs) on sites of replication origin requires low Cdk activity, which is ensured by continued cyclin destruction by APC/C. Moreover, degradation of geminin, a protein that prevents the full assembly of Pre-RCs, is also a prerequisite for DNA replication in vertebrates and is mediated through the activity of APC/C^{Cdh1}. Taken together, the roles of the APC/C^{Cdh1} during G1 phase make a significant contribution to preparing the cell for the next round of DNA replication (Diffley, 2004; Geley et al., 2001; McGarry and Kirschner, 1998; Sivaprasad et al., 2007; van Leuken et al., 2008).

1.2.3 Substrate recognition of APC/C

Certain amino acid sequences, or degradation motifs, are present in APC/C substrates and play an important role for APC/C recognition. The destruction box, or D-box (defined as R-X-X-L-X-X-X-D/E), was initially identified in cyclin B and can be found

in the majority of APC/C substrates (Glotzer et al., 1991; Pfleger et al., 2001). Another degradation motif, the KEN box (defined as K-E-N-X-X-X-D/E), was first characterized in Cdc20 and is also present in many APC/C substrates, including Aurora A and Plk1 (Burton and Solomon, 2001; Pfleger and Kirschner, 2000). Both domains are recognized by the WD40 domains of the APC/C activators Cdc20 and Cdh1. The D-box was initially proposed to be recognized by Cdc20 and the KEN box by Cdh1. However, recent studies have shown that both motifs can be efficiently utilized by either activator to drive the ubiquitylation of the substrates. In addition to these two motifs, other non-canonical motifs, such as the A-box or U-box, have also been identified, most of which are relatively poorly understood (Castro et al., 2003; Hatakeyama et al., 2001; Littlepage and Ruderman, 2002).

Numerous pieces of evidence have established that the roles played by the activators do not complete the whole story of substrate recruitment by APC/C. In early studies, removal of APC10/Doc1 was observed to increase the dissociation of substrate from the APC/C and decrease the processivity of ubiquitylation reactions, suggesting its role in promoting substrate binding (Carroll et al., 2005; Carroll and Morgan, 2002; Passmore et al., 2003). Recently, sites on the APC/C core subunits (APC3 and APC8) that are required for activator binding have been identified and mutation of these sites was observed to decrease activator binding without affecting the processivity of the substrate ubiquitylation (Matyskiela and Morgan, 2009). This finding strongly argues for the

direct association between core APC/C subunits and the substrate; it also supports a model in which the APC/C, activator and substrate form a trimolecular complex with each component interacting with the other two.

1.2.4 Regulation of the APC/C

1.2.4.1 The regulation of Cdc20 and Cdh1

The activity of APC/C requires association with its co-activators, which are tightly regulated by phosphorylation. Cdc20 is transcribed and synthesized in interphase, but it can only interact efficiently with APC/C in M phase, when several core subunits of APC/C are phosphorylated by mitotic kinases including Cdc2/cyclin B and Plk1 (Eckerdt and Strebhardt, 2006). On the contrary, Cdh1 cannot bind to APC/C in S, G2 and M phases due to its phosphorylation by different Cdk/cyclin pairs. Only when a cell exits M phase and Cdk activity drops can the dephosphorylated form of Cdh1 actively complex with APC/C and facilitate the ubiquitylation reactions of G1 phase substrates (Acquaviva and Pines, 2006; Baker et al., 2007; Kraft et al., 2003; Kramer et al., 2000; Lahav-Baratz et al., 1995; Pesin and Orr-Weaver, 2008).

This oscillation between APC/C^{Cdc20} and APC/C^{Cdh1} activity serves as one of the key mechanisms underlying cell cycle regulation (Fuller and Stukenberg, 2009; van Leuken et al., 2008). High Cdc2 activity promotes assembly of the APC/C^{Cdc20} in M phase and leads to the decrease of cyclin B levels and M phase exit. The activation of phosphatases, combined with decreased kinase activity, inactivates Cdc20 and enables

the formation of the APC/C^{Cdh1} complex, preparing the cell for the next round of DNA synthesis. The fast and irreversible switch between Cdc20 and Cdh1 at M phase exit is not only attributed to the sudden drop of Cdc2/cyclin B kinase activity, but also to the degradation of Cdc20 by APC/C^{Cdh1} in early G1 phase (Fig. 6).

1.2.4.2 The regulation of UbcH10

After S phase entry, the active Cdk2/cyclin A kinase complex can turn off the APC/C^{Cdh1} by promoting the dissociation of Cdh1 by phosphorylation. In theory, another level of inhibition is needed to restrain APC/C^{Cdh1} activity before G1/S transition so that the newly synthesized cyclin A can be accumulated in the first place. The controlled degradation of UbcH10, the APC/C specific E2, has been demonstrated to be at least partly accountable for the stabilization of cyclin A before S entry. As the rate of cyclin A degradation prior to the G1/S transition closely correlates with levels of UbcH10, the APC/C^{Cdh1} triggers its own inactivation in G1 phase by ubiquitylating UbcH10 and targeting it for destruction, thereby enabling the stabilization of cyclin A and allowing S phase entry (Peters, 2006; Rape and Kirschner, 2004). It should be noted that this is not the only instance of the APC/C triggering its own inactivation to promote cell cycle progression into the next phase. In Chapter Two we describe an additional auto-inhibitory loop of APC/C that is essential for the MI-MII transition in *Xenopus* oocytes.

1.2.4.3 The inhibitors of APC/C

Timely activation of the APC/C is of pivotal importance for normal cell cycle progression, which is ensured by a number of APC/C inhibitors acting at different cell cycle stages. Early mitotic inhibitor-1 (Emi1) is expressed right before the G1/S transition and provides an additional mechanism of APC/C^{Cdh1} inhibition to allow cyclin A stabilization. Emi1 remains active throughout S phase and is degraded at the onset of M phase by the E3 ligase SCF□^{TrCP}. The function of Emi1 during S phase is critical in preventing rereplication of DNA. With the accumulation of cyclin A, Pre-RC formation is inhibited by the increased level of Cdk2 kinase activity, preventing the refiring of the replication origin. In parallel, Emi1 also blocks the degradation of the APC/C^{Cdh1} substrate geminin, which further blocks Pre-RC formation and ensures that DNA replication occurs once and only once per cell cycle. Consequently, dysregulation of Emi1 has been reported to accompany genomic instability and contribute to the development of cancer, illustrating the significance of the proper timing of APC/C activity (Di Fiore and Pines, 2007; Gutgemann et al., 2008; Hsu et al., 2002; Machida and Dutta, 2007; Reimann et al., 2001a; Reimann et al., 2001b).

After a cell enters M phase, APC/C^{Cdc20} is potentially active, as high Cdk1 activity leads to the phosphorylation of APC/C and assembly of APC/C^{Cdc20}. However, anaphase cannot be initiated until the bipolar spindle is completely formed and all of the chromosomes are properly aligned at the metaphase plate. Before these criteria are met,

APC/C^{Cdc20} is kept inactive by the Spindle Assembly Checkpoint (SAC). Intuitively, aberrant SAC signaling is closely associated with chromosome segregation defects and aneuploidy, which are commonly found in many types of human cancer. A number of protein factors have been identified to be involved in the SAC signaling pathway, including *m*itotic-*a*rrest *d*eficient homologue (Mad)2 and *b*udding *u*ninhibited by *b*enzimidazole (Bub)R1, both of which were among the earliest discovered SAC proteins in yeast. Both Mad2 and BubR1 can directly bind to APC/C *in vitro* and inhibit its ability to catalyze ubiquitylation reactions (Diaz-Martinez and Yu, 2007; May and Hardwick, 2006; Musacchio and Hardwick, 2002; Musacchio and Salmon, 2007; Yu, 2002). SAC signaling will be discussed in detail later in this chapter.

In meiosis, APC/C is regulated by additional mechanisms and several inhibitor proteins are reported to be involved in different species. In yeast, the activity of Acm1 and Mes1 is crucial in preventing the complete inactivation of Cdk through APC/C inhibition between MI and MII. In vertebrates, the Emi1-related protein Emi2 (or Erp1 in *Xenopus*) has been demonstrated to be essential to the oocyte maturation process. A more complete list of APC/C inhibitors and their roles can be found in Table 2 (Irniger, 2006; Madgwick et al., 2006; Tung et al., 2005; Wu and Kornbluth, 2008).

1.3 Emi2 and the cytostatic factor

Vertebrate eggs are arrested at metaphase of meiosis II. Upon fertilization, this arrest is released and eggs enter their first interphase of the embryonic cell cycle. In the

1970s, researchers found that injection of an extract prepared from an egg into a two-cell-stage embryo could artificially create a similar M phase arrest in the injected blastomere; this observed activity was termed cytostatic factor (CSF) (Masui and Markert, 1971). In 2005, three groups independently identified Emi2/Erp1, the meiotic APC/C inhibitor, as the key component of CSF in *Xenopus* (Liu et al., 2006; Liu and Maller, 2005; Rauh et al., 2005; Tung et al., 2005). Since its discovery, there have been numerous studies on Emi2 regulation in the *Xenopus* system and its critical roles in mouse oocytes have also been recognized. The major findings regarding the regulation of Emi2 are briefly summarized below.

1.3.1 Degradation of Emi2 at fertilization

Three criteria were set for CSF activity when it was initially proposed: first, it should emerge as an oocyte matures and reach its peak activity during MII; second, it should be capable of inducing M phase arrest once introduced in embryonic blastomeres; third, it should be quickly inactivated upon fertilization, when the cytoplasmic Ca^{2+} level is elevated. Emi2 was known to be quickly degraded upon egg activation in a Plx1 (*Xenopus* Plk1)-dependent manner. Further characterization of the Emi2 degradation pathway at MII exit has revealed that Emi2 is a substrate for the calcium/Calmodulin-depent kinase II (CaMKII). Phosphorylation of Emi2 residue Thr195 by CaMKII provides a docking site for Plx1. Plx1-mediated phosphorylation of Emi2 creates a phosphodegron site for the SCF□^{TrCP}E3 ligase, leading to its

polyubiquitylation and further degradation by the S26 proteasome (Fig. 7). In summary, the sequential phosphorylation by CaMKII and Plx1 promotes the destruction of Emi2 and APC/C activation at MII exit (Hansen et al., 2006; Liu et al., 2007; Liu and Maller, 2005; Rauh et al., 2005).

The pathway described above was elucidated in the *Xenopus* system. Studies in mice have found that Emi2 is also present in maturing and MII mouse oocytes (Madgwick et al., 2006; Shoji et al., 2006). Depletion of Emi2 in MII oocytes causes M phase release, suggesting an evolutionary conserved role of Emi2. However, significant discrepancies may exist between the two systems and to date we are still far from understanding the signaling pathways that regulate Emi2 in the mammalian oocytes. For example, *Xenopus* Emi2 degradation at fertilization requires Plx1-mediated phosphorylation, but the phosphodegron targeted by Plk1 is absent in mammalian Emi2 without proximal substitutes. Clearly, additional studies are needed to fully elucidate the roles and regulation of mammalian Emi2.

1.3.2 Regulation of Emi2 by Cdc2 and PP2A

Emi2 can directly interact with the APC/C and this interaction is critical for its inhibitory function. Previous studies in our laboratory have demonstrated that the binding of Emi2 and APC/C is a dynamic process controlled by phosphorylation. The Cdc2/cyclin B kinase can phosphorylate Emi2 on two residues in the C-terminal region, Thr545 and Thr551, resulting in the dissociation of Emi2 from the APC/C. This reversible

inactivation of Emi2 allows transient activation of APC/C. Since cyclin B is under constant synthesis in CSF-arrested eggs, the Cdc2-mediated inactivation of Emi2 provides a mechanism to prevent unrestrained accumulation of cyclin B, which is essential in maintaining a relatively constant level of Cdc2 kinase activity in eggs. In addition, Cdc2-mediated phosphorylation of Emi2 can be antagonized by protein phosphatase 2A (PP2A). When cyclin B decreases to a level where the kinase activity of Cdc2 is exceeded by the phosphatase activity of PP2A, the phosphorylation of the two sites are removed and Emi2 is reactivated to bind and inhibit the APC/C. By controlling Emi2 activity, the balance between Cdc2 and PP2A allows homeostatic control of cyclin B levels to both maintain CSF arrest and allow for rapid M-phase exit at fertilization (Hansen et al., 2007; Wu et al., 2007b).

In addition to being regulated by C-terminal phosphorylation, Emi2 stability is also controlled by Cdc2-mediated phosphorylation on a cluster of four residues in the N-terminus (Ser213, Thr293, Thr252, and Thr267). Under physiological situations, with constant dephosphorylation by PP2A, these four sites remain unmodified and Emi2 is stable in CSF arrested eggs (Wu et al., 2007a). Only when Cdc2 kinase activity spikes beyond a certain threshold (about double the physiological level, which does not normally occur due to the function of the feedback loop described above) does Emi2 become phosphorylated at the N-terminal sites and is subjected to degradation through the $SCF^{\beta TrCP}$ E3 ligase. Cdc2-mediated phosphorylation and degradation of Emi2

provides an additional level of cyclin B homeostasis regulation in CSF arrested eggs; this also serves as the primary mechanism of Emi2 regulation during MI, which will be discussed in detail in Chapter Two.

1.3.3 Regulation of Emi2 by the Mos-MAPK pathway

Long before Emi2 was characterized, Mos had been identified as a master regulator of the oocyte maturation process and the Mos-MAPK pathway had been demonstrated to be essential for CSF maintenance (Frank-Vaillant et al., 1999; Gotoh and Nishida, 1995; Guadagno and Ferrell, 1998; Kosako et al., 1994a, b; Sagata et al., 1988; Sagata et al., 1989). Rsk, the downstream kinase of the Mos-MAPK pathway, was also shown to be crucial for CSF arrest (Bhatt and Ferrell, 1999; Gross et al., 1999; Gross et al., 2000). However, the link between Mos or Rsk activity and APC/C inhibition was not identified until 2007, when two groups independently reported that Emi2 was required for the CSF activity of Mos and that Rsk could directly phosphorylate Emi2 (Inoue et al., 2007; Nishiyama et al., 2007). Studies in our laboratory have further contributed to this model. In short, Mos-activated Rsk can phosphorylate Emi2 at Ser335 and Thr336. This phosphorylation promotes Emi2-PP2A association, enhancing Emi2 dephosphorylation at both the N- and C-terminal Cdc2 phosphorylation sites. Dephosphorylation of the N-terminal sites is essential to ensure Emi2 stability, while that of the C-terminal sites promotes its activity to inhibit APC/C (Fig. 8). Taken together, these findings delineate a model in which Mos, through its downstream kinases, promotes CSF arrest by

regulating the meiosis specific APC/C inhibitor Emi2 (Wu et al., 2007a; Wu and Kornbluth, 2008).

1.4 Xnf7 and the Spindle Assembly Checkpoint

Spindle Assembly Checkpoint (SAC) is one of the most important self-surveillance systems engaged by eukaryotes to ensure the fidelity of chromosome segregation in M phase (Fig. 9). The mechanisms of SAC activation and silencing have been extensively investigated since the 1990s and additional factors involved in this process are still being identified (Musacchio and Salmon, 2007).

1.4.1 Spindle Assembly Checkpoint: from activation to silencing

In all of the examined systems, the kinetochore appears to be the source of the SAC signal; although the exact mechanism by which it triggers SAC activation remains to be elucidated. It is generally accepted that the checkpoint monitors kinetochore-microtubule attachment and possibly the inter-kinetochore tension created by amphitelic attachments (Pinsky and Biggins, 2005). The fact that a single unattached kinetochore can generate a "wait" signal that can keep all APC/C complexes in check implies that some type of an amplification mechanism must be utilized to diffuse the signal throughout the cell. Several models have been proposed to explain this process, including a "Mad2-template" model, which is supported by investigations of kinetochore dynamics using fluorescence recovery after photobleaching (FRAP). Mad2 exists in two forms in the cell, either as O-Mad2 (the open form) or C-Mad2 (the closed

form). O-Mad2 usually present as monomers in the cytosol, but can be transformed into C-Mad2 under an active checkpoint where it tightly associates with Cdc20. The Mad2 template model describes a process in which an unattached kinetochore recruits Mad2 and triggers a conformational change from O-Mad2 to C-Mad2. The C-Mad2 can then catalyze other O-Mad2 in the cytosolic pool to transform into C-Mad2, rapidly amplifying the signal across the cytoplasm. $P31^{comet}$ is another protein that has been shown to compete for the binding of C-Mad2 to O-Mad2, thus stopping amplification of the signal once all kinetochores have become properly attached (May and Hardwick, 2006; Musacchio and Hardwick, 2002; Musacchio and Salmon, 2007; Nasmyth, 2005; Pinsky and Biggins, 2005).

A number of kinases involved in SAC signaling have been identified through RNA interference (RNAi): 1) BubR1 phosphorylates CENP-E to promote chromosome alignment and CENP-E is required for sustaining SAC signaling when only a few unattached kinetochores exist (Chan et al., 1999; Weaver et al., 2003); 2) Bub1 and Mps1 are both involved in the kinetochore recruitment of SAC proteins, including Mad1 and Mad2 (Abrieu et al., 2001; Johnson et al., 2004). In addition, Bub1 has also been proposed to sensitize Cdc20 to its SAC inhibition through phosphorylation (Tang et al., 2004a); and, 3) Aurora-B kinase is required for the SAC signals that are generated from faulty attachments of microtubules to the kinetochores rather than lack of attachment (Biggins and Murray, 2001). Together, these and many other kinases function in a highly

coordinated manner to promote both the kinetochore recruitment of the SAC proteins and the generation of robust SAC activity (Musacchio and Salmon, 2007; Nigg, 2001).

Once activated, the SAC executes its APC/C inhibitory function mainly through the Mitotic Checkpoint Complex (MCC). The MCC is consists of four proteins: Mad2, Cdc20, Bub3 and BubR1 (also known as Mad3 in yeast). While Mad2 and BubR1 are both capable of inhibiting the APC/C *in vitro*, studies have shown that BubR1 has a binding site on Cdc20 distinct from Mad2 and that these two proteins work synergistically in promoting APC/C inhibition. In fact, a partially purified MCC prepared from mitotic HeLa cells was found to exert 3,000-fold stronger APC/C inhibition as compared to Mad2 or BubR1 alone (Braunstein et al., 2007; Diaz-Martinez and Yu, 2007; Herzog et al., 2009; Sudakin et al., 2001). The mechanism by which MCC inhibits the APC/C is not completely understood, but Mad3 is reported to act at least in part by preventing substrate binding of the APC/C (Burton and Solomon, 2007).

Cdc20 is definitely the focus of regulation by SAC signaling (Yu, 2002). Recent studies have shown that Cdc20 can be ubiquitylated by the APC/C in a SAC-dependent manner (Reddy et al., 2007; Stegmeier et al., 2007). This ubiquitylation could decrease the binding of Cdc20 to both Mad2 and the APC/C, which is facilitated by the E2 UbcH10 and antagonized by the deubiquitinase Usp44. This process has been proposed to be necessary for SAC inactivation based on two observations: first, UbcH10 knockdown by RNAi strengthened the Mad2-Cdc20 association and delayed the

initiation of anaphase; second, the loss of Usp44 in cells resulted in a SAC defect phenotype, explained by the uncontrolled ubiquitylation of Cdc20 leading to the premature disassembly of the MCC. However, this model has been challenged by the finding that cells overexpressing lysine-less Cdc20 could inactive the SAC normally, which strongly argues against the roles of Cdc20 ubiquitylation in SAC silencing. Instead, ubiquitylated Cdc20 was found to be degraded, suggesting a dual inhibitory mechanism on Cdc20 under an active checkpoint. Not only is its activity tightly restrained by SAC proteins, it is also maintained at a low level to prevent the premature activation of APC/C (Chen, 2007; Nilsson et al., 2008).

1.4.2 Identification of Xnf7

Xenopus nuclear factor-7 (Xnf7) is an 80 kDa protein that belongs to a family of proteins possessing a tripartite motif (TRIM) containing RING and B-Box zinc-fingers and a coiled-coil domain. This family, also referred as the RBCC family, contains proteins implicated in various cellular processes, such as developmental regulation and oncogenesis. Xnf7 was first identified as a developmental regulator of dorsal-ventral patterning in *Xenopus* embryos. Based on its DNA binding ability, Xnf7 was speculated to function as a transcription factor, although no targeted genes have been identified to date. Moreover, Xnf7 was also reported to have microtubule bundling activity, which contributes to the integrity of spindle microtubules (El-Hodiri et al., 1997; Etkin et al., 1997; Maresca et al., 2005).

Although Xnf7 was isolated in our laboratory via its affinity for cyclin B, it demonstrated the unexpected function of inhibiting the ligase activity of APC/C. In CSF extract, overexpression of Xnf7 can block Ca^{2+}-induced degradation of cyclin B and securin, and immunodepletion of Xnf7 or addition of a neutralizing antibody results in an acceleration of this process. *In vitro* APC/C assays further confirmed that Xnf7 can independently inhibit APC/C-mediated ubiquitylation reactions (Casaletto et al., 2005).

1.4.3 Ligase activity of Xnf7 and its role in SAC

Further studies on Xnf7 have revealed that its mechanism of APC/C inhibition differs significantly from other inhibitors known at that time. Behaving as a RING E3 ligase itself, purified recombinant Xnf7 can undergo auto-ubiquitylation *in vitro,* activity that depends on an intact RING domain. Unlike other APC/C inhibitors (e.g., Mad2, which functions through Cdc20), Xnf7 directly binds to the core subunits of APC/C. Moreover, the intrinsic ligase activity of Xnf7 is required for its APC/C inhibitory function, as Xnf7 can only inhibit the APC/C *in vitro* when the specific E2 that supports its ligase activity is provided.

Depleting Xnf7 from CSF-arrested egg extract does not lead to cyclin B degradation, suggesting that it is not involved in APC/C inhibition by CSF activity. Nonetheless, loss of Xnf7 activity in a SAC-arrested egg extract causes its spontaneous release, strongly arguing for a role of Xnf7 in SAC signaling. As Xnf7 can inhibit the APC/C independently, it is likely to act as an SAC effector (Casaletto et al., 2005). As part

of my dissertation study, I have identified Trim39 to be the human homologue of Xnf7. The roles and regulations of Xnf7 and Trim39 will be further discussed in Chapter Four.

1.5 Studying the cell cycle using Xenopus egg extract

Stage VI *Xenopus* oocytes are arrested at the G2/M transition of meiosis I in the female ovary. Upon progesterone stimuli, the oocyte enters MI with high Cdc2/cyclin B activity, which is characterized by the breakdown of the oocyte nucleus, or germinal vesicle (germinal vesicle breakdown, or GVBD). Over the next couple of hours, the maturing oocyte will degrade cyclin B, exit M I and subsequently enter meiosis II, where it will eventually arrest in metaphase II, awaiting fertilization. At this point, the oocyte is a mature, fertilizable egg. Fertilization triggers the release of internal stores of Ca^{2+} within the egg, triggering the degradation of cyclin B and exit from M phase into the first mitotic cell cycle. The fertilized egg will then undergo multiple cell divisions without any transcriptional input from the zygotic DNA. These early embryonic cell cycles will be driven completely by maternal stores of mRNA and protein and will oscillate very rapidly between interphase and mitosis, without any intervening gap phases (Figure 10).

Extracts made from these matured eggs can recapitulate many of the biological events of the cell, including those that may be otherwise difficult to reconstitute *in vitro*. Moreover, extracts can be obtained in large quantities and easily manipulated. Overexpression can be achieved by the addition of proteins (recombinant or *in vitro*

translated) or mRNAs; factors can also be removed via either affinity or immunodepletion from the extracts. All of these characteristics make the *Xenopus* egg extract a powerful system to study the mechanisms underlying cellular processes, such as the cell cycle or apoptosis (Belmont et al., 1990; Blow and Laskey, 1986; Hutchison et al., 1987; Hutchison et al., 1988; Murray, 1991; Smythe and Newport, 1991; Verde et al., 1990).

To make egg extract, freshly laid eggs are collected, dejellied, washed with buffer, and lysed by centrifugation. Extracts that recapitulate different cell cycle stages can be generated depending on the constituents of the buffer and the approaches used to prepare the lysates.

Interphase extracts (S extracts): Crushing eggs by centrifugation causes the release of Ca^{2+} from internal stores, activation of APC/C and transition from meiosis II to interphase. Extracts are maintained at interphase by the addition of cycloheximide to prevent synthesis and reaccumulation of cyclin B. Addition of demembranated sperm chromatin and an ATP-regenerating system allows the assembly of nuclei which undergo a single round of DNA replication. Interphase extracts can be driven into M phase by the addition of recombinant cyclin B.

CSF extracts: *Xenopus* eggs are arrested at metaphase of meiosis II by CSF, which prevents cyclin B degradation. Eggs crushed in the presence of the Ca^{2+} chelator

EGTA do not activate CaMKII, preventing the destruction of Emi2, thus maintaining the metaphase II arrest. This prolonged meiotic arrest makes these extracts useful for the analysis of chromatin condensation and spindle formation. The addition of Ca^{2+} to CSF extracts triggers Emi2 degradation and cyclin B degradation, making these extracts an ideal system for studying the control of APC/C activation and M phase exit. Moreover, the addition of high concentrations of sperm chromatin and nocodazole (a microtubule depolymerizing drug) to CSF extracts can trigger the SAC, making these extracts useful for studying the molecular events under active the active checkpoint (Minshull et al., 1994).

Cycling extracts: During natural fertilization, sperm entry promotes the destruction of CSF, permitting commencement of the normal embryonic cell cycles. To generate extracts that recapitulate these early embryonic divisions, electrical stimulation is used to mimic the fertilization process. This stimulation triggers an influx of Ca^{2+} through voltage-gated channels and leads to the rapid and synchronous destruction of cyclin B and exit of MII. Extracts made from these eggs faithfully simulate the embryonic cell cycle, exhibiting nuclear envelope formation and breakdown, DNA replication, cyclin accumulation and degradation, and spindle formation.

Table 1 APC/C subunits

	Structure motifs	*Functions*
Subunit		
APC1	RPN1/2 homology*	
APC2	Cullin homology	APC10 and APC11 binding
APC3/cdc27	TPRs	Cdh1 binding
APC4	WD40 repeats	
APC5	TPRs	
APC6/cdc16	TPRs	
APC7	TPRs	
APC8/cdc26	TPRs	
APC10/Doc1	Doc domain	Substrate binding
APC11	RING finger	E2 recruiting
Cdc26		
APC13		
Activator		
Cdc20	C-box WD40 repeats IR tail	Substrate binding
Cdh1	C-box WD40 repeats IR tail	Substrate binding

*RPN1 and RPN2 are subunits of the 26S proteasome. Adapted from (Peters et al., 2006).

Table 2 APC/C inhibitors

Inhibitor	*Species*	*Proposed to inhibit*	*Proposed mechanism*
Cdk1	Sc, Sp, Hs, Xl	APC/C^{Cdh1} in S and G2	Cdh1 phosphorylation
APC/C^{Cdh1}	Hs, Sc	APC/C^{Cdc20} in anaphase	Cdc20 degradation
SCF	Hs	APC/C^{Cdh1} in S phase	Cdh1 degradation
Mad2	Sc, Hs	APC/C^{Cdc20} in prometaphase	Inhibition of Cdc20 substrate release
Mad2B	Xl, Hs	APC/C^{Cdh1}	Inhibition of Cdh1 substrate release
BubR1	Hs	APC/C^{Cdc20} in prometaphase	Cdc20 sequestration
Bub1	Hs	APC/C^{Cdc20} in prometaphase	Cdc20 phosphorylation
MAPK	Xl	APC/C^{Cdc20} in prometaphase	Cdc20 phosphorylation
Emi1	Xl, Hs	APC/C^{Cdch1} in S and G2 phases	Substrate binding competition
Rca1	Dm	APC/C^{Cdc20} in S and G2 phases	Unknown
Emi2	Xl, Hs	APC/C^{Cdc20} in meiosis II	Unknown
Mes1	Sp	APC/C^{Cdc20} during meiosis exit	Substrate binding competition
Mnd2	Sc	APC/C^{Ama1} in meiosis I	Unknown
Rassf1A	Hs	APC/C^{Cdc20} in mitosis	Cdc20 binding
Xnf7	Xl	APC/C^{Cdc20} in mitosis	Unknown, ligase activity required
RAE1	Mm	APC/C^{Cdc20} in prometaphase	Unknown
Apoptin	CAV	APC/C^{Cdc20} and APC/C^{Cdh1}	APC1 binding
Unknown	HCMV	APC/C^{Cdh1} in G0 phase	Inhibiting APC/C and Cdh1 interaction
E4orf4	HAV	APC/C^{Cdc20}	PP2A recruitment

Mad2B is also known as MAD2L1. CAV, chicken anemia virus. Dm, Drosophila melanogaster. HAV, human adenovirus. HCMV, human cytomegalovirus. *Hs, homo sapiens*. MAP, mitogen-activated protein. *Mm, Mus musculus*. Mnd2, meiotic nuclear division protein-2. RAE1, Rab escort protein-1. RCA1, regulator of cyclin A-1. Sc, *Saccharomyces cerevisiae*. Sp, *Schizosaccharomyces pombe*. Xl, *Xenopus laevis*. Xnf7, *Xenopus* nuclear factor 7. Adapted from (Peters et al., 2006).

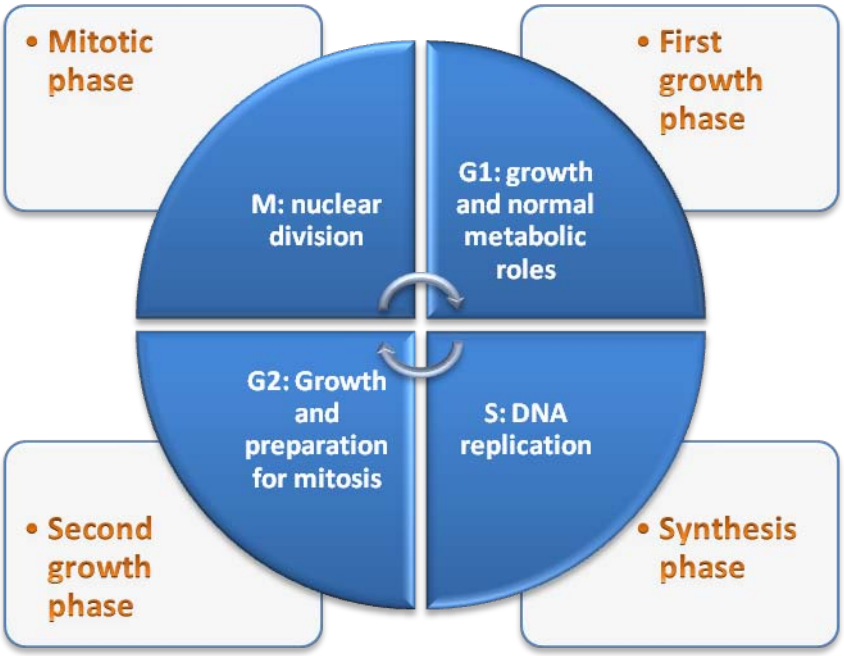

Figure 1 The cell cycle

A cell cycle is divided into interphase and M phase. Interphase can be sub-divided into G1, S and G2 phases, during which the cell grows and duplicates its contents, including DNA. The duplicated chromosomes are subsequently divided during M phase. Adapted from Alberts (2002).

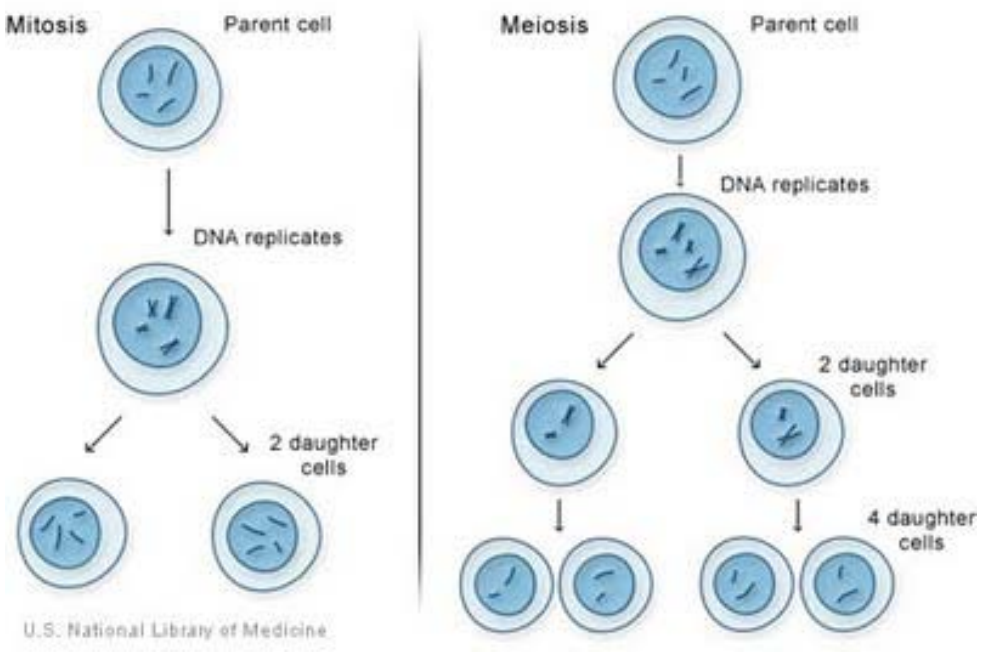

Figure 2 Mitosis and meiosis

During mitosis (left), a cell replicates its genome once and divides once, with the new daughter cell containing exactly the same number of chromosomes and information as the parent cell. During meiosis (right), one cell replicates its genome once but divides twice, generating four new daughter cells. Each new gamete cell contains only one-half of the number of chromosomes of the parent cell. Adapted from U.S. National Library of Medicine.

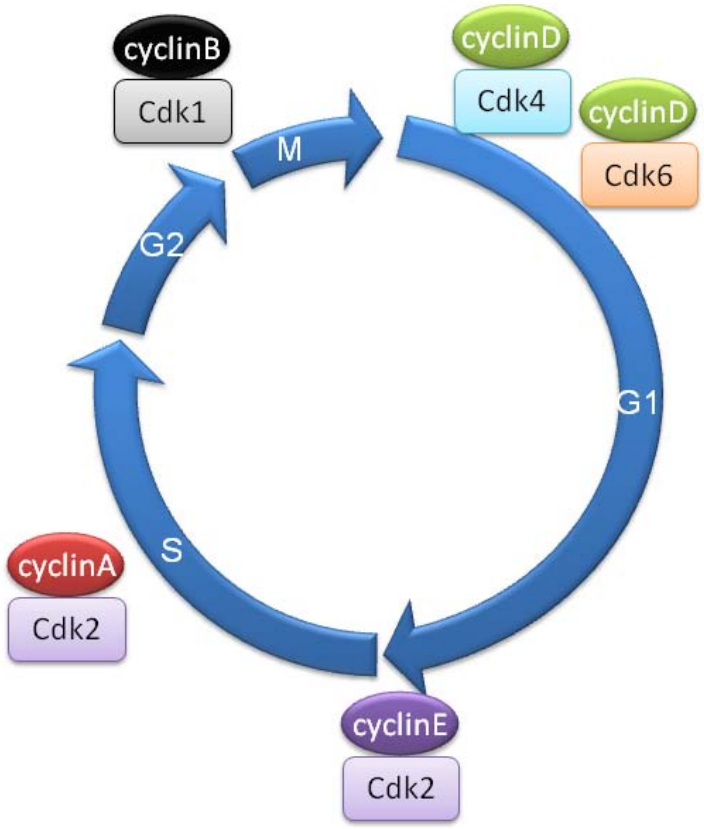

Figure 3 Cdks and cyclins

Progression through the cell cycle is catalyzed by cyclin-dependent kinases bound to their respective cyclin binding partners. Cdk6 and Cdk4 bind to D-type cyclins during G1, which promotes the expression of cyclin E. Cdk2 binds cyclin E to promote the G1 to S phase transition and then binds to cyclin A to move the cell through S phase. Cdc2, or Cdk1, binds to B-type cyclins to promote entry into mitosis.

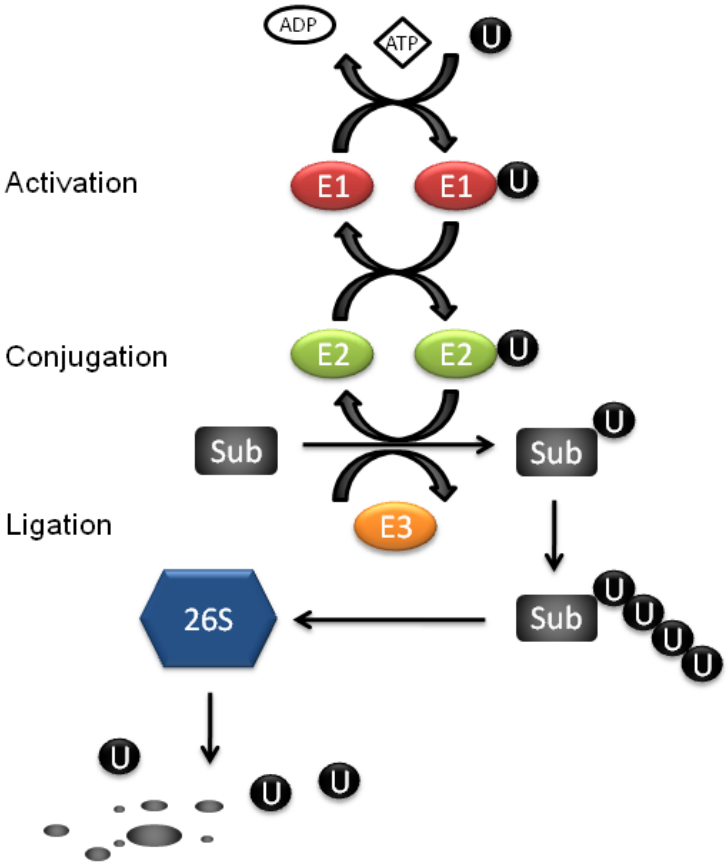

Figure 4 Ubiquitin-dependent proteolysis

A critical process governing cell cycle progression is the destruction of key substrates by ubiquitin-dependent proteolysis. During this process, ubiquitin is activated by the formation of a thioester bond with an ubiquitin-activating enzyme (E1), where it is then transferred to an ubiquitin-conjugating enzyme (E2). Ubiquitin ligase (E3) bonds the activated ubiquitin to a lysine residue on the targeted substrate. Several rounds of ubiquitylation (polyubiquitylation) mark the substrate for degradation by the 26S proteasome. Adapted and modified from (Wasch and Engelbert, 2005).

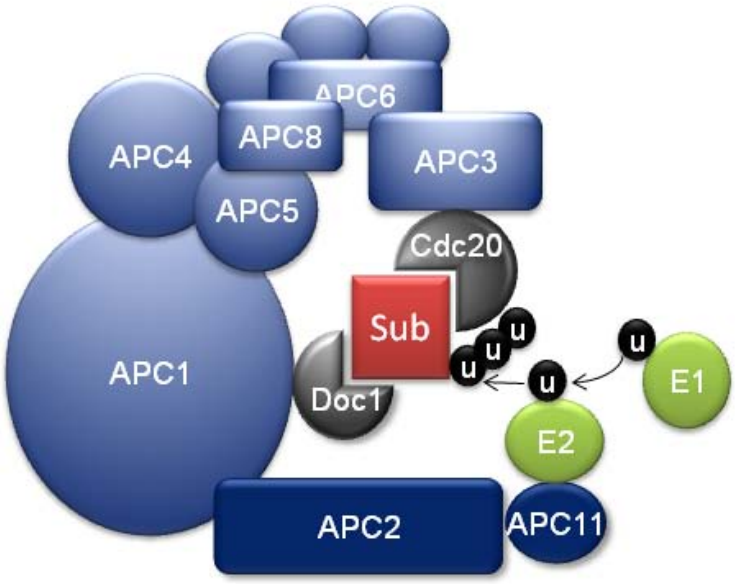

Figure 5 APC/C structure

The vertebrate APC/C is composed of twelve core subunits. The catalytic core of the complex consists of APC2 and APC11, containing a Cullin domain and a RING finger, respectively. The Cullin domain of APC2 associates with the RING finger on APC11, which is directly responsible for E2 recruitment. The Tetratricopeptide Repeat (TRP)-containing subunits (D'Andrea and Regan, 2003), including APC3, APC6, APC7 and APC8, are found to mediate the binding of APC/C to its activators. APC10/Doc1 cooperates with the APC/C activators to recruit substrates. APC1, APC4 and APC5 are proposed to act in part as a bridge and connect the catalytic core of APC/C and the TRP subunits. Adapted and modified from (Peters et al., 2006).

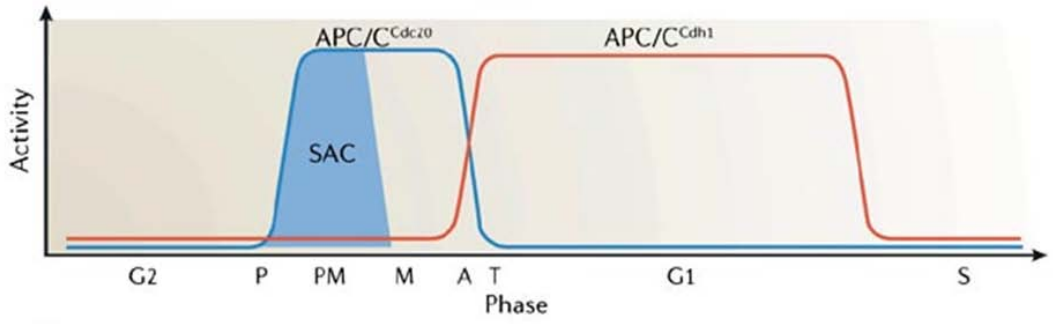

Figure 6 Activation of APC/C by Cdc20 and Cdh1 during the cell cycle

Anaphase promoting complex/cyclosome Cdc20 (APC/C^{Cdc20}) is thought to be assembled in prophase (P) and initiates the degradation of cyclin A already in prometaphase (PM). Proteolysis of cyclin B and the separase inhibitor securin also depends on APC/C^{Cdc20} but is delayed until metaphase (M) by the spindle-assembly checkpoint (SAC). During anaphase (A) and telophase (T), APC^{Cdh1} is activated, contributes to the degradation of securin and cyclin B, and mediates the destruction of additional substrates such as Polo-like kinase-1 (Plk1) and Cdc20, which leads to the inactivation of APC/C^{Cdc20}. In G1 phase, APC/C^{Cdh1} mediates the destruction of the ubiquitin-conjugating (E2) enzyme UbcH10, and thereby allows for the accumulation of cyclin A, which contributes to the inactivation of APC/C^{Cdh1} at the transition from G1 to S phase. Adapted from (Peters et al., 2006).

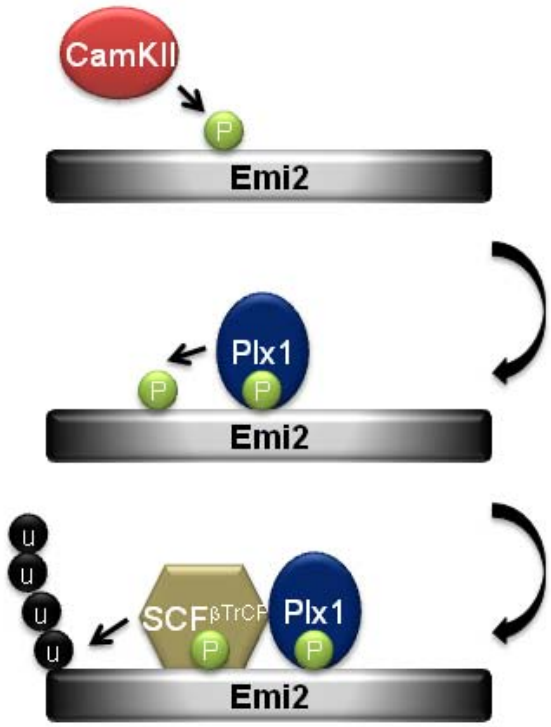

Figure 7 The Emi2 degradation pathway at fertilization

CSF arrest is controlled by Emi2-mediated APC/C inhibition. Upon fertilization, CaMKII kinase activity increases, leading to Emi2 phosphorylation, which creates a Plx1-binding motif on Emi2. Emi2-bound Plx1 then phosphorylates Emi2, allowing Emi2 to be recognized by the β-TrCP E3 ubiquitin ligase. Emi2 ubiquitylation primes Emi2 for degradation, leading to APC/C activation and, eventually, exit from CSF arrest. Adapted and modified from (Wu and Kornbluth, 2009).

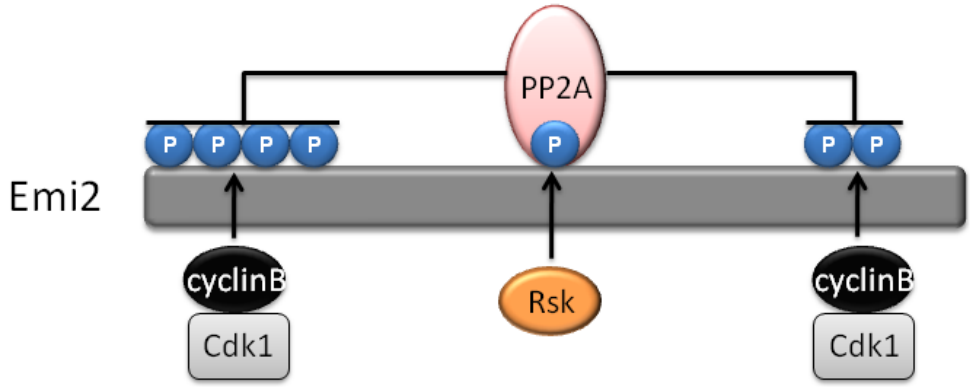

Figure 8 Regulation of Emi2 activity and stability by phosphorylation

The ability of Emi2 to bind and inhibit the APC/C is modulated by Cdc2–cyclin-B mediated phosphorylation. Specifically, phosphorylation at the Emi2 C-terminus weakens the Emi2-APC/C interaction, promoting dissociation of the Emi2-APC/C complex and activation of APC/C. During CSF arrest, Cdc2–cyclin-B-mediated Emi2 phosphorylation is antagonized by the PP2A dependent dephosphorylation. Rsk-mediated phosphorylation promotes recruitment of PP2A to Emi2, keeping Emi2 dephosphorylated. This allows Emi2 activation and APC/C inhibition. Bound PP2A can also promote Emi2 stabilization by dephosphorylating Emi2 at its N-terminus – otherwise, Cdc2–cyclin-B-mediated phosphorylation at this terminus would trigger Emi2 ubiquitylation and degradation. Adapted and modified from (Wu and Kornbluth, 2009).

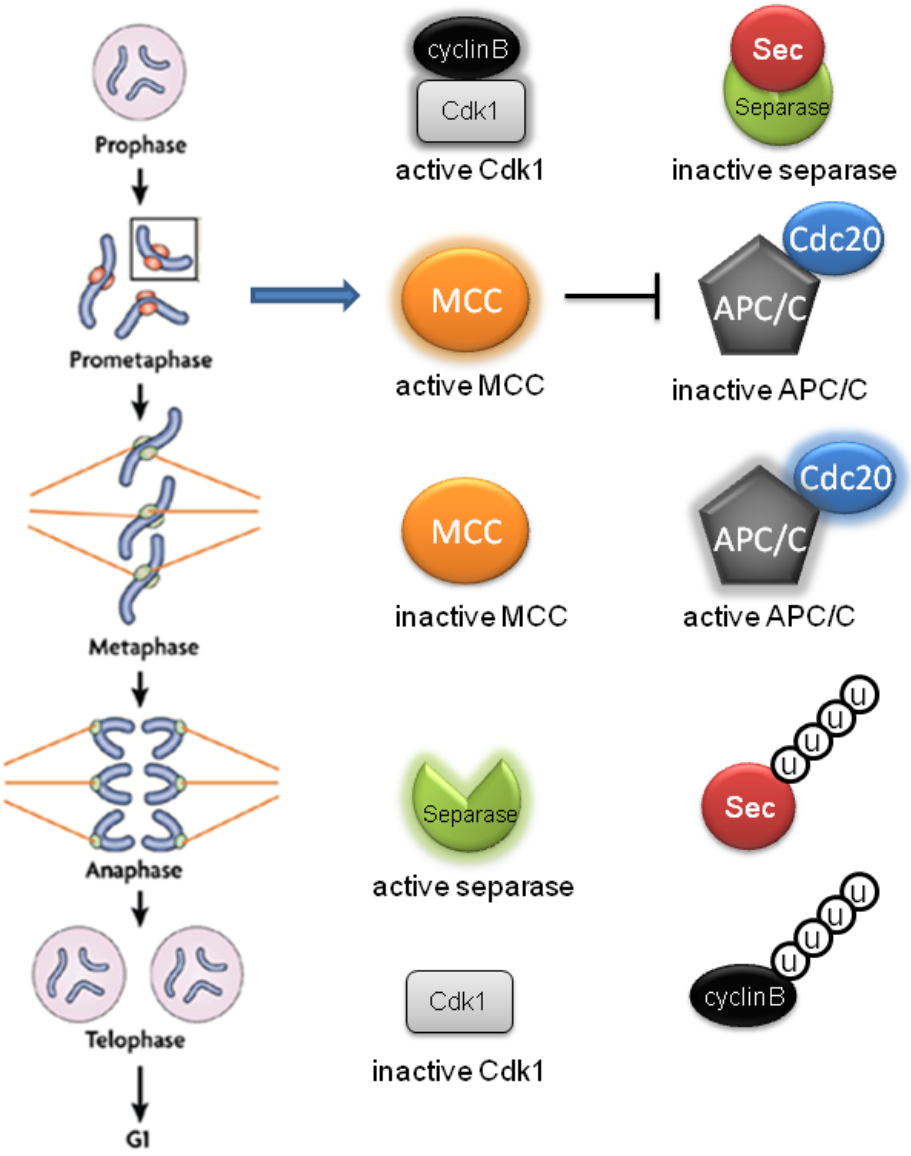

Figure 9 The Spindle Assembly Checkpoint

Figure 9 The Spindle Assembly Checkpoint

To enter mitosis, the cell requires the activity of the master mitotic kinase, cyclin-dependent kinase-1 (CDK1), which depends strictly on the binding of cyclin B to CDK1. Separase is a protease which is responsible for cohesion degradation at the metaphase-anaphase transition. Prior to anaphase, separase is kept inactive by the binding of a protein known as securin (SEC). Unattached kinetochores contribute to the activation of the mitotic checkpoint complex (MCC), which inhibits the activity of APC/C^{Cdc20}. The attachment of all sister-kinetochore pairs to kinetochore microtubules, and their bi-orientation silences the spindle-assembly checkpoint (SAC) signal, which leads to the activation of the APC/ C^{Cdc20}. This results in the polyubiquitylation of anaphase substrates such as cyclin B and securin, and their subsequent proteolytic destruction by the proteasome. The degradation of SEC results in the activation of separase, which targets the cohesin ring that is holding the sister chromatids together, thus causing the separation of sister chromatids. The degradation of cyclin B at this stage also inactivates the master mitotic kinase CDK1–cyclin B, initiating cytokinesis and the mitotic-exit program. Adapted and modified from (Musacchio and D. Salmon, 2007).

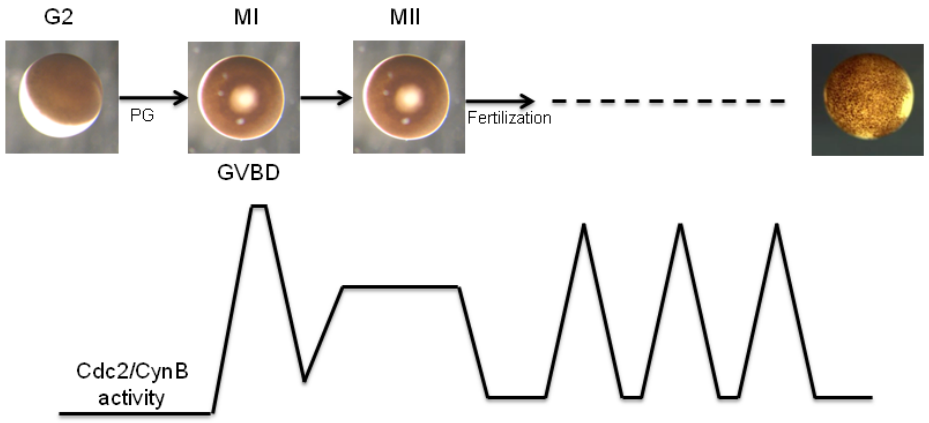

Figure 10 *Xenopus* oocyte maturation

Immature stage VI *Xenopus* oocytes are arrested in G2 of the meiotic cell cycle. Progesterone stimulates maturation and the activation of Cdc2/cyclin B and the progression into meiosis I (MI) as evidenced by the breakdown of the germinal vesicle (nuclear envelope). This is followed by a transient decline in Cdc2/cyclin B activity and entry into meiosis II (MII). The egg will remain arrested in metaphase of MII until fertilization. Upon fertilization, the egg will exit MII and enter into the rapid embryonic cell cycles. Adapted and modified from Tunquist and Maller (2003).

2. Regulation of Emi2 stability to promote the meiosis transition

2.1 Summary

The transition of oocytes from Meiosis I (MI) to Meiosis II (MII) requires partial cyclin B degradation to allow MI exit without S phase entry. Rapid reaccumulation of cyclin B allows direct progression into MII, producing a cytostatic factor (CSF)-arrested egg. It has been reported that dampened translation of the Anaphase Promoting Complex/Cyclosome (APC/C) inhibitor Emi2 at MI allows partial APC/C activation and MI exit. We have detected active Emi2 translation at MI and show that Emi2 levels in MI are mainly controlled by regulated degradation. Emi2 degradation in MI depends not on Ca^{2+}/CaMKII, but on Cdc2-mediated phosphorylation of multiple sites within Emi2. As in MII, this phosphorylation is antagonized by Mos-mediated recruitment of PP2A to Emi2. Higher Cdc2 kinase activity in MI than MII allows sufficient Emi2 phosphorylation to destabilize Emi2 in MI. At MI anaphase, APC/C–mediated degradation of cyclin B decreases Cdc2 activity, enabling Cdc2-mediated Emi2 phosphorylation to be successfully antagonized by Mos-mediated PP2A recruitment. These restults suggest a model of APC/C auto-inhibition mediated by stabilization of Emi2; Emi2 proteins accumulate at MI exit and inhibit APC/C activity sufficiently to prevent complete degradation of cyclin B, allowing MI exit while preventing interphase prior to MII entry.

2.2 Introduction

The process of vertebrate oocyte maturation, which produces a haploid gamete, is characterized by two consecutive M phases, Meiosis I (MI) and Meiosis II (MII), without an intervening interphase (Ohsumi et al., 1994). To generate an egg competent for fertilization, the nascent oocyte must undergo entry into MI, transit from MI to MII, and finally, an arrest in metaphase of MII. Failure to complete any of these key cell cycle events prevents normal egg production. MI entry is driven by the Cdc2/cyclin B kinase, the molecular components of maturation promoting factor (MPF). In the well-characterized *Xenopus* oocyte system, progesterone treatment initiates the translation of several proteins that trigger maturation, including cyclin B and the Mos kinase (Frank-Vaillant et al., 1999; Haccard and Jessus, 2006). Mos facilitates MI entry through activation of the ERK-MAPK pathway, which promotes Cdc2 activation by antagonizing its inhibitory kinase, Myt1, and by enhancing the activity of its activating phosphatase, Cdc25 (Palmer et al., 1998; Peter et al., 2002; Sagata et al., 1988). An additional Cdc2 activator, Ringo, has also been implicated in MI entry; this protein both drives maximal Cdc2 activation and renders Cdc2 less susceptible than cyclin-bound Cdc2 to the inhibitory action of Myt1 kinase (Ferby et al., 1999; Karaiskou et al., 2001).

In addition to playing a role in MI, Mos kinase is a critical component of cytostatic factor (CSF), an activity required for arrest in MII (Haccard et al., 1993; Sagata et al., 1989). Although Mos has been long known to act as a constituent of CSF (through

activation of the MAPK pathway, as in MI), its precise mechanism of action was not clear. Recently, it was shown that Mos helps to maintain MII arrest by inhibiting degradation of substrates of the APC/C, including cyclin B and a key regulator of chromosome segregation, securin. This APC/C inhibitory activity of Mos is exerted through the APC/C, Emi2. In MII, Mos promotes both the stability and activity of Emi2; the ability of Emi2 to inhibit the APC/C is regulated through phosphorylation of its C-terminus by Cdc2 and Mos enhances Emi2 function by facilitating its PP2A-mediated dephosphorylation (Inoue et al., 2007; Nishiyama et al., 2007; Wu et al., 2007a). Moreover, Mos helps to maintain steady state levels of Emi2 by promoting the dephosphorylation of multiple Cdc2 sites on Emi2 N teriminus, which trigger slow degradation of Emi2 when Cdc2/cyclin B kinase levels rise above a certain threshold. At fertilization, a transient increase in cellular Ca2+ level activates CaMK II, which primes Emi2 for docking of the Polo-like kinase 1 (Plx1 in *Xenopus*) and subsequent Plx1-mediated Emi2 phosphorylation. This creates a phosphodegron for the E3 ligase SCFβTrcp, leading to proteasomal degradation (Hansen et al., 2006; Liu and Maller, 2005; Rauh et al., 2005). When Emi2 is degraded, the APC/C is fully activated, releasing eggs from MII into the first embryonic cell cycle (Wu and Kornbluth, 2008).

In comparison to MI entry and the CSF-induced MII arrest, the MI-MII transition is not well understood. However, errors in this transition, including both inappropriate MI arrest and failure to enter MII following MI exit are not uncommon and can lead to

parthenogenesis and/or teratoma formation if abnormal oocytes are not properly eliminated (Eppig et al., 1996; Hashimoto et al., 1994). It is generally accepted that Mos is required for the MI-MII transition (Dupre et al., 2002; Hashimoto et al., 1994; Kanki and Donoghue, 1991; Phillips et al., 2002) because ablation of Mos translation clearly results in a failure of MII entry (and a consequent artificial interphase). Moreover, maintaining residual Cdc2 kinase activity at MI exit is necessary, as complete inhibition, either by chemical inhibitors or by overexpression of the inhibitory kinase Wee l promotes an artificial interphase (Gorr et al., 2006; Iwabuchi et al., 2000). One unsettled question, however, concerns the role of the APC/C in the MI-MII transition. Early studies in *Xenopus* oocytes indicated that the APC/C was dispensable for this transition, as neither antibody neutralization of the APC/C nor overexpression of its natural inhibitor, Mad2, inhibited the first Meiotic anaphase (Peter et al., 2001; Taieb et al., 2001). This idea was challenged more recently by the discovery that activation of the spindle assembly checkpoint (SAC), that targets the APC/C in MI, could lead to MI arrest (Homer et al., 2005). Moreover, in 2006, studies in both the *Xenopus* and murine oocyte systems demonstrated a requirement for Emi2 in the MI-MII transition and suggested that not only is the APC/C activated at MI anaphase, but also that its timely inhibition by Emi2 is required to promote entry into MII (Madgwick et al., 2006; Ohe et al., 2007). Although Emi2 protein has been remarkably difficult to detect in MI oocytes, it was shown that ablation of Emi2 message using antisense morpholino oligonucleotides could promote

exit from MI into interphase. Moreover, overexpression of Emi2 in the immature oocyte will promote an MI arrest upon progesterone treatment. These findings argued that Emi2 is most likely present at the end of MI to prevent complete APC/C-mediated degradation of cyclin B, which would lead to parthenogenetic activation of the oocytes. Conversely, in order to prevent the MI arrest that would occur if cyclin B degradation were to be completely inhibited, Emi2 levels must be tightly controlled to allow only partial APC/C inhibition.

Though it was initially reported that Emi2 protein was present throughout oocyte maturation, multiple groups have subsequently determined that significant accumulation of Emi2 protein is prevented during MI (Liu et al., 2006; Ohe et al., 2007; Tung et al., 2007). One of these groups reported that Emi2 levels are kept appropriately low in MI through the dampening of Emi2 mRNA translation (Ohe et al., 2007). Rapid translation at the onset of MII would then allow efficient CSF arrest. However, Tung et al found that Emi2 mRNA polyadenylation, which governs the timing of translation, was controlled by Cdc2 and began immediately following MI entry, though Emi2 protein did not accumulate until the onset of MII (Tung et al., 2007). In our study, we demonstrate that translation of Emi2 does indeed occur during MI and that regulation of Emi2 levels in MI is exerted mainly at the level of protein stability. Throughout MI, Emi2 protein undergoes continuous and rapid turnover. Interestingly, we demonstrate that the same degron that controls precipitous degradation of Emi2 at exit from MII also

regulates the continuous degradation of Emi2 prior to MI exit (Rauh et al., 2005). Moreover, this degradation is required to prevent inappropriate MI arrest. However, unlike degradation at MII exit, MI Emi2 degradation does not appear to require Ca^{2+}/CaMKII. Rather, phosphorylation of four sites in the Emi2 N-terminus (213/239/252/267) primes the protein for degradation through the degron site. Moreover, Mos facilitates MII entry, in large part, by promoting Emi2 stabilization through PP2A-mediated dephosphorylation of these sites, a pathway similar to that which controls slow Cdc2-mediated Emi2 degradation during MII. In concert with these observations, we have found that overall Cdc2/cyclin B kinase activity (and consequent Emi2 phosphorylation) is higher in MI than MII, providing an explanation for the instability and low abundance of Emi2 in MI that is necessary to avoid inappropriate MI arrest. Additionally, the reduction in Cdc2 kinase activity at MII, relative to MI, allows Mos/PP2A-mediated dephosphorylation of Emi2 to predominate, enhancing Emi2 stability, and allowing the prolonged arrest characteristic of MII.

2.3 Material and methods

2.3.1 Cloning, protein expression, mRNA preparation

Emi2 mutants including S213A/T239A/T252A/T267A, T545/551A, DS32AA, T195A were cloned as previously described (Wu et al., 2007a), as were Myc_6 tagged Emi2 ORF including its own 3' UTR (WT and DS32AA) in pCS2+ vector (Tung et al., 2007). For mRNA synthesis, Emi2 ORFs (Emi2 aa 489-651, Emi2 wildtype, Emi2 DS32AA, Emi2 4A)

were PCR amplified and subcloned into the NotI site of the pSP64T vector. Constructs were digested with XbaI and mRNAs were produced using mCAP RNA capping kit (Stratagene).

^{35}S-labeled Emi2 proteins were generated using the TNT Quick Coupled Transcription/Translation System (Promega) in the presence of ^{35}S-labeled Methionine/Cysteine (MP Biomedicals).

Recombinant GST-Emi2 proteins (aa 489-651 T545/551A; aa 319-375; aa 319-375 ST335AA) were prepared as previously described (Wu et al., 2007b).

To ablate translation, 20μM morpholino was injected to oocytes. Mos morpholino (AAGGCATTGCTGTGTGACTCGCTGA) and inverted Mos morpholino (AGTCGCTCAGTGTGTCGTTACGGAA) were purchased from Gene Tools. Emi2 morpholino was prepared as previously described (Wu et al., 2007b).

2.3.2 Oocyte injections and lysate preparation

Stage VI oocytes were treated with 2.8 units of liberase in OR-2 buffer (82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES [pH 7.5]) for 1.5 hr at room temperature, washed extensively with OR-2 buffer, and stored in OR-2 buffer with 10% fetal bovine serum and 0.5% gentamicin at 18°C. Oocyte lysate was made by crushing oocytes in oocyte lysis buffer (20 mM Hepes KOH, pH 7.5, 20 mM ß-glycerophosphate, 15 mM $MgCl_2$, 20 mM EGTA, 1 mM PMSF, and 5 ng/μl aprotinin/leupeptin). Lysate was clarified by centrifugation to remove insoluble material. For western blot analysis, two

oocytes equivalent was loaded to each lane. For autoradiography analysis, eight oocytes equivalent was loaded. To make MI extract, oocytes were treated with progesterone and lysate was made immediately after GVBD. To make MII extract, lysate was made 4 hours after GVBD.

2.3.3 Immunoblot and immunoprecipitation analysis

The antibodies used for immunoblotting were as follows: mouse anti-cyclin B2 (Casaletto et al., 2005), rabbit anti-Emi2 (Tung et al., 2005), mouse anti-Cdc27 (Transduction), mouse anti-PP2A (Upstate), rabbit anti-phospho-MAPK (Cell Signaling Technology), rabbit anti-phospho-Cdc2 (Cell Signaling Technology), mouse anti-Myc (Santa Cruz), mouse anti-Rsk (Santa Cruz).

Myc-Emi2 was immunoprecipitated using anti-Myc-tag-Agarose (MBL). Lambda phosphatase (New England Biolabs) treatment was performed according to the manufacturer's instructions.

2.3.4 HHI Kinase assay

Oocytes lysate was made from 5 oocytes per sample and flash frozen until processed. 15 μl of HI kinase reaction mix (final concentrations, 10 mM Hepes KOH [pH 7.2], 5 mM $MgCl_2$, 50 mM NaCl, 83 μM ATP, 4.2 mM DTT, 5 μg of histone HI) and 2 μCi [γ-^{32}P] ATP were added to the extract and the reaction was incubated at room temperature for 10 min. Samples were resolved by SDS-PAGE and the bands corresponding to HHI were quantified with a phosphorimager (Molecular Dynamics).

2.4 Results

2.4.1 Tight regulation of Emi2 levels is critical for ensuring a smooth MI-MII transition.

The very rapid accumulation of Emi2 observed upon transition of oocytes from MI into MII led us re-examine the notion that translation inhibition was the primary mechanism for restraining Emi2 abundance in MI. To monitor Emi2 translation, G2/prophase-arrested oocytes were injected with Myc-tagged Emi2 mRNA containing its own 3'-UTR, which is known to regulate endogenous Emi2 translation (Tung et al., 2007). After treatment with progesterone, oocytes were soaked in the proteasome inhibitor MG132 to inhibit any possible proteasomal degradation. Within one hour following GVBD, (Note that oocytes typically reached anaphase of MI 1.5h-2.5h after visual GVBD) we were able to detect a significant accumulation of Emi2 protein (Fig. 11A). Consistent with the idea that Emi2 could, indeed, be translated at MI, we found that newly synthesized endogenous Emi2 could be immunoprecipitated from MI oocytes that had been soaked in 35S-methionine/cysteine and treated with MG132 (Fig. 11B). These data suggested that Emi2 translation could occur in MI, potentially resulting in sufficient Emi2 protein accumulation to prevent exit from MI (see further, below). These data also raised the possibility that controlled Emi2 degradation might be necessary at MI to prevent inappropriate Emi2 accumulation. To test this, we injected oocytes with 35S-labeled, *in vitro* translated Emi2 and monitored its stability. As shown in Fig. 11C, Emi2 was quickly degraded following GVBD in progesterone-treated

oocytes, but remained stable in the untreated controls. To demonstrate the relevance of Emi2 regulation in the MI-MII transition, we injected oocytes with Emi2 (489-651) mRNA encoding a fragment of Emi2 known to be non-degradable in MII, but able to inhibit the APC/C (Wu et al., 2007b). In control oocytes (either uninjected or injected with β-globin control mRNA), cyclin B degradation, indicative of APC/C activation, occurred approximately two hours after visual GVBD, though it was not complete (as is characteristic of the MI-MII boundary). cyclin B then reaccumulated as oocytes progressed into MII. In contrast, expression of Emi2 (489-651) protein in oocytes led to an MI arrest, as evidenced by the maintenance of high cyclin B levels (Fig. 11D). Conversely, ablation of Emi2 translation by injection of Emi2-directed antisense morpholino oligonucleotides led to complete and rapid degradation of cyclin B at MI exit, causing an inappropriate exit into interphase and a failure to reaccumulate cyclin B (Fig. 11E). These data indicate that the partial inhibition of the APC/C (and the resulting partial degradation of cyclin B typical of the MI-MII transition) depends upon Emi2. Strict control over Emi2 levels appears to be critical for the MI-MII transition, as either overexpression or underexpression results in abnormal maturation.

2.4.2 Emi2 degradation in MI is mediated through Cdk-mediated phosphorylation of S213/T239/T252/T267

Rapid degradation of Emi2 is required for the release of eggs from MII arrest following fertilization by a well characterized degradative pathway. In order to elucidate the mechanism underlying regulated Emi2 degradation in MI, we first wished

to know if the determinants were the same in MI and MII. As shown in Fig. 12A (left panel), mutation of the previously-identified degron at sites known to abrogate the required Plx-mediated phosphorylation that occurs in MII (changing D32 and S33 to alanine) also prevented Emi2 degradation in MI. Indeed, microinjection of this degron mutant Emi2 mRNA containing the Emi2 3'UTR led to a significant accumulation of Emi2 protein only one hour after GVBD, confirming the rapid synthesis of Emi2 at the time of passage through MI (Fig. 12A, right panel). In contrast, mutation of the known site of CaMKII phosphorylation, T195, required for degradation at the time of fertilization, did not stabilize the protein, suggesting that CaMKII is not involved in the MI degradation pathway. We have also confirmed this conclusion using several CaMKII inhibitors, all of which failed to stabilize Emi2 in MI (data not shown). However, in the course of exploring this issue, we tested other kinase inhibitors and found that the CDK inhibitor, roscovitine, could fully stabilize Emi2, which strongly suggested a requirement for CDK activity (Cdc2 or Cdk2) in the MI Emi2 degradative pathway (Fig. 12B).

We reported previously that when Cdc2 kinase activity exceeds a certain threshold, Emi2 undergoes slow degradation in MII, which is distinct from the precipitous degradation observed following fertilization. This slow degradation is triggered by phosphorylation of Emi2 by Cdc2 on four N-terminal sites in Emi2 (Wu et al., 2007a). To determine if these sites might also be relevant to MI Emi2 degradation, we

generated 35S-labeled mutant Em2 in which the four Cdc2 phosphorylation sites had been mutated into alanine. As shown in Fig. 12C, this mutant (hereafter referred to as 4A; note that these sites have been verified as Cdc2 sites previously (Wu et al., 2007a)) was almost entirely stabilized in MI. To address the physiological relevance of phosphorylation at these four sites, we injected oocytes with either wild type or 4A Emi2-encoding mRNA appended to the β-globin poly A tail (to abrogate any possible Emi2-specific translational control). Wild type and 4A mutant Emi2-expressing mRNA accelerated GVBD, consistent with the existence of basal APC/C activity that limits cyclin B levels before MI entry (data not shown). Although expression of wild type Emi2 somewhat delayed the MI-MII transition (most likely due to saturation of the Emi2 degradative pathway) expression of the 4A mutant Emi2 led to MI arrest, as evidenced by both a constant high level of cyclin B (Fig. 12D) and phenotypic observation of the oocytes (Data not shown). To further demonstrate that Emi2 degradation was indeed required for the onset of MI, we investigated the effects of replacing endogenous Emi2 mRNA with non-degradable Emi2. First, we monitored oocyte maturation following microinjection of oocytes with an antisense Emi2 morpholino oligonucleotide along with various levels of wildtype Emi2 mRNA appended to its own 3'UTR (Data not shown). With this approach, we were able to determine that the minimal level of wildtype Emi2 mRNA able to prevent the parthenogenesis resulting from ablation of Emi2 was 11 pg/oocyte. We then microinjected oocytes with the same amount of mRNA encoding

non-degradable Emi2 (11pg/oocyte) together with the Emi2-directed morpholino oligonucleotide. As shown in Fig. 12E, the non-degradable Emi2 clearly prevented APC/C activation at MI anaphase, while wild type Emi2 did not. These data are fully consistent with the notion that degradation of Emi2 is indeed required for the exit from MI.

2.4.3 Mos promotes MII entry by promoting stabilization of Emi2

With the knowledge that Emi2 levels are controlled at the level of stability, we wanted to determine whether Mos controlled Emi2 stability in MI as it does in MII. As shown in Fig. 13A, the Mos pathway is turned on soon after progesterone treatment, as indicated by activation of MAPK. Moreover, we could largely abrogate its activity by ablating its translation through morpholino oligonucleotide injection (based on multiple experiments, we have routinely achieve a reduction in Mos activity of ~75-95%, judging by the degree of MAPK phosphorylation). In the absence of Mos, oocytes were still able to initiate the maturation process (Fig. 13B), though entry into MI (as indicated by GVBD) was significantly delayed (data not shown). However, oocytes lacking Mos were unable to transition appropriately to MII. Rather, oocytes exhibited complete cyclin B degradation, as was seen when Emi2 was ablated (Fig. 13B). Conversely, when Emi2 expression was inhibited by injection with Emi2-directed morpholino oligonucleotides, Mos activity was unaffected, yet oocytes still failed to enter MII. These data demonstrate that the Mos-MAPK pathway itself is not sufficient to promote MII entry, and strongly

suggest that the role of Mos in promoting MII entry is mediated through Emi2. As Mos had been previously shown to regulate Emi2 stability in MII, we wished to examine the effect of Mos on Emi2 stability at the end of MI. Accordingly, we again injected oocytes with Myc-tagged Emi2 mRNA appended to its own 3'UTR, but then performed a second injection with either control or Mos-directed morpholino oligonucleotide. As shown in Fig. 13D, loss of Mos resulted in a failure of Emi2 accumulation, most likely accounting for the failure in MII entry (note that samples in the right hand panel of Fig. 13D were treated with lambda phosphatase prior to SDS-PAGE to collapse the phosphorylated species into a single electrophoretic species). These data suggest that one crucial function of Mos in both blocking S phase initiation after MI and promoting entry into MII is to enable timely accumulation of Emi2 , thereby allowing only partial, rather than full, cyclin B degradation.

2.4.4 Differential Emi2 stability in MI and MII is controlled by different levels of Cdc2 activity.

Taken together, our data suggested that Emi2 does not normally cause an arrest in MI because its levels are suppressed through Cdc2-mediated phosphorylation. Since our previous work demonstrated that this Cdc2-mediated pathway could also be operative during an MII arrest ((Wu et al., 2007b)), we were left with the perplexing question of why Emi2 was maintained at a level sufficient to produce an arrest in MII, but not in MI. We initially hypothesized that this difference might reflect differential activity of Mos or its effectors in MI and MII. From previous work, we knew that Mos

could stabilize Emi2 by promoting its binding to PP2A (to catalyze dephosphorylation of the Cdc2 phosphorylation sites), and thus it was possible that decreased recruitment of PP2A to Emi2 in MI could enhance Emi2 turnover. To address this issue, we used the GST- Emi2 PP2A binding domain (Emi2 aa 319-375) to retrieve PP2A from both MI and MII extract (Wu et al., 2007a). As shown in Fig. 14A, PP2A bound similarly to Emi2 in MI and MII. Consistent with these observations, Emi2-directed Rsk kinase activity, was also similar, based on *in vitro* kinase assays using Rsk immunoprecipitated from MI and MII extracts (Fig. 14A, right). (A mutant lacking the Rsk phosphorylation site necessary for PP2A recruitment, AM, served as a negative control). Alternatively, we considered the possibility that the magnitude of Cdc2 kinase activity might differ in these two consecutive phases. To address this, we treated oocytes with progesterone and followed the maturation process by withdrawing oocytes at different time points, preparing oocyte extract, and then examining the activity of Cdc2 using histone HI as an exogenous substrate. Surprisingly, we consistently observed a two-fold difference in histone HI-directed kinase activity between MI and MII, which had not been previously reported (Fig. 14B). More importantly, we could fully recapitulate in MII extracts the rapid degradation of Emi2 observed in MI extracts by adding recombinant non-degradable cyclin B to induce Cdc2 kinase activity comparable to that observed in MI (note that these extracts were also supplemented with a C-terminal non-degradable fragment of Emi2 to prevent the Cdc2-induced activation of the APC/C, which would

promote degradation of endogenous cyclin B and thus down regulate Cdc2 kinase activity as we previously reported (Wu et al., 2007b)(Fig. 14C). Based on these data, we conclude that the differential stability of Emi2 in MI and MII results from the combined facts that it is Cdc2 and not CaMKII that is the major determinant of Emi2 stability in MI and that Cdc2 kinase activity is sufficiently higher in MI than in MII to accelerate this degradative pathway (see model, Fig. 15A). Whether there are other physiological consequences of differing kinase activity in MI and MII is an intriguing question that merits further investigation.

2.5 Discussion

It was previously reported that low Emi2 levels were maintained at MI through translational suppression. Rather, we report here that this control is exerted through Cdc2-regulated Emi2 degradation, which is required for exit from MI. Although some of the same determinants of Emi2 degradation appeared to operate in MI and MII, including a role for Mos in recruiting PP2A to Emi2, the absence of a role for CaMKII in controlling Emi2 stability in MI, coupled with the differential levels of Cdc2 kinase activity at these two cell cycle stages appears to allow for subtle, but important differences in control of Emi2 abundance. These differences account, at least in part, for the ability of oocytes to exit MI, enter MII without an intervening interphase, and arrest for long periods in MII.

2.5.1 Cdc2 kinase activity in the control of Emi2 stability

Although we have not excluded the possibility that the translation of Emi2 is differentially regulated during MI and MII, it is clear that the rapid degradation of Emi2 is required to allow MI exit. This degradative process relies upon the high Cdc2 kinase activity characteristic of MI. When Cdc2 kinase activity was raised in MII to mimic the higher levels found in MI, Emi2 was commensurately destabilized, consistent with this being a key determinant of Emi2 destabilization in MI. Although the Cdc2 kinase activity differed in MI and MII, we found that levels of cyclin B were very similar in MI and MII oocytes (Fig. 13A; compare 1.5 and 5.5 hour time points). We speculate that the differing Cdc2 kinase activities at these two developmental stages could stem from higher levels of Ringo protein in MI than in MII (Gutierrez et al., 2006). Alternatively, regulation of Cdc2 by Cks proteins may also dictate differential kinase activity. It was reported that knock-out of Cks2 in mouse promoted an MI arrest, though the reason for this was unclear (Spruck et al., 2003). It is possible that loss of Cks2 in the knock-out lowered Cdc2 kinase activity in meiosis I, leading to inappropriate Emi2 stabilization. Finally, differential Wee1 levels may be another critical factor in determining Cdc2 activity (Kosaka et al., 2000). Since premature Wee1 expression arrests oocytes at MI, the MII–specific appearance of Wee1A could potentially lessen to Emi2-directed Cdc2 kinase activity sufficiently to allow PP2A to prevail; this would maintain Emi2 in the stable and active configuration necessary for MII arrest.

2.5.2 Differential control of the APC/C in MI and MII

In MII, when cyclin B levels rise through de novo synthesis, there is a feedback loop operative in which elevated Cdc2 kinase activity leads to Emi2 dissociation from the APC/C. This dissociation allows cyclin degradation sufficient to restore Cdc2 kinase activity to the baseline levels characteristic of CSF arrest. When Cdc2 kinase activity drops sufficiently, Emi2 reassociates with the APC/C, maintaining its inhibition. On the face of it, it is perplexing that all of the components critical for operation of this loop could potentially be present in MI, yet this feedback pathway does not appear to operate at this cell cycle stage. This is most likely due to the fact that the spindle checkpoint is operative in MI (Wassmann et al., 2003), but not during MII arrest (Tsurumi et al., 2004). This checkpoint results in profound APC/C inhibition until the metaphase I plate is formed, allowing constitutively high Cdc2 kinase activity and consequently rapid Emi2 turnover. Once the metaphase plate is formed and chromosomes are properly attached to the spindle, the checkpoint signal dissipates, leading to APC/C activation and cyclin B degradation. Only then is Emi2 able to accumulate, preventing complete degradation of cyclin B. This prevents interphase entry, allowing transition directly into MII.

2.5.3 Mos and the MI-MII transition

Although Mos had been implicated in regulating the MI-MII transition, the mechanism was not clear. We have provided a distinct mechanism to explain the role of Mos in regulating this transition: Mos helps to stabilize Emi2 at the end of MI, thus

maintaining Emi2 at levels that partially inhibit the APC/C, allowing incomplete cyclin B destruction. The ability of Mos to modulate Emi2 in MI appears to reside, as in MII, with the Rsk-mediated recruitment of PP2A to Emi2. Although Mos has been implicated in suppressing the Cdc2 inhibitor MytI at MI entry, because Mos-Rsk kinase activities appeared to be similar in MI and MII, Mos is unlikely to account for the observed differences in Cdc2 kinase activity between MI and MII (Palmer et al., 1998). In addition, similar Mos-mediated targeting of PP2A to Emi2 was observed in MI and MII. Thus, it is likely that similar phosphatase activities appear to be differentially effective given the different levels of antagonistic Cdc2 kinase activity. Taken together, these findings suggest that the smooth transition from MI to MII is a finely balanced process wherein higher Cdc2 kinase levels in MI than in MII renders Emi2 unstable in MI, but at the anaphase of MI, decreased kinase activity can be counterbalanced by Mos activity, which is critical to allow timely accumulation of Emi2 and the partial cyclin B degradation characteristic of the MI to MII transition.

2.5.4 An auto-inhibitory loop regulates APC/C activity during the MI-MII transition

Although exit from MI requires cyclin B degradation, residual cyclin B/Cdc2 kinase activity is known to be required for transiting from MI to MII. However, the mechanism underlying the delicate control of cyclin B degradation at MI-MII has not been clear. With the finding that Cdc2 and Mos coordinately control Emi2 stability, we now propose a model of APC/C–directed APC/C inhibition to ensure a smooth MI-MII

transition (Fig. 15B). Before the onset of MI anaphase, Emi2 protein levels are held in check by Cdc2 kinase-mediated destabilization. APC/C activation then results in decreased Cdc2 kinase activity as cyclin B levels drop. With the Mos-PP2A pathway promoting Emi2 dephosphorylation, Emi2 can accumulate and effectively inhibit the APC/C. This leads to appropriately-timed stabilization of cyclin B and subsequent entry into MII without an intervening interphase. Although other regulatory pathways no doubt contribute to ensuring the smooth progression from MI to MII, this auto-inhibitory regulation loop of APC/C plays critical role in regulating the meiotic transitions.

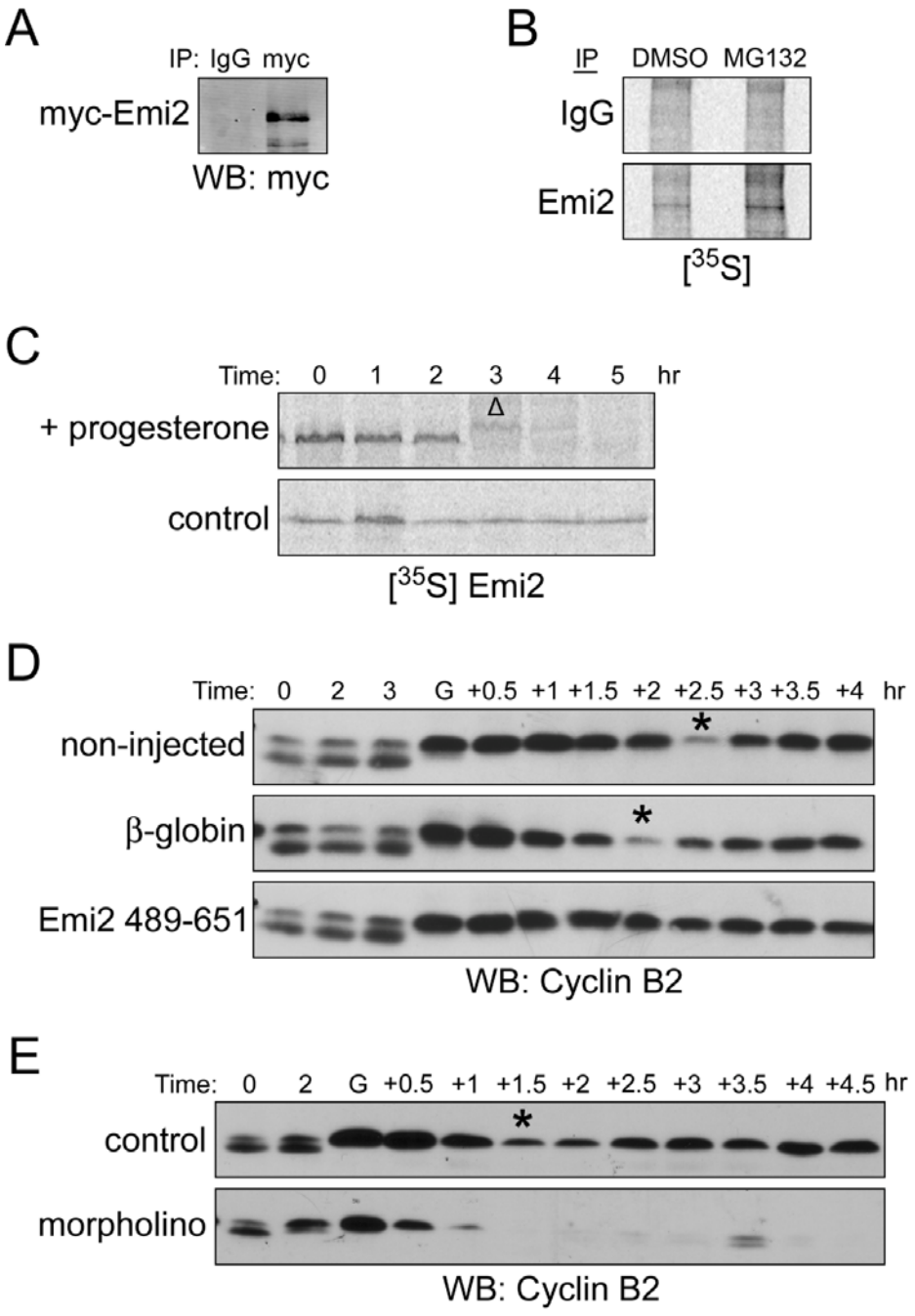

Figure 11 Translation and degradation of Emi2 in MI

Figure 11 Translation and degradation of Emi2 in MI

(A) Oocytes were injected with Myc_6-Emi2-3′ UTR mRNA and incubated overnight (0.3 ng/oocyte). Oocytes were treated with progesterone and monitored visually for GVBD. One hour after GVBD, oocytes were lysed and lysates were incubated with anti-Myc or IgG coupled to Protein A Sepharose beads for 2 hours at 4°C. The beads were retrieved, washed and treated with lambda phosphatase before Western Blot analysis. (B) 100 oocytes were treated with 200 μM Mg132 or DMSO in the presence of 400 μCi ^{35}S - methionine/cystein. 1 h after GVBD, oocytes were lysed, and lysates were incubated with anti-Emi2 or IgG coupled to Protein A Sepharose beads for 2 hours at 4°C. The beads were retrieved, washed and treated with lambda phosphatase before analysis by autoradiography. (C) ^{35}S-labeled Emi2 was injected into oocytes that were subsequently treated with or without progesterone. At the indicated times, lysates were made and analyzed by autoradiography following SDS-PAGE. GVBD was monitored visually. Δ: GVBD (D) Oocytes were injected with either β-globin or Flag-Emi2 (489-651) mRNA appended with β-globin 3′UTR (0.3 ng/oocyte). After overnight incubation, oocytes were treated with progesterone. At the indicated times, lysates were made and analyzed by Western blotting. Asterisks indicate the transition from MI to MII. GVBD was monitored visually. G: GVBD (E) Oocytes were injected with either Emi2 morpholino (20 μM) or control morpholino (20 μM). After 1h incubation, oocytes were treated with progesterone. At the indicated times, lysates were made and analyzed by Western blotting. Asterisks indicate the transition from MI to MII. GVBD was monitored visually. G: GVBD

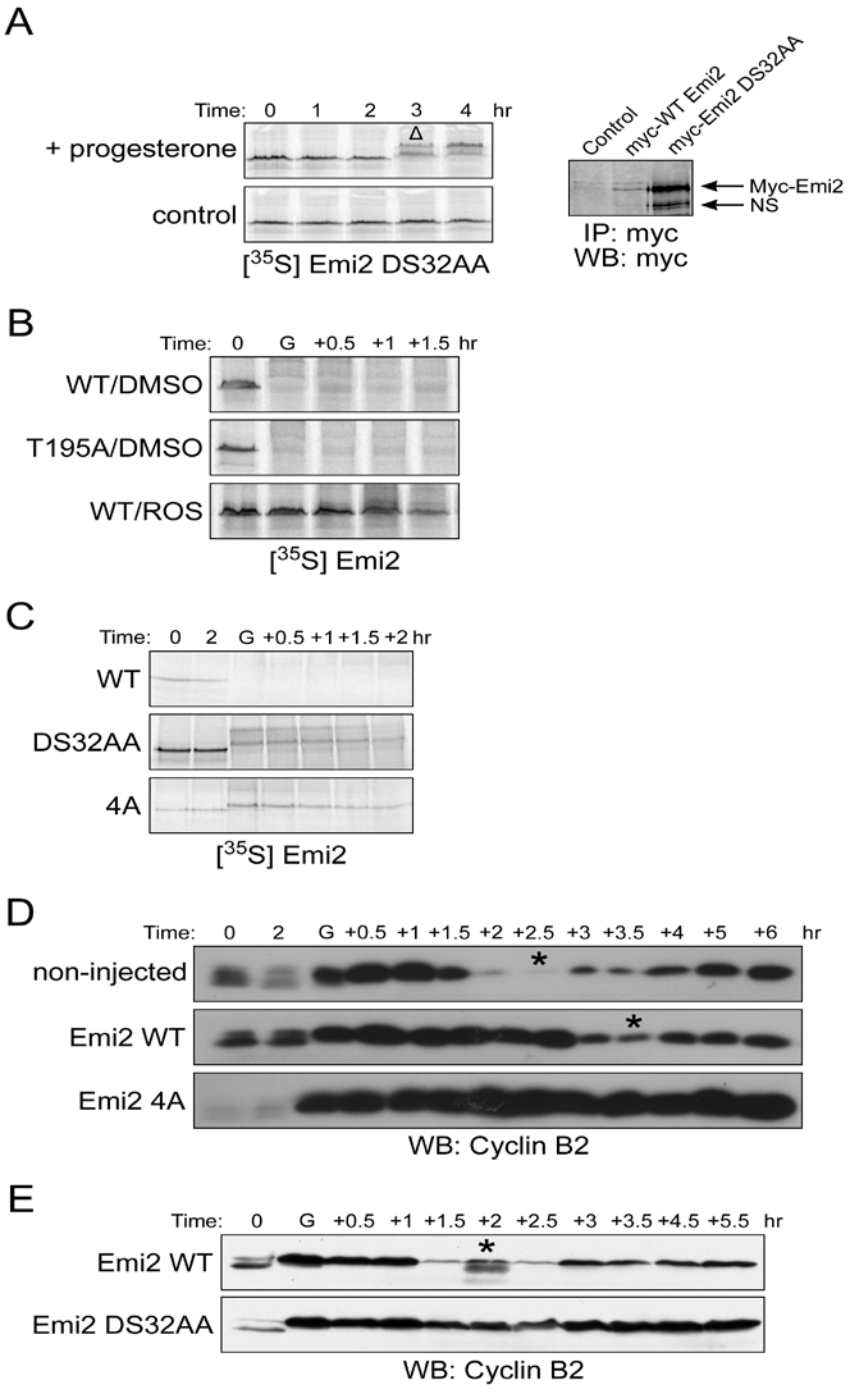

Figure 12 Emi2 degradation is mediated through Cdc2 phosphorylation on 213/239/252/267

Figure 12 Emi2 degradation is mediated through Cdc2 phosphorylation on 213/239/252/267 sites

(A) (left) ^{35}S-labeled DS32AA Emi2 protein was injected into oocytes and samples were processed as in Fig. 11C. GVBD was monitored visually. Δ: GVBD (right) Oocytes were injected with Myc_6-Emi2-3′ UTR mRNA (wildtype or DS32AA). Samples were processed same as in Fig. 11A. NS: non-specific band. (B) Oocytes were injected with ^{35}S-labeled Emi2 (WT or T195A). One hour after injection, oocytes were treated with progesterone in the presence or absence of 300 μM roscovitine. At the indicated times, lysates were made and analyzed by SDS-PAGE and autoradiography. GVBD was monitored visually. G: GVBD (C) Oocytes were injected with ^{35}S-labeled Emi2 (wildtype, DS32AA or 4A). One hour after protein injection, oocytes were treated with progesterone and samples were processed as in Fig. 11B. (D) Oocytes were injected with Flag-Emi2 mRNA appended with β-globin 3′ UTR (wildtype or 4A) (0.3 ng/oocyte). After overnight incubation, oocytes were treated with progesterone and samples were processed as in Fig. 11D. (E) Oocyte were injected with Myc_6-Emi2-3′ UTR mRNA(0.3 ng/oocyte) and incubated for 1 hour. before progesterone treatment. Samples were processed as in Fig. 11D.

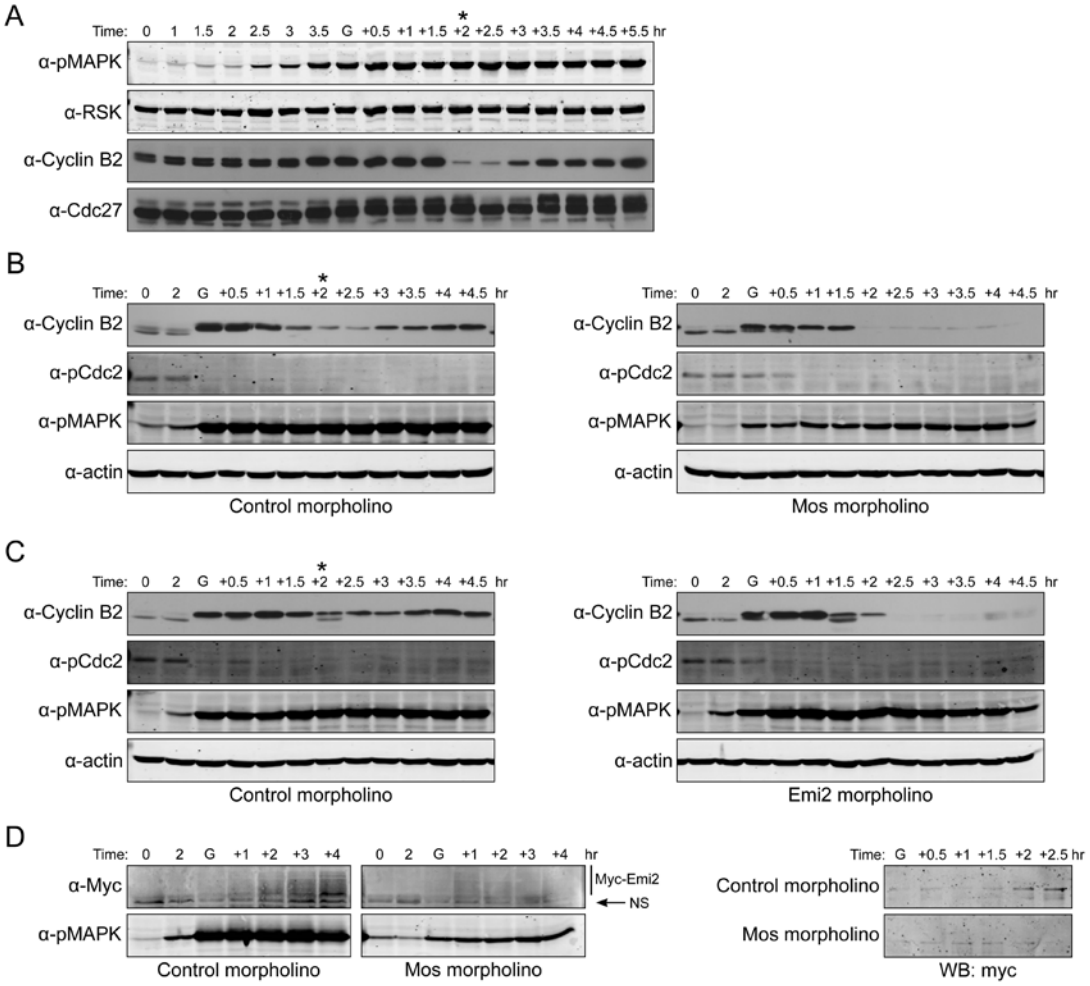

Figure 13 Mos ensures MII entry by promoting Emi2 stability

Figure 13 Mos ensures MII entry by promoting Emi2 stability

(A) Oocytes were treated with progesterone. At the indicated times, oocytes were lysed and analyzed by Western blotting. Asterisks indicate the transition from MI to MII. GVBD was monitored visually. G: GVBD (B) Oocytes were injected with either Mos morpholino (20 μM) or control morpholino (20 μM). After 1h incubation, oocytes were treated with progesterone. At the indicated times (G: GVBD), lysates were made and analyzed by Western blotting. In Mos morpholino injected oocytes in this experiment, phosphorylation of MAPK was reduced by 83%. Asterisks indicate the transition from MI to MII. GVBD was monitored visually. G: GVBD (C) Oocytes were injected with either Emi2 morpholino (20 μM) or control morpholino (20 μM). Samples were taken at indicated times and analyzed by Western blotting. Asterisks indicate the transition from MI to MII. GVBD was monitored visually. G: GVBD (D) (left) Oocytes were first injected with Myc_6-Emi2-3′ UTR mRNA (11 pg/oocyte). After overnight incubation, they were divided into two groups and injected with either control morpholino or Mos morpholino. In Mos morpholino injected oocytes in this experiment, phosphorylation of MAPK was reduced by 86%. One hour later, oocytes were treated with progesterone and samples were taken at indicated times and analyzed by Western blotting. (right) Before Western blot analysis, Myc-Emi2 was immunoprecipitated and treated with lambda phosphatase. GVBD was monitored visually. G: GVBD; NS: non-specific band.

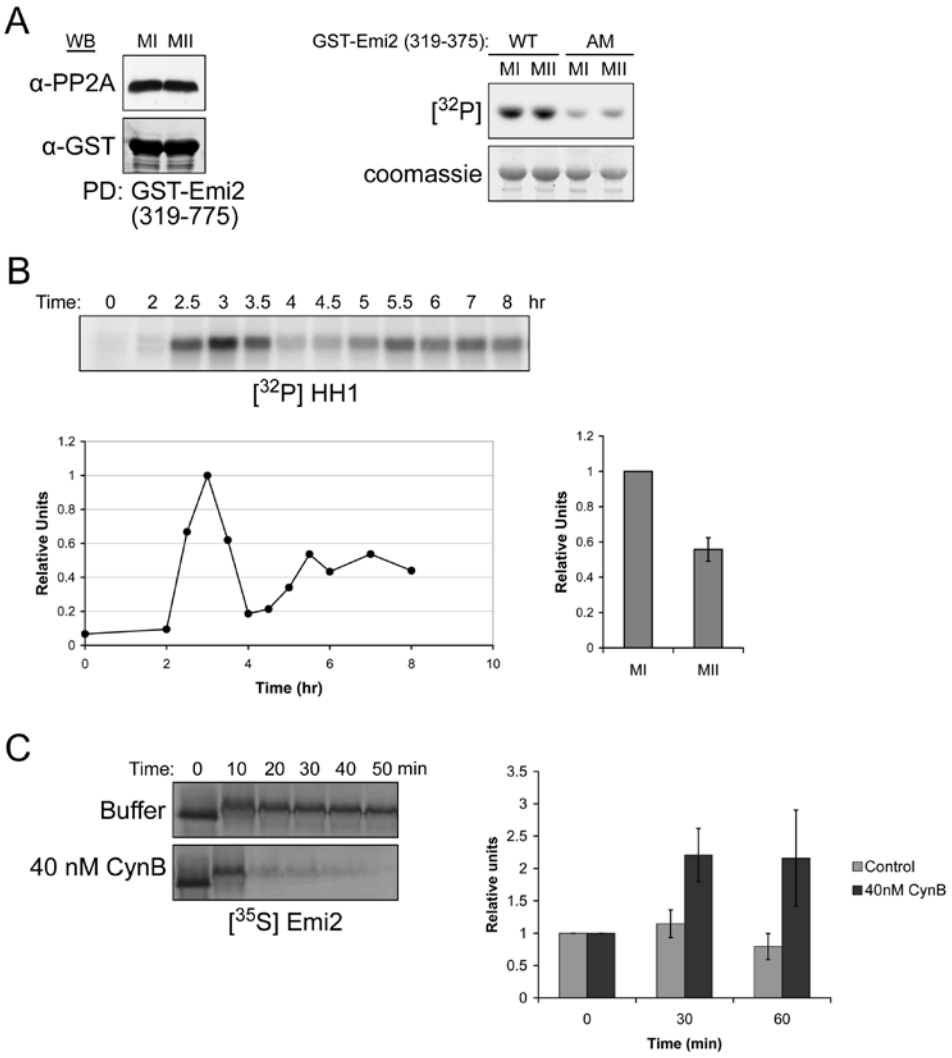

Figure 14 Instability of Emi2 in MI results from high Cdc2 kinase activity.

Figure 14 Instability of Emi2 in MI results from high Cdc2 kinase activity.

(A) Oocytes were treated with progesterone and MI and MII lysates were prepared. Recombinant GST-Emi2 protein (aa 319-375), conjugated to glutathione Sepharose beads, was (left) incubated in either MI or MII extract for one hour at 4 °C. Beads were washed five times with PBS (supplemented with 300 mM NaCl and 0.1% Triton). The amount of bound PP2A was detected by immunoblotting. (right) Rsk kinase was immunoprecipitated from both MI and MII extracts, washed five times with PBS (supplemented with 300 mM NaCl and 0.1% Triton) and its activity was measured by *in vitro* kinase assay using recombinant GST-Emi2 (aa 489-651) wild type or T545/551A as substrate. (B) Oocytes were treated with progesterone and lysates were made at the indicated times. Cdc2 kinase activity was measured by *in vitro* HHI kinase assay followed by autoradiography (top). Results were quantified by phosphorimager (bottom, left). Quantification of the average Cdc2 kinase activity in MII relative to MI is shown on the bottom right; error bar represents standard deviation of three independent experiments. (C) (left) CSF extract supplemented with recombinant GST-Emi2 (aa 489-651) T545/551A mutant protein was treated with or without recombinant non-degradable cyclin B (40 nM). ^{35}S-labeled Emi2 protein was added and samples were taken at the indicated times and analyzed by SDS-PAGE and autoradiography. (right) Cdc2 kinase activity was measured by *in vitro* HHI kinase assay. Quantification of kinase activity was shown; error bar represents standard deviation of three independent experiments.

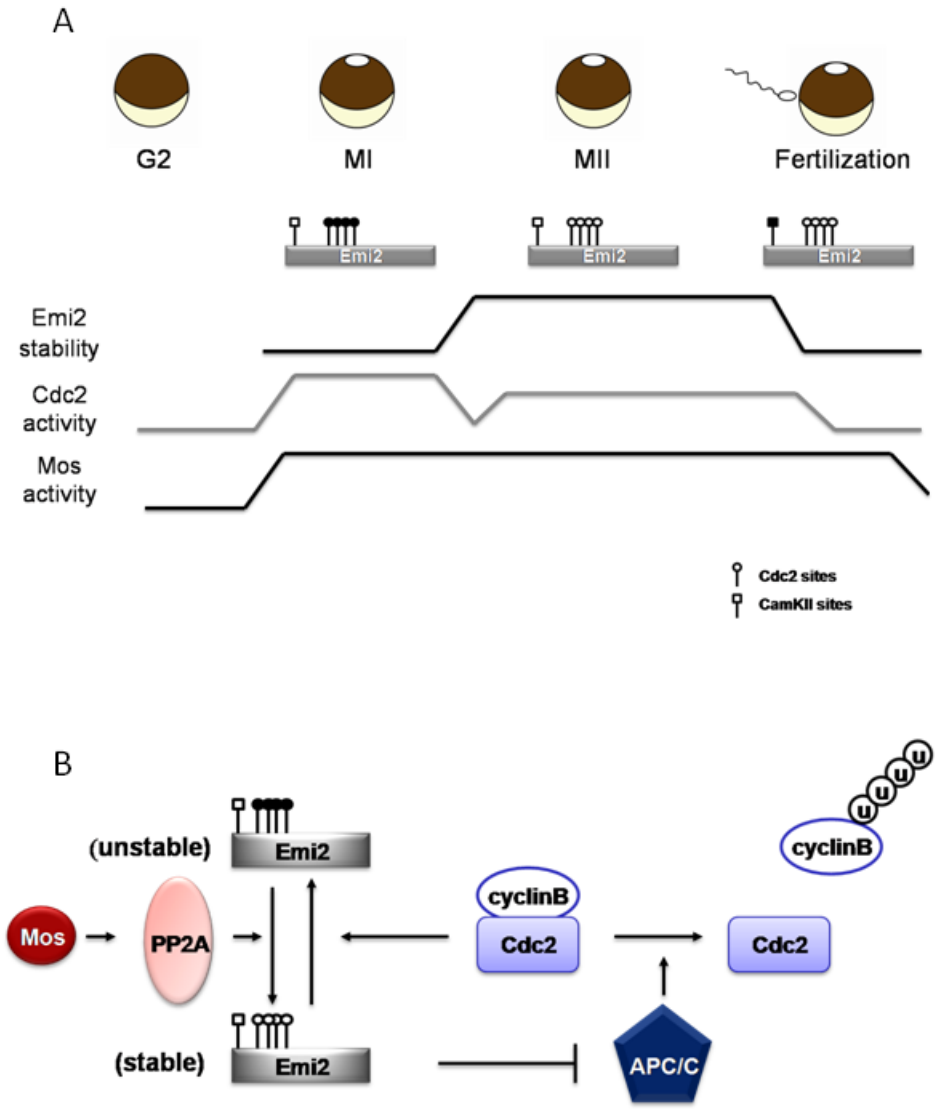

Figure 15 Regulation of Emi2 and the APC during the MI-MII transition

Figure 15 Regulation of Emi2 and the APC during the MI-MII transition

(A) Control of Emi2 stability during oocyte maturation. Emi2 stability is regulated by phosphorylation throughout the oocyte maturation process. Upon MI entry, high levels of Cdc2 kinase activity result in phosphorylation of four N-terminal sites (S213/T239/T252/T267) on Emi2, which leads to its degradation. Rapid degradation of Emi2 in MI is essential for MI exit, as its stabilization would lead to MI arrest. At the MI anaphase, Cdc2 kinase activity decreases as cyclin B is degraded by the APC. With Mos promoting its dephosphorylation, Emi2 is stabilized and accumulates, resulting in APC inhibition, which is necessary for S phase block and MII entry. In MII, Cdc2 kinase activity remains relatively low as compared to MI through an APC-mediated feedback loop we reported previously (Wu *et al.*, 2007b). Emi2 is stable in MII, which is essential for CSF arrest. At fertilization, Emi2 is quickly degraded through the CaMKII mediated pathway, which allows full activation of APC and exit from MII. (B) Auto-inhibitory regulation loop of APC. consequent decrease in Cdc2 Kinase activity. With the active Mos-PP2A pathway prevailing, Emi2 protein is dephosphorylated and stabilized, preventing the APC from completely degrading cyclin B; this is essential for the inhibition of S phase characteristic of the MI-MII transition.

3. Mechanism of APC/C inhibition by Emi2

3.1 Summary

Vertebrate eggs are arrested at Metaphase II by Emi2, the meiotic Anaphase Promoting Complex/Cyclosome (APC/C) inhibitor. Although the importance of Emi2 during oocyte maturation has been widely recognized and its regulation extensively studied, its mechanism of action remained elusive. Many APC/C inhibitors have been reported to act as pseudosubstrates, inhibiting the APC/C by preventing substrate binding. Here we show that a previously identified zinc-binding region is critical for the function of Emi2 while its D-box is largely dispensable. In addition, instead of acting through a "pseudosubstrate" mechanism as previously hypothesized, Emi2 inhibits Cdc20-dependent activation of the APC/C by blocking ubiquitin transfer from the ubiquitin-charged E2 to the substrate. We further demonstrate that Emi2 itself functions as an E3 ligase and this ligase activity depends upon a previously characterized zinc-binding region. Through its catalytic activity, Emi2 substoichiometrically inhibits the APC/C. These findings suggest that the APC/C may be inhibited by Emi2-mediated ubiquitylation and also provide a novel mechanism of APC/C inhibition wherein the final step of ubiquitin transfer is targeted.

3.2 Introduction

Anaphase is initiated by the activation of a large multi-subunit protein complex known as the APC/C. The APC/C is a 12 subunit E3 ubiquitin ligase that mediates poly-

ubiquitylation of numerous proteins, targeting them for proteasomal degradation. Many APC/C substrates are critical cell cycle regulators, including cyclin B and Securin, whose degradation is essential for the metaphase-anaphase transition. Although the functions of all 12 subunits are not fully understood, it is known that the APC/C recruits an E2 (UbcH5 or UbcH10, charged by an E1) to the core subunit APC11, recruits substrates destined for polyubiquitylation (facilitated by an activator), and transfers ubiquitin from the charged E2 to the substrate (Castro et al., 2005; Peters, 2006; Sullivan and Morgan, 2007). During the transfer step, the APC/C promotes the release of ubiquitin from the charged E2 before linking ubiquitin to the substrate (Ozkan et al., 2005).

Two E2 enzymes are known to support APC activity, UbcH5 and UbcH10 (UbcX in *Xenopus*) (Peters, 2006). The N terminus of UbcH10 has also been implicated in the regulation of APC/C ubiquitylation activity (Summers et al., 2008). Although APC/C core subunits confer a basal level of substrate interaction, the APC/C activators Cdc20 and Cdh1 can significantly enhance substrate binding to the APC/C by recognizing specific sequence motifs within substrates, such as the Destruction box (D-box) and KEN box (Matyskiela and Morgan, 2009; Peters, 2006; Yu, 2007). A recent study of APC/CCdc20 identified an additional important activator function; while the C-terminal WD40 domain of Cdc20 was required for substrate binding, the C- Box within the N terminus was required for activation of intrinsic APC/C ligase activity, a previously unrecognized step in APC/C-mediated substrate ubiquitylation (Kimata et al., 2008a).

APC/C activity is tightly controlled by a variety of inhibitors, such as Mad2 and BubR1 at active SAC, Emi1 during interphase, as well as Emi2, the meiotic specific inhibitor. The actions of these APC/C inhibitors are also governed by a variety of regulatory mechanisms, to ensure appropriately timed APC/C activation. Recently, a number of APC/C inhibitors have been shown to function as APC/C pseudosubstrates. Human Emi1 was the first APC/C inhibitor shown to compete with substrates for APC/C binding in a Destruction Box (D-Box) dependent manner, though this mechanism could not fully explain the essential role played by the Zinc Binding Region (ZBR) found at the C-terminus of the protein (Miller et al., 2006). Later, Mad3 (Burton and Solomon, 2007), and Acm1 in budding yeast (Choi et al., 2008), Securin in mouse oocyte (Marangos and Carroll, 2008), Mes1(Kimata et al., 2008b) in fission yeast and the also BubR1 in interphase mammalian cells (Malureanu et al., 2009) were all found capable of competing with substrates for APC/C binding. These findings, taken together with the observation that Emi2 also contains an apparent D-Box, have led to the strong suggestion that Emi2 might also act as a pseudosubstrate inhibitor. However, we show here that the D-Box of Emi2 is dispensable for its APC/C inhibitory activity. Examination of each step in substrate ubiquitylation by the APC/C has revealed that Emi2 exerts its APC/C inhibitory effect by precluding the transfer of ubiquitin from the charged E2 to its substrate, and that it is APC/C activation, rather than substrate binding, that is inhibited by Emi2. We also now show that Emi2 functions as an E3 ubiquitin ligase. This

ligase activity is required for its ability to inhibit the APC/C, providing a satisfying explanation for the critical role of the ZBR in Emi2 function. The demonstration of an enzymatic function for Emi2 also explains how Emi2 can so effectively inhibit the APC/C during a CSF arrest when present at levels substoichiometric to the APC/C.

3.3 Materials and methods

3.3.1 Plasmids and proteins preparation

Recombinant GST or MBP fusion Emi2 fragments (aa 489-651) were prepared as previously described (Wu et al., 2007a; Wu et al., 2007b). Emi2 mutants (C583A; R529A, L532A; F608A; F610A) were prepared using the QuickChange Site-Directed Mutagenesis Kit (Stratagene). hCdc20 (aa 1-151) was cloned into the BamH1 and NotI sites of pEnter3C vector and recombined into pDest15 vector (Invitrogen) for the bacterial production of recombinant protein. hCyclin B1 (aa 1-106) was cloned into the NotI sites of pEnter3C vector containing hCdc20 (aa 1-151). The DB of cyclin B1 (aa 1-106) on the chimera protein was mutated (R42A, L45A) with the same kit described above. The constructs were recombined into the pCDNA3 vector for production of *in vitro* translated proteins, which were generated using the TNT Quick Coupled Transcription/Translation System (Promega) in the presence of 35S-labeled Methionine and Cysteine (MP Biomedicals).

The plasmids used to make recombinant His-xUBCX and GST-APC3 for quantification were gifts from Dr. Hongtao Yu, and Dr. Hiro Yamano, respectively. The

plasmid to make GST-cyclin B N terminus (aa 1-70, 2×) was a gift from Dr. Tim Hunt. The plasmid used to make IVT APC11 was a gift from Dr. Peter Jackson.

3.3.2 Extracts and cyclin B degradation assay

CSF extracts and interphase extracts were prepared as previously described (Murray, 1991; Smythe and Newport, 1991). M phase extract were prepared by incubating the interphase extract with purified His-hCyclin B1 lacking the first 13 amino acids. This protein was made as described previously in baculovirus-infected Sf9 cells (Wu et al., 2007b).

For the cyclin B degradation assay, extracts were incubated with various Emi2 mutants at room temperature for 10 min before Ca2+ addition; 0.8 mM Ca2+ was added to extracts to induce release from CSF arrest.

3.3.3 Antibodies, immunoprecipitation and immunodepletion

Antibodies used in this study were as follows: anti-cyclin B1 as previously described (Hochegger et al., 2001); anti-Cdc27 (Santa Cruz) for immunoprecipitation; anti-Cdc27 (Transduction Laboratories) for immunoblotting; anti-flag (Sigma); anti-MBP (Cell Signaling); anti-GST (Santa Cruz); anti UbcH10 (Boston Biochem); and anti-xUBCx was a gift from Dr. Hongtou Yu.

For Cdc27 immunoprecipitation, 4 μg of antibodies were coupled to Protein A Sepharose beads and incubated in 100 μl of *Xenopus* egg extracts for 2 hours at 4 ^{o}C.

The antibody used for Cdc20 immunodepletion from *Xenopus* egg extracts was a gift from Dr. Hiroyuki Yamano and the depletion procedure was performed as described previously (Hayes et al., 2006).

3.3.4 *In vitro* ubiquitylation assay and APC/C assay

For the Emi2 ubiquitylation assay, 2 μg of Emi2 WT or mutants were incubated (2 h at 37 °C) with 50 ng of hE1, 0.5 μg UbcH5a and UbcH10, 10 μg ubiquitin (recombinant E1, E2 and ubiquitin were all purchased from Bioston Biochem), and 3 mM ATP brought to 40 μl with buffer (25 mM Hepes, pH 7.5, 10 mM NaCl, 3 mM MgCl2, 10 mM DTT, and 0.05% Triton X-100). Reactions were stopped with SDS sample buffer, resolved by SDS-PAGE, and analyzed by immunoblotting.

Unless otherwise specified, immunoprecipitated APC/C was incubated with 4 μl of *in vitro* translated human Cdc20 or Cdh1 at 22°C for 30 min in our APC/C assays. Then 50 ng of hE1, 0.5 μg UbcH10, 10 μg of ubiquitin, and an energy regenerating system were added together with 2 μl of *in vitro* translated 35S-labeled human cyclin B1 fragment (aa 1–106) as a substrate. Reactions were incubated at 22°C for 45 min with constant shaking, quenched with SDS sample buffer, analyzed by SDS-PAGE and autoradiography, and quantified by phosphorimager.

3.4 Results

3.4.1 Characterization of D-Box and ZBR regions within the Emi2 C-terminus

The C-terminus (aa 489-651) of Emi2 was previously shown to be essential for its APC/C inhibitory activity (Wu et al., 2007b) (see diagram, Fig. 16A). As confirmed in Fig. 16B, the recombinant Emi2 C-terminus could inhibit either Cdc20 or Cdh1-dependent APC/C activity *in vitro*. Analysis of the Emi2 C-terminal fragment revealed two potential functional domains, a putative D-box, found in a number of APC/C substrates (DB: aa 529-536) and a previously reported Zinc Binding Region (ZBR: aa 583-624). To evaluate the relative importance of these two domains for Emi2 function, we mutated them and assayed the mutants for both APC/C binding and APC/C inhibition. As shown in Fig. 16C, mutations in either the DB (DBM; R529A, L532A) or ZBR (ZBRM; C583A) compromised APC/C binding, with the DB mutant having a more profound effect. Combination of both mutants (2M: the double mutant) further decreased binding to undetectable levels, indicating that the DB and ZBR domains cooperated to achieve optimal APC/C binding. CSF extracts supplemented with either recombinant Emi2 ZBRM or the double mutant exhibited very similar kinetics of cyclin B degradation to the buffer control, consistent with a complete loss of APC/C inhibitory activity for these mutants (Fig. 16D). In contrast, the DB mutant was considerably less impaired in its ability to inhibit cyclin B degradation (Fig. 16D). Similar results were obtained in an *in vitro* APC/C assay; using Emi2 variants at similar concentrations, the ZBRM was more

markedly impaired than the DBM in its ability to prevent cyclin B ubiquitylation (Fig. 16E). These data strongly suggest that while the DB may contribute to APC/C inhibition, there is a particularly critical role for the intact ZBR in APC/C inhibition by Emi2.

3.4.2 DB promotes binding of Emi2 to APC/C, while ZBR, is critical for its APC/C inhibitory activity

Although binding of both the ZBR and DB Emi2 mutants was impaired, the amount of APC/C associated with Emi2 in the CSF extract could be restored to endogenous levels by simply elevating the levels of mutant protein (Fig. 17A). Interestingly, even at concentrations that restored APC/C binding, the ZBR mutant failed to delay cyclin B degradation (Fig. 17B, bottom panel). In contrast, the inhibitory activity of DB mutant was largely restored when binding was restored, increasing in a dose-dependent manner as more protein was added to the extract (Fig. 17B, middle panel). These data indicate that although both domains contributed to APC/C binding, the APC/C inhibitory activity resided mainly in the ZBR of Emi2, while the function of the D-Box was simply to promote the binding required for inhibition by another function/domain of Emi2. To further test this hypothesis, we directly added recombinant mutant proteins into CSF extract and found that ZBR mutant, which contained an intact D-Box, worked in a dominant negative manner, causing cyclin B degradation without addition of Ca2+. At the same time, the DB mutant Emi2 did not have such an effect (Fig. 17C). This finding argued strongly against a D-box-dependent pseudosubstrate mechanism of APC/C inhibition by Emi2. Indeed, addition of a large excess of WT

recombinant Emi2 (24 μM; Note that the endogenous concentration of Emi2 was estimated to be 15 nM) to M phase extract did not reduce the cyclin B-APC/C binding (Fig. 17D), reinforcing the conclusion that substrate competition could not account for APC/C inhibition by Emi2. Indeed, in an *in vitro* APC/C assay with Emi2 substoichiometric to APC/C, we found that 10 nM and 500 nM Emi2 achieved similar degrees of inhibition on 340 nM APC/C (Fig. 17E; the quantification of APC/C in the assay was shown in Fig. 17F), which renders the substrate competition model implausible. Instead, we hypothesized that Emi2 might act catalytically to inhibit the APC/C in a ZBR dependent manner.

3.4.3 Emi2 inhibits Cdc20 dependent activation of APC/C activity

A recent study showed that Cdc20 could not only promote substrate binding to the APC/C but could also activate the APC/C's E3 ubiquitin ligase activity (Kimata et al., 2008a). Although Emi2 did not prevent the substrate binding step, we considered it possible that it might inhibit the Cdc20-dependent APC/C activation step. To address this, we produced a chimeric protein consisting of the hCdc20 N terminus (aa 1-151) directly linked to cyclin B (Fig. 18A). Consistent with a previous report, when physically linked together, this chimeric protein could bind to the APC/C in a D Box-independent manner (Fig. 18B). An assay to monitor degradation of the chimeric protein was then performed in a Cdc20-depleted CSF extract. While endogenous cyclin B remained stable after addition of Ca^{2+} (data not shown), the chimeric protein was quickly degraded.

More importantly, the degradation could be completely blocked by addition of exogenous Emi2 into the extract, suggesting that Emi2 was indeed capable of inhibiting the Cdc20-dependent activation of APC/C ligase activity (Fig. 18C). The experiment was repeated with the D-box mutant chimeric protein, which, as predicted, could also be degraded upon Ca2+ addition to the CSF extract and the degradation of the DB mutant chimeric protein was also prevented by exogenous Emi2 (Fig. 18D). To exclude the possibility that Emi2 is promoting the dissociation of the chimeric protein from APC/C, we performed a binding experiment and found that the amount of GST-C-C (both WT and DB mutant) associated with the APC/C was not affected by the presence of Emi2 (Fig. 18E). These data confirmed the conclusion that Emi2 could not work by blocking substrate recruitment and suggested that either some aspect of Cdc20-mediated APC/C activation or a fundamental feature of APC/C E3 ligase activity was inhibited by Emi2.

3.4.4 Emi2 inhibits APC/C by blocking the transfer of ubiquitin from activated E2 to substrates

In an attempt to identify the binding site(s) of Emi2 on APC/C, we tested binding between recombinant Emi2 and IVT radiolabeled individual APC/C subunits and found that Emi2 could interact directly with APC6, APC10 and APC11 in the absence of other APC/C components (quantitation of binding of the radiolabeled APC subunits to MBP/MBP-Emi2 is shown in Fig. 19A). As the APC/C recruits E2s through subunit APC11, the observed binding to this APC subunit prompted us to hypothesize that Emi2 might inhibit the APC/C by preventing E2 binding. In this regard, we were interested to

find that UbcH10 and Emi2 competed for APC/C binding *in vitro* as preincubation of APC/C with Emi2 prevented UbcH10 binding (Fig. 19B); however, this effect was not observed under ubiquitylation conditions (in the presence of E1, E2, ubiquitin and an energy regenerating system). UbcH10, in either its unmodified or ubiquitylated form, associated with APC/C equally well in the presence or absence of Emi2 (Fig. 19C). Furthermore, we tested and confirmed that Emi2 did not affect the charging of E2 (Fig. 19D) or the binding of Cdc20 to APC/C (Fig. 19E). Indeed, Emi2 could inhibit polyubiquitin chain formation on cyclin B in an *in vitro* APC/C assay lacking E1 and ubiquitin if E2 pre-charged with ubiquitin was added to the reaction (Fig. 19F), strongly suggesting that it is ubiquitin transfer from charged E2 to the substrate that is targeted by Emi2. (Note again that the amount of cyclin B bound to the APC/C was not affected by Emi2.)

As reported previously, ubiquitin transfer by APC/C from a charged E2 to its substrate first requires the release of ubiquitin from the activating site on E2 (Ozkan et al., 2005). Based on the observation that APC/C dependent poly-ubiquitylation of E2 itself, which would also require ubiquitin release was not affected by Emi2, we conclude that it is the latter step in which ubiquitin is transferred to the substrate that is inhibited by Emi2 (Fig. 19G).

3.4.5 Emi2 binds to E2 and acts as an E3 ubiquitin ligase, catalytically inhibiting the APC/C

Based on the hypothesis that Emi2 could inhibit efficient ubiquitin transfer from the E2 to the substrate by affecting either the E2 or APC/C, we first tested the E2-Emi2 association and detected a direct interaction *in vitro* (Fig. 20A). It has been suggested that E2 binding to ZBR containing proteins may be mediated by hydrophobic residues (Pickart, 2001; Zheng et al., 2000). In order to examine the role of Emi2-E2 interaction, we analyzed the sequence within the ZBR again and found two evolutionarily conserved phenylalanines (F608 and F610; Fig. 20B). Indeed, mutating F608 (FA mutant) or both F608 and F610 to alanine (2FA mutant) significantly reduced the interaction between Emi2 and UbcH10 (Fig. 20C). Moreover, APC/C assays with these E2 binding mutants showed that they were non-functional at inhibiting the APC/C *in vitro* (Fig. 20D). A cyclin B degradation assay in CSF extract also confirmed that these E2-binding deficient mutants of Emi2 could not inhibit APC/C activity in the extract (Fig. 20E).

Although these data were superficially consistent with the possibility that Emi2 might act as a direct binder and inhibitor of E2 function, this interpretation was somewhat difficult to reconcile with the previously reported observations that the concentration of Emi2 in CSF extract was estimated to be 15 nM, far less than that of APC/C (80~160 nM, based on quantification of the APC3 subunit) and of UbcX (1 μM), making it unlikely that Emi2 could act as a stoichiometric E2 inhibitor. Therefore, we considered another possible interpretation of our data: In that a major class of E3 ligases

(Ring domain E3s) are characterized by the presence of ZBR that bind to E2s (Pickart, 2001), we hypothesized that Emi2 might, like the APC/C itself, function as an E3 ligase that could act catalytically to inhibit the APC/C. To test this, we performed an *in vitro* ubiquitin ligase assay with Emi2, E1, E2 and ubiquitin. As shown in Fig. 20F, we were able to detect autoubiquitylation of Emi2 *in vitro*. Moreover, this ligase activity was lost when the ZBR structure was disrupted (ZBRM), or the E2 binding ability was abrogated (FA mutant), potentially explaining the inability of these mutants to inhibit APC/C function. Therefore, these data were consistent with the possibility that Emi2 might act enzymatically, as expected of an E3 ligase, rather than as a stoichiometric inhibitor of APC/C function.

If Emi2 was indeed working catalytically, then pre-incubation of Emi2 with APC/C, which would allow time for any modification (such as ubiquitylation of APC/C components) to occur, should result in a more profound inhibition. As shown in Fig. 20G, increasing the time of pre-incubation of Emi2 with APC/C did correlate with a stronger *in vitro* inhibition. In addition, as predicted by the hypothesis that the inhibition was mediated by ubiquitylation, we found that the increase in inhibition failed to occur when the pre-incubation was carried out under non-ubiquitylating conditions (Fig. 20H). In an additional experiment, Emi2 and an APC/C preparation were pre-incubated for a fixed time period, while E1, E2 and an energy regenerating system were supplied to the reaction at various time points to allow ubiquitylation to proceed (Fig. 20I). We

found that the earlier components required for ubiquitylation were provided, the more marked was the inhibition obtained. These data argue strongly for a requirement for Emi2 ubiquitylating activity in inhibiting the APC/C, supporting a critical role for Emi2 E3 ligase activity.

3.5 Discussion

3.5.1 Mechanism of APC/C inhibition by Emi2

The importance of APC/C inhibition by Emi2 during oocyte maturation and CSF maintenance is well recognized (Wu and Kornbluth, 2008). However, the mechanism of Emi2 action has not been elucidated. In this study, we show that unlike many other APC/C inhibitors, including a closely related protein, Emi1, Emi2 does not act as a pseudosubstrate inhibitor. Instead, Emi2 targets the last step of substrate ubiquitylation by APC/C, where the ubiquitin is transferred from charged E2 to the substrate. In particular, it blocks the reception of ubiquitin by the substrate rather than the release of ubiquitin from charged E2. Indeed, when linked to the Cdc20 N terminus, cyclin B degradation became D-Box independent, yet still inhibitable by Emi2, completely ruling out the model wherein Emi2 prevents the D-Box dependent binding of substrates to prohibit their ubiquitylation.

3.5.2 The substrate specificity of APC/C

As previously shown, Emi2 mediated APC/C inhibition selectively targets cyclin B but not cyclin A (Wu et al., 2007a). In the course of this study, we observed that, similar to cyclin A, the APC/C dependent degradation of Nek2A is also not inhibitable by Emi2 (data not shown). The substrate specificity of APC/C is a fascinating topic and recent studies have yielded significant insights into this phenomenon. APC/C^{Cdc20} and APC/C^{Cdh1} are known to have different, albeit overlapping, sets of substrates and the two activators are differently regulated (Peters, 2006). In addition, the APC/C has been demonstrated to be a processive enzyme and substrate-dependent processivity helps to determine the order of destruction when more than one substrate is available (Rape et al., 2006). The observations on Emi2 have raised the interesting possibility that some APC/C inhibitors may have independent means to modulate APC/C substrate specificity, which has not yet been explored. For instance, in theory, Emi2 could alter the APC/C such that it only allows ubiquitylation of a specific subset of substrates.

3.5.3 Roles of ubiquitylation in APC/C inhibition

Phosphorylation is the only known post-translational modification event that regulates the activity of APC/C to date. However, the activity of its close kin SCF, the other Cullin-RING E3 ligase complex, was found to be stimulated by linkage to Cullin of the ubiquitin-like protein Nedd8 (Hori et al., 1999; Ohh et al., 2002). No evidence of APC/C regulation by neddylation has been reported, but one exciting possibility

remains that the APC/C can be regulated by other post-translational modifications. To date, two APC/C inhibitors, Xnf7 (Casaletto et al., 2005) and now, Emi2, have been identified as RING domain E3 ligases and their ligase activities appear to be essential for their function. It may be that Xnf7 and Emi2 share similar mechanisms of action in response to SAC and CSF signals, respectively. Consistent with the idea that Emi2 inhibits the APC/C via its catalytic activity rather than through physical association, it is worth noting that majority of Emi2 is not present in the same fractions as APC/C by gel filtration (Wu et al., 2007b). Nonetheless, this model is not incompatible with the notion that being able to bind APC/C is a critical step in Emi2-mediated inhibition as it is probably a prerequisite to allow any APC/C modification to occur (by close juxtaposition of Emi2 and the APC/C). This provides a potential explanation for the partial defect in action of the D-box mutant observed in Fig. 16D and 16E. Since Emi2-mediated APC/C inhibition could be fully recapitulated *in vitro* with defined components, we suspect that the relevant substrate(s) must be among those protein factors: Emi2 itself, the activator, APC/C core subunits, or APC/C-associated proteins. A requirement for Emi2 autoubiquitylation to activate its inhibitory activity is unlikely to explain a requirement for its ligase activity since this would not help solve the stoichiometry issue. The activator is also not likely to be the relevant substrate as no ubiquitylation on purified Cdc20 could be detected in an *in vitro* ubiquitylation assay using recombinant Emi2 (data not shown), though it remains possible that such a modification requires the

presence of APC/C or other protein(s) that co-immunoprecipitate with the APC/C. In that Emi2 could directly interact with APC/C core subunits in the absence of an activator and could inhibit the basal activity of the APC/C, we favor the model that Emi2 modifies APC/C subunit(s). In particular, we speculate that ubiquitylation of APC/C subunits may prevent an APC/C conformational change required for transfer of ubiquitin from E2 to the substrate. Since Emi2 appears capable of binding APC11, another intriguing possibility is that a RING-RING interaction between Emi2 and APC11 creates an E3 ligase of novel specificity able to ubiquitylate an APC/C component. As we begin to search for targets of Emi2 inhibition on the APC/C, it will be of interest to compare potential APC/C autoubiquitylation targets, Emi2 targets, and possible Emi2/APC/C novel targets.

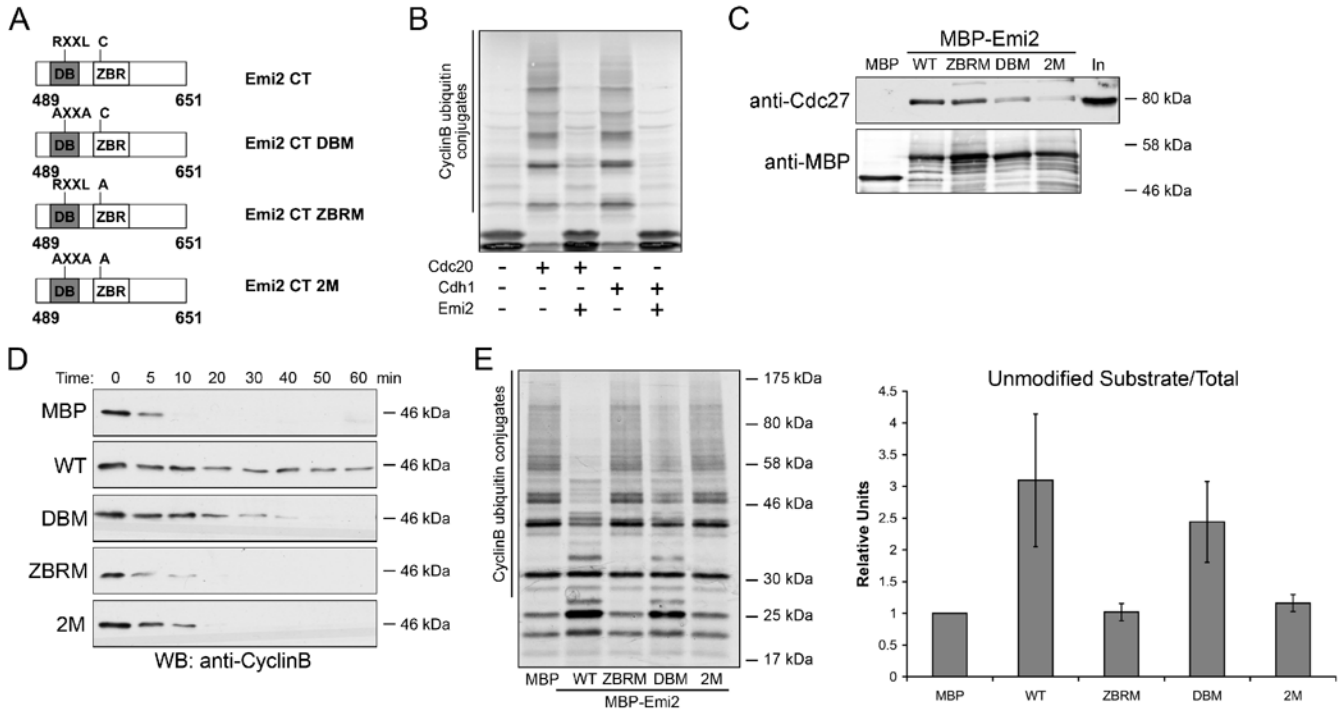

Figure 16 Characterization of D-Box and ZBR regions within the Emi2 C-terminus

Figure 16 Characterization of D-Box and ZBR regions within the Emi2 C-terminus

(A) Emi2 C-terminus (CT) mutants. DBM: D-Box mutant; ZBRM: Zinc Binding Region mutant; 2M: double mutant. All experiments were performed with Emi2 CT and its mutant variants. (B) *In vitro* APC/C assays were performed in the presence or absence of 600 nM GST-Emi2 with either Cdc20 or Cdh1 as the activator. The conversion of radiolabeled cyclin B to ubiquitylated forms was monitored by autoradiography. (C) 50 nM of recombinant MBP-Emi2 (wildtype or mutants) was conjugated to Amylose beads and incubated in Crude S extract for 20 min at 22°C. Beads were washed five times with PBS (supplemented with 300 mM NaCl and 0.1% Triton). The amount of associated Cdc27 was detected by western blotting. In: Input (D) 50 nM of recombinant MBP or MPB-Emi2 (wildtype or mutants) was added to CSF extracts supplemented with Ca^{2+}. Aliquots removed at the indicated times were analyzed by SDS-PAGE and immunoblotted for cyclin B. (E) 800 nM of recombinant MBP or MPB-Emi2 (wildtype or mutants) was added to *in vitro* APC/C assays with IVT Cdc20 as the activator. The immunoprecipitated APC/C was pre-incubated with Emi2 and Cdc20 together with E1, E2 and ubiquitin for 1h before the addition of substrates. Conversion of radiolabeled cyclin B to ubiquitylated forms was monitored by autoradiography and phosphorimager. Four independent experiments were further quantified with ImageQuant 5.0.

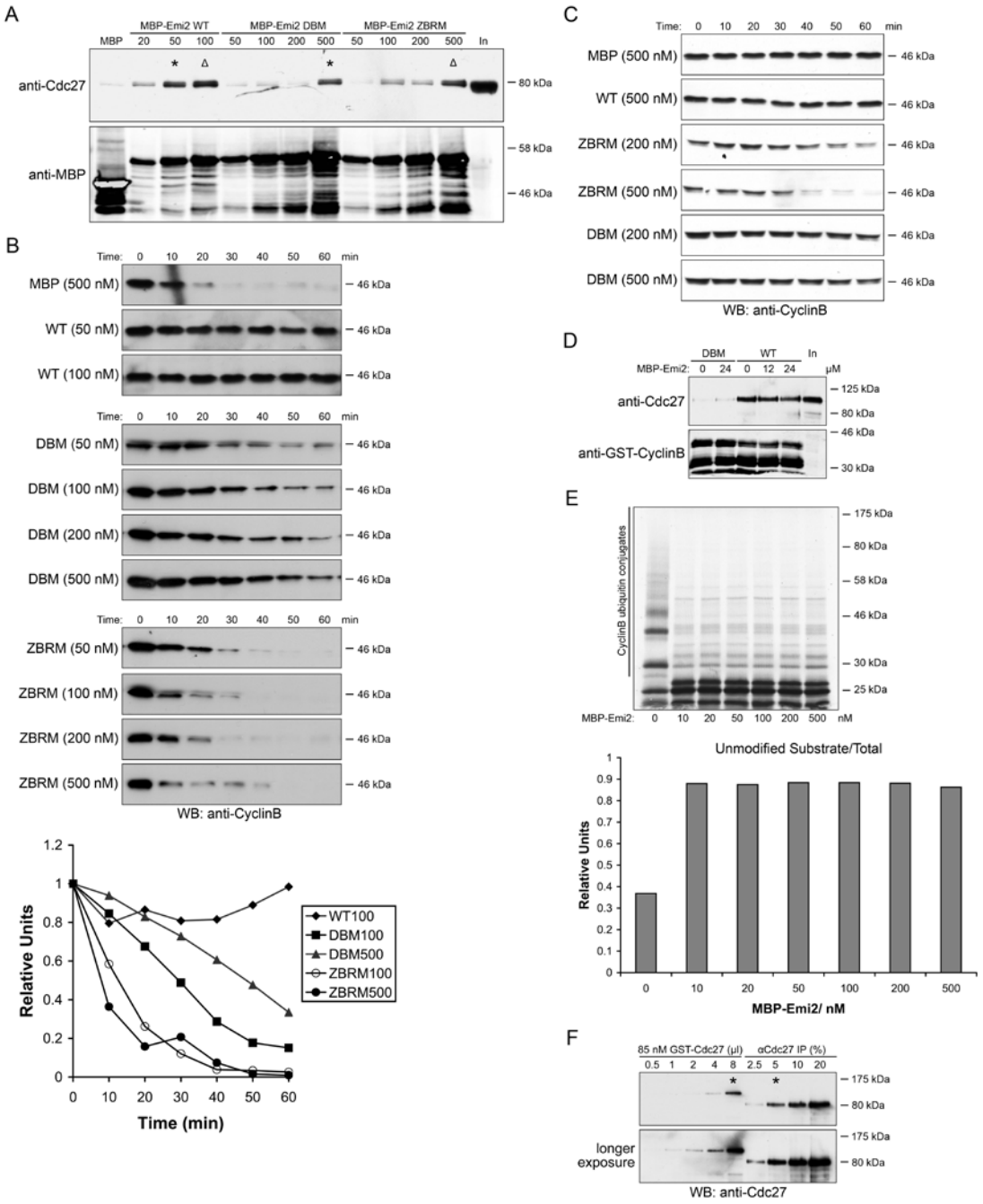

Figure 17 DB promotes binding of Emi2 to APC/C, while ZBR is critical for its APC/C inhibitory activity

Figure 17 DB promotes binding of Emi2 to APC/C, while ZBR is critical for its APC/C inhibitory activity

(A) Recombinant MBP or MBP-Emi2 (wildtype or mutant) was incubated in Crude S extract at the indicated concentrations for 20 min at 22°C and pulled out on Amylose beads. Samples were analyzed as in Fig. 16C. Asterisks indicate that the amount of APC/C associated with 500 nM DBM was equal to that with 50 nM WT; Triangles indicate that the amount of APC/C associated with 500 nM ZBRM was equal to that with 100nM WT. In: Input(B) Recombinant MBP or MBP-Emi2 (wildtype or mutant) at the indicated concentrations were added to CSF extracts supplemented with Ca2+. Samples were analyzed as in Fig. 16D; five samples as indicated were quantified with ImageQuant 5.0.(C) Recombinant MBP or MPB-Emi2 proteins (wildtype or mutants) were added to CSF extracts at the indicated concentrations. Aliquots removed at the indicated times were analyzed by SDS-PAGE and immunoblotted for cyclin B. (D) GST-CyclinB (aa 1-70, 2×; WT or DBM) conjugated to glutathione beads was incubated in M phase extract at the indicated concentrations in the absence or presence of recombinant MBP-Emi2 for 20 min at 22°C. GST-cyclin B was retrieved from the extract by centrifugation, washed and immunoblotted for Cdc27 or GST. In: Input. (E) The APC/C were immunoprecipitated from M phase extract and incubated with 1 μM recombinant His-Cdc20 and MBP-Emi2 at the indicated concentrations for 45 min prior to substrate addition. The conversion of radiolabeled cyclin B to ubiquitylated forms was monitored by autoradiography and phosphorimager. Results were further quantified with ImageQuant 5.0. (F) APC/C was immunoprecipitated from the same egg extract used in Fig. 17E (Note that in experiments shown in Fig. 17E, extract was driven into M phase as described in the Materials and Methods) and quantified (using recombinant GST-Cdc27 as a standard) by immunoblotting. Asterisks indicate that 5% of the total APC/C (as represented by Cdc27) from one sample of the APC/C assay in Fig. 17E is equivalent to the amount of Cdc27 detected in 8 μl of the recombinant GST-Cdc27 preparation (85nM). As the total volume of each sample in the APC/C assay was 40 μl, the concentration of APC/C in the APC/C assays shown in Fig. 17E was calculated to be 340 nM.

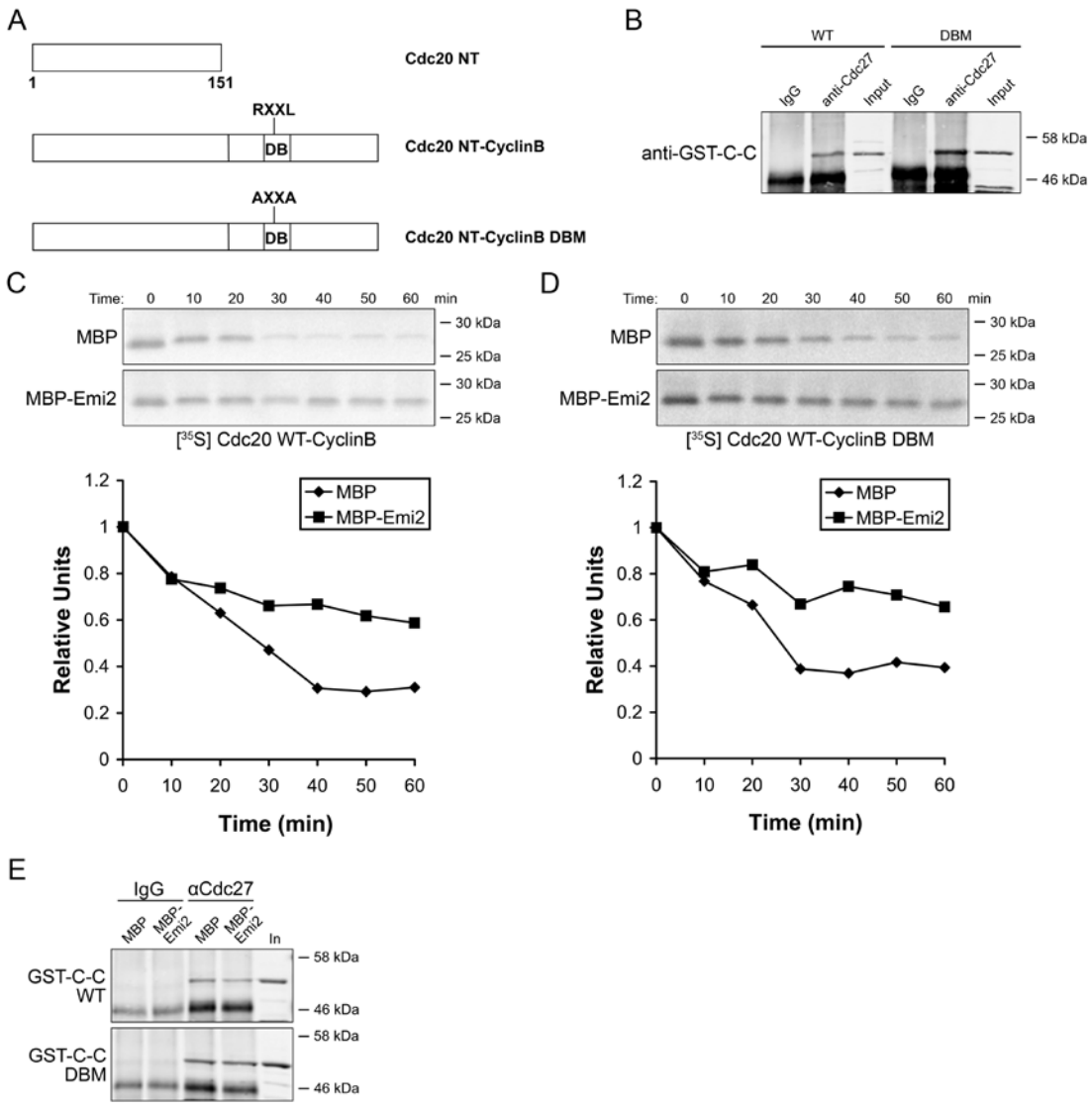

Figure 18 Emi2 inhibits Cdc20-dependent activation of APC/C activity

Figure 18 Emi2 inhibits Cdc20-dependent activation of APC/C activity

(A) Diagram of the chimeric Cdc20/cyclin B proteins. hCdc20 aa 1-151 was linked with hCyclin B1 1-106 (Cdc20 NT-cyclin B). Cdc20 NT-cyclin B DBM: Cdc20 NT-cyclin B with R42A, L45A mutation on cyclin B. (B) APC/C immunoprecipitated from M phase extracts was incubated in XB buffer with 250 nM recombinant GST-Cdc20 NT-cyclin B (GST-C-C; WT or DBM) for 20 min at 22°C. APC/C beads were retrieved and washed. The amount of associated GST-C-C was detected by western blotting. (C) IVT ^{35}S labeled Cdc20 NT-cyclin B was added to Cdc20-depleted CSF extract supplemented with 60 nM recombinant MBP or MBP-Emi2. After Ca^{2+} addition, aliquots removed at the indicated times were subjected to autoradiography for IVT Cdc20 NT-cyclin B as well as to western blotting for endogenous cyclin B (Data not shown) Results were quantified with ImageQuant 5.0. (D) Same as in Fig. 18C except that Cdc20 NT-cyclin B DBM was analyzed. (E) APC/C immunoprecipitated from M phase extracts was incubated in XB buffer with 250 nM recombinant GST-Cdc20 NT-cyclin B (GST-C-C) in the presence of 500 nM MBP or MBP-Emi2 for 20 min at 22°C. APC/C beads were retrieved and washed. The amount of associated GST-C-C was detected by western blotting.

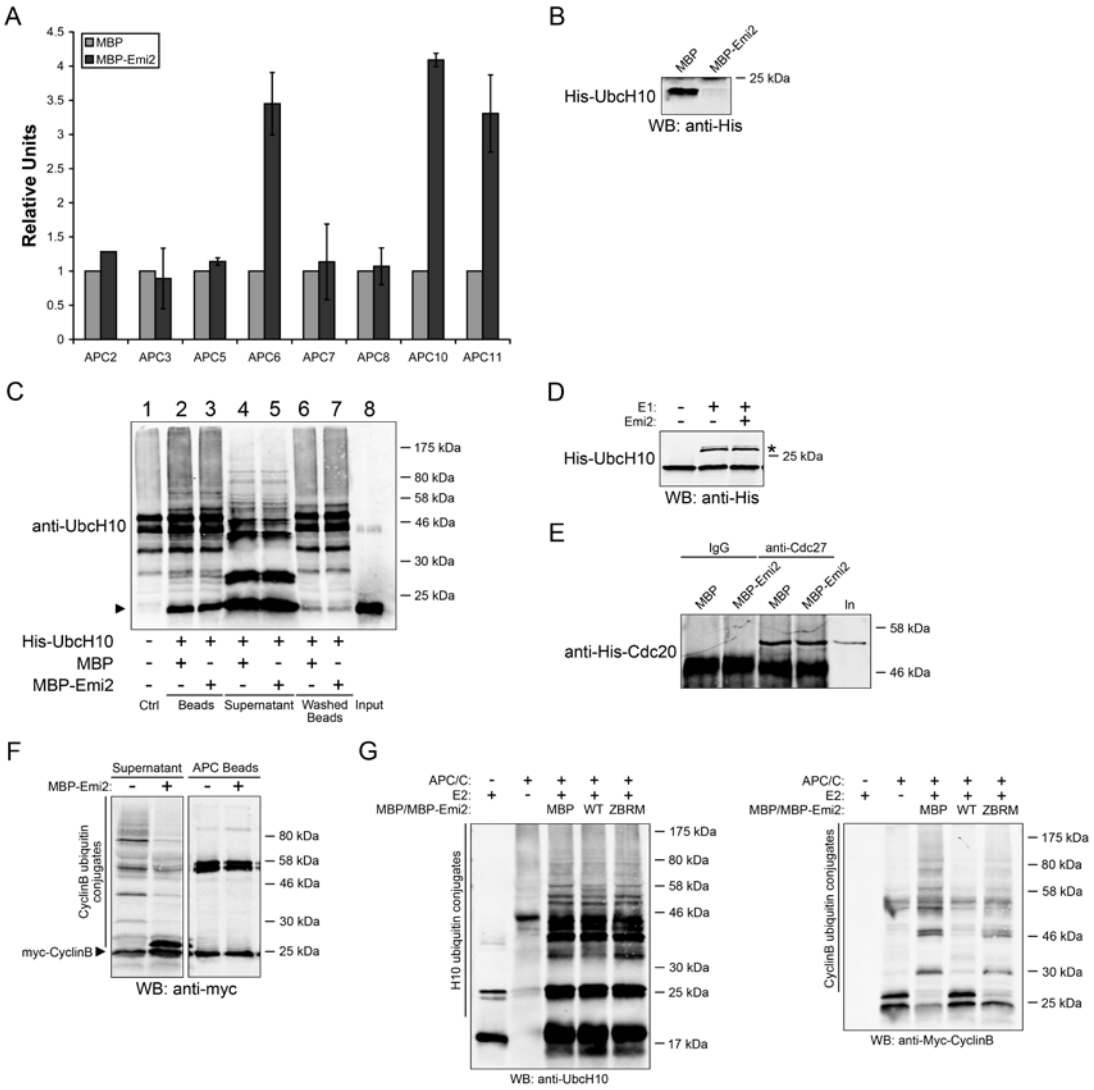

Figure 19 Emi2 inhibits APC/C by blocking the transfer of ubiquitin from activated E2 to substrates

Figure 19 Emi2 inhibits APC/C by blocking the transfer of ubiquitin from activated E2 to substrates

(A) MBP or MBP-Emi2 conjugated to Amylose beads were incubated with ^{35}S labeled IVT APC subunits for 1 h at 22°C. Beads were washed and bound IVT APC subunits were examined by SDS-PAGE and autoradiography. Results from at least three independent experiments for each subunit were quantified with ImageQuant 5.0. (B) APC/C immunoprecipitated from mitotic extracts was pre-incubated with MBP or MBP-Emi2 (600 nM) for 15 min at 22°C. Purified His-UbcH10 was added to both samples and incubated for 1 h at 22°C. APC/C beads were washed and the bound UbcH10 was analyzed by western blotting. (C) APC/C was immunoprecipitated from mitotic extracts and incubated with purified His-UbcH10 in the presence of MBP or MBP-Emi2 (0.6 μM) for 1h at 22°C. E1, ubiquitin and an energy regeneration system were also added to the reactions. Beads (washed or not with PBS supplemented with 300 mM NaCl and 0.1% Triton-100) and supernatant were separated and analyzed by UbcH10 western blotting. APC/C immunoprecipitant without UbcH10 is represented in lane 1. The arrow indicates unmodified His-UbcH10. (D) His-UbcH10 was incubated with E1, ubiquitin and an energy regenerating system in the presence or absence of 600 nM Emi2 for 30 min at 22°C. Reactions were stopped with addition of sample buffer and charging of UbcH10 was analyzed by His immunoblotting. The asterisk indicates charged/activated E2. (E) APC/C immunoprecipitated from M phase extracts was incubated in XB buffer with recombinant His-Cdc20 in the presence of 500nM MBP or MBP-Emi2 for 20 min at 22°C. APC/C beads were retrieved and washed. The amount of associated His-Cdc20 was detected by western blotting. (F) APC/C immunoprecipitated from mitotic extracts was incubated with pre-charged UbcH10, cyclin B and an energy regenerating system in the presence or absence of MBP-Emi2 (600 nM) for 1 h at 22°C. Sample buffer was added to the supernatant and APC/C beads separately. The formation of ubiquitin conjugates on cyclin B was analyzed by Myc western blotting. (G) *In vitro* APC/C assays were performed in the presence of 500 nM MBP or MBP-Emi2 (wildtype or ZBRM) and the formation of ubiquitin conjugates on both UbcH10 and cyclin B were analyzed by western blotting for UbcH10 or Myc.

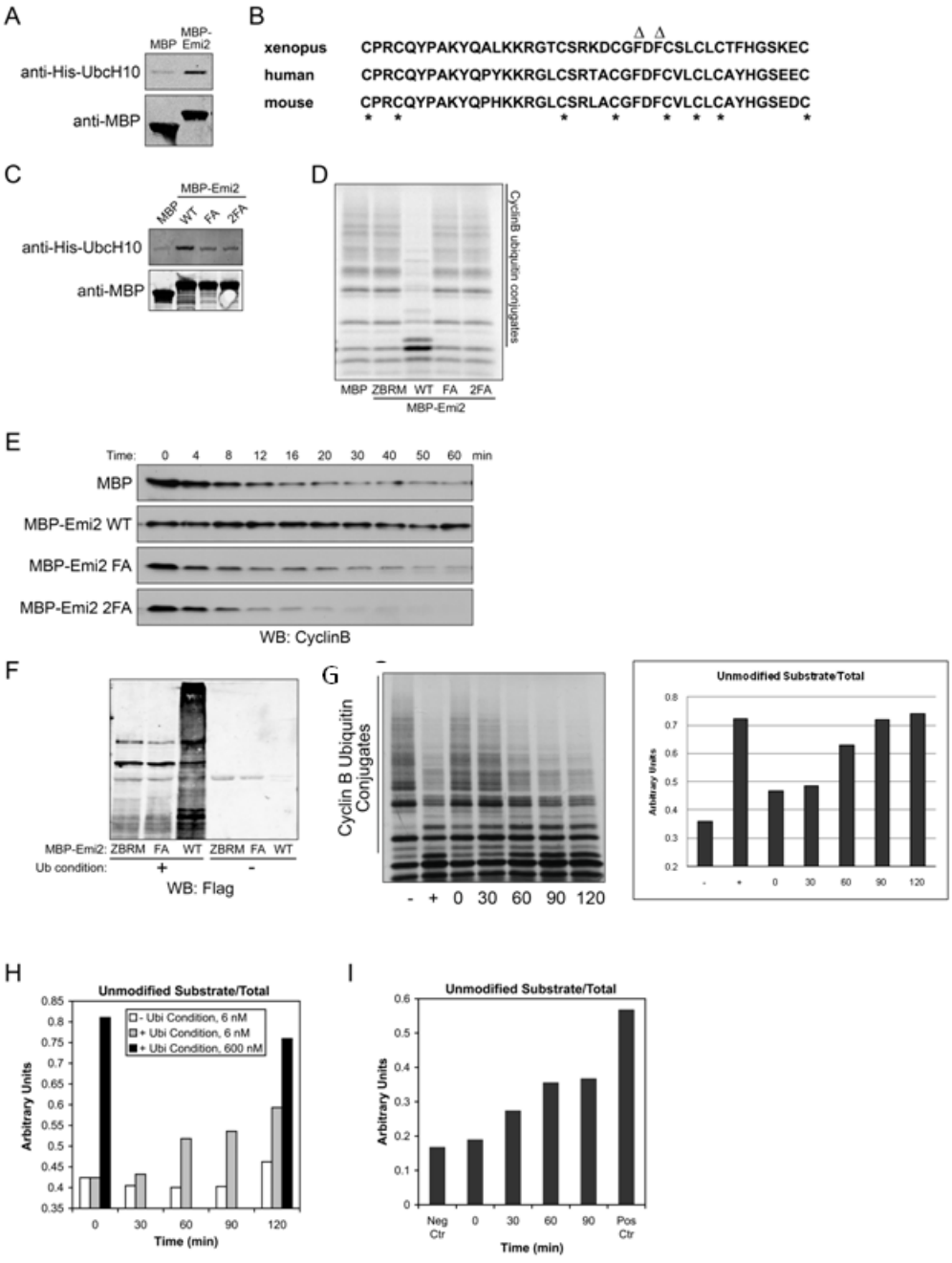

Figure 20 Emi2 is a RING ligase and the ligase activity is required for its function.

Figure 20 Emi2 is a RING ligase and the ligase activity is required for its function.

(A) MBP or MBP-Emi2 conjugated to Amylose beads was incubated with purified His-UbcH10 for 1 h at 22°C. Beads were washed and immunoblotted for MBP or His. (B) Alignment of Zinc Binding Region of *Xenopus*, human and mouse Emi2. Asterisks indicate conserved cysteines and the triangles indicate conserved phenylalanines. (C) MBP or MBP-Emi2 (wildtype or mutant) conjugated to Amylose beads were incubated with purified His-UbcH10 for 1 h at 22°C. Beads were washed and immunoblotted for MBP or His. (D) Same as in Fig. 16E except that different Emi2 mutants were analyzed. (E) Same as in Fig. 17C except that different Emi2 mutants were analyzed. (F) MBP-Emi2 (wildtype or mutant) was incubated with E1, E2 (UbcH5a and UbcH10), Flag-ubiquitin and ATP for 2 h at 37°C. Reactions were stopped by addition of sample buffer and samples were analyzed by Flag immunoblotting. (G) APC immunoprecipitated from mitotic extracts were incubated with 6 nM MBP-Emi2, Cdc20, ubiquitin together with E1, UbcH10 and energy regenerating mix (ubiquitylating mixture) for indicated times. After incubation, substrates were added and the reactions were incubated for 45 min at 22°C. Conversion of radiolabeled cyclin B to ubiquitinated forms was monitored by autoradiography and quantified by phosphorimager.-: no Emi2 was added; +: 600nM of Emi2 was added (H) APC immunoprecipitated from mitotic extracts was incubated with 6 nM or 600 nM MBP-Emi2, Cdc20 and ubiquitin for indicated times in the presence or absence of the ubiquitylating mixture. After incubation, ubiquitylating mixtures were added to the rest of samples and substrates were added to all samples and the reactions were incubated for 45 min at 22°. (I) APC immunoprecipitated from mitotic extracts were incubated with 6 nM MBP-Emi2, Cdc20 and ubiquitin for 90 minutes. Ubiquitylating mixtures were added at different times. The time that Emi2 had been incubated with the presence of the ubiquitylating mixture was indicated. Substrates were added and the reactions were incubated 45 min at 22°C. Result was analyzed as in Fig. 20G. Neg Ctr: Emi2 was not added in this sample; Pos Ctr: 600 nM MBP-MBP was used in this sample.

4. Trim39 and its roles in Spindle Assembly Checkpoint signaling

4.1 Summary

The Spindle Assembly Checkpoint (SAC) inhibits the APC/C–mediated ubiquitylation of securin and cyclin B to avoid the mis-segregation of chromosomes and premature initiation of anaphase. Consequently, a compromised SAC can contribute to genomic instability and tumorigenesis. Previous research from our laboratory has demonstrated that Xnf7, a RING E3 ligase, is required for SAC to function properly in *Xenopus* egg extracts. Here we show that human Trim39, which shares 42% sequence identity with Xnf7, is also a RING E3 ligase that plays an essential role in SAC signaling. Knockdown of Trim39 in RPE cells by RNAi overrides nocodazole-induced M phase arrest, which could be rescued by exogenously expressed wildtype Trim39 but not RING mutants. Like many other SAC proteins, the activity of Trim39 appears to be regulated by the SAC. The polyubiquitylated form of Trim39 is only observed in cells that are arrested in M phase by nocodazole, indicating that the E3 ligase activity of Trim39 may be triggered by an active checkpoint. Finally, although Trim39 resembles Xnf7 in many respects, the mechanism of action may differ, as Xnf7, but not Trim39, can inhibit the APC/C-mediated ubiquitylation reaction *in vitro*. The roles and regulation of Trim39 in SAC are still undergoing investigation.

4.2 Introduction

In mature vertebrate eggs, Emi2, the key component of CSF activity, maintains APC/C inhibition prior to fertilization. During the mitoses of somatic cells, the APC/C is kept inactive by the Spindle Assembly Checkpoint (SAC) in order to prevent the precocious separation of chromosomes (Musacchio and Salmon, 2007; Wu and Kornbluth, 2008). Impairing the SAC signaling pathway typically leads to errors in chromosome segregation and aneuploidy, which could further result in the loss of genetic integrity and cancer development. Elucidating the molecular events underlying the SAC signaling pathway is necessary to better understand how its dysregulation is linked to human carcinogenesis. In addition, since chemotherapeutic agents frequently target the SAC, identification and characterization of new SAC proteins may provide novel opportunities for the development of more efficient cancer treatments (Bharadwaj and Yu, 2004; Chi and Jeang, 2007; Heilman et al., 2005; Liu et al., 2009).

The SAC signal is sensed at the kinetochores and efficiently amplified across the cell, culminating in the inhibition of the APC/C^{Cdc20}. After the last chromosome achieves bipolar attachment to the mitotic spindle, the SAC is immediately silenced and all of the chromosomes simultaneously separate. A number of proteins involved in this process have been identified, many of which are evolutionarily conserved, including factors in the mitotic checkpoint complex (MCC). The MCC, consisting of four proteins (Mad2, Bub3, BubR1 and Cdc20), has emerged as the major SAC effector mediating APC/C

inhibition within the past few years. The MCC is capable of directly binding to the APC/C to prevent the polyubiquitylation of substrates and undergoes dynamic assembly (accelerated by active SAC) and disassembly throughout the cell cycle. In addition to the MCC-mediated direct inhibition of the APC/C^{Cdc20}, it has also been reported that the APC/C-dependent degradation of Cdc20 can also contribute to a stable SAC. While the significance of these different pathways are still being evaluated, the list of SAC proteins also continues to grow (May and Hardwick, 2006; Musacchio and Salmon, 2007).

As previously described, Xnf7 was initially identified in our laboratory in a screen for cyclin B interacting proteins and was later demonstrated to be a *bona fide* APC/C inhibitor. Recombinant Xnf7 is capable of directly inhibiting APC/C-mediated ubiquitylation reactions *in vitro* as well as decreasing the cyclin B degradation rate in CSF extracts upon Ca^{2+} addition. Although Xnf7 does not appear to be required for the maintenance of CSF, its function is indispensible for SAC signaling in *Xenopus* egg extract. Antibody neutralization of Xnf7 can override SAC signaling, permitting cyclin B degradation and the reformation of interphase nuclei under an activated SAC; both events are inhibited in control extract (Casaletto et al., 2005).

When first identified, Xnf7 appeared quite special compared to other known SAC-activated APC/C inhibitors like Mad2 or BubR1. First, it directly interacted with the core subunits of APC/C but not Cdc20; second, it had intrinsic RING-dependent ligase

activity that was required for its APC/C inhibitory function. Because Xnf7-mediated APC/C inhibition could be achieved in a proteasome-free *in vitro* system, we concluded that inhibition was not exerted through the degradation of certain APC/C subunits or co-factors. To determine if the role of Xnf7 in controlling the SAC was also evolutionarily conserved, we searched for Xnf7 homologs in human sequence databases and identified two related proteins, Trim39 and Trim69, that are 42% and 46% identical, respectively, to *Xenopus* Xnf7. *Tri*partite *m*otif (Trim) proteins consist of RING finger, B-box, and coiled coil domains and have been defined as a subgroup of RING finger proteins based on their conserved structure. Recently, some proteins from the Trim family have been identified as E3 ligases, which are involved in a number of cellular processes such as cell proliferation, tumorigenesis, immune modification, and neuronal function (Balint et al., 2004; Cuykendall and Houston, 2009; Kano et al., 2008; Kong et al., 2007; Lee et al., 2009; Miyajima et al., 2008; Miyajima et al., 2009; Quaderi et al., 1997; Stremlau et al., 2004; Tanji et al., 2010). However, the function of many other Trim proteins has not yet been defined. In this study, we demonstrate that Trim39 plays a crucial role in SAC signaling in mammalian cells and suggest it to be the mammalian Xnf7 homolog. As with other members in the Trim family, Trim39 is also a RING E3 ligase whose activity correlates with its APC/C inhibitory function. Results presented in this chapter of the dissertation are preliminary and the research is ongoing.

4.3 Material and methods

4.3.1 Tissue culture

RPE cells (retinal pigmented epithelium cells) were obtained from the Duke University Medical Center Cell Culture Facility and maintained under an atmosphere of 5% CO_2 at 37°C in DMEM (Dulbecco's modified Eagle's medium):F12 medium modified to contain 2.5 mM L-glutamine, 15 mM HEPES, 0.5 mM sodium pyruvate, 1200 mg/L sodium bicarbonate (ATCC) and supplemented with 10% fetal bovine serum (Gibco).

4.3.2 Cloning and mutagenesis

Image clones of human Trim39 and Trim69 were purchased from ATCC. Both genes were PCR amplified with a FLAG tag and subcloned into the SalI and NotI sites of pEnter3C vector (Invitrogen). Using LR Clonase™ II enzyme mix (Invitrogen), our inserts were recombined into pcDNA3 vector for overexpression in mammalian cell lines, pDest15 vector (Invitrogen) for bacterial production of GST-conjugated recombinant proteins and pLenti7.3 vector (Invitrogen) for lentivirus production. Trim39 was also subcloned into the EcoRI and Xbal sites of pMalc2X vector for bacterial production of MBP-conjugated recombinant protein. Trim39 mutants (C44A and C52A) were prepared using the QuikChange Site-Directed Mutagenesis Kit (Stratagene).

4.3.3 Recombinant protein and antibody production

MBP-tagged Trim39 proteins (wildtype, C44A and C52A) were expressed in BL21-DE3 cells and purified using amylose beads (BioMol). GST-tagged Trim39

wildtype protein was expressed in BL21-AI cells (Invitrogen) and purified over glutathione beads (Amersham). A polyclonal anti-hTrim39 antibody was generated by immunizing rabbits with recombinant MBP-Trim39 and purified using GST-Trim39 conjugated and DMP crosslinked to glutathione beads.

4.3.4 RNAi, transfection and lentivirus infection

Plasmids were transfected into RPE cells with Fugene6 (Roche). siRNA transfection was carried out using Lipofectamine RNAiMAX (Invitrogen). SMARTpool siRNAs targeting human Trim39, Trim69, BubR1 and a non-targeting control siRNA pool were purchased from Dharmacon.

For lentivirus production, pLenti7.3 vector containing Trim39 (wildtype, C44A, C52A) was transiently transfected into HEK293FT cells using the pLenti7.3/V5-DEST Gateway vector kit (Invitrogen) that generates lentivirus that expresses the gene of interest and GFP. Virus supernatants were collected at 48 h post-transfection and viral titers were determined by the percentage of GFP-positive cells. For our addback experiments, cells were infected 24 h after siRNA transfection.

4.3.5 Immunoblotting, immunoprecipitation and immunofluorescence

Cell lysates were prepared by lysing cells in a solution containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, leupeptin (10 mg/ml), 1 mM phenylmethylsulfonyl fluoride, 400 μM Na_3VO_4, 400 μM EDTA, 10 mM NaF, and 10 mM sodium pyrophosphate. After incubating on ice for 15 min, the cells were sonicated,

centrifuged at 16,000 x g for 15 min at 4°C, and the protein concentration of the cell lysates determined by Bradford assay. Unless otherwise specified, 50 μg of protein were loaded in each lane for western blot analysis.

For co-immunoprecipitation experiments, antibodies were incubated with Protein A-Sepharose beads (Pierce) in PBS buffer overnight at 4°C; the resins were then washed with cell lysis buffer and incubated with the cell lysates for 2 h at 4°C. The beads were washed and the co-immunoprecipitated proteins were analyzed by western blot.

For immunofluorescence, RPE cells were grown on coverslips, fixed with 4% formaldehyde, and blocked with 3% goat serum albumin in phosphate-buffered saline. Cells were stained with mouse-anti-FLAG antibody (Sigma) or rabbit-anti-pH3 antibody (Cell Signaling), followed by incubation with Alexa-488-conjugated anti-mouse antibody (Invitrogen) and Alexa-568-conjugated anti-rabbit antibody (Invitrogen), respectively. Nuclei were counterstained with 4', 6-diamidino-2-phenylindole. Coverslips were mounted with Vetashield mounting medium (Vector laboratories). All images were obtained with a Zeiss Axio Imager microscope.

Antibodies used for western blots in this study included: mouse anti-cyclin B1 as previously described [26]; mouse anti-FLAG (Sigma); mouse anti-ubiquitin (Santa Cruz Biotechnology, Inc.); mouse anti-Cdc27 (Transduction Laboratories); mouse anti-BubR1 (Abcam).

4.3.5 Cell cycle analysis

To determine the cell cycle stage, RPE cells were harvested, fixed in ice-cold 70% ethanol overnight, pelleted by centrifugation at 1,000 x g for 5 min, treated with RNase at 37^{o}C for 15 min, stained with 50 μg/ml propidium iodide and subjected to fluorescence-activated cell sorting (FACS) analysis for DNA content.

4.3.6 *In vitro* ubiquitylation assay

MBP-Trim39 (2 μg; wildtype or mutants) were incubated (2 h at 37^{o}C) with 50 ng of hE1, 0.5 μg UbcH5a and UbcH10, 10 μg ubiquitin (recombinant E1, E2 and ubiquitin were all purchased from Boston Biochem), and 3 mM ATP brought up to 40 μl with buffer (25 mM Hepes, pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, 10 mM DTT, and 0.05% Triton X-100). Reactions were stopped with SDS sample buffer, resolved by SDS-PAGE, and analyzed by immunoblotting.

4.3.7 CSF extract and cyclin B degradation assay

CSF extracts were prepared as previously described [24, 25]. For the cyclin B degradation assays, extracts were incubated with MBP-Trim39 (wildtype or mutants) at room temperature for 10 min before Ca^{2+} addition; 0.8 mM Ca^{2+} was added to extracts to induce release from CSF arrest.

4.4 Results

4.4.1 Identification and characterization of Trim39 as the human Xnf7 homolog

As illustrated by the diagram in Fig. 21A, *Xenopus* Xnf7 and human Trim39 not only contain the same conserved domains (RING finger, B-Box and PRY/SPRY [*Sp*la kinase and *ry*anodine receptor]), but also share similar domain arrangement and spacing. To facilitate our studies of endogenous Trim39, we have raised a polyclonal antibody against MBP-Trim39 purified from *E. coli* and tested it with both recombinant protein and cell lysates overexpressing FLAG-Trim39 (Fig. 21B). Meanwhile, a series of experiments were performed to characterize the protein. As shown in Fig. 21C, Trim39 acts as an E3 ligase and autoubiquitylates *in vitro* when supplemented with ubiquitin, ATP, E1 and E2; an activity typical of Trim family proteins. More importantly, addition of recombinant MBP-Trim39 to CSF extract significantly delayed cyclin B degradation upon Ca^{2+} treatment (Fig. 21D), suggesting that it is capable of APC/C inhibition. Finally, since many SAC proteins are regulated by subcellular localization, we performed immunostaining against FLAG-Trim39 to examine its distribution inside cells. Consistent with a previous published result, FLAG-Trim39 was mainly localized in cytoplasm (Fig. 21E). Whether this result accurately reflects the localization of endogenous protein, and whether this localization is under checkpoint regulation, remains to be examined.

4.4.2 The RING-dependent ligase activity of Trim39 is required for its role in controlling M phase exit

Given that Trim39 is a functional E3 ligase, we continued to examine the relevance of its ligase activity. As shown in Fig. 22A, we have generated two mutated forms of Trim39 in which the conserved Cys residues that coordinate zinc binding are replaced by Ala. Trim39 C44A contains a mutation at position three of the RING finger and Trim 39 C52A contains a mutation at position six. As expected, wildtype Trim39 was able to undergo autoubiquitylation *in vitro*, while the mutation in the RING domain completely abrogated this activity (Fig. 22B; same result with C44A but not shown here). In order to determine whether Trim39's ligase activity is required for its cell cycle effect, we added recombinant MBP-Trim39 (wildtype or mutants) to CSF extracts supplemented with Ca^{2+} and examined their effects on preventing M phase exit. As demonstrated in Fig. 22C, excess wildtype Trim39 was capable of blocking cyclin B degradation, while the addition of C44A or C52A MBP-Trim39 did not affect the kinetics of cyclin B degradation as compared to the MBP control, indicating that the ligase activity of Trim39 was required for its effect on M phase exit.

4.4.3 Trim39 is required for SAC signaling in RPE cells

To determine if Trim39 plays an essential role in SAC signaling in mammalian cells, as Xnf7 does in *Xenopus* egg extract, we used siRNA to knockdown endogenous Trim39 and examined the behavior of cells under nocodazole-induced SAC. Direct observation of the cell cultures (which were evaluated for cell rounding, evidence of

mitotic cells) demonstrated that Trim39 ablation was as effective as knockdown of BubR1 (an established SAC mediator) in allowing cells to escape a nocodazole-induced cell cycle arrest (Fig. 23A). The loss of endogenous Trim39 was confirmed by western blot (Fig. 23B). In addition, we stained nocodazole-treated cells with anti-phospho-histone H3 antibody and found that both Trim39 and BubR1 siRNAs could abrogate the mitotic arrest (Fig. 23C). To verify that the loss-of-checkpoint phenotype was indeed a result specific of Trim39 ablation, we have repeated the experiment with a siRNA construct that targets the 3'UTR of Trim39 and observed a similar phenotype (Fig. 23D). Since this 3'UTR targeting siRNA does not target the exogenously expressed protein, a rescue experiment was performed in which FLAG-Trim39 (wildtype or mutants) was overexpressed using a lentiviral infection-based system after endogenous Trim39 was knocked down. As shown in Fig. 23E, wildtype FLAG-Trim39 was largely able to restore the checkpoint-induced M phase arrest while both the RING finger mutants (C44A, C52A) failed to rescue, again underscoring the critical role played by the RING finger. It remained possible that instead of causing a failure in M phase arrest upon nocodazole treatment, the loss of Trim39 simply affected earlier phases in the cell cycle so that majority of the cells failed to enter M phase. If this were true, we would expect to see a change in cell cycle profile following Trim39 RNAi along with growth arrest of these cells. As neither of the two predicted outcomes were observed (data now shown), we conclude that the observed phenotype indeed reflects a failure in M phase arrest.

Together, these results strongly support the idea that Trim39 plays an essential role in SAC signaling in mammalian cells and that its RING domain needs to be intact for this function.

4.4.4 Possible activation of Trim39 ligase activity by the SAC

To act as an efficient SAC protein that can translate the signal from inadequately attached kinetochores to APC/C silencing, the activity of Trim39 itself ought to be tightly regulated by the checkpoint. Interestingly, we noticed that the level of exogenously expressed FLAG-Trim39 was significantly elevated in SAC-arrested cells (Fig. 24A), suggesting that the protein might be stabilized under an active checkpoint. In order to confirm this hypothesis, we blotted for endogenous Trim39 to check its levels in the presence or absence of the checkpoint. To our surprise, the difference in proteins levels was not as striking as seen with FLAG-Trim39; instead, the endogenous Trim39 in SAC arrested cells showed slower migrating bands that were likely to be ubiquitylated forms of Trim39 (Fig. 24B). This observation is particularly interesting considering that the ligase activity of Trim39 is required for the APC/C inhibitory function, which then raises the exciting possibility that the Trim39 ligase is only activated as an APC/C inhibitor when cells are under active SAC signaling. We reason that the striking difference in the expression levels of FLAG-Trim39 could be partially attributed to the fact that Trim39 overexpression can lead to M phase arrest, causing the transfected cells with high FLAG-Trim39 expression to be enriched in the M phase arrested population. As a result, the

difference does not truly reflect checkpoint regulation of Trim39. We are currently attempting to verify the change in Trim39 protein levels and are working to elucidate the mechanism of activation.

4.4.5 Trim39 associates with BubR1 as well as APC/C core subunits

Although the complete picture of how APC/C inhibition is achieved under active SAC is not yet in sight, it is commonly agreed that the MCC plays an essential role. Given the possibility that Trim39 is indeed the human Xnf7 homolog, we speculate that Trim39 may work in a similar fashion as Xnf7 (i.e., by directly inhibiting the APC/C) while also acknowledging the possibility that it may have other roles in the SAC signaling pathway. In fact, the same recombinant MBP-Trim39 that blocks cyclin B degradation at CSF exit could not inhibit the ubiquitylation reactions mediated by APC/C *in vitro* (Fig. 25A, note that recombinant MBP-Emi2 completely inhibited poly-ubiquitylation on cyclin B by APC/C). In order to understand how Trim39 functions to achieve SAC-directed APC/C inhibition, we first examined its association with MCC proteins. Interestingly, BubR1 could be co-immunoprecipitated with Trim39 in RPE cell lysates (Fig. 25B) as well as the APC/C core subunit Cdc27 (Fig. 25C), suggesting that Trim39 may be involved in MCC formation upon SAC activation. Moreover, we did not detect a significant amount of co-immunoprecipitated Cdc20 with Trim39 (data not shown), implying that Trim39 might also contribute to the SAC-triggered degradation of Cdc20. Both of these possibilities will be pursued in future studies.

4.5 Discussion

4.5.1 Roles of Trim39 and other Trim family protein in mitosis

Prior to our identification of Trim39 as an indispensible protein for the SAC signaling pathway in human cells, another protein from the Trim family, Trim36, was reported to interact with the kinetochore protein CENP-H and was demonstrated to be capable of delaying cell cycle progression (Balint et al., 2004; Kano et al., 2008). Although the exact roles played by Trim36 in cell cycle control are still unknown, the protein is likely to be involved in either mitotic spindle formation or chromosome segregation considering its co-localization at the mitotic spindle. Interestingly, Xnf7 has also been characterized as a microtubule binding protein that has microtubule bundling activity; it is possible that Trim36 shares a similar function with Xnf7 in this regard. In addition, a candidate we had previously considered a potential human Xnf7 homolog based on sequence similarity, Trim69, also appears to be engaged in the SAC signaling pathway. Knockdown of Trim69 by RNAi also generated SAC defects in RPE cells (Fig. 26), although the phenotype was not as pronounced as that with Trim39 knockdown. For this reason, we chose to focus on Trim39 at this stage of investigation. However, as we begin to uncover the roles and regulation of Trim39 in SAC, it would be valuable to examine if Trim39 and Trim69 have redundant or independent functions. We are eventually interested in understanding how the Trim family proteins coordinate and regulate the SAC signaling pathway in concert.

4.5.2 Regulation of Trim39 by SAC

Endogenous Trim39 appears to be ubiquitylated under an active SAC, implicating the activation of its ligase activity. Consistent with this, we have observed mitotic band shifts of Xnf7 by SDS-PAGE analysis that resemble polyubiquitylation (data not shown). As recombinant Trim39 protein purified from *E. coli* can autoubiquitylate *in vitro* when supplemented with E1, E2, ubiquitin and an energy regenerating system (i.e., it does not require additional factors for activation), it is possible that the activity of endogenous Trim39 (and also Xnf7) is restrained by either inhibitor binding or post-translational modification in the absence of an active checkpoint. There is a cluster of TP sites (Thr391, Thr414 and Thr417) at the C-terminus of Trim39 that indicates possible mitotic phosphorylation. Additionally, the B-Box and PRY/SPRY domains of Trim39 are known to mediate protein-protein interaction. At present, we are generating site-directed mutations to evaluate the possible effects of mitotic phosphorylation, as well as deletion mutants of Trim39 that lack the PRY/SPRY domain or the B-Box, in order to study their functional relevance.

4.5.3 Mechanism of Trim39-mediated APC/C inhibition

The fact that recombinant Trim39 was capable of delaying cyclin B degradation and M phase exit but could not inhibit immunoprecipitated APC/C *in vitro* suggests that Trim39 might not be inhibiting APC/C directly; although we still can not exclude the possibility that Trim39 may need to be modified or requires an auxiliary factor from the

extract to exert its APC/C inhibition. This scenario appears unlikely, as Trim39 that had been dipped into CSF extract to acquire any possible modifications or binding partners did not demonstrate an inhibitory effect in *in vitro* APC/C assays (data not shown). On the other hand, Trim39 could also function through an indirect mechanism. It was reported that the formation of the MCC could be regulated by ubiquitylation, which could potentially be regulated by Trim39. In addition, APC/C-dependent ubiquitylation and degradation of Cdc20 is required for the maintenance of SAC, and SAC-dependent BubR1 association of Cdc20 has been suggested to be the key for its transition from an activator to a substrate. The interaction of Cdc20 with BubR1 suggests that Trim39 might be involved in this pathway. For instance, it could mediate the SAC-dependent binding between BubR1 and Cdc20, or the APC/C-dependent ubiquitylation of Cdc20. Furthermore, since expression of lysine-less Cdc20 can override the checkpoint while the tenfold overexpression of wildtype Cdc20 cannot, ubiquitylation of Cdc20 has also been proposed to directly inactive Cdc20 in a non-degradative manner. It would be interesting to determine if Trim39 contributes to the ubiquitylation-mediated inactivation of Cdc20.

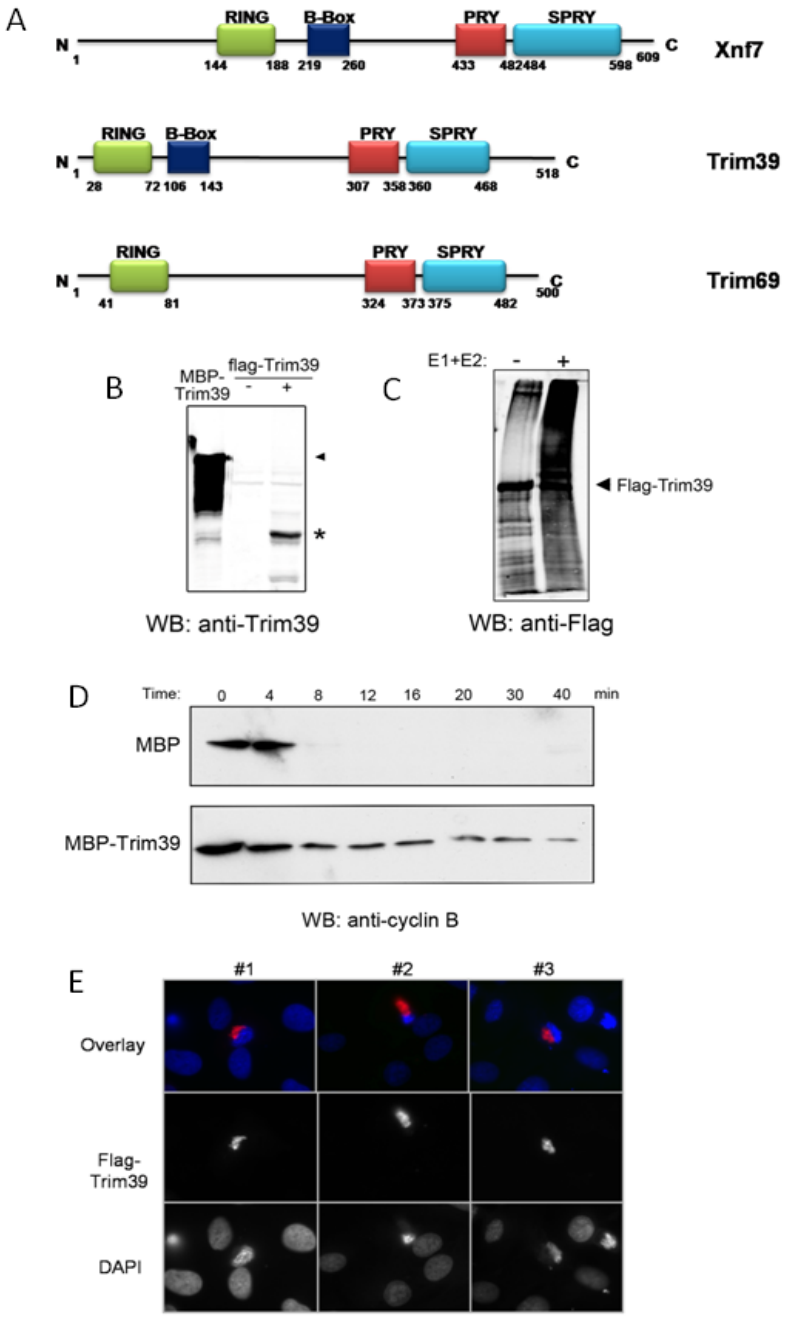

Figure 21 Identification of Trim39 as the Xnf7 homolog

Figure 21 Identification of Trim39 as the Xnf7 homolog

(A) A comparison of the domain structures of Xnf7, Trim39 and Trim69. (B) Verification of the Trim39 antibody. Recombinant MBP-Trim39 (2 μg) and 50 μg of RPE cell lysates with either control or FLAG-Trim39 overexpression were blotted with the rabbit-anti-Trim39 antibody. The triangle indicates MBP-Trim39 and the asterisk indicates FLAG-Trim39. (C) FLAG-Trim39 was transfected into RPE cells and immunoprecipitated with FLAG antibody. *In vitro* ubiquitylation assays were performed in the presence or absence of E1 and E2. The autoubiquitylation of Trim39 was examined by western blotting with FLAG antibody. (D) 200 nM of recombinant MBP or MPB-Trim39 was added to CSF extracts supplemented with Ca^{2+}. Aliquots removed at the indicated times were analyzed by SDS-PAGE and immunoblotted for cyclin B. (E) RPE cells were transfected with FLAG-Trim39 and immunostained with DAPI and anti-FLAG. Three different representative fields are shown.

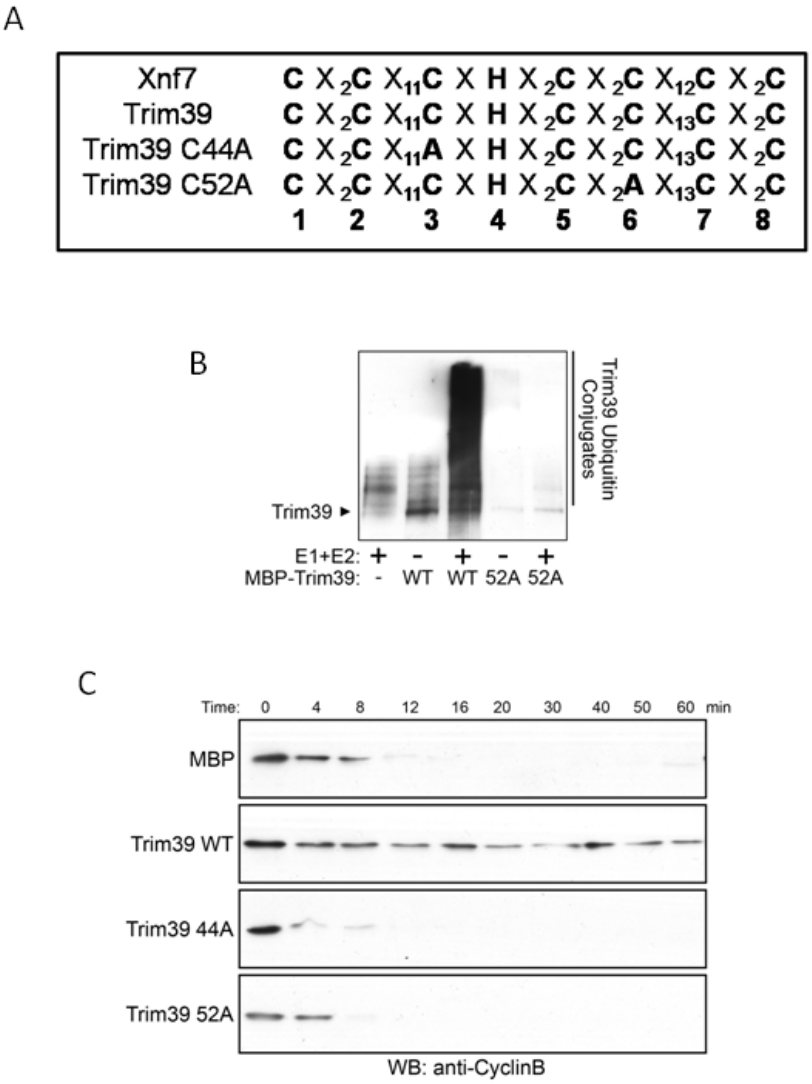

Figure 22 Trim39 is a RING E3 ligase that is required for controlling M phase exit

Figure 22 Trim39 is a RING E3 ligase that is required for controlling M phase exit

(A) Alignment of the RING finger sequences of Xnf7 and Trim39. The eight amino acids in bold are the zinc-coordinating sites of the RING finger. The third and sixth Cys is mutated in Trim39 C44A and C52A, respectively. (B) Trim39 has ubiquitin ligase activity. Trim39 (wildtype of C52A) was incubated with E1, E2 (UbcH10 and UbcH5a), ubiquitin and an energy regenerating mixture. Reactions were incubated at 37°C for 2 h and were analyzed by western blotting using anti-ubiquitin antibody. (C) 200 nM of recombinant MBP or MPB-Trim39 (wildtype or mutant) was added to CSF extracts supplemented with Ca^{2+}. Aliquots removed at the indicated times were analyzed by SDS-PAGE and immunoblotted for cyclin B.

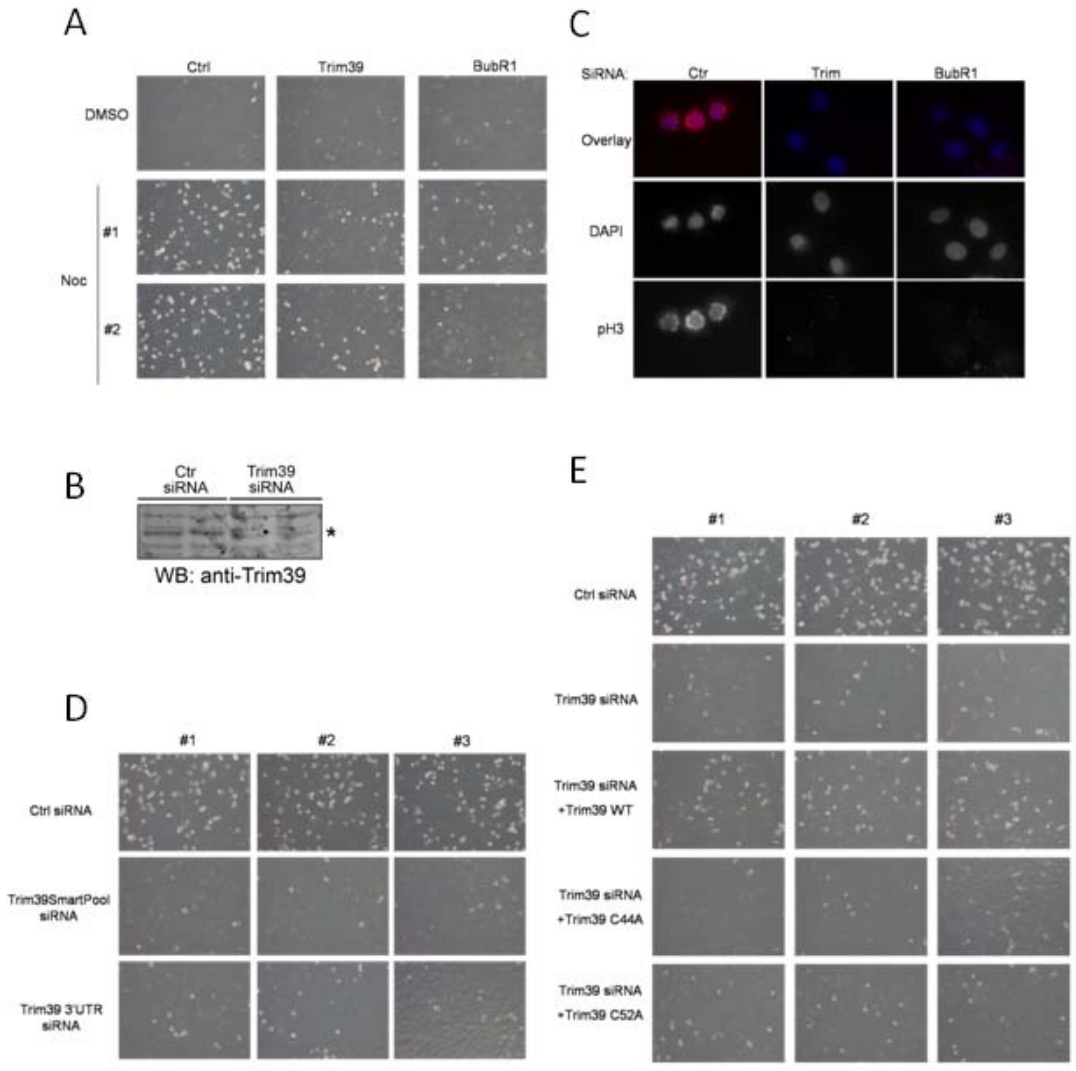

Figure 23 Trim39 is required for the proper function of SAC in RPE cells

Figure 23 Trim39 is required for the proper function of SAC in RPE cells

(A) RPE cells were transfected with 50 nM SMARTpool siRNA (Ctrl, Trim39 or BubR1) for 72 h. Photomicrographs were taken following treatment with 40 nm/ml nocodazole or DMSO for 18 h. (B) Same as in (A), the cells were lysed and analyzed by western blotting with Trim39 antibody. (C) Same as in (A), after nocodazole treatment, cells were immunostained with DAPI and anti-pH3. (D) RPE cells were transfected with 50 nM siRNA (Ctrl, Trim39 SMARTpool siRNA, or Trim39 3'UTR targeting siRNA) for 72 h. Photomicrographs were taken following treatment with 40 nm/ml nocodazole for 18 h. (D) RPE cells were transfected with 50 nM siRNA (Ctrl, Trim39 SMARTpool siRNA, or Trim39 3'UTR targeting siRNA) for 72 h. Photomicrographs were taken following treatment with 40 nm/ml nocodazole for 18 h. (E) RPE cells were transfected with 50 nM siRNA (Ctrl or Trim39 3'UTR targeting siRNA) for 48 h, followed by lentiviral infection for 24 h to produce either wildtype or mutant FLAG-Trim39. Photomicrographs were taken following treatment with 40 nm/ml nocodazole for 18 h.

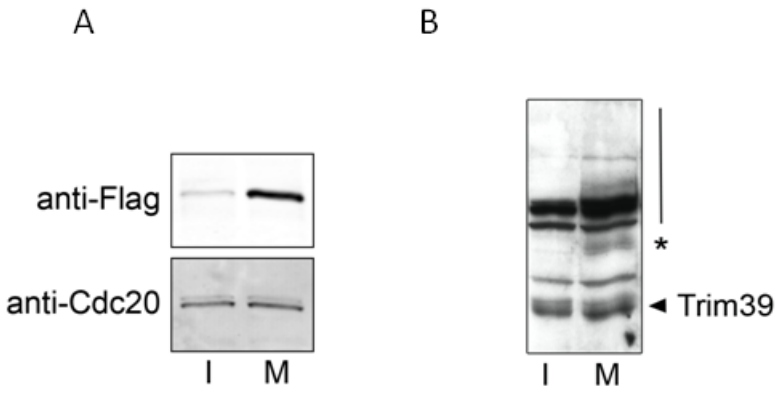

Figure 24 Regulation of Trim39 by the SAC

(A) RPE cells were transfected with FLAG-Trim39 for 72 h and treated with 40 ng/ml nocodazole for 18 h. M arrested cells (M) were separated from non-arrested cells (I) by mitotic shake-off. Cells were then lysed and analyzed by western blotting with both Cdc20 and FLAG antibodies. (B) Same as in (A) except that cells were not transfected and endogenous Trim39 was examined instead. Asterisk indicates possible mono-ubiquitylated Trim39; the line indicates possible ubiquitylated forms of Trim39.

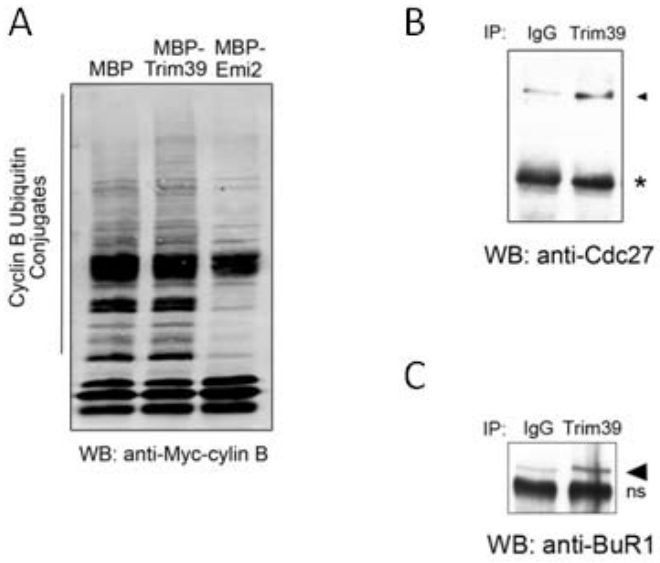

Figure 25 Roles of Trim39 in the SAC signaling pathway

(A) 600 nM of recombinant MBP, MPB-Emi2 or MBP-Trim39 was added to *in vitro* APC/C assays with IVT Cdc20 as the activator. The immunoprecipitated APC/C was pre-incubated with Emi2 and Cdc20 together with E1, E2 and ubiquitin for 1 h before the addition of substrates. Conversion of radiolabeled cyclin B to ubiquitylated forms was monitored by western blotting with Myc antibody. (B) and (C) The RPE cell lysates were immunoprecipitated with either rabbit IgG or Trim39 antibody. Associated protein factors were analyzed by western blotting.

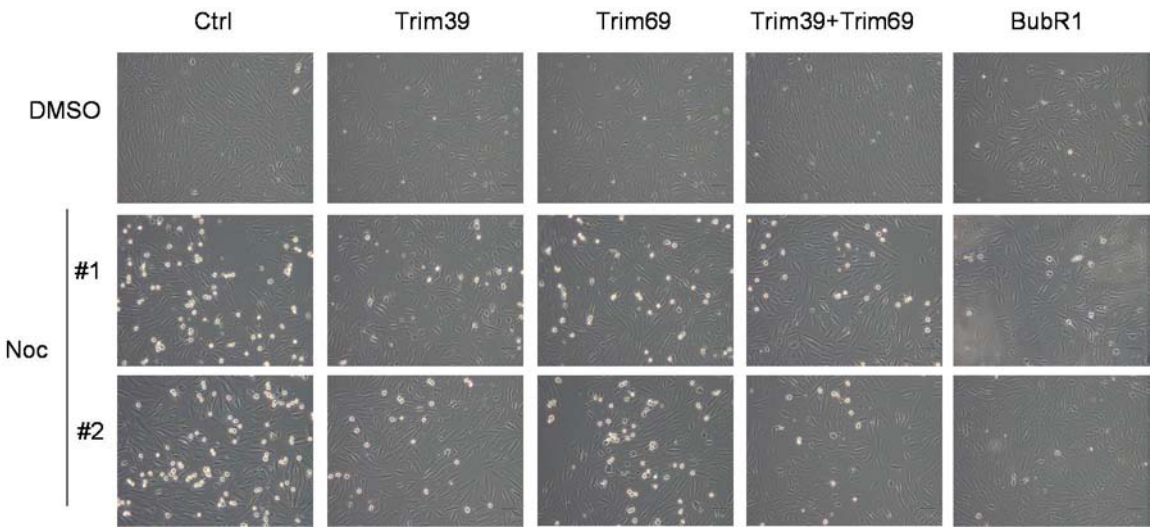

Figure 26 Roles of Trim69 in the SAC signaling pathway

RPE cells were transfected with 50 nM of SMARTpool siRNA as indicated for 72 h. Photomicrographs were taken following treatment with 40 nm/ml nocodazole or DMSO for 18 h.

5. Conclusions and perspectives

Since the discovery of the multi-subunit E3 ligase—the Anaphase Promoting Complex/Cyclosome—in the *Xenopus* egg extracts over 15 years ago, its roles in controlling the degradation of key cell cycle regulators during mitosis and DNA replication have been well established, and it has been identified as the central target of both CSF activity and the SAC signaling pathway. Recent studies have discovered additional functions of the APC/C outside of the cell cycle, including a role in the regulation of cell death pathways as well as neuronal activities. It has become clear that understanding APC/C regulation will not only expand our knowledge about these cellular processes, but also help us develop treatments against certain human diseases, including cancer.

A major component of APC/C regulation is attributed to its inhibitor proteins, which restrain the function of the APC/C to ensure the perfect timing of its activation. A number of APC/C inhibitors have been identified and characterized, including the CSF component Emi2 and the SAC protein Xnf7; the studies detailed in this dissertation have concentrated on elucidating the roles and mechanisms of these proteins. In summary, we have found that: 1) an auto-inhibitory loop of the APC/C mediated by Emi2 is critical for MI-MII transition; 2) Emi2 inhibits the E2-substrate ubiquitin transfer catalyzed by APC/C; 3) Trim39, the human Xnf7 homolog, is required for functional SAC signaling; 4)

Emi2, Xnf7 and Trim39 are all E3 ligases and their ligase activities are required for their APC/C inhibitory function.

5.1 An Emi2-mediated auto-inhibitory loop of the APC/C at the MI-MII transition

A hallmark of the meiotic cell cycle is the two consecutive M phases without an intervening interphase. This special feature is critical for its role in generating mature gametes that contain half of the genetic information of the progenitor cell and also necessitates additional regulatory events. Homologous chromosomes are separated during anaphase of MI, a process dependent on APC/C-mediated degradation of the securin subunit. Similar to a regular mitotic cell cycle, the APC/C is inhibited by the SAC before the homologous chromosomes are properly aligned in MI. However, in mitosis, the APC/C will remain active throughout anaphase, telophase, and the G1 phase of the next cycle in order to prepare the cell for new DNA synthesis. In meiosis I, the activity of the APC/C needs to be restrained in a timely fashion to prevent the complete degradation of cyclin B, as maintaining residual levels of Cdc2 kinase activity during the MI-MII transition is necessary to prevent unwanted DNA synthesis and parthenogenetic activation of the oocyte. For this purpose, inhibition of the APC/C by Emi2 is critical to ensure the MI-MII transition of vertebrate oocytes and the regulation by SAC pathway.

The timing of Emi2 activity must be tightly controlled to not only allow MI exit, but to also prevent interphase entry through fine tuning of APC/C function. Through careful examination of Emi2 protein levels during MI, we concluded that the protein is

continuously synthesized upon MI entry, while its destruction is regulated by Cdc2-mediated phosphorylation (an activity that could be antagonized by the phosphatase activity of PP2A). After an oocyte enters MI, Cdc2 triggers the phosphorylation of Emi2 at four N-terminal residues (213/239/252/267), which prime it for Plx1 binding and phosphorylation. Through the same proteasome-mediated degradation pathway that functions at MII exit, Plx1-phosphorylated Emi2 is then recognized by the F-box protein βTrCP and gets subsequently polyubiquitylated by $SCF^{\beta TrCP}$ complex. The key to Emi2 instability during MI was found to be the kinase activity of Cdc2, whose activity is significantly higher in MI compared to MII (almost double as judged by histone HI phosphorylation assay). High Cdc2 activity ensures that Emi2 does not accumulate prior to anaphase of MI and that APC/C activity is only controlled by the SAC before MI exit. Once activated, the APC/C triggers the separation of homologous chromosome as well as cyclin B degradation, leading to a decrease in Cdc2 kinase activity and the stabilization of Emi2. The activity of the APC/C decreases rapidly with the accumulation of Emi2 protein due to its ability to act in catalytic manner and inhibit APC/C sub-stoichiometrically. As a result, the newly synthesized cyclin B is stabilized before its protein level decreases beyond the threshold necessary to permit DNA synthesis. Meanwhile, the increasing kinase activity of Cdc2/cyclin B promotes the progression of the oocyte directly to MII for the separation of sister chromatids. Cdc2 kinase activity is maintained at a level insufficient to cause Emi2 degradation during MII due to a

feedback loop that controls cyclin B levels. Taken together, it appears that the APC/C can trigger its own inactivation by stabilizing Emi2 at the MI-MII transition and that this small window of APC/C activity is crucial for preventing parthenogenesis and death of oocytes (Tang et al., 2008).

5.2 Trim39 is required for the Spindle Assembly Checkpoint in PRE cells

Dysregulation of the Spindle Assembly Checkpoint has long been known to contribute to genomic instability and tumorigenesis. Studies employing approaches utilizing yeast genetics, biochemical analysis, structure analysis and mammalian cell biology have significantly expanded our views about this complex cellular process. However, many questions remain unsolved and new proteins involved in the pathway are still being identified.

Xnf7, a Trim family protein found in *Xenopus,* is a crucial component of a functional SAC in egg extracts. As many proteins in the SAC signaling pathway are highly conserved, we tried to identify a human homolog that would function in manner similar to Xnf7. Two protein candidates were selected based on sequence similarities: Trim39 and Trim69. Interestingly, knockdown of either protein abrogated SAC function to certain a degree in the RPE cells. While Trim39 knockdown almost completely overrode the M phase arrest induced by nocodazole, Trim69 knockdown had a relatively mild phenotype. Our recent investigations have focused on the activity of

Trim39; however, we are interested in delineating the roles of Trim69 in future studies to elucidate how these proteins cooperate to promote SAC in human cells.

Trim39 resembles Xnf7 in that it is required for the SAC pathway, and it also shares other functional aspects that are similar to Xnf7—overexpression of either protein in *Xenopus* egg extract was able to block cyclin B degradation at CSF release and both proteins are RING E3 ligases whose ligase activity is required for their function. However, we did observe some interesting variations between the two proteins. We found that Xnf7 was capable of inhibiting APC/C-mediated ubiquitylation reactions *in vitro* while Trim39 could not. It is possible that Trim39 plays a role in the human SAC pathway that is quite different from that of Xnf7 in *Xenopus*. Perhaps additional protein factors have evolved to compensate for Trim39's lack of APC/C inhibition in the human cells (it should be noted that such a hypothetical protein should be present in *Xenopus* as Trim39 is able to inhibit the APC/C in the egg extracts).

Besides the downstream events that link Trim39 to APC/C inhibition, another angle we have been pursuing is the upstream events that transduce the SAC signal from kinetochores to Trim39. Preliminary results suggest that the ligase activity of Trim39 is stimulated by active SAC. Whether the activation is mediated through subcellular localization changes or post-translational modifications remain to be decided. We are also interested in identifying additional Trim39 interacting proteins with known SAC functions in order to better integrate Trim39 into the SAC pathway. Taken together, we

believe that our studies focusing on the roles and regulations of Trim39 will help us gain a more complete picture of the SAC pathway in human cells.

5.3 Mechanism of APC/C inhibition by E3 ligases

When we first began to examine the mechanism of APC/C inhibition by Emi2, we focused on the "pseudosubstrate" model, as a closely related protein, Emi1, was reported to inhibit the APC/C by preventing substrate association (Miller et al., 2006). In addition, the binding of Emi2 and the APC/C appeared to be critical for its activity, and the D-Box on Emi2 was found to contribute to this binding, consistent with Emi2 acting as a pseudosubstrate. However, we soon realized that this model was unable to explain two basic facts: 1) 150 nM APC/C was completely inhibited by 15 nM Emi2 in CSF extract (which is largely homogenous); and, 2) The RING finger of Emi2 is critical for its inhibitory function, as a RING finger mutant of Emi2 (which has an intact D-Box) acts as a dominant negative to cause CSF release. Moreover, we could not detect a decrease in binding between cyclin B and the APC/C in the presence of excessive recombinant Emi2, which strongly argues against the pseudosubstrate model. Instead, a close examination of each step in the APC/C-mediated ubiquitylation reaction revealed that it was the final step (where ubiquitin was transferred from the E2 to substrate) that was prevented by Emi2.

The discovery of Xnf7 has raised the intriguing possibility that the APC/C could be inhibited by ligase activity derived from another E3. As Emi2 is also a RING E3

ligase, we hypothesized that Emi2 could inhibit the APC/C in a catalytic manner, which would provide a satisfying explanation to both the stoichiometry issue and the critical role played by the RING finger. Guided by this idea, we found that pre-incubation of Emi2 with the APC/C *in vitro* could strengthen the inhibition when performed under ubiquitylating conditions, supporting the idea that a certain factor needs to be modified in order to inhibit the APC/C.

As inhibition of the APC/C by Xnf7 and Emi2 both require ligase activity, it is reasonable to postulate that they both function by similar mechanisms in response to SAC or CSF signaling, respectively, to inhibit the APC/C. Our efforts to identify substrates that mediate the APC have mainly focused on Emi2; although, we have not completely ruled out the hypothesis that an activator or E2 serves as the relevant substrate. We are currently in favor of a model in which an APC/C core subunit is modified by an E3 in such a way that the whole E3 ligase is rendered inactive. For example, it has been reported that the APC/C undergoes a conformational change when associated with an activator. In theory, Emi2 could modify the core APC/C such that it becomes refractory to an activator-induced conformation change.

In order to identify the APC/C subunit that is potentially modified by Emi2 (or Xnf7), we decided to employ additional model systems, as the reagents in *Xenopus* system are limited. Studies have indicated that the only essential function of the APC/C in budding yeast is to degrade securin and cyclin B. If yeast are genetically manipulated

to override this need, then they can proliferate in the absence of the otherwise necessary APC/C subunits (Thornton and Toczyski, 2003). Since active APC/C cannot be constructed *in vitro* (so far), the yeast model has provided an opportunity for us to eliminate single APC/C subunits and test for its response to Emi2 (or Xnf7)-mediated inhibition. We constructed plasmids to overexpress Emi2 and Xnf7 in *S. cerevisiae* and expected to observe a proliferation deficient phenotype in transformed cells (which was observed in Cdc20 null yeasts). To our surprise, yeast cells overexpressing Emi2 or Xnf7 had no phenotype in growth deficiency as compared to the control group, indicating that these inhibitors could not inhibit the yeast APC/C. This finding was particularly exciting as the major difference that distinguishes yeast APC/C from vertebrate APC/C is its lack of the subunit APC7 (Peters, 2006). This particular lack of phenotype would make perfect sense if APC7 had only evolved in vertebrate cells to mediate APC/C inhibition by Emi2/Xnf7. (It should be noted that Mnd2, a stoichiometric subunit of APC/C in yeast, has been identified to specifically inhibit APC/C activity when associated with the activator Ama1 [Oelschlaegel et al., 2005].) In addition, APC7 was found to be down-regulated in breast cancer cells (Park et al., 2005), implicating a role in controlling cell proliferation, probably through APC/C regulation. However, as promising as it was, we could not detect a modified form of APC7 after ubiquitylating immunoprecipitated human APC/C with Emi2 *in vitro*. More importantly, APC/C purified from cells in which APC7 was ablated by siRNA was still inhibitable by Emi2

(data not shown), suggesting that APC7 does not play a critical role in Emi2-induced inhibition.

Another approach we have taken to search for substrates is based on the interaction profile of Emi2 to the APC/C subunits. As shown in Fig. 19A, Emi2 can specifically interact with APC6, APC10 and APC11 of all subunits we have examined. Interestingly, after ubiquitylating human APC/C with Emi2 *in vitro*, we observed a band that was recognized by an APC6 antibody and corresponded to monoubiquitylated APC6 by molecular weight (Fig. 27). We are still in the process of verifying this finding and investigating the relevance of the ubiquitylation.

Besides the approaches taken above, we have also adopted several unbiased strategies in pursuit of the substrate. First, we have performed mass spectrometry to identify any ubiquitylated proteins from an *in vitro* reaction. However, we need to eliminate the background signals of autoubiquitylation from both Emi2 and the APC/C to get any meaningful information. Second, we were able to knockdown a number of APC/C subunits from human cells and obtain the APC/C complex lacking a particular subunit. In theory, APC/C that lacks the relevant subunit should become resistant to Emi2, which gives us an easy readout *in vitro*. However, this approach requires the APC/C to be active in the absence of the deleted subunit and we will be unable to find the substrate if it happens to be essential for the activity of the APC/C. The third approach we are taking is to examine each of the APC/C subunits purified from bacteria

to see if they could act as a substrate of Emi2 *in vitro*. The weakness of this approach is that a candidate subunit may only act as the substrate when it is incorporated into the whole APC/C complex, and the examination of individual subunits may risk false negative results. By combining these approaches, we hope to identify the substrate that underlies ubiquitylation-induced APC/C inhibition in the near future.

5.4 APC/C and cell death pathways

5.4.1 MI arrest induces non-apoptotic cell death

Vertebrate eggs are arrested at metaphase of meiosis II prior to fertilization, an arrest that can last for days, weeks, or even decades. It is of critical importance that the apoptotic pathway is suppressed in eggs, and research in our laboratory has uncovered two pathways that are at least, in part, accountable for the suppression of apoptosis. First, Cdc2-mediated phosphorylation of the initiator caspase, caspase 2, prevents its activation and subsequent cytochrome c release from mitochondria (Andersen et al., 2009). Second, Rsk-mediated phosphorylation of Apaf1 leads to a decrease in its ability to associate, and be activated by, cytochrome c, preventing the formation of apoptosome. (Please note that CSF arrest is characterized by high Mos activity, the upstream kinase that activates Rsk.) Based on this information, it was both perplexing and interesting to us to notice that oocytes arrested in MI instead of MII by Emi2 overexpression showed clear signs of cell death within hours following MI entry (Fig. 28A). The same cell death phenotype was observed when oocytes were injected with

non-degradable cyclin B or treated with MG132 (data now shown), the proteasome inhibitor that causes MI arrest by inhibiting cyclin B degradation at anaphase of MI. Taken together, these data strongly suggested that cell death was indeed caused by MI arrest rather than ectopic Emi2 expression.

No matter what mechanism underlies MI arrest-induced cell death, it is clear that it bears crucial physiological relevance for reproduction. During MI, homologous chromosomes undergo recombination and exchange genetic materials, a process closely monitored by the SAC (Stein et al., 2007; Tang et al., 2004b) . Under normal circumstances, MI takes only 1-1.5 h to complete in *Xenopus* oocytes. However, if problems occur during this process and remain unsolved for a prolonged period of time, it is important for those MI-arrested oocytes to be eliminated properly rather than going on to be fertilized and start their embryonic divisions. Obviously, defects in this death pathway could have detrimental consequences as the development of birth defects or terotoma (Eppig et al., 1996; Ohama et al., 1985; Reis et al., 2007).

In order to elucidate the mechanism of death, we first wanted to verify if apoptosis was responsible for the phenotypic presentation of MI arrest-induced death in oocytes. Although both pathways that prohibit apoptosis in MII (mediated by Cdc2 and Rsk, respectively) are also supposed to be active in MI, it is possible that they are overpowered by other apoptotic signals. To examine this, we injected *in vitro* translated S^{35} labeled caspase 3 into MI arrested oocytes and detected no processing (the sign of

activation) when the oocytes showed visible signs of death (date not shown). *In vitro* caspase assays with oocyte lysates were performed to check the activity of endogenous caspases. We consistently obtained negative results for the MI-arrested oocytes in this assay, while the cytochrome c injected oocytes demonstrated robust caspase activity, strongly indicating that the oocyte death caused by MI arrest was not mediated by apoptosis (Fig. 28B).

While exploring the possible pathways that underlie MI arrest-induced oocyte death, a clue that we cannot ignore is that the factor/activity that mediates this pathway, whatever it could be, is eliminated or antagonized in MII oocytes, which need to survive a long period of time. One feature that is known to distinguish MI from MII is the high level of Cdc2 kinase activity described previously. Indeed, we noticed that high Cdc2 kinase activity in MI arrested oocytes would last for at least 5 h after MI entry, while it only existed transiently (less than 30 min) in oocytes that undergo the normal maturation process (data now shown). This has raised the exciting possibility that the abnormally prolonged high kinase activity triggered some kind of "energy crisis" status within the oocytes, which eventually led to the death. In recent years, autophagy has been characterized as a form of controlled cell death under energy shortage situations and we suspected that it might contribute to MI arrest-induced oocyte death. Taken together, we conclude that MI arrested oocytes, which probably results from unsolved problems with homologous recombination, are efficiently eliminated through a caspase-

independent pathway. The nature of this pathway is still unknown, but we hypothesize it to be related to the abnormally prolonged high level of Cdc2 kinase activity in MI arrested oocytes. Although this project is still in a preliminary stage, continued investigation into the mechanism of MI arrest-induced oocyte death could help us better understand the quality control process in vertebrate gamete production, as well as how the impaired pathway may contribute to certain reproduction defects.

5.4.2 Roles of Trim39 in apoptosis: involvement of APC/C?

Around the same time we identified Trim39 as the human Xnf7 homolog required for SAC to function properly in human cells, another group reported their findings from a totally different line of research that Trim39 was required for etoposide-induced apoptosis in HEK293T cells. Etoposide triggers apoptosis through a Bax-induced cytochrome c release pathway, and Bax activation requires the stabilization of another protein, modulator of apoptosis 1 (MOAP1). MOAP1 is normally a shorted-lived protein that undergoes constant degradation through the ubiquitin-proteasome pathway; however, it can be stabilized and accumulated with etoposide treatment. The E3 ligase responsible for the degradation of MOAP1, and exactly how etoposide treatment leads to its stabilization, are still unknown. Interestingly, however, this study showed that Trim39 was required for the stabilization process, as Trim39 knockdown by shRNA overrode etoposide-induced MOAP1 stabilization and apoptosis (Lee et al., 2009).

As an E3 ligase itself, it is unlikely that Trim39 ubiquitylates MOAP1 and targets it for degradation; it is more likely that it functions to inhibit the E3 that mediates MOAP1 degradation. We already know from our SAC studies that Trim39 can prevent the activation of APC/C, which leads us to the hypothesis that the APC/C is the E3 that is responsible for MOAP1 instability under normal situations. We are currently testing this hypothesis by examining the protein levels of MOAP1 to see if they are affected by APC/C inhibition through Cdh1 RNAi. If the APC/C is indeed the relevant E3 ligase, MOAP1 should stabilize and accumulate when Cdh1 is knocked down and may either trigger apoptosis or sensitize the cells to apoptotic stimuli.

The link between APC/C^{Cdh1} and apoptosis is particularly interesting in the field of neurodegenerative diseases. Several years ago, the activity of the APC/C^{Cdh1} was found to be required for the survival of post-mitotic neurons. Cyclin B and Cdc2 levels were shown to be elevated in neurons from Alzheimer's disease patients and the phosphorylation and inactivation of Cdh1 by Cdc2/cyclin B was proposed to be at least part of the mechanism underlying the disease. Moreover, in a cell culture model where the death of primary cortical neurons was triggered by exposure to β-amyloid, Cdh1 overexpression could significantly rescue β-amyloid-induced neuronal death, suggesting a role in pro-neuronal survival. It has been proposed that the major role of Cdh1 in post-mitotic neurons is to maintain a stable G1, as previously described. Consequently, death of post-mitotic neurons has been observed as an indirect result of

their re-entry into the cell cycle (Almeida et al., 2005; Aulia and Tang, 2006; Kim and Bonni, 2007). However, if APC/C^{Cdh1} targets pro-apoptotic factors for degradation, it would provide a simpler and more direct explanation of its roles in neuronal survival. Interestingly, another Trim family protein, Trim9, has been reported to be localized in neurons of normal mouse and human brain, and the level of Trim9 protein is severely decreased in brain areas affected by Parkinson's disease and dementia (Tanji et al., 2010). At this stage, we do not know whether the protein has a significant role in neuron survival or if the activity of the APC/C is involved. In any event, since many neurodegenerative diseases and vascular dementia all exhibit an increased cyclin B level and decreased Cdh1 activity, it would be of great interest to further explore the roles of the APC/C^{Cdh1} in apoptosis and identify the relevant targets including MOAP1.

5.5 Concluding remarks

The studies presented in this dissertation have advanced our understanding of both the functions and mechanisms of APC/C inhibitors. We have characterized the regulatory events of the CSF effector Emi2 during *Xenopus* oocyte maturation process. We have also demonstrated that Emi2 inhibits the APC/C by preventing the last step of the ubiquitylation reaction, where the ubiquitin is transferred from a charged E2 to its substrate. In addition, we have provided strong evidence demonstrating that the E3 ligase activity of Emi2 is required for its function. Finally, we have identified another E3 ligase, Trim39, to be an essential SAC protein in RPE cells. Identification and

characterization of these APC/C inhibitors will not only aid our understanding of the regulatory events during cell cycle and other cellular events, but also help us develop more efficient therapies against certain human diseases.

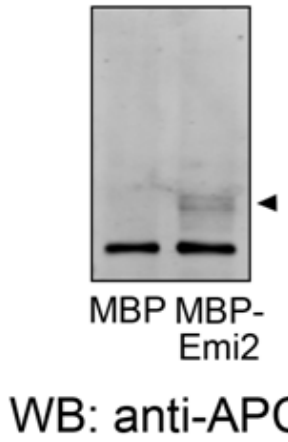

Figure 27 Possible modification of APC6

Human APC/C immunoprecipitated from HeLa lysates was *in vitro* ubiquitylated with either 600 nM MBP or MBP-Emi2 at 37°C for 2 h. The reaction was quenched with SDS sample buffer and analyzed by western blotting with APC6 antibody.

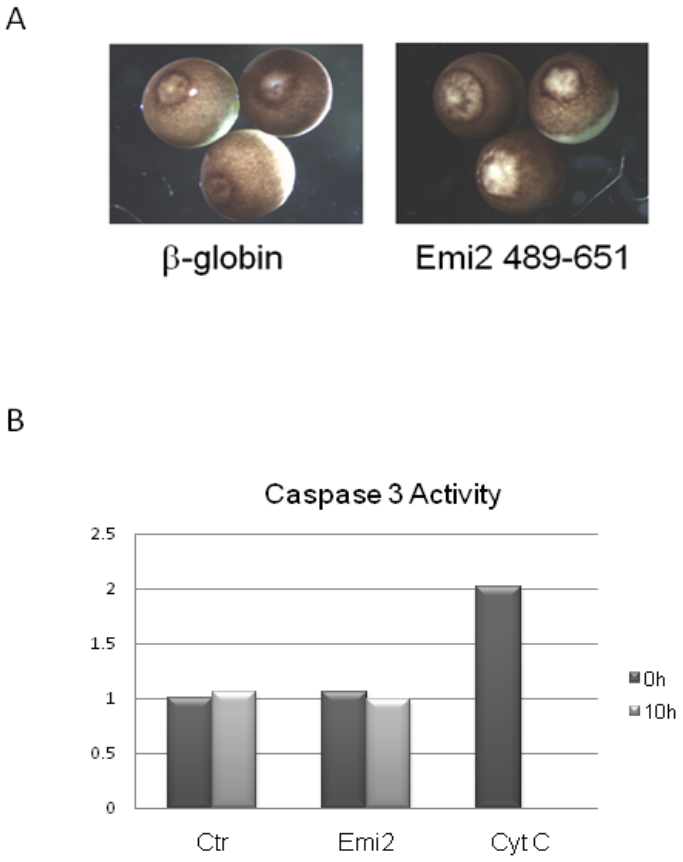

Figure 28 Non-apoptotic death of MI arrested oocytes

(A) Oocytes were injected with either β-globin or FLAG-Emi2 (489-651) mRNA appended with β-globin 3'UTR (0.3 ng/oocyte). After overnight incubation, oocytes were treated with progesterone. Pictures were taken 5 h after GVBD. (B) Same as in (A), oocyte samples for β-globin Ctrl and Emi2 injection were taken 0 h and 10 h after progesterone treatment. For a positive control, 100 nM cytochrome c was injected into oocytes and samples were taken after 30 min. Lysates were made and assayed for caspase 3 activity.

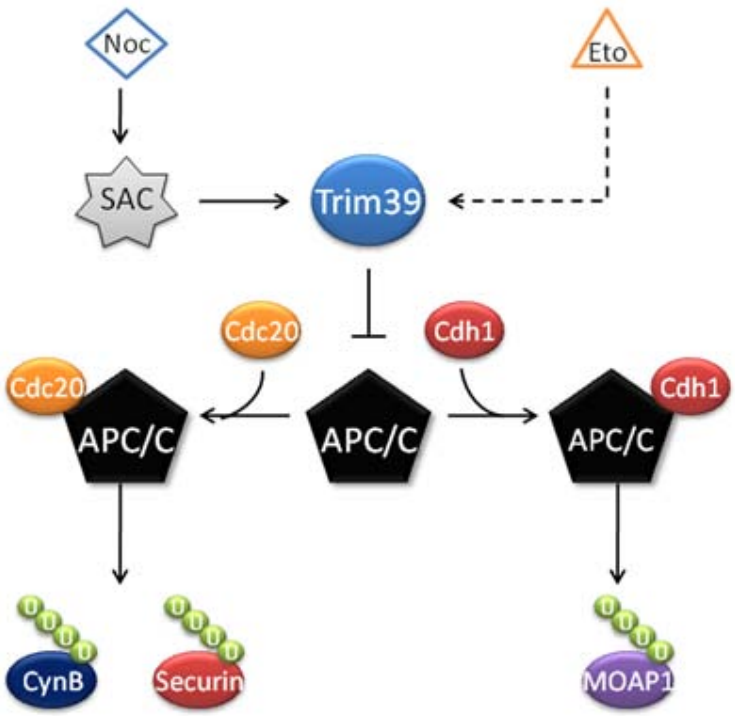

Figure 29 Hypothetical model of Trim39-mediated regulation of SAC and apoptotic pathways by inhibiting APC/C

Upon nocodazole-induced SAC, Trim39 is activated and inhibits APC/C^{Cdc20}-dependent ubiquitylation of the mitotic substrates cyclin B and securin, thus preventing anaphase inhibition until all of the chromosomes achieve bipolar alignment. When the cells are treated with etoposide, Trim39 is also activated and inhibits APC/C^{Cdh1}-dependent ubiquitylation of the Bax activator MOAP1, thus leading to the accumulation of MOAP1, the activation of Bax, and the initiation of the apoptotic cascade. Noc: Nocodazole; Eto:Etoposide; CynB: Cyclin B

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Biography

Wanli Tang

Born to Xiaoyuan Cheng and Youcheng Tang on November 7, 1983 in Sichuan, China.

Education

Ph.D. Molecular Cancer Biology, Certificate in Cell and Molecular Biology
Duke University, Durham, NC, USA, May 2010

B.S. Biological Science
Tsinghua University, Beijing, China, May 2005

Publications

Tang W*, Wu JQ*, Chen C, Yang CS, Guo JY, Freel CD, Kornbluth S. Inhibition of E2-substrate ubiquitin transfer by the APC mediated by a D box-independent Emi2 catalytic activity. (under review)

Yang CS, Gan E, Thomenius MJ, **Tang W**, Freel CD, Nutt LK, and Kornbluth S Metabolic Regulation of Drosophila apoptosis through the inhibitory phosphorylation of initiator caspase Dronc. (under review)

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*: co-equal first author

Honors and Awards

Chinese Government Award for Outstanding Self-Financed Student Abroad	2010
Robert FitzGerald Scholar	2009
NIH training grant	2008-present
Duke University Graduate School Fellowship	2006-2008
University of Southern California Graduate School Fellowship	2005
National Scholarship for Academic Excellence	2002&2003