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Emi1 Is a Mitotic Regulator that Interacts with Cdc20 and Inhibits the Anaphase Promoting Complex

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Summary

We have discovered an *e*arly *m*itotic *i*nhibitor, Emi1, which regulates mitosis by inhibiting the anaphase promoting complex/cyclosome (APC). Emi1 is a conserved F box protein containing a zinc binding region essential for APC inhibition. Emi1 accumulates before mitosis and is ubiquitylated and destroyed in mitosis, independent of the APC. Emi1 immunodepletion from cycling *Xenopus* extracts strongly delays cyclin B accumulation and mitotic entry, whereas nondestructible Emi1 stabilizes APC substrates and causes a mitotic block. Emi1 binds the APC activator Cdc20, and Cdc20 can rescue an Emi1-induced block to cyclin B destruction. Our results suggest that Emi1 regulates progression through early mitosis by preventing premature APC activation, and may help explain the well-known delay between cyclin B/Cdc2 activation and cyclin B destruction.

Introduction

Mitotic entry is regulated by maturation promoting factor (MPF), a kinase complex composed of cyclin B and Cdc2 (Meijer et al., 1989; Minshull et al., 1989; Murray and Kirschner, 1989). Mitotic exit requires MPF inactivation, which is achieved by cyclin B destruction. Cyclin B is degraded by ubiquitin-dependent proteolysis, triggered by the anaphase promoting complex/cyclosome (APC) ubiquitin ligase (reviewed in Zachariae and Nasmyth, 1999). However, activated MPF must mediate chromatin condensation, nuclear envelope breakdown, and spindle formation before cyclin B is degraded. How this critical delay between MPF activation and APC activation is achieved remains unclear.

The vertebrate APC is composed of at least eleven subunits, including APC2, a cullin family member, and APC11, a RING-H2 finger protein (Gmachl et al., 2000; Yu et al., 1998). The APC shares homology with other ubiquitin ligases, including the SCF (*S*kp1, *C*ullin, and *F* box protein) ubiquitin ligase, which contains a cullin and a RING-H2 finger protein as its catalytic core (reviewed in Jackson et al., 2000). In the SCF, substrate recognition appears to be mediated by F box proteins. Substrate recognition by the APC is less well understood.

Although present throughout the cell cycle, the APC is inactive while cyclin B accumulates in S, G2, and early M phase. The APC is activated by binding the WD-repeat-containing proteins Cdc20 or Cdh1 (reviewed in Page and Hieter, 1999). In somatic cells, Cdc20 and Cdh1 binding to the APC is differentially regulated, resulting in a peak of APC^{Cdc20} activity in mitosis and APC^{Cdh1} activity in G_1 (Kramer et al., 2000; Shirayama et al., 1998; Zachariae et al., 1998). In the *Xenopus* embryo, however, Cdh1 is not expressed, and only APC^{Cdc20} is active (Kramer et al., 2000; Lorca et al., 1998). Cdc20 and Cdh1 may serve as substrate adaptors for the APC, much like the proposed function for F box proteins in the SCF, although how they activate the APC is not understood.

The APC targets proteins containing a destruction box or a KEN box motif for ubiquitin-mediated proteolysis (Pfleger and Kirschner, 2000). Nonetheless, the timing of APC substrate destruction is varied, suggesting that additional factors control the timing of APC activity. For example, cyclin A destruction begins in prometaphase, inhibitors of sister chromatid separation (securins) are destroyed at the metaphase-anaphase transition, and proteolysis of the mitotic kinase Plk1 and the spindle-associated protein Ase1 occurs as cells exit mitosis (Geley et al., 2001; den Elzen and Pines, 2001; Charles et al., 1998; Juang et al., 1997; Shirayama et al., 1998).

Mitotic kinases, including cyclin B/Cdc2, Plk1, and PKA, regulate mitotic APC activity (reviewed in Page and Hieter, 1999; Zachariae and Nasmyth, 1999). Notably, mitotic APC phosphorylation promotes its activation by Cdc20 (Kramer et al., 2000; Rudner and Murray, 2000; Shteinberg et al., 1999). However, mitotic APC phosphorylation is not sufficient to explain the timing of APC activity, because APC from interphase extract can be activated in vitro by Cdc20 or Cdh1 (Fang et al., 1998a; Kotani et al., 1999), or in vivo by overexpression of Cdc20 or Cdh1 at any stage in the cell cycle (Schwab et al., 1997; Visintin et al., 1997).

APC^{Cdc20} activity is also restrained by the spindle checkpoint (SC), a regulatory pathway conserved in yeast and vertebrates (reviewed in Straight and Murray, 1997). The SC protein Mad2 functions on unattached kinetochores in prometaphase to inhibit the APC until chromosomes align at the metaphase plate. Mad2 binds Cdc20 to inhibit APC activity (Alexandru et al., 1999; Fang et al., 1998b; Hwang et al., 1998; Kallio et al., 1998; Kim et al., 1998; Li et al., 1997; Wassmann and Benezra, 1998), although how Mad2 inhibits APC^{Cdc20} is not clear. The APC also localizes to centrosomes and the mitotic spindle (Tugendreich et al., 1995), where it may direct the local degradation of critical substrates (Clute and Pines, 1999; Huang and Raff, 1999).

We have identified an APC regulator called Emi1. Emi1 shares homology with the *Drosophila* protein *r*egulator of cyclin *A* (Rca1), a positive regulator of cyclin A (Dong et al., 1997). Loss of Rca1 blocks embryos in G_2 of cell cycle 16. Rca1 overexpression in G_1 causes precocious cyclin A/Cdk2 activation by increasing cyclin A protein levels without affecting its transcription. How Rca1 mediates this effect is not known.

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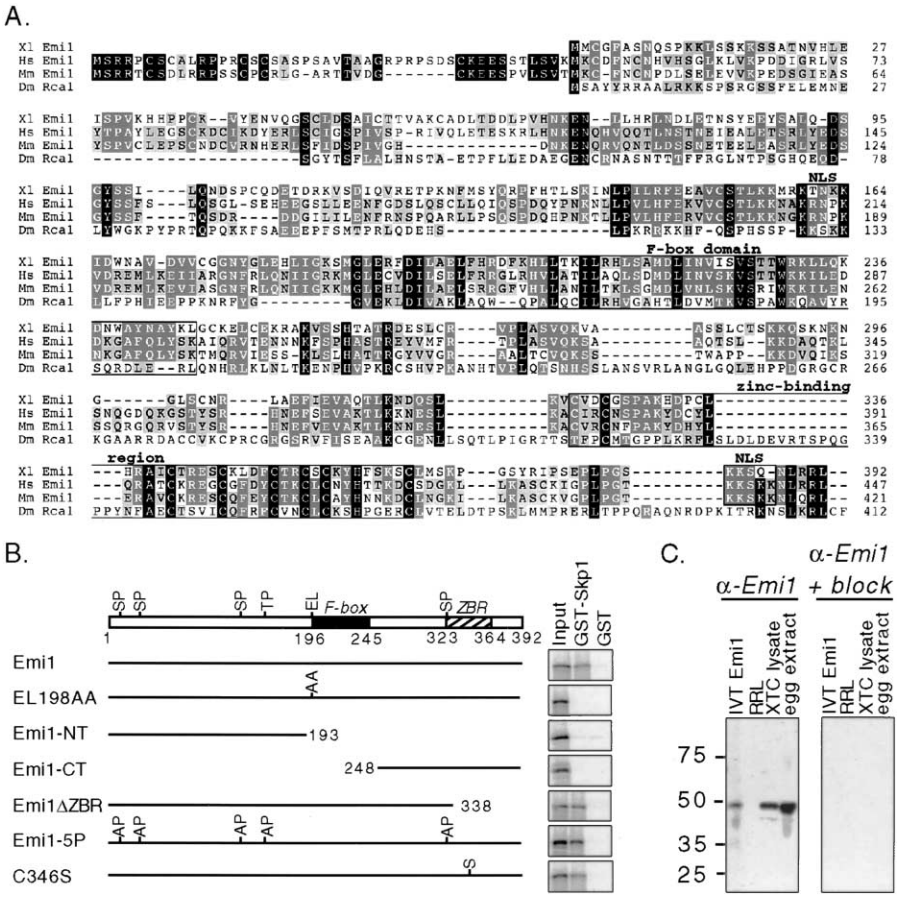

Figure 1. Emi1 Is an F Box Protein Related to *Drosophila* Rca1

(A) Clustal W alignment of Emi1 and homologs. Xl, *Xenopus laevis*; Dm, *Drosophila melanogaster*; Hs, *Homo sapiens*; and Mm, *Mus musculus*. Black = identity, dark gray = highly conserved, and light gray = less highly conserved. The F box, zinc binding region (ZBR), and possible NLS sequences are boxed.

(B) Emi1 protein (accession # AF319594) schematic, key features, and variant proteins. Emi1 = wild type; EL198AA = mutated in 2 conserved F box residues; Emi1-N-terminus (NT) = amino acids 1–193; Emi1-C-terminus (CT) = amino acids 248–392; Emi1-ΔZBR = amino acids 1–338; Emi1-5P = substitution of alanine for serine or threonine in all five SP/TP sites; and C346S = substitution of cysteine 346 with serine. GST-Skp1 or GST was incubated with ^{35}S-labeled, in-vitro-translated (IVT) proteins, bound to glutathione agarose, and analyzed by SDS-PAGE and autoradiography (right).

(C) Characterization of Emi1 antibodies. Rabbit reticulocyte lysate (RRL) programmed with Emi1 (lane 1), unprogrammed RRL (lane 2), *Xenopus* XTC cell lysate (lane 3), and interphase *Xenopus* egg extract (lane 4) were resolved by SDS-PAGE and immunoblotted with affinity-purified anti-Emi1 or MBP-Emi1 blocked antibodies.

We show here that Emi1 promotes mitotic cyclin stabilization by inhibiting APC activity. Emi1 accumulates in S phase and is destroyed in mitosis independent of the APC, but dependent on Cdk phosphorylation. Emi1 overexpression blocks cells in mitosis with high cyclin B levels and inhibits cyclin B ubiquitylation in vitro. Emi1 immunodepletion from egg extracts prevents cyclin B accumulation and mitotic entry. Emi1 binds Cdc20 and Cdc20 can rescue Emi1-induced cyclin B stabilization in vitro. The zinc binding region of Emi1 interacts with Cdc20 and is required for Emi1 to inhibit APC^{Cdc20} in vitro. Collectively, our data indicate that Emi1 controls the timing of APC activity through Cdc20 regulation.

Results

Xenopus Emi1 Is a Cell Cycle Regulated Protein Related to *Drosophila* Regulator of Cyclin A (Rca1)

We initially isolated Emi1 in a yeast two-hybrid screen for Skp1 binding proteins. Details of this screen are discussed elsewhere (Regan-Reimann et al., 1999). We cloned a full-length *Xenopus* Emi1 oocyte cDNA (see Experimental Procedures). The predicted Emi1 protein is 392 residues long with an F box, a zinc binding region (ZBR), and five possible Cdk phosphorylation sites (Figures 1A and 1B). There are two potential nuclear localization sequences. BLAST search revealed that Emi1 has homology to the *Drosophila* protein Rca1 (Figure 1A). Emi1 and Rca1 are similar in size, placement of functional domains, and share 25% similarity (16% identity). Emi1 is 43% similar (35% identical) to human Fbx5, a recently identified F box protein of unknown function (Cenciarelli et al., 1999). Mutation or deletion of the Emi1 F box abrogates binding to Skp1 in vitro (Figure 1B).

Xenopus Emi1 and its homologs contain 8 cysteines and a histidine in the C terminus that are highly conserved and may comprise two zinc binding domains (Figure 1A). The spacing of the cysteines and histidine in Emi1/Rca1, C-x(2)-C-x(14–30)-C-x(4)-C-x(4)-C-x(2)-C-x(4)-H-x(4)-C, is similar but not identical to the recently

described DRIL (TRIAD) cysteine-rich motif (van der Reijden et al., 1999).

Affinity purified antibodies against *Xenopus* Emi1 recognize a protein of the expected molecular mass (44 kDa) in egg extracts and *Xenopus* XTC lysates, which is blocked by preincubation of the antibodies with Emi1 protein (Figure 1C). Antibodies also recognize in vitro translated (IVT) Emi1, but fail to detect a protein in unprogrammed reticulocyte lysate.

Emi1 Protein Levels Oscillate in a Cell Cycle-Dependent Manner

We examined the Emi1 protein in the cell cycle of the early embryo. In fertilized eggs, Emi1 levels increase in S phase and decrease in M phase (Figure 2A). Emi1 is present in CSF-arrested eggs and persists after fertilization through the longer first interphase, during pronuclear migration.

Extracts made from activated eggs reproduce cell cycle events in vitro (reviewed in Murray, 1991). Both endogenous Emi1 and exogenous IVT Emi1 added to these extracts are ubiquitylated in mitosis (Figure 2B). Emi1 destruction requires the proteasome because IVT Emi1 is stabilized when the proteasome inhibitor MG-132 is added to mitotic egg extracts (data not shown).

Because Emi1 is mitotically destroyed, we tested whether it is an APC substrate. IVT Emi1 or an N-terminal cyclin B fragment was incubated in *Xenopus* egg extracts stabilized in mitosis by addition of nondestructible cyclin B (Δ90). In these Δ90 extracts, the APC is active and cyclin B is degraded (King et al., 1995). IVT Emi1 protein is destroyed in Δ90 extracts, but not interphase-arrested extracts. APC immunodepletion or addition of a peptide containing the cyclin B destruction box, known to inhibit APC-mediated proteolysis (Holloway, 1993; King et al., 1995), prevented cyclin B destruction, whereas a control peptide did not (Figure 2C). However, Emi1 was destroyed with similar kinetics whether the APC was depleted or blocked by destruction box peptides or a control peptide (Figure 2C). Thus, Emi1 does not appear to be an APC substrate in the egg.

To investigate the sequence requirements for Emi1 destruction, we constructed N- or C-terminal Emi1 fragments (Figure 1B). IVT Emi1 N terminus (Emi1-NT) was destroyed with kinetics similar to full-length Emi1 in Δ90 extracts ($t_{1/2} \approx 10$ min), whereas the C terminus (Emi1-CT) was stable ($t_{1/2} > 100$ min; Figure 2D). Because the N terminus contains four of five possible Cdk phosphorylation sites in Emi1, we mutated serine or threonine to alanine in all five sites and found that this Emi1-5P mutant was stable in Δ90 extracts compared to wild type (Figure 2E). Interestingly, the N terminus of Emi1 identified *Xenopus* cyclins B1 and B2 as interacting proteins several times in a yeast two-hybrid screen (data not shown). We do not yet know whether Emi1 is a Cdk substrate in vivo, but we found that full-length Emi1 and Emi1-NT were efficient in vitro cyclin B/Cdc2 substrates, although neither the Emi1-CT nor Emi1-5P mutants were phosphorylated (Figure 2E). Further, Emi1 binds the mitotic cyclins A and B in vitro and Emi1 is a phosphoprotein in egg extracts (data not shown). Thus, a plausible model is that phosphorylation of Emi1 by mitotically active kinases triggers the APC-independent destruction of Emi1.

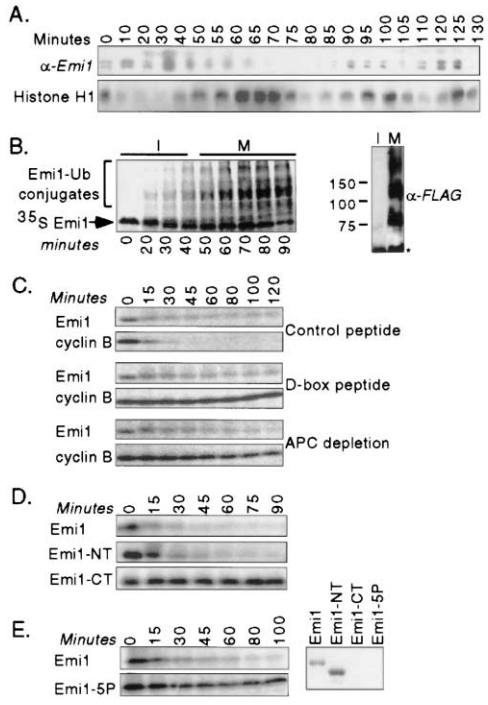

Figure 2. Emi1 Is Destroyed in Mitosis

(A) Emi1 levels fluctuate in the embryonic cell cycle. Fertilized eggs were incubated at 23°C, equal numbers of embryos removed at the indicated times, and processed for immunoblotting with anti-Emi1 antibodies (upper panel) and for histone H1 kinase activity of immunoprecipitated cyclin B1 (lower panel).

(B) Emi1 is ubiquitylated in cycling extracts. Left: Activated *Xenopus* cycling egg extracts with added ^{35}S-labeled IVT Emi1 were incubated at 23°C. Aliquots were removed at the indicated times and analyzed by SDS-PAGE and autoradiography . I = interphase and M = mitosis, as determined by cyclin B ubiquitylation and histone H1 kinase activity. Right: Interphase and mitotic extract with added FLAG-tagged ubiquitin were incubated (23°C, 60 min), immunoprecipitated with anti-Emi1 sera, and analyzed by immunoblotting for FLAG-ubiquitin. * = IgG band.

(C) Emi1 destruction does not require the APC. ^{35}S-labeled IVT Emi1 or N-terminal cyclin B fragment was added to Δ90 extracts treated with either destruction box (D-box) peptide, scrambled peptide (control), or depleted of the APC with anti-Cdc27 antibodies. Aliquots were removed at the indicated times and analyzed by SDS-PAGE and autoradiography.

(D) In mitotic extracts, Emi1 and its N terminus are unstable; the C terminus is stable. ^{35}S-labeled IVT full-length, N-terminal, or C-terminal Emi1 was added to Δ90 extracts and assayed for stability as in (C).

(E) Mutation of the five possible Cdk phosphorylation sites stabilizes Emi1. ^{35}S-labeled IVT wild-type Emi1 or a mutant in all five SP/TP sites (Emi1-5P) was added to Δ90 extracts and assayed for stability as in (C), left. Equimolar amounts of purified MBP-Emi1, MBP-Emi1-NT, MBP-Emi1-CT, or MBP-Emi1-5P were incubated with purified cyclin B/Cdc2 in the presence of [^{32}P]-γATP. Proteins were analyzed by SDS-PAGE and autoradiography (right).

Emi1 Inhibits APC Activity in *Xenopus* Egg Extracts

The oscillation of Emi1 in *Xenopus* embryos and the G2 arrest seen in Rca1-deficient *Drosophila* embryos suggested that like cyclin B, Emi1 accumulation may be important for mitotic entry and that Emi1 destruction may be necessary for mitotic exit. To test whether Emi1 destruction is required for mitotic exit, we analyzed the effect of Emi1 addition to *Xenopus* extracts. Addition of purified MBP-Emi1 protein to cycling extracts prevented

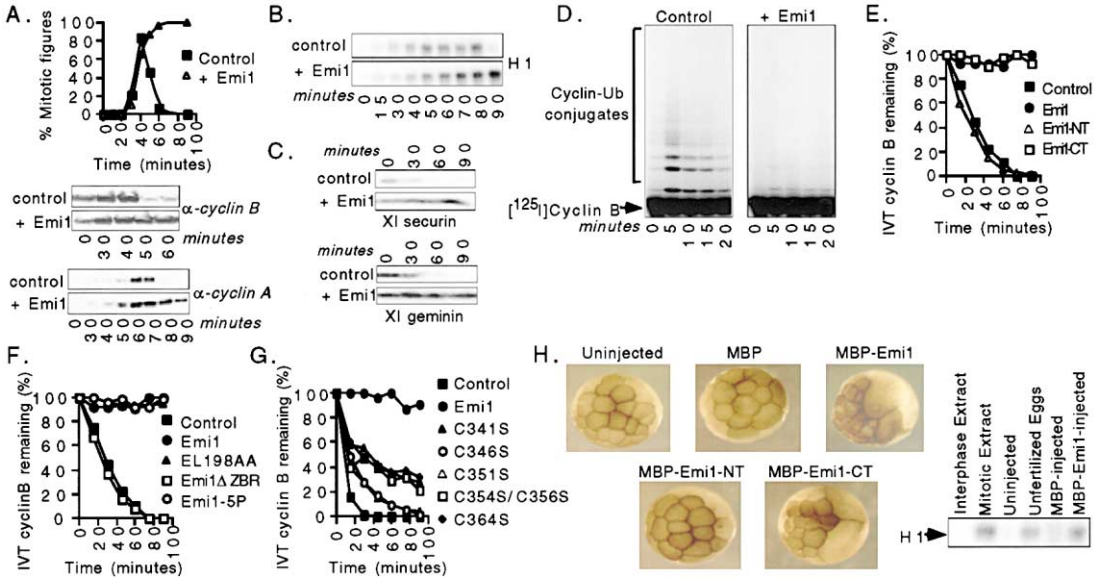

Figure 3. Emi1 Prevents the Ubiquitin-Mediated Destruction of APC Substrates and Inhibits Mitotic Exit

(A) Emi1 prevents cyclin A and B destruction and mitotic exit in cycling egg extracts. Activated *Xenopus* cycling egg extracts were incubated with either buffer (■) or 1 μM purified MBP-Emi1 (Δ). Aliquots were removed at the indicated times and assayed for DNA morphology (graph) or *Xenopus* cyclins A and B by immunoblotting (lower panels).

(B) Excess Emi1 does not affect the kinetics of cyclin B/Cdc2 activation in egg extracts. Activated *Xenopus* cycling egg extracts were incubated with buffer or 1 μM purified MBP-Emi1. Aliquots were removed at the indicated times and processed for the histone H1 kinase activity of immunoprecipitated cyclin B1.

(C) Emi1 stabilizes securin and geminin. ^{35}S-labeled IVT *Xenopus* securin or geminin protein was incubated in Δ90 extracts treated with buffer (control) or 1 μM purified MBP-Emi1. Aliquots were removed at the indicated times and analyzed by SDS-PAGE and autoradiography.

(D) Emi1 inhibits cyclin B ubiquitylation in mitotic extracts. An ^{125}I-labeled cyclin B N-terminal fragment was incubated in Δ90 extracts treated with 2.5 μM purified MBP (left) or MBP-Emi1 (right). Aliquots were removed at the indicated times and analyzed as in (C).

(E–G) ^{35}S-labeled IVT cyclin B N-terminal fragment was added to Δ90 extracts treated with Emi1 variants. Aliquots were removed at indicated times, resolved by SDS-PAGE and quantitated by Phosphorimager.

(E) The Emi1 C terminus is sufficient to block cyclin B destruction. Additions: buffer (■) or 1 μM purified MBP-Emi1 (●), MBP-Emi1-NT (Δ), or MBP-Emi1-CT (□). (F) The Emi1 ZBR but not the F box domain is required to block cyclin B destruction. Additions: buffer (■), 1 μM purified MBP-Emi1 (●), MBP-EL198AA (▲), MBP-Emi1ΔZBR (□), or MBP-Emi1–5P (○). (G) ZBR mutations fail to inhibit cyclin B destruction. Additions: buffer (■), 1 μM purified MBP-Emi1 (●), MBP-C341S (▲), MBP-C346S (○), MBP-C351S (Δ), MBP-C354S/C356S (□), or MBP-C364S (◆).

(H) Injection of Emi1 blocks *Xenopus* embryos in mitosis with high Cdk kinase activity. One pmol purified MBP-Emi1, MBP-Emi1-NT, MBP-Emi1-CT, or MBP was injected into one blastomere (right side) of two-cell stage *Xenopus* embryos. Embryos were photographed 2.5 hr after injection (left panel). For kinase assays, both blastomeres of two-cell stage embryos were injected and extracts from injected embryos assayed for histone H1 kinase activity 2.5 hr post-injection (right panel). Unfertilized eggs and equivalent aliquots of interphase and Δ90 extracts were assayed as controls.

the destruction of endogenous cyclins A and B and mitotic exit (Figure 3A). Addition of equimolar amounts of MBP or another *Xenopus* F box protein had no effect on cyclin stability or mitosis (data not shown). Excess Emi1 did not affect the timing of mitotic entry or MPF activation in egg extracts, as analyzed by DNA morphology (Figure 3A) or cyclin B/Cdc2 kinase activity (Figure 3B). By quantitative immunoblotting, we estimate Emi1 to be ~300 nM in interphase egg extracts. As little as 100 nM additional Emi1 protein stabilizes cyclins A and B. However, we see a stronger delay between cyclin B/Cdc2 activation and cyclin B destruction with 300 nM to 1 μM Emi1 protein concentrations, likely because Emi1 is itself destroyed in mitosis.

We found that Emi1 also inhibits the destruction of two other known APC substrates, securin and geminin, in Δ90 extracts (Figure 3C). To test whether Emi1 directly affects APC substrate ubiquitylation, we measured cyclin B ubiquitylation in Δ90 extracts treated with purified MBP or MBP-Emi1 protein. Addition of MBP-Emi1 strongly reduced the ubiquitylation of an iodinated amino-terminal fragment of cyclin B containing the destruction box, whereas MBP did not (Figure 3D).

To determine which domains of Emi1 are required to block cyclin B destruction, we tested several Emi1 mutants (schematic, Figure 1B). Cyclin B was destroyed in Δ90 extracts treated with buffer (control) or an MBP-Emi1-NT fusion protein, but was stabilized in the presence of MBP fusions to wild-type Emi1, Emi1-5P, the F box mutant (EL198AA), or Emi1-CT (Figures 3E and 3F). Therefore, the Cdk sites, the F box, and the region N-terminal to the F box are not required for Emi1 to stabilize cyclin B; however, the C terminus is both necessary and sufficient. An Emi1 truncation mutant missing the C-terminal ZBR (Emi1-ΔZBR) was incapable of stabilizing cyclin B (Figure 3F). Further, mutations of conserved ZBR residues cysteine 341 (C341S) or cysteine 346 (C346S) to serine greatly reduced the ability of Emi1 to inhibit cyclin B destruction (Figure 3G). Thus, the ZBR is necessary for Emi1 to inhibit APC activity.

To test whether Emi1 affects the cell cycle in vivo, we injected the protein into one blastomere of a two-cell stage *Xenopus* embryo. Emi1 caused a stable cell cycle arrest in the injected blastomere, whereas the uninjected blastomere continued to divide normally (Figure 3H). Embryos injected in both blastomeres with Emi1 had a

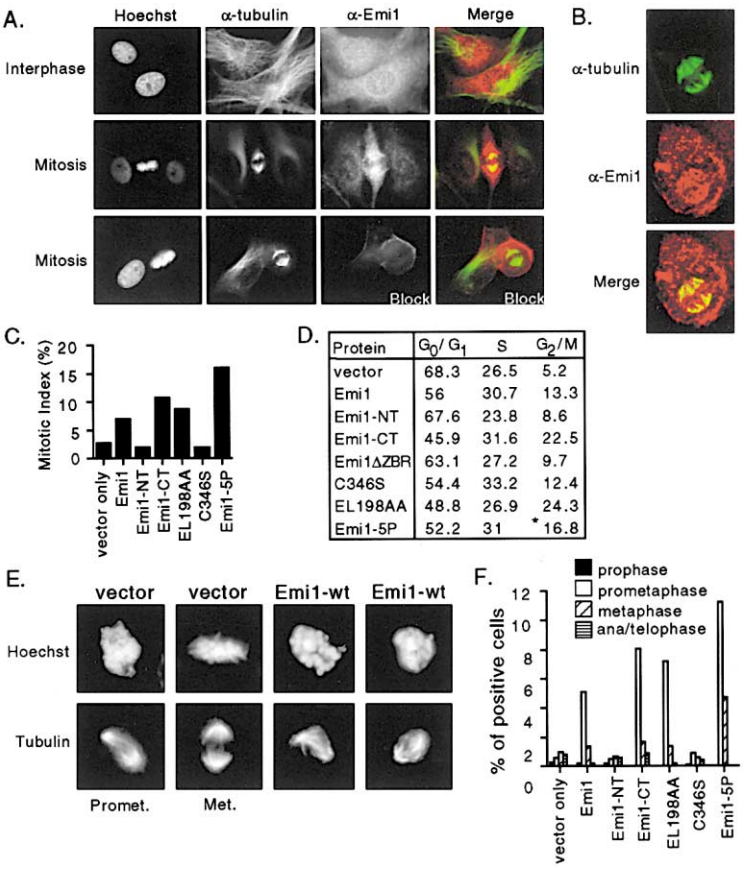

Figure 4. Transfection of Emi1 into XTC Cells Causes a Mitotic Block

(A) Emi1 localization. XTC cells were labeled with affinity-purified antibodies to Emi1, anti-α-tubulin, and Hoechst 33258 dye. Anti-Emi1 antibodies were blocked with MBP-Emi1 protein ("Block"). The Emi1 staining (red) and α-tubulin (green) images were merged (Merge) to show Emi1 spindle localization.

(B) Deconvolution image of Emi1 spindle localization. XTC cells were labeled as in (A), and the Emi1 staining (red) and α-tubulin (green) images were merged (Merge) to show the Emi1 spindle localization.

(C) Emi1 overexpression causes a mitotic index increase. XTC cells were cotransfected with GFP and myc-tagged constructs expressing Emi1 variants. Cells were fixed and stained with anti-α-tubulin antibody and Hoechst 33258 dye. The number of GFP positive mitotic cells was quantitated based on DNA and spindle morphology.

(D) Flow cytometric analysis of Emi1-transfected XTC cells. Cells were fixed, labeled with propidium iodide, and analyzed by flow cytometry. The table lists the % GFP positive cells in each cell cycle stage for each transfection. "*" = The percentage mitotic for the Emi1-5P mutant is likely an underestimate because many cells expressing this mutant undergo apoptosis.

(E and F) Emi1 overexpression blocks cells in prometaphase. XTC cells were transfected with either myc vector or myc-tagged Emi1 and processed for immunofluorescence to visualize α-tubulin and DNA. Promet. = normal prometaphase cell, met. = normal metaphase cell (E). The number of GFP-positive cells in each mitotic phase was quantitated as in (C) (shown in [F]).

high level of histone H1 kinase activity similar to that detected in Δ90 extracts, whereas uninjected and control-injected embryos had H1 kinase levels similar to interphase extracts (Figure 3H). As in cycling extracts, the Emi1 C terminus with an intact ZBR was also necessary and sufficient to mediate the mitotic block in vivo and wild-type and N-terminal Emi1 are unstable in vivo (Figure 3H and data not shown). In summary, Emi1 blocks the cell cycle at mitosis both in vitro and in vivo and prevents the ubiquitin-mediated destruction of known APC substrates in vitro.

Emi1 Overexpression in Somatic Cells Causes a Mitotic Block

To examine Emi1 subcellular localization, we stained *Xenopus* XTC cells with affinity-purified antibodies to Emi1. In interphase, the protein localizes in a punctate pattern in the nucleus and the cytoplasm, with some perinuclear concentration (Figure 4A). In mitotic cells, Emi1 localized throughout the cell and particularly at the spindle (Figures 4A and B).

Because Emi1 can stabilize several APC substrates, which are each destroyed at specific times in mitosis, we tested more precisely when in mitosis Emi1 blocks by overexpressing epitope-tagged Emi1 variants in somatic cells. Because Emi1 is unstable in mitotic XTC cells (data not shown), the myc-tagged Emi1 variants were cotransfected with a GFP expression construct to mark transfected cells. Transfection of wild-type Emi1, EL198AA, Emi1-5P, or Emi1-CT caused a mitotic index increase compared to vector, whereas neither Emi1-NT nor the C346S point mutant had a significant effect (Figure 4C). The mitotic block was confirmed by flow cytometric analysis of DNA content (Figure 4D). The stable Emi1 mutants (Emi1-CT and Emi1-5P) caused a stronger mitotic delay than the unstable wild-type Emi1. Overexpression of the APC inhibitor Mad2 in XTC cells caused a mitotic index increase similar to Emi1 (~9%; data not shown).

DNA and spindle morphology examination revealed that cells transfected with Emi1, EL198AA, Emi1-5P, or Emi1-CT accumulated predominantly in prometaphase (Figures 4E and 4F). Cyclin A destruction (which is blocked by Emi1), occurs in prometaphase and cyclin A overexpression causes a prometaphase delay in human cells (den Elzen and Pines, 2001) and in XTC cells (our unpublished data). In contrast, Mad2 does not stabilize cyclin A and blocks predominantly in metaphase when transfected into XTC cells (J.D.R.R. and P.J., unpublished data).

Emi1 Depletion Prevents Cyclin B Accumulation and Mitotic Entry

If Emi1 normally inhibits APC activity in interphase, then Emi1 depletion from cycling egg extracts might block cyclin B accumulation and prevent mitotic entry. Following Emi1 immunodepletion (Figure 5D), we examined cyclin B accumulation and DNA morphology as markers

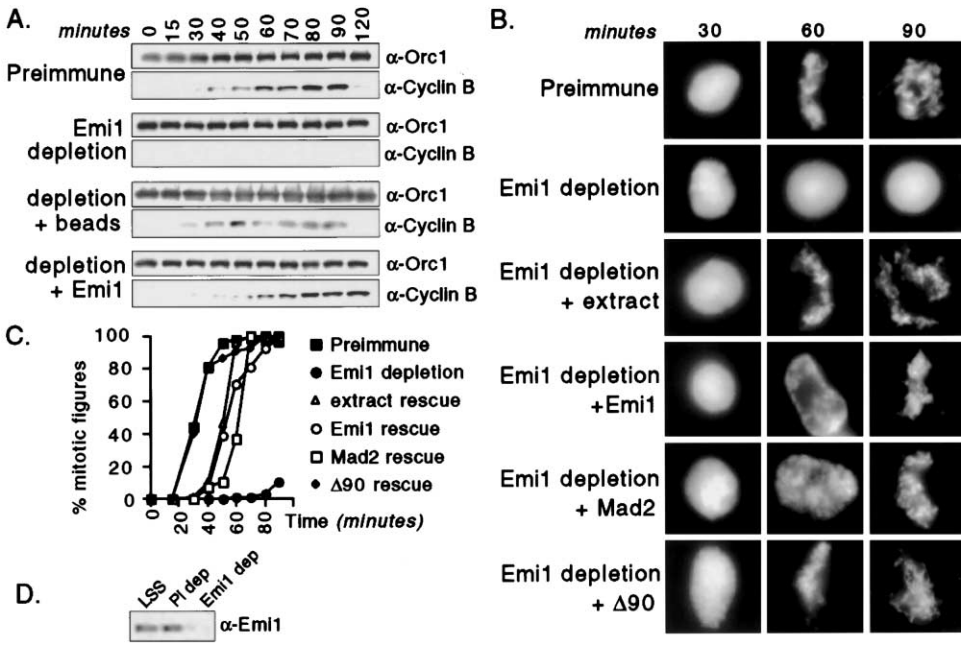

Figure 5. Emi1 Depletion Prevents Mitotic Entry in Egg Extracts

(A) Emi1 depletion prevents cyclin B accumulation in *Xenopus* cycling extracts. Equal aliquots were removed at the indicated times from preimmune sera-depleted, Emi1-depleted, or Emi1-depleted cycling extracts preincubated with either 300 nM MBP-Emi1, 0.13 volumes extract, or beads from the Emi1 depletion. Samples were processed for immunoblotting with anti-cyclin B2 and anti-Orc1 antibodies (as a loading control). Exposure time is the same for all blots.

(B and C) Emi1-depleted cycling extracts fail to enter mitosis. Sperm DNA was added to preimmune sera-depleted, Emi1-depleted, or Emi1-depleted cycling extracts preincubated with either 300 nM MBP-Emi1, 0.2 volumes extract, 6 μM GST-Mad2, or 60 μg/ml GST-Δ90 cyclin B. Aliquots were removed at the indicated times, fixed onto slides, and DNA visualized by Hoechst 33258 staining (B). The number of interphase and mitotic figures was quantitated (C).

(D) Equal amounts of undepleted, preimmune sera-depleted and Emi1-depleted extracts were resolved by SDS-PAGE and processed for immunoblotting with anti-Emi1 antibodies. Emi1 depletion removes >80% of the protein.

of mitotic entry. In cycling extracts, cyclin B normally peaks by 80 min and is destroyed by 120 min. In Emi1-depleted extracts, cyclin B levels fail to accumulate (Figure 5A). Addition of beads from the Emi1 immunodepletion rescued the accumulation and subsequent destruction of cyclin B. Addition of 300 nM purified Emi1 protein rescued cyclin B accumulation but blocked its destruction (Figure 5A). This is likely because excess Emi1 is not completely destroyed, thus inhibiting the APC and stabilizing cyclin B.

The effect of Emi1 depletion on mitosis was verified by examining DNA morphology in cycling extracts. In control extracts, demembranated sperm DNA was highly condensed by 60 min, indicating onset of mitosis, and typically displayed anaphase or telophase morphology by 90 min (Figures 5B and 5C). In Emi1-depleted extracts, nuclei remained intact with DNA decondensed (Figures 5B and 5C). Addition of undepleted extract or purified Emi1 to Emi1-depleted extracts rescued mitotic entry. Although Emi1-depleted extracts rescued with undepleted extract progressed past metaphase, extracts rescued with Emi1 protein did not, presumably because Emi1 blocks APC-dependent securin destruction and thus sister chromatid separation. It is not clear whether our assay in egg extracts can easily distinguish prometaphase and metaphase or whether, like the spindle checkpoint, a mechanism necessary to block in prometaphase fails to function in egg extracts.

If Emi1 depletion prematurely activates the APC, then addition of the APC inhibitor Mad2 should also rescue mitotic entry. Mad2 addition to Emi1-depleted extracts did rescue mitotic entry (Figures 5B and 5C), although, much like rescue with Emi1 protein, the extracts did not progress beyond metaphase. To test whether the inability of Emi1-depleted extracts to enter mitosis was primarily due to their failure to accumulate cyclin B, we also tested whether nondestructible Δ90 cyclin B addition rescued mitotic entry. Δ90 addition to depleted extracts rescued nuclear envelope breakdown and mitotic DNA condensation, indicating that nondestructible cyclin B can overcome the requirement for Emi1 in mitotic entry (Figures 5B and 5C).

Emi1 Interacts with the APC Activator Cdc20

To better understand how Emi1 controls APC activity, we looked for interacting proteins by yeast two-hybrid screens of a *Xenopus* oocyte library using Emi1 as the bait. Screening with full-length Emi1 identified only Skp1, therefore we tested Emi1-NT and Emi1-CT for interacting proteins as well (see Experimental Procedures). As previously mentioned, the Emi1-NT bait identified cyclin B.

Importantly, Emi1-NT also identified the APC activator Cdc20. To validate this interaction, we took several approaches. First, Emi1 and Cdc20 coimmunoprecipitate from egg extracts (Figure 6A). This interaction appears to be APC independent, since we were unable to detect the APC subunit APC2 in the precipitate. Second, in-

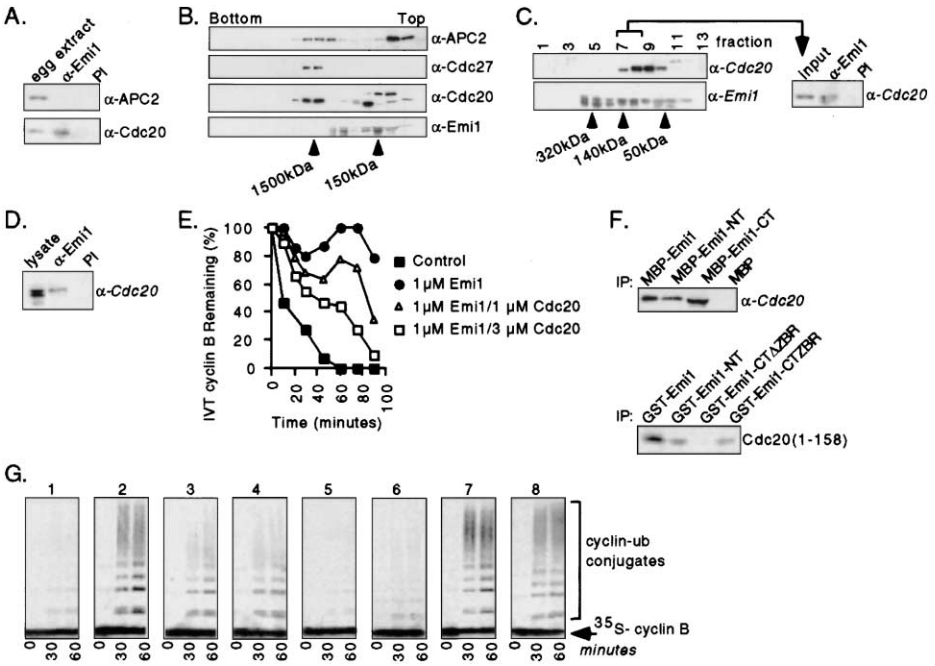

Figure 6. Emi1p Interacts with Cdc20 and Inhibits APC Activation by Cdc20

(A) Emi1 coimmunoprecipitates with Cdc20 but not APC2. Preimmune (PI) or anti-Emi1 immunoprecipitates from interphase egg extract were assayed by immunoblotting for APC2 (upper panel) and Cdc20 (lower panel).

(B) Sucrose gradient cosedimentation of Emi1 and Cdc20. Interphase egg extract was fractionated on a 10%–40% sucrose gradient, and fractions analyzed by immunoblotting with antibodies to the indicated proteins.

(C) Emi1 and Cdc20 cofractionate during gel filtration chromatography. Interphase egg extract was resolved on a Resource Q anion exchange column and fractions containing Emi1 chromatographed on an S-300 gel filtration column and immunoblotted for Emi1 and Cdc20 (left panel). Preimmune (PI) or anti-Emi1 immunoprecipitates from a 100 kDa–200 kDa fraction were assayed by immunoblotting for Cdc20 (right).

(D) Emi1 and Cdc20 associate in baculovirus coinfection. SF9 cells were coinfected with baculovirus-expressed Emi1 and Cdc20, precipitated with preimmune (PI) or anti-Emi1 antisera, and analyzed for Cdc20 by immunoblotting.

(E) Cdc20 rescues cyclin B destruction. ^{35}S-labeled IVT N-terminal cyclin B was added to mitotic *Xenopus* egg extracts treated with buffer (■), 1 μM purified MBP-Emi1 (●), 1 μM MBP-Emi1 plus 1 μM His-Cdc20 (Δ), or 1 μM MBP-Emi1 plus 3 μM His-Cdc20 (□). Aliquots were removed at the indicated times, resolved by SDS-PAGE and quantitated on a Phosphorimager.

(F) Cdc20 interacts with both the N terminus and the ZBR of Emi1 in vitro. Purified MBP-Emi1 fusion protein variants and purified baculovirus-expressed Cdc20 were incubated together in binding buffer, bound to amylose beads, washed, and assayed for Cdc20 by immunoblotting (upper panel). Purified GST-Emi1, GST-Emi1-NT, GST-Emi1-CTΔZBR (residues 248–334), or GST-Emi1-CTZBR (residues 335–364) was incubated with ^{35}S-labeled in vitro translated (IVT) Cdc20(1–158), bound to glutathione agarose, and analyzed by SDS-PAGE and autoradiography (lower panel).

(G) Inhibition of Cdc20-mediated activation of the APC by Emi1. IVT Cdc20 (2–8) or rabbit reticulocyte lysate (1) was incubated for 30 min with buffer (1 and 2) or purified bacterially expressed 1 μM MBP-Emi1 (3), 3 μM MBP-Emi1 (4), 6 μM MBP-Emi1 (5), 3 μM MBP-Emi1-CT (6), 6 μM MBP (7), or 20 μM GST-Emi1-CTΔZBR (8). APC was immunopurified from mitotic egg extracts with anti-Cdc27 beads, then incubated with the Cdc20/protein mixtures for 1 hr. APC beads were washed, and assayed for cyclin ubiquitylation activity using a ^{35}S-labeled IVT N-terminal *Xenopus* cyclin B substrate.

terphase extracts separated on a sucrose gradient or gel filtration column showed that Emi1 and Cdc20 cofractionate (Figures 6B and 6C). Emi1 is found in two higher molecular weight pools, ~100–200 kDa and ~300–500 kDa. Cdc20 cofractionates in the ~100–200 kDa complex and Cdc20 coimmunoprecipitates with Emi1 from these fractions (Figure 6C). The Cdc20 protein that does not cofractionate with Emi1 cofractionates with the ~1.5 MDa APC complex (Figure 6B); however, Cdc20 binds weakly to the inactive interphase APC (Fang et al., 1998a; Kramer et al., 2000). Interestingly, a slower migrating form of Cdc20 is consistently seen in some of the fractions containing Emi1. The nature and significance of this modification is not known. We can also reconstitute the interaction between Emi1 and Cdc20 with baculovirus or using purified proteins (Figures 6D and 6F), further supporting a direct interaction.

Emi1 Can Block Cdc20-Dependent APC Activation In Vitro

If Emi1 inhibits Cdc20 activation of the APC, then Cdc20 protein should rescue the Emi1 block to cyclin B destruction. Baculovirus-expressed Cdc20 addition to mitotic extracts rescued the Emi1-induced block to cyclin B destruction in a dose-dependent manner (Figure 6E), supporting the hypothesis that Emi1 prevents Cdc20 from activating the APC. This result also indicates that the APC is competent for activation by Cdc20 even when Emi1 is present, reinforcing our other observations that Emi1 does not directly inhibit the APC enzymatic complex (see discussion).

We knew that the Emi1 N terminus interacts with Cdc20 from our two-hybrid screen, but the C terminus of Emi1 (Emi1-CT) also bound Cdc20 in vitro (Figure 6F). We confirmed the Cdc20-Emi1-CT interaction in the

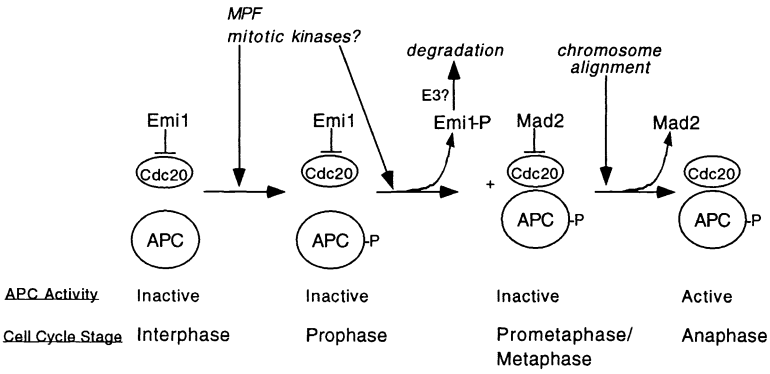

Figure 7. A Model for Emi1 Regulation of the Anaphase Promoting Complex

yeast two-hybrid system. Interestingly, we also observed in both yeast two-hybrid and in vitro binding assays, that the Cdc20 N terminus from residues 1–158, but not the WD repeat domain, is sufficient for binding to Emi1 (data not shown). Because the C-terminal ZBR is required for Emi1 to inhibit APC activity, we tested the ability of C-terminal Emi1 fragments to bind the Cdc20 N terminus. A C-terminal subfragment containing the most conserved region of the ZBR, GST-Emi1-CTZBR (residues 335–364), interacts with the Cdc20-NT, whereas the C-terminal fragment without the ZBR, GST-Emi1-CTΔZBR (residues 248–334), does not (Figure 6F).

We utilized this binding information to test whether the interaction between Emi1 and Cdc20 is required for Emi1 to inhibit Cdc20 from activating the APC in a reconstituted system. Addition of full-length Emi1 protein prevented Cdc20 from activating APC immunopurified from mitotic egg extract in a dose-dependent fashion (Figure 6G). The Emi1 C terminus, which contains the ZBR, is sufficient to inhibit cyclin B ubiquitylation in this purified system whereas the Emi1-CTΔZBR, which fails to bind Cdc20, does not inhibit.

Discussion

We have identified an APC inhibitor called Emi1, which is required for mitotic entry. Emi1 is unstable in mitosis and expression of nondestructible versions of the protein or overexpression of the wild-type protein causes a mitotic block in embryos and somatic cells. Emi1 destruction is APC-independent in the egg and appears to require phosphorylation by mitotically active Cdks. Emi1 binds the APC activator Cdc20 and Cdc20 can rescue the Emi1-induced block of cyclin B degradation, indicating that Cdc20 is the target of Emi1-APC regulation.

Identification of an Independent Cell Cycle Oscillator that Controls APC Activity

Like cyclin B, Emi1 must accumulate for mitotic entry and be destroyed for mitotic exit. Emi1 is destroyed in mitosis by ubiquitin-mediated proteolysis and its destruction likely requires phosphorylation by mitotic kinases, including cyclin B/Cdc2. We are currently testing the mechanism of Emi1 destruction in mitosis, but it does not require the APC. Several F box proteins are unstable in mitosis, and the SCF has been implicated in their destruction. Because many SCF substrates require prior phosphorylation for degradation (reviewed in Jackson et al., 2000), mitotically phosphorylated Emi1 may be an SCF substrate.

Emi1 destruction may be influenced by its association with Cdc20. An interesting possibility is that phosphorylation by cyclin B/Cdc2 triggers the dissociation of Emi1 and Cdc20, thereby promoting Emi1 destruction. Indeed, we found that Cdc20 addition not only rescued the Emi1 block of APC activity, but also stabilized Emi1 in mitotic extracts (J.D.R.R. and P.J., unpublished data) suggesting that Emi1 is more stable when complexed with Cdc20.

Emi1 Inhibits the APC^{Cdc20} Complex

Cdc20 exists in high molecular weight complexes both with and independent of the APC (Kramer et al., 1998; Lorca et al., 1998). Emi1 and Cdc20 coimmunoprecipitate from interphase extracts in a complex independent of the APC, suggesting a model where Emi1 sequesters Cdc20 from the APC (Figure 7). We do not yet know if Emi1 is associated with E3 activity, but one possibility is that Cdc20 is a substrate of an Emi1-containing SCF complex. However, addition of Emi1 to egg extracts does not destabilize Cdc20 (J.D.R.R. and P.J., unpublished data), and the F box is not required for Emi1 to block cyclin B destruction, making it unlikely that Emi1 directs Cdc20 destruction. Cdc20 is also stable in the early embryo (Kramer et al., 2000; Lorca et al., 1998) so the ability of Emi1 to regulate the cell cycle is unlikely to require Cdc20 destruction.

Our in vitro APC inhibition assays and rescue experiments indicate that Emi1 is a direct Cdc20 inhibitor. The Emi1 ZBR is required to inhibit the APC and binds to Cdc20 in vitro. The ZBR appears to cooperate with the Emi1 N terminus to bind Cdc20 and may prevent the interaction of Cdc20 with APC substrates. Importantly, Emi1 does not inhibit the substrate and Cdc20-independent ubiquitylation activity of the APC2/APC11 core complex (Gmachl et al., 2000), further indicating that Emi1 inhibits APC activity through Cdc20 and not at the level of the APC enzymatic machinery (J.D.R.R., B. Gardner, and P.J., unpublished data). Further indicating its specificity, Emi1 does not inhibit SCF ubiquitin-ligase activity in vitro, or SCF-dependent events (DNA replication and mitotic entry) in egg extracts.

Emi1 as a Mitotic Timer and Potential Checkpoint Protein for APC Activation

Cyclin B ubiquitylation activity of APC immunoprecipitated from synchronized HeLa cells increases significantly before cyclin B levels decrease, and the APC

subunit Cdc27 is phosphorylated well before cyclin B levels decrease (Kramer et al., 2000). This delay in APC activation even when the APC is phosphorylated by MPF suggests the presence of an inhibitor that restrains full APC activation until nuclear envelope breakdown, spindle assembly, and chromatin condensation have occurred. The delay might be explained in part by Mad2, which is required for APC inhibition in prometaphase until chromosomes have been properly aligned at the metaphase plate (Gorbsky et al., 1998; Taylor and McKeon, 1997). However, although anti-Mad2 antibody injection affects progression through metaphase, it does not affect progression through prophase, when MPF is also active and Cdc20 is present (Gorbsky et al., 1998).

We considered whether Emi1 cooperates with Mad2 to inhibit Cdc20 as part of the SC pathway. However, Emi1 does not bind to Mad2 in vitro and Emi1 competes with Mad2 for binding to Cdc20 in vitro (J.D.R.R. and P.J., unpublished data). Further, cyclin A destruction is not inhibited when Mad2 is activated (Geley et al., 2001; den Elzen and Pines, 2001), whereas Emi1 stabilizes cyclin A in cycling extracts. In human cells, cyclin A normally is destroyed in prometaphase (Geley et al., 2001; den Elzen and Pines, 2001), at a time when Emi1 levels drop (J.Y.H., J.D.R.R., and P.J., unpublished data). Emi1 likely causes a prometaphase and not a metaphase delay like Mad2 because Emi1 stabilizes cyclin A, which is not seen with Mad2 overexpression. Consistently, coexpression of the cyclin A/Cdk2 inhibitor $p21^{Cip1}$ with Emi1 can reverse the Emi1-induced mitotic block in XTC cells (our unpublished data). However, it is possible that the prometaphase delay induced by Emi1 results from the stabilization of other as yet unidentified APC substrate(s) that must normally be destroyed in early mitosis.

The observation that Emi1 immunodepletion delays cyclin B accumulation and mitotic entry further indicates that Emi1 inhibits the APC in interphase and in early mitosis, before Mad2 begins to function. Similarly, loss of the likely Emi1 homolog Rca1 prevents mitotic entry in *Drosophila* embryos (Dong et al., 1997). Because APC inhibition by Mad2 or proteasome inhibition by addition of MG132 (J.D.R.R. and P.J., unpublished data), rescued mitosis in Emi1-depleted extracts, Emi1 most likely affects cyclin B ubiquitylation and destruction, rather than, for example, its translation. Nonetheless, we cannot exclude that Emi1 may have additional roles in promoting mitotic entry.

Recent studies indicate APC activation is spatially as well as temporally restricted. Notably, cyclin B proteolysis begins first at the spindle poles (Clute and Pines, 1999; Huang and Raff, 1999) and Mad2 activation at kinetochores restrains securin destruction to prevent chromosome segregation. Are there sensing mechanisms other than the SC that regulate the APC? Mitotic events other than kinetochore capture by microtubules, namely chromatin condensation, centrosome separation, nuclear envelope breakdown, and spindle formation must occur sequentially before APC activation. It is therefore possible that these critical prophase and prometaphase events are controlled by sensing mechanisms that involve Emi1.

Experimental Procedures

Emi1 Cloning and Yeast Two-Hybrid Screen

A partial cDNA isolated in a Skp1 yeast two-hybrid screen (Regan-Reimann et al., 1999) was used to screen a *Xenopus* ovary cDNA library (Stratagene). A 1.9 kb clone was sequenced on both strands and contains stop codons upstream of the 5′ start codon and a 3′ poly-A tail. In vitro translation produces a 44 kDa species in reticulocyte lysate.

Emi1-NT (1–193), Emi1-CT (233–392), or Emi1 full-length (fl) were cloned into pAS2 and used to screen (2.5 million clones each) a *Xenopus* oocyte library (Clontech) in strain Y190. Interacting proteins were verified with fl Emi1 by filter lift β-galactosidase assay.

Preparation of Emi1 Full-Length and Mutant Constructs and Proteins

Emi1 and variants were cloned into pCS2-5mt (myc-tagged), pMAL or pGEX vectors and Cdc20(1–158) into pCS2 vector. Emi1-pCS2-5mt site-directed mutants (E198A and L199A [EL198AA], S10A, S29A, S105A, T123A, S328A [Emi1-5P], C341S, C346S, C354S, and C356S [C354S/C356S], and C364S), were verified by sequencing. Emi1 baculovirus was generated using the BAC-TO-BAC system (Gibco).

Emi1 variants were produced as MBP fusion proteins and purified by standard protocols. Human Cdc20 baculovirus protein was as described (Kramer et al., 2000).

Antibody Preparation

Bacterially produced MBP-Emi1 was used to raise polyclonal antibodies in rabbits and mice (Josman Laboratories). Rabbit antibodies were affinity purified on a GST-Emi1 column.

Binding Assays and Chromatography

In vitro GST-fusion protein binding reactions were as described (Bai et al., 1996). In vitro MBP protein binding assays: 100 nM purified MBP-Emi1, MBP-Emi1-NT, MBP-Emi1-CT, or MBP was incubated with 100 nM His-Cdc20 in Buffer 1 (50 mM Tris [pH 7.5], 100 mM NaCl, and 0.1% NP-40), supernatants bound to amylose beads, washed 4 times with buffer 2 (50 mM Tris [pH 7.5], 300 mM NaCl, and 1% NP-40), and bound proteins resolved by SDS-PAGE and anti-MBP immunoblots.

Baculovirus Reconstitution

SF9 cells coinfected with Emi1 and Cdc20 baculoviruses were lysed in RIPB (100 mM NaCl, 50 mM β-glycerophosphate, 5 mM EDTA, 0.1% Triton X-100, and 1 mM DTT), and lysates precleared with protein G Sepharose. Supernatants were incubated with mouse anti-Emi1 or preimmune (PI) sera, bound to protein G Sepharose, washed 4 times in RIPB, and analyzed by anti-Cdc20 immunoblots.

Sucrose Gradient

Interphase egg extract was diluted 1:5 in buffer (100 mM KOAc [pH 7.2], 2.5 mM $Mg(OAc)_2$, 5 mM EGTA, 2 mM DTT, 10 mM Tris [pH 7.2], 80 mM β-glycerophosphate, and 100 mM sucrose), and cleared at 40,000 rpm (SW50.1 rotor, 1 hr, 4°C). Lysate was resolved on a 10%–40% w/v sucrose gradient, centrifuged in an SW40.1 rotor (30,000 rpm, 18 hr, 4°C), and fractions analyzed by SDS-PAGE and immunoblotting.

High speed interphase *Xenopus* egg extract supernatants were fractionated on a Resource Q column, and eluted with a 0–0.5 M NaCl gradient. Pooled Emi1-containing fractions were separated on an S-300 gel filtration column. Egg extract or the 100–200 kDa fraction immunoprecipitated with anti-Emi1 or PI sera (as above) were analyzed by anti-Cdc20 or anti-APC2 immunoblot.

Kinase Assays

Histone H1 kinase activity (Murray, 1991) and cyclin B kinase activity (Jackson et al., 1995) were analyzed as described. In vitro cyclin B phosphorylation experiments: 1 μM purified MBP-Emi1 or MBP-Emi1 variants were incubated with 2 units cyclin B/Cdc2 (NEB) in kinase buffer plus 66 μM ATP and 0.25 μCi/μl [^{32}p]-γATP) (15min, RT), reactions quenched with sample buffer, and resolved by SDS-PAGE.

Xenopus Extracts and Embryos

Interphase and cycling extracts (Murray, 1991) were made from eggs activated with calcium ionophore A23187. To assay DNA morphology, sperm nuclei were added, fixed at various times, and DNA labeled (Hoechst 33258). Endogenous cyclin A and B levels were assayed by immunoblots with anti-Xl cyclin B2 or anti-Xl cyclin A1 mouse monoclonal antibodies (S. Geley and T. Hunt). Mitotic extracts were made by addition of nondegradable Δ90 sea urchin

cyclin B to interphase extracts. *Xenopus* eggs were fertilized in vitro, 10 eggs isolated per time point, lysed in RIPB, and assayed for cyclin B-associated kinase activity and Emi1 protein levels by immunoblot.

Embryo Injection Experiments

9.2 nl of 100 μM protein was injected into one blastomere at the two-cell stage. Injected embryos were transferred to 0.1× MMR with 3% Ficoll. H1 kinase activity in injected embryos was assayed as described (Fang et al., 1998b).

Degradation and Ubiquitylation Assays

Emi1 Ubiquitylation

Interphase or mitotic extracts were incubated at 23°C for 60 min with 4.6 ng/μl FLAG-ubiquitin, 1 μM ubiquitin aldehyde, and 2 mM MG-132. Time points were diluted in RIPB, immunoprecipitated with mouse anti-Emi1 sera or PI sera and analyzed by anti-FLAG (Sigma) immunoblots. Substrate degradation in Δ90 or cycling extracts: ^{35}S-labeled IVT protein was added and extracts incubated (23°C). Aliquots were removed, resolved by SDS-PAGE, and quantitated on a Phosphorimager. The cyclin B substrate was an N-terminal sea urchin cyclin B fragment (aa 13–91)-protein A fusion (Glotzer et al., 1991). Extracts were treated with 1 mM Hs cyclin B destruction box peptide or a scrambled version, or depleted of the APC with anti-Cdc27 antisera to assay the effect of APC inhibition on Emi1 stability. To assay Emi1's effect on APC activity, 1 μM MBP fusion protein, 1 μM control protein, or buffer was added. To assay Emi1's effect on cyclin B ubiquitylation, 2.5 μM MBP-Emi1 or MBP was incubated in Δ90 extracts (20 min), with iodinated sea-urchin cyclin-B fragment as described (Kramer et al., 2000).

In Vitro APC Assay

Mitotic extract anti-Cdc27 immunoprecipates were incubated (1hr, 4°C) with IVT hCdc20 preincubated with Emi1, control protein, or buffer, washed in XB^-, and assayed for cyclin ubiquitylation as described (Fang et al., 1998a), using a ^{35}S-labeled IVT Xl cyclin B1 (aa 2–97) fragment as substrate.

Immunodepletions

Anti-Emi1 rabbit polyclonal or PI sera were covalently coupled to protein A Affiprep beads (Bio-Rad). Beads were washed (20 mM HEPES [pH 7.7] and 100 mM KCl), incubated with cycling extracts (0.3 μl beads/μl extracts, 45 min, 4°C), and samples cleared (3 min, 3000 rpm, 4°C). The process was repeated 2 more times (30 min, 4°C), and the triple-depleted extracts set to cycle at 23°C. For rescue, depleted extracts were preincubated with 0.2 volumes undepleted extract, 300 nM MBP-Emi1, 0.3 μl depletion beads/μl extract, 60 ng/μl Δ90, or 0.3 mg/ml GST-Mad2 (10 min, 4°C) prior to cycling.

Tissue Culture, Immunofluorescence, and Flow Cytometry

Xenopus XTC cells were maintained as described (Freed et al., 1999). Fugene 6 reagent was used for transfections (Roche Molecular Biochemicals). pEGFP-C1 (Clontech), and myc-Emi1 constructs were cotransfected (1:10). Ninety-eight percent of GFP-positive interphase cells were also myc labeled. Cells were processed for immunofluorescence or flow cytometry 72 hr post-transfection. Immunofluorescence: cells were grown on cover slips and fixed in methanol (−20°C) or 2% paraformaldehyde with similar results. Cover slips were washed in immunofluorescence wash buffer (IFWB) (Freed et al., 1999), and blocked in IFWB with 5% normal donkey serum. 1° Antibodies were: affinity-purified anti-Emi1 (1.5 μg/ml); anti-α-tubulin (Serotec rat anti-α-tubulin mAb, Clone YL1/2 supernatant; 1:1); anti-myc mAb 9E10 (1 μg/ml). Texas Red or fluorescein-conjugated donkey 2° antibodies (Jackson Immunoresearch) were used at 1:150, and Hoechst dye at 5 μg/ml. Fluorescent cells were visualized and digitally imaged or examined by deconvolution microscopy (Freed et al., 1999). Flow cytometric analysis of PI-stained cells was performed with a Beckman Coulter ALTRA flow cytometer, using MultiCycle AV software (Phoenix Flow Systems, Inc.)

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References

Alexandru, G., Zachariae, W., Schleiffer, A., and Nasmyth, K. (1999). Sister chromatid separation and chromosome re-duplication are regulated by different mechanisms in response to spindle damage. EMBO J. *18*, 2707–2721.

Bai, C., Sen, P., Hofmann, K., Ma, L., Goebl, M., Harper, J.W., and Elledge, S.J. (1996). SKP1 connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F box. Cell *86*, 263–274.

Cenciarelli, C., Chiaur, D.S., Guardavaccaro, D., Parks, W., Vidal, M., and Pagano, M. (1999). Identification of a family of human F box proteins. Curr. Biol. *9*, 1177–1179.

Charles, J.F., Jaspersen, S.L., Tinker-Kulberg, R.L., Hwang, L., Szidon, A., and Morgan, D.O. (1998). The Polo-related kinase Cdc5 activates and is destroyed by the mitotic cyclin destruction machinery in S. cerevisiae. Curr. Biol. *8*, 497–507.

Clute, P., and Pines, J. (1999). Temporal and spatial control of cyclin B1 destruction in metaphase. Nat. Cell Biol. *1*, 82–87.

Dong, X., Zavitz, K.H., Thomas, B.J., Lin, M., Campbell, S., and Zipursky, S.L. (1997). Control of G1 in the developing Drosophila eye: rca1 regulates Cyclin A. Genes Dev. *11*, 94–105.

den Elzen, N., and Pines, J. (2001). Cyclin A is destroyed in prometaphase and can delay chromosome alignment and anaphase. J. Cell Biol. *153*, 121–135.

Fang, G., Yu, H., and Kirschner, M.W. (1998a). Direct binding of CDC20 protein family members activates the anaphase-promoting complex in mitosis and G1. Mol. Cell *2*, 163–171.

Fang, G., Yu, H., and Kirschner, M.W. (1998b). The checkpoint protein MAD2 and the mitotic regulator CDC20 form a ternary complex with the anaphase-promoting complex to control anaphase initiation. Genes Dev. *12*, 1871–1883.

Freed, E., Lacey, K.R., Huie, P., Lyapina, S.A., Deshaies, R.J., Stearns, T., and Jackson, P.K. (1999). Components of an SCF ubiquitin ligase localize to the centrosome and regulate the centrosome duplication cycle. Genes Dev. *13*, 2242–2257.

Geley, S., Kramer, E., Giefferes, C., Gannon, J., Peters, J.-M., and Hunt, T. (2001). Anaphase-promoting Complex/Cyclosome-dependent proteolysis of human cyclin A starts at the beginning of mitosis and is not subject to the spindle assembly checkpoint. J. Cell Biol. *153*, 137–147.

Glotzer, M., Murray, A.W., and Kirschner, M.W. (1991). Cyclin is degraded by the ubiquitin pathway. Nature *349*, 132–138.

Gmachl, M., Gieffers, C., Podtelejnikov, A.V., Mann, M., and Peters, J.M. (2000). The RING-H2 finger protein APC11 and the E2 enzyme UBC4 are sufficient to ubiquitinate substrates of the anaphase-promoting complex. Proc. Natl. Acad. Sci. USA *97*, 8973–8978.

Gorbsky, G.J., Chen, R.H., and Murray, A.W. (1998). Microinjection of antibody to Mad2 protein into mammalian cells in mitosis induces premature anaphase. J. Cell Biol. *141*, 1193–1205.

Holloway, S.L., Glotzer, M., King, R.W., and Murray, A.W. (1993). Anaphase is initiated by proteolysis rather than by the inactivation of maturation-promoting factor. Cell *73*, 1393–1402.

Huang, J., and Raff, J.W. (1999). The disappearance of cyclin B at the end of mitosis is regulated spatially in Drosophila cells. EMBO J. *18*, 2184–2195.

Hwang, L.H., Lau, L.F., Smith, D.L., Mistrot, C.A., Hardwick, K.G., Hwang, E.S., Amon, A., and Murray, A.W. (1998). Budding yeast Cdc20: a target of the spindle checkpoint. Science *279*, 1041–1044.

Jackson, P.K., Chevalier, S., Philippe, M., and Kirschner, M.W. (1995). Early events in DNA replication require cyclin E and are blocked by p21CIP1. J. Cell Biol. *130*, 755–769.

Jackson, P.K., Eldridge, A.G., Freed, E., Furstenthal, L., Hsu, J.Y., Kaiser, B.K., and Reimann, J. D. (2000). The lore of the RINGs: substrate recognition and catalysis by ubiquitin ligases. Trends Cell Biol. *10*, 429–439.

Juang, Y.L., Huang, J., Peters, J.M., McLaughlin, M.E., Tai, C.Y., and Pellman, D. (1997). APC-mediated proteolysis of Ase1 and the morphogenesis of the mitotic spindle. Science *275*, 1311–1314.

Kallio, M., Weinstein, J., Daum, J.R., Burke, D.J., and Gorbsky, G.J. (1998). Mammalian p55CDC mediates association of the spindle checkpoint protein Mad2 with the cyclosome/anaphase-promoting complex, and is involved in regulating anaphase onset and late mitotic events. J. Cell Biol. *141*, 1393–1406.

Kim, S.H., Lin, D.P., Matsumoto, S., Kitazono, A., and Matsumoto, T. (1998). Fission yeast Slp1: an effector of the Mad2-dependent spindle checkpoint. Science *279*, 1045–1047.

King, R.W., Peters, J.M., Tugendreich, S., Rolfe, M., Hieter, P., and Kirschner, M.W. (1995). A 20S complex containing CDC27 and CDC16 catalyzes the mitosis-specific conjugation of ubiquitin to cyclin B. Cell *81*, 279–288.

Kotani, S., Tanaka, H., Yasuda, H., and Todokoro, K. (1999). Regulation of APC activity by phosphorylation and regulatory factors. J. Cell Biol. *146*, 791–800.

Kramer, E.R., Gieffers, C., Hölzl, G., Hengstschläger, M., and Peters, J.M. (1998). Activation of the human anaphase-promoting complex by proteins of the CDC20/Fizzy family. Curr. Biol. *8*, 1207–1210.

Kramer, E.R., Scheuringer, N., Podtelejnikov, A.V., Mann, M., and Peters, J.M. (2000). Mitotic regulation of the APC activator proteins CDC20 and CDH1. Mol. Biol. Cell *11*, 1555–1569.

Li, Y., Gorbea, C., Mahaffey, D., Rechsteiner, M., and Benezra, R. (1997). MAD2 associates with the cyclosome/anaphase-promoting complex and inhibits its activity. Proc. Natl. Acad. Sci. USA *94*, 12431–12436.

Lorca, T., Castro, A., Martinez, A.M., Vigneron, S., Morin, N., Sigrist, S., Lehner, C., Dorée, M., and Labbé, J.C. (1998). Fizzy is required for activation of the APC/cyclosome in Xenopus egg extracts. EMBO J. *17*, 3565–3575.

Meijer, L., Arion, D., Golsteyn, R., Pines, J., Brizuela, L., Hunt, T., and Beach, D. (1989). Cyclin is a component of the sea urchin egg M-phase specific histone H1 kinase. EMBO J. *8*, 2275–2282.

Minshull, J., Blow, J.J., and Hunt, T. (1989). Translation of cyclin mRNA is necessary for extracts of activated xenopus eggs to enter mitosis. Cell *56*, 947–956.

Murray, A.W. (1991). Cell cycle extracts. Methods Cell Biol. *36*, 581–605.

Murray, A.W., and Kirschner, M.W. (1989). Cyclin synthesis drives the early embryonic cell cycle. Nature *339*, 275–280.

Page, A.M., and Hieter, P. (1999). The anaphase-promoting complex: new subunits and regulators. Annu. Rev. Biochem. *68*, 583–609.

Pfleger, C.M., and Kirschner, M.W. (2000). The KEN box: an APC recognition signal distinct from the D box targeted by Cdh1. Genes Dev. *14*, 655–665.

Regan-Reimann, J.D., Duong, Q.V., and Jackson, P.K. (1999). Identification of novel F box proteins in Xenopus laevis. Curr. Biol. *9*, R762–R763.

Rudner, A.D., and Murray, A.W. (2000). Phosphorylation by Cdc28 activates the Cdc20-dependent activity of the anaphase-promoting complex. J. Cell Biol. *149*, 1377–1390.

Schwab, M., Lutum, A.S., and Seufert, W. (1997). Yeast Hct1 is a regulator of Clb2 cyclin proteolysis. Cell *90*, 683–693.

Shirayama, M., Zachariae, W., Ciosk, R., and Nasmyth, K. (1998). The Polo-like kinase Cdc5p and the WD-repeat protein Cdc20p/fizzy are regulators and substrates of the anaphase promoting complex in Saccharomyces cerevisiae. EMBO J. *17*, 1336–1349.

Shteinberg, M., Protopopov, Y., Listovsky, T., Brandeis, M., and Hershko, A. (1999). Phosphorylation of the cyclosome is required for its stimulation by Fizzy/cdc20. Biochem. Biophys. Res. Commun. *260*, 193–198.

Straight, A.F., and Murray, A.W. (1997). The spindle assembly checkpoint in budding yeast. Methods Enzymol. *283*, 425–440.

Taylor, S.S., and McKeon, F. (1997). Kinetochore localization of murine Bub1 is required for normal mitotic timing and checkpoint response to spindle damage. Cell *89*, 727–735.

Tugendreich, S., Tomkiel, J., Earnshaw, W., and Hieter, P. (1995). CDC27Hs colocalizes with CDC16Hs to the centrosome and mitotic spindle and is essential for the metaphase to anaphase transition. Cell *81*, 261–268.

van der Reijden, B.A., Erpelinck-Verschueren, C.A., Löwenberg, B., and Jansen, J.H. (1999). TRIADs: a new class of proteins with a novel cysteine-rich signature. Protein Sci. *8*, 1557–1561.

Visintin, R., Prinz, S., and Amon, A. (1997). CDC20 and CDH1: a family of substrate-specific activators of APC-dependent proteolysis. Science *278*, 460–463.

Wassmann, K., and Benezra, R. (1998). Mad2 transiently associates with an APC/p55Cdc complex during mitosis. Proc. Natl. Acad. Sci. USA *95*, 11193–11198.

Yu, H., Peters, J.M., King, R.W., Page, A.M., Hieter, P., and Kirschner, M.W. (1998). Identification of a cullin homology region in a subunit of the anaphase-promoting complex. Science *279*, 1219–1222.

Zachariae, W., and Nasmyth, K. (1999). Whose end is destruction: cell division and the anaphase-promoting complex. Genes Dev. *13*, 2039–2058.

Zachariae, W., Schwab, M., Nasmyth, K., and Seufert, W. (1998). Control of cyclin ubiquitination by CDK-regulated binding of Hct1 to the anaphase promoting complex. Science *282*, 1721–1724.

Accession Numbers

The sequence of the Emi1 protein reported in this paper has been deposited in the Protein Data Bank with accession number AF319594.