Emi1 is needed to couple DNA replication with mitosis but does not regulate activation of the mitotic APC/C

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Ubiquitin-mediated proteolysis is critical for the alternation between DNA replication and mitosis and for the key regulatory events in mitosis. The anaphase-promoting complex/cyclosome (APC/C) is a conserved ubiquitin ligase that has a fundamental role in regulating mitosis and the cell cycle in all eukaryotes. In vertebrate cells, early mitotic inhibitor 1 (Emi1) has been proposed as an important APC/C inhibitor whose destruction may trigger activation of the APC/C at mitosis. However, in this study, we show that the degradation of Emi1 is not required to activate the APC/C in mitosis. Instead, we uncover a key role for Emi1 in inhibiting the APC/C in interphase to stabilize the mitotic cyclins and geminin to promote mitosis and prevent rereplication. Thus, Emi1 plays a crucial role in the cell cycle to couple DNA replication with mitosis, and our results also question the current view that the APC/C has to be inactivated to allow DNA replication.

Introduction

Ubiquitylation and proteolysis of cell cycle regulators is essential for control of the cell cycle. In particular, the anaphase-promoting complex/cyclosome (APC/C) ubiquitin ligase regulates mitosis by directing the degradation of key substrates at different times during mitosis. The APC/C is regulated at several levels to ensure that (1) its activity is restricted to mitosis and G1 phase and that (2) it recognizes specific substrates at specific times. This is achieved through its phosphorylation in mitosis, through its interaction with coactivators (including Cdc20 and Cdh1), and by inhibitors such as regulator of cyclin A 1 (Rca1)/early mitotic inhibitor 1 (Emi1; Harper et al., 2002; Peters, 2002; Castro et al., 2005).

Interaction between the APC/C and its two coactivators is regulated during the cell cycle (for review see Peters, 2006). Cdc20 can only interact with the phosphorylated form of APC/C that is specific to mitosis and is itself degraded after metaphase, thereby restricting its activity to the first part of mitosis. In contrast, Cdh1 can interact with both the interphase and mitotic forms of the APC/C, and its binding depends on its phosphorylation status. In budding yeast, Cdh1 is phosphorylated by B-type cyclin–Cdk complexes from S phase to anaphase, and this prevents it from binding to the APC/C. The Cdc14 phosphatase subsequently dephosphorylates Cdh1 at mitotic exit (Zachariae et al., 1998; Jaspersen et al., 1999), and the Cdh1-bound form of the APC/C (APC/C^{Cdh1}) aids mitotic exit and regulates G1 phase. Cdh1 is phosphorylated in metazoans too, and this also prevents its binding to APC/C (Lukas et al., 1999; Kramer et al., 2000; Sorensen et al., 2001). However, how Cdh1 phosphorylation is regulated during the cell cycle is less clear.

In metazoans, APC/C^{Cdh1} is negatively regulated in interphase in *Drosophila melanogaster* by Rca1 and in mammalian and *Xenopus laevis* cells by the orthologous protein Emi1. The *Drosophila* protein Rca1 was originally identified as a regulator of cyclin A whose mutation induces an arrest in G2 phase of cell cycle 16, which is similar to that observed in cyclin A mutants (Dong et al., 1997). Rca1 mutant cells block in G2 phase and prematurely degrade mitotic cyclins in a Cdh1-dependent manner (Grosskortenhaus and Sprenger, 2002). Similarly, in *Xenopus* extracts, Emi1 immunodepletion prevents mitotic entry, and cyclins are prematurely degraded (Reimann et al., 2001a).

Emi1 was originally identified in a yeast two-hybrid screen for F-box proteins using Skp1 as bait (Reimann et al., 2001a). Emi1 also contains a zinc-binding domain that is important for APC/C inhibition. Emi1 levels are regulated during the cell cycle: in mammalian cells, its transcription is induced at the G1/S

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Abbreviations used in this paper: APC/C, anaphase-promoting complex/cyclosome; DIC, differential interference contrast; Emi1, early mitotic inhibitor 1; IRES, internal ribosome entry site; MCM, minichromosome maintenance; NEBD, nuclear envelope breakdown; Rca, regulator of cyclin A; RPE, retinal pigment epithelium.

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transition under the control of E2F, and this has been reported to be required to stabilize cyclin A and allow cells to begin S phase (Hsu et al., 2002). Overexpressing Rca1 in G1-phase cells has also recently been shown to promote S phase in *Drosophila* embryos in an F-box–dependent manner (Zielke et al., 2006). Emi1 is subsequently degraded at the entry to mitosis (Reimann et al., 2001a; Castro et al., 2005) by the $SCF^{\beta\text{-}TrCP}$ ubiquitin ligase (Guardavaccaro et al., 2003; Margottin-Goguet et al., 2003). $SCF^{\beta\text{-}TrCP}$ recognizes Emi1 only after it has been phosphorylated by Plk1 (Hansen et al., 2004; Moshe et al., 2004), which creates the phosphodegron DSGxxS. Recently, the Evi5 protein has been reported to stabilize Emi1 in S and G2 phases by blocking its phosphorylation by Plk1 (Eldridge et al., 2006).

Emi1 has been proposed to have a wider role than Rca1 by acting on APC/C^{Cdc20} as well as APC/C^{Cdh1} and, thus, to play a key role in controlling activation of the APC/C in mitosis. Emi1 has been reported to inhibit APC/C^{Cdc20} when cells begin mitosis such that its degradation is essential for APC/C^{Cdc20} activation and the degradation of mitotic regulators (such as cyclins A and B and securin; Reimann et al., 2001a; Margottin-Goguet et al., 2003). Excess Emi1 inhibits cyclin B ubiquitylation when added to an in vitro ubiquitylation assay, and excess Emi1 added to *Xenopus* egg extracts prevents cyclins A and B, securin, and geminin degradation (Reimann et al., 2001a). Furthermore, overexpressing Emi1 in vertebrate cells induces a delay in mitosis (Reimann et al., 2001a; Hsu et al., 2002), which has been interpreted as evidence that it inhibits Cdc20. In contrast, genetic interactions in *Drosophila* are not consistent with a role for Rca1 in the inhibition of Cdc20 (Grosskortenhaus and Sprenger, 2002).

In this study, we have investigated the role and regulation of Emi1 in mammalian cells in vivo. We find that Emi1 is normally degraded in prophase but that a nondegradable version of Emi1 has no effect on the timing or extent of cyclin A destruction nor on the destruction of the majority of cyclin B1 and securin. Thus, we show that Emi1 does not regulate activation of the APC/C at mitosis. Rather, we find that the major requirement for Emi1 is to allow the accumulation of mitotic cyclins and geminin in G2 phase in order to allow cells to enter mitosis and to prevent rereplication.

Results

Emi1 is degraded in prophase before the APC/C is activated

We set out to test the role of Emi1 in regulating the APC/C at mitosis. We used a commercial antibody to characterize Emi1 in human cells. We confirmed that the antibody is specific for Emi1 because it recognized a band of the correct size (~50 kD) on an immunoblot of whole cell lysate (Fig. 1 and Fig. S1 A, lane 1; available at http://www.jcb.org/cgi/content/full/jcb.200611166/DC1) that decreased after treating cells with siRNA oligonucleotides specific for Emi1 (Fig. S1 A, lanes 4–5). Furthermore, it recognized higher molecular mass bands of the correct size after transfecting plasmids encoding HA-tagged or fluorescent protein-tagged forms of Emi1 into human tissue culture cells (Fig. S1 A, lanes 2–3). A minor faster migrating band was also reproducibly detected whose significance is unknown but seemed to be related to Emi1, as it also disappeared after siRNA treatment (Fig. S1 A). Using this antibody, Emi1 protein levels were analyzed in HeLa cells synchronized by a thymidine/aphidicolin block and release regime (see Materials and methods); samples were collected during S and G2 phases and mitosis. The cell cycle phase was monitored by flow cytometry, which showed that most of the cells passed through mitosis between 12 and 15 h after release from the aphidicolin block. Emi1 levels decreased 11 h after release (Fig. 1 A), when most of the cells were in late G2 phase or mitosis, and before cyclin B1 levels decreased at 13 h, which is consistent with a previous study (Hsu et al., 2002). This was ~2 h after we could detect an increase in Plk1 activity as judged by phosphorylation on S133 of cyclin B1, a known Plk1 phosphorylation site (Fig. 1 A; Jackman et al., 2003).

The degradation of Emi1 had been reported to be essential for activation of the APC/C at mitotic entry and, thus, for the degradation of early mitotic regulators, including cyclin A. Therefore, it was important to characterize the exact timing of Emi1 degradation. With this aim, we generated a chimeric protein in which the Venus fluorescent protein (Nagai et al., 2002) was fused to the N terminus of Emi1. A plasmid encoding Venus-Emi1 was injected into HeLa cells in G2 phase, and the fluorescence levels were measured as a read-out for Emi1 degradation. Venus-Emi1 started to be degraded in prophase usually before or occasionally at nuclear envelope breakdown (NEBD; Fig. 1 C), as determined by differential interference contrast (DIC) images (Fig. 1 B). The earliest time we observed Emi1 proteolysis to start was 20 min before NEBD. We also analyzed Emi1 degradation in the nontransformed hTert–retinal pigment epithelium (RPE) cell line and observed very similar timings (Fig. S1 B). In vitro, Emi1 has to be phosphorylated by Plk1 before it can be degraded (Hansen et al., 2004; Moshe et al., 2004), and the timing that we observe for Emi1 degradation is consistent with the time when Plk1 is activated (Golsteyn et al., 1995).

A β-TrCP recognition site (DSGxxS) had been identified in Emi1 (Guardavaccaro et al., 2003; Margottin-Goguet et al., 2003), and mutating either Asp or Gly in this motif prevented the recognition of other substrates by β-TrCP (Liu et al., 2002). Therefore, we generated two different Emi1 mutants (D144A and G146V) in the β-TrCP recognition site and tested their stability. Both mutants were stable in HeLa (Fig. 1 D) and hTert-RPE (Fig. S1 C) cells progressing through G2 phase and mitosis, confirming that the β-TrCP site was required for the degradation of Emi1 in prophase. We also generated a mutant (S145A/S149A) to ablate phosphorylation by Plk1 and found that this also stabilized Emi1 in mitosis (Fig. 1 D), which is consistent with previous studies (Guardavaccaro et al., 2003; Margottin-Goguet et al., 2003; Hansen et al., 2004). In most of the experiments described in this and the next section, each of the D144A and G126V degradation mutants and the S145A/S149A phosphorylation mutants were used to rule out any effects specific to only one mutant. We could also demonstrate that the S145A/S149A mutant retained its biological activity by using it to rescue cells depleted of endogenous Emi1 by siRNA (unpublished data).

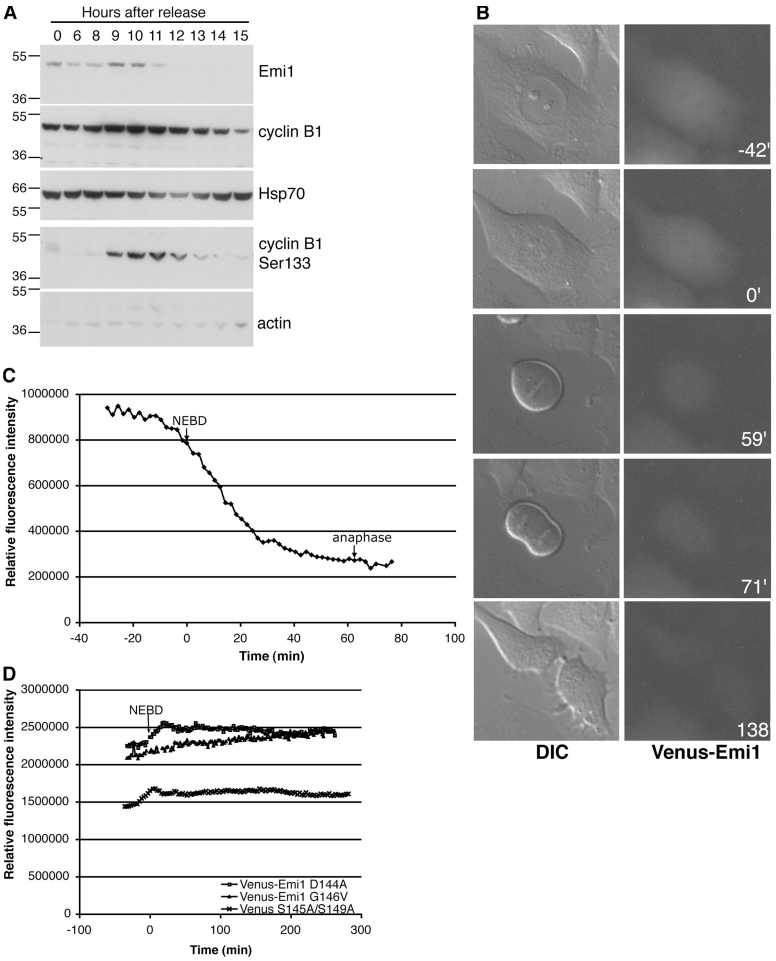

Figure 1. **Emi1 is degraded in prophase.** (A) Emi1 levels were analyzed by Western blotting in extracts from HeLa cells synchronized at the G1/S transition by thymidine/aphidicolin block and then released and followed through S and G2 phases and mitosis. Cyclin B1 levels were also analyzed. Anti–cyclin B1 S133 phosphospecific antibody was used as a read-out of Plk1 activity. Hsp70 and actin were used as loading controls. Molecular mass markers (given in kilodaltons) are shown. (B) HeLa cells injected with Emi1 were followed by video microscopy through G2 phase and mitosis. The DIC images were used to determine the different phases and mitotic stages. Time (given in minutes) is shown taking NEBD as t = 0. In general, cells expressing ectopic Emi1 took slightly longer to complete mitosis than control cells. (C) Quantification of fluorescence levels of the cell shown in B. Time of NEBD and anaphase are indicated. Representative of 39 cells from 11 experiments. (D) Two different mutants in the β-TrCP recognition site (D144A and G146V) and the S145A/S149A phosphorylation site mutant are stable after injection in HeLa cells. Representative of 43 cells from 10 experiments for D144A mutant, 44 cells from eight experiments for G146V mutant, and 30 cells from five experiments for the S145A/S149A mutant.

The timing of Venus-Emi1 degradation with respect to NEBD was variable and, in some cases, started 20 min before NEBD; in comparison, the earliest mitotic APC/C substrates (e.g., cyclin A) are degraded at or after NEBD (den Elzen and Pines, 2001; Geley et al., 2001). When we compared the timing of Emi1 and cyclin A degradation in the same cell using Venus-Emi1 and cyclin A–CFP, we found that these were also variable and that Emi1 began to be degraded anywhere between 6 and 45 min (mean of 26 min; $n = 8$), before cyclin A. This made it unlikely that Emi degradation was the trigger that directly activated APC/C^{Cdc20} at mitosis, although it could still be a prerequisite to activate APC/C^{Cdc20}.

Emi1 degradation is not required to activate the APC/C

To determine whether Emi1 had to be degraded to activate APC/C^{Cdc20}, we injected wild-type and nondegradable Emi1 constructs into cells and assayed the effect on the degradation of the three most well-characterized substrates of APC/C^{Cdc20}: cyclin A, cyclin B1, and securin. We assayed both the timing of degradation in vivo and the level of endogenous proteins remaining in cells arrested in mitosis after the injection of Emi1.

When we injected RNA encoding nondegradable Emi1 at 1 μg/μl, this produced levels of Emi1 similar to those found in previously published transient transfection experiments, and we found that as reported, cells delayed in mitosis (Reimann et al., 2001a; Hsu et al., 2002; Margottin-Goguet et al., 2003). However, in these experiments, Emi1 was expressed at 10–30-fold more than its endogenous level (see Materials and methods; Lehman et al., 2006). Thus, the delay in mitosis might not be apparent if we expressed Emi1 closer to its normal levels, which would be consistent with cells lacking β-TrCP (and thus unable to degrade Emi1) that only exhibit a slight delay (∼30 min) in exit from mitosis after release from a nocodazole block (Guardavaccaro et al., 2003). Therefore, we reduced the amount of RNA encoding nondegradable Emi1 between 2- and 10-fold (0.1 and 0.5 μg/μl) and found that 51% of cells ($n = 45$) expressing lower amounts of Emi1 were able to perform anaphase successfully and reenter interphase. This indicated that the cells must have activated the APC/C and degraded most or all of their securin and mitotic cyclins.

The delay in mitosis seen with high levels of nondegradable Emi1 had been attributed to inhibition of the APC/C and, in particular, protecting cyclin A from destruction. To determine whether the timing of cyclin A degradation was affected by high levels of nondegradable Emi1, we analyzed cyclin A degradation in living cells after coinjection with high levels of any of the three nondegradable Emi1 mutants. We found that cyclin A–CFP degradation was unaffected by the presence of Emi1 (Fig. 2 A). To rule out any possible artifact caused by the presence of the

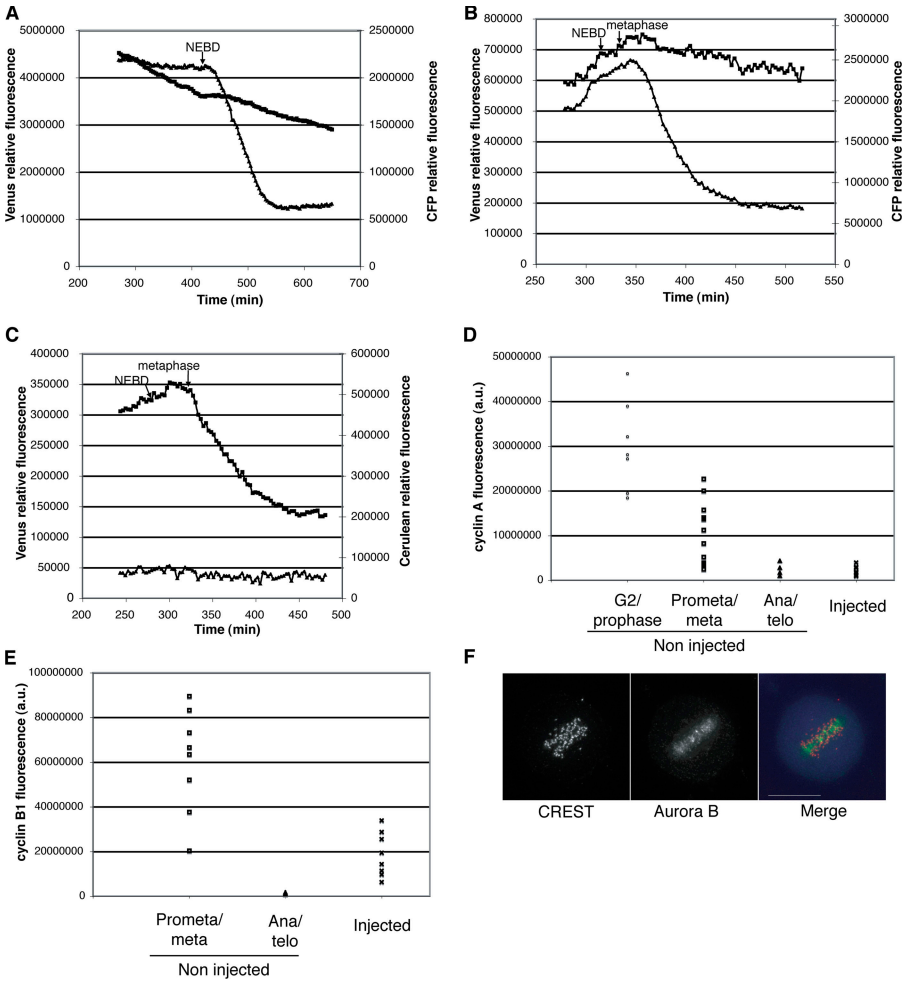

Figure 2. **The timing of degradation of cyclins A and B1 and securin is not affected by the presence of Emi1.** (A) Cyclin A–CFP: HeLa cells were coinjected in G2 phase with Venus-Emi1 D144A and cyclin A–CFP and followed by time-lapse microscopy. The fluorescence levels of both proteins were measured and plotted over time. NEBD was determined from the DIC images. This result is representative of four cells from two experiments with Venus-Emi1 D144A, 14 cells from three experiments with Venus-Emi1 G146V, and eight cells from five experiments with Venus-Emi1 S145A/S149A. The apparent decrease of Venus-Emi1 D144A fluorescence is the result of photobleaching. (B) Cyclin B1–CFP: HeLa cells were coinjected in G2 phase with Venus-Emi1 D144A and cyclin B1–CFP and analyzed as in A. This result is representative of 15 cells in six experiments with Venus-Emi1 D144A and six cells in three experiments with Venus-Emi1 G146V. (C) Securin-Cerulean: HeLa cells were coinjected in G2 phase with Venus-Emi1 D144A and securin-Cerulean followed by time-lapse fluorescence microscopy, and the fluorescence levels were measured and analyzed as in A and B. This result is representative of seven cells in three experiments with Venus-Emi1 D144A. Note that in these experiments, lower amounts of Venus-Emi1 D144A were injected. (D) Cyclin A: HeLa cells injected with Venus-Emi1 D144A in G2 phase were followed by time-lapse microscopy and fixed after they had delayed in metaphase. Cells were stained for cyclin A, and the levels were quantified in the injected arrested cells and in control noninjected cells at different stages. The quantification shown is of PFA-fixed cells, and similar results were obtained after methanol/acetone fixation. Similar results were obtained with two different cyclin A antibodies. Results are representative of four experiments. (E) Cyclin B1 levels analyzed on cells injected, filmed, and stained as in D. The quantification shown is of cells fixed in methanol/acetone, and similar results were obtained after PFA fixation. Results are representative of five experiments. (F) HeLa cells that delay in mitosis after Venus-Emi1 D144A injection (blue in the merge) were fixed and stained with CREST serum (red in the merge) and aurora B antibodies (green in the merge). Representative of 11 cells analyzed. Bar, 15 μm.

tag on Emi1, we also analyzed cyclin A degradation in cells coinjected with untagged Emi1 D144A expressed from an internal ribosome entry site (IRES) vector coexpressing GFP and obtained similar results (Fig. S2 A, available at http://www.jcb.org/cgi/content/full/jcb.200611166/DC1). Similarly, the timing of cyclin B1 and securin degradation, which starts at metaphase (Clute and Pines, 1999; Hagting et al., 2002), was unaffected when nondegradable Emi1 mutants were coinjected with cyclin B1–CFP (Fig. 2 B) or securin-Cerulean (Fig. 2 C). Similar results were obtained when the wild-type version of Venus-Emi was coinjected with cyclins A and B1 or securin (unpublished data). Thus, APC/C^{Cdc20} is activated upon entry to mitosis regardless of the presence of even supraphysiological levels of Emi1. Although the timing of securin destruction was unaffected, we did find that its extent was more sensitive to the levels of Emi1 than either cyclin A or B1 destruction.

We also analyzed the levels of the endogenous cyclins and securin in cells arrested in mitosis by high levels of nondegradable Emi1. Each of the three nondegradable Emi1 mutants were injected separately into G2-phase HeLa cells, and cells were followed by time-lapse DIC microscopy. After the cells were delayed for >2 h in mitosis, they were fixed and processed for immunofluorescence. Although immunofluorescence is not a truly quantitative approach, the relative levels of cyclins A and B1 and securin in noninjected control cells at different stages of mitosis were consistent with the known timings of their degradation. In noninjected cells, cyclin A levels were maximal in G2 phase and prophase, decreased in prometaphase and metaphase, and were almost undetectable in anaphase and telophase cells (Fig. 2 D), which is consistent with cyclin A degradation starting in prometaphase (den Elzen and Pines, 2001; Geley et al., 2001). The immunofluorescence signal of endogenous cyclin A in cells expressing high levels of a nondegradable Emi1 that had delayed in a metaphase-like state was similar to the intensity of noninjected anaphase/telophase cells (Fig. 2 D), indicating that, like the cyclin A–CFP marker, endogenous cyclin A was degraded completely as normal. The three nondegradable Emi1 mutants produced identical results.

Cyclin B1 degradation normally starts when the spindle checkpoint is satisfied at metaphase (Clute and Pines, 1999). Consistent with this, we observed maximum cyclin B1 levels in prometaphase control noninjected cells, whereas the signal was

almost undetectable in anaphase and telophase cells (Fig. 2 E). When we analyzed the cells delayed by high levels of Emi1, we found that cyclin B1 levels were lower than in control prometaphase or metaphase cells but not as low as in control anaphase and telophase cells (Fig. 2 E). This showed that the bulk of cyclin B1 had been degraded but that a fraction of cyclin B1 remained, accounting for the cells delaying in mitosis. To rule out any artifact caused by the presence of the tag, we quantified the levels of either endogenous cyclins A (Fig. S2 B) or B1 (Fig. S2 C) in cells that had delayed in mitosis after injecting untagged Emi1 D144A expressed from an IRES vector. For both cyclins, results were identical to those obtained with Venus-D144A injection (Fig. 2, D and E).

Staining Emi1-arrested cells for endogenous securin showed that some securin also remained (unpublished data), which was consistent with the cells delaying in metaphase, and we confirmed that the sister chromatids were still attached by staining the cells with anti–Aurora B and autoimmune CREST antibodies to visualize the kinetochores and centromeres (Fig. 2 F).

To assay the effect of Emi1 on mitosis in nontransformed cells, we used hTert-RPE cells. Cells were stimulated to reenter in the cell cycle after serum starvation and were injected with nondegradable Emi1 when they had reached G2 phase. With high levels of Emi1, these cells also delayed in mitosis. We fixed the cells, measured the levels of endogenous cyclins A and B1 by immunofluorescence, and compared these with the levels in control noninjected cells. We found that as in HeLa cells, endogenous cyclins A and B1 had been degraded (Fig. S2, D and E). Thus, in both normal and transformed cells, Emi1 did not have to be degraded for all of cyclin A and the bulk of cyclin B1 to be destroyed. Although a fraction of cyclin B1 remained in cells expressing supraphysiological levels of Emi1, cyclin B1 destruction began at its normal time. In all, these results are clearly inconsistent with the model that Emi1 degradation activates APC/C^{Cdc20} and with the proposal that Emi1 is required to stabilize cyclin A in early mitosis.

Emi1 regulates mitotic entry, stabilizing cyclins A and B1

Because Emi1 did not appear to control activation of the mitotic APC/C, we investigated its role in the cell cycle by depleting it by siRNA. Two different siRNA duplexes specific for Emi1 (siEmi1_1 and siEmi1_2) were effective in reducing Emi1 protein levels, although to different extents (see Fig. 5 A). To analyze the effects of reducing Emi1 levels during the cell cycle, synchronized cells were treated with siRNA, and cells were analyzed by flow cytometry. Emi1 siRNA–treated cells arrested at the G1/S transition in an aphidicolin block and progressed through S and G2 phase after release from aphidicolin in a similar manner to that of control siRNA–treated cells (Fig. 3 A). Labeling cells with short pulses of BrdU to reveal the pattern of replication foci in S phase showed that Emi1-depleted cells also resembled control cells in having many evenly distributed foci in early S phase and fewer foci that tend to be closer to the nuclear envelope in mid- to late S phase (Fig. 3 B; Nakayasu and Berezney, 1989). These changes likely reflect the firing of early, mid-, and late origins of replication. However, Emi1-depleted

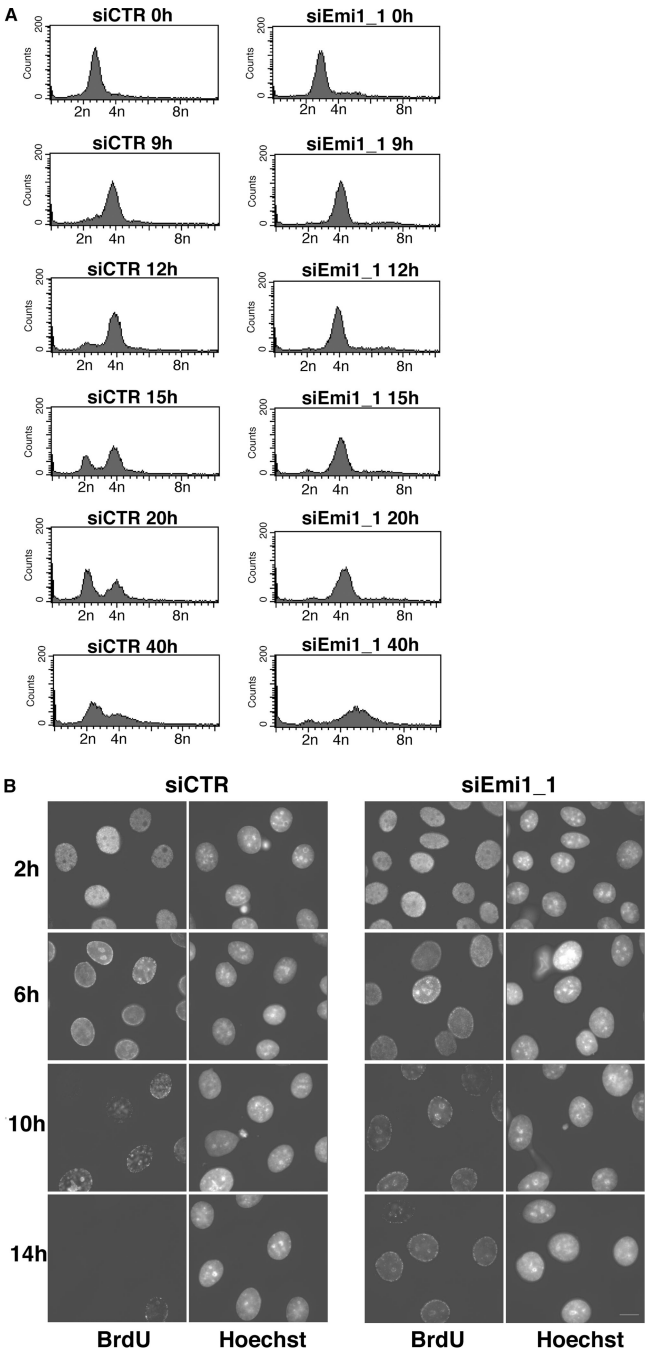

Figure 3. **Emi1 is required for mitotic entry.** (A) Thymidine/aphidicolin-synchronized cells were transfected during the release from thymidine with either control (left) or Emi1_1 (right) siRNA oligonucleotides. Cells were analyzed at various times after aphidicolin release by propidium iodide staining and flow cytometry. The time indicates hours after aphidicolin release. Representative of two experiments. (B) Cells were transfected as in A, labeled with BrdU for 30 min before fixation, and stained with anti-BrdU antibodies at the indicated times after release from an aphidicolin block. Total DNA was stained with Hoechst 33342. Bar, 10 μm.

cells differed from controls in that the foci persisted after cells had reached a 4N DNA content as judged by flow cytometry.

Around 15 h after release from the aphidicolin block, most of the control cells had progressed through mitosis, and the bulk of the cells had entered the following G1 phase (2N DNA content) after 20 h (Fig. 3 A). However, at these time

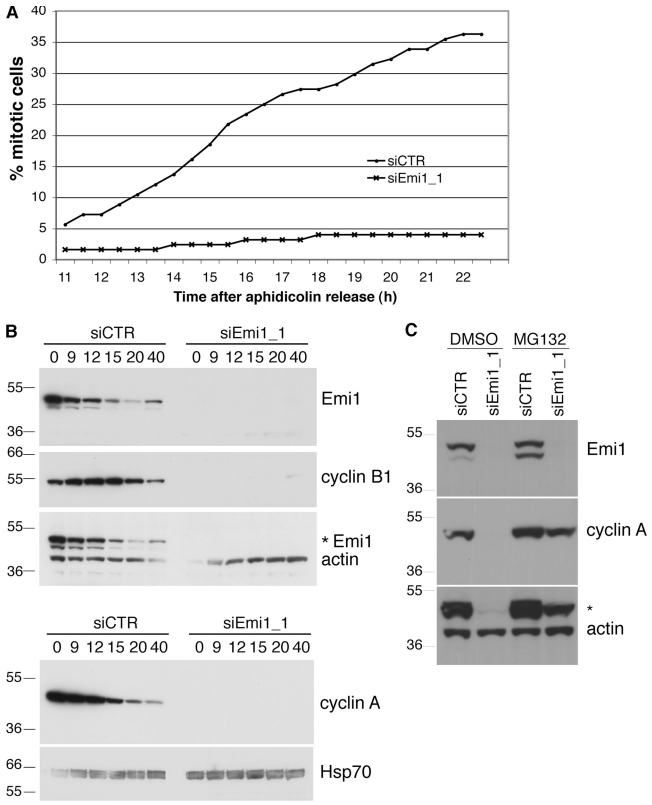

Figure 4. **Emi1 is required to accumulate cyclins A and B1.** (A) Cumulative number of cells entering mitosis (NEBD) plotted over time, as observed by time-lapse DIC microscopy of cells transfected with either control or Emi1_1 siRNA. Representative of three experiments. (B) Emi1 and cyclins A and B1 levels in cells transfected with either control or siEmi1_1 siRNA were analyzed by Western blotting. These samples correspond to the time points after release from a G1/S block as shown in Fig. 3 A. Actin and Hsp70 are shown as loading controls. The asterisk marks residual Emi1 signal. The same extracts were run on two different gels (top and bottom), and a loading control is shown for each gel. Results are representative of two experiments. (C) HeLa cells were transfected with either control (siCTR) or Emi1 (siEmi1_1) siRNA oligonucleotides, and, 24 h after transfection, MG132 was added for 6 h. Actin is shown as a loading control. The asterisk denotes residual cyclin A signal.

points, cells treated with siRNA targeting Emi1 accumulated with a 4N DNA content. At 40 h after release from the G1/S block, only a minor fraction of these cells had entered the next cell cycle (Fig. 3 A); most of the cells had increased in ploidy compared with control cells (Fig. 3 A and see Fig. 5).

To determine whether depleting Emi1 caused a block in G2 phase or mitosis, siRNA-treated cells were followed by time-lapse microscopy 11 h after release from an aphidicolin block. Fig. 4 A shows the cumulative total of cells that entered mitosis as judged by NEBD. Only a minor fraction of cells transfected with siRNA against Emi1 entered mitosis (Fig. 4 A), indicating that the cells accumulating in the 4N DNA peak observed by flow cytometry were delayed in G2 phase and not in mitosis. Similar results were obtained with an independent siRNA oligonucleotide specific for Emi1 (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200611166/DC1).

In *Drosophila* cells and *Xenopus* egg extracts, mitotic entry was blocked when Rca1 or Emi was mutated or depleted (Reimann et al., 2001a; Grosskortenhaus and Sprenger, 2002), and this correlated with a failure to accumulate mitotic cyclins. To test whether this was also the case in mammalian cells, we collected samples at the same time points as for flow cytometry analysis and analyzed the levels of Emi1 and cyclins A and B1 by immunoblotting. In cells treated with the control siRNA oligonucleotides, Emi1 levels decreased 12–15 h after release from a G1/S block, when most of the cells were entering mitosis. As expected, cyclin A levels decreased with a similar timing, whereas cyclin B1 levels decreased later (Fig. 4 B). In stark contrast, in Emi1-depleted cells, cyclin A and B1 levels were almost undetectable (Fig. 4 B). Treating Emi1-depleted cells with a proteasome inhibitor caused cyclin A to accumulate, showing that Emi1 was indeed required to stabilize it (Fig. 4 C). Thus, the impairment in mitotic entry in Emi1-depleted cells correlated with a failure to accumulate cyclins A and B1.

Emi1 is required to prevent rereplication

In budding and fission yeasts and in *Drosophila* cells, the mitotic cyclins have an important role in preventing rereplication, which might provide an explanation for the increase in ploidy in Emi1-depleted cells after 40 h (Fig. 3 A). However, in human cells, geminin has been implicated as the primary block to rereplication (Melixetian et al., 2004; Zhu et al., 2004). To study this further, we depleted Emi1 by siRNA in asynchronous HeLa and hTert-RPE cells and harvested the cells 24, 48, and 72 h after transfection. We used two different siRNA oligonucleotides to deplete Emi1, one of which (Emi1_2) was less effective than the other (Emi1_1), particularly at later time points (Fig. 5 A). Flow cytometry analysis showed that in contrast to cells treated with the control oligonucleotides, which continued to proliferate normally, most of the cells treated with the Emi1 siRNA oligonucleotides accumulated with a 4N DNA peak 24 h after transfection (Fig. 5 B), as we had observed in synchronized cells (Fig. 3 A). At later time points (48 and 72 h after transfection), a substantial number of cells became polyploid (Fig. 5 B), and this correlated with the appearance of enlarged nuclei (Fig. 5 C). Nuclei became even larger 72 h after transfection, indicating that the cells were actively replicating their DNA. We confirmed that the increase in polyploidy and enlarged nuclear size were caused by extra rounds of DNA replication by labeling cells with BrdU and measuring DNA synthesis by flow cytometry (Fig. S4 A, available at http://www.jcb.org/cgi/content/full/jcb.200611166/DC1).

The rereplication phenotype was more pronounced with oligonucleotide siEmi1_1 than with siEmi1_2, which is consistent with the more efficient knockdown observed by immunoblotting (Fig. 5 A). Cells treated with the siEmi1_2 siRNA oligonucleotides showed a partial phenotype both by flow cytometry and the number of enlarged nuclei visible after Hoechst staining. In particular, the rereplication phenotype seemed to be reduced 72 h after transfection, most likely because the proliferating nontransfected cells diluted out the rereplicating cells that were still visible as large nuclei (Fig. 5 C).

We confirmed these results in nontransformed hTert-RPE cells. Here, the phenotype was slightly less pronounced, which is consistent with a less efficient reduction in Emi1 levels (Fig. S4 B). Nevertheless, reducing Emi1 levels caused hTert-RPE cells to become polyploid (Fig. S4 C) with greatly enlarged

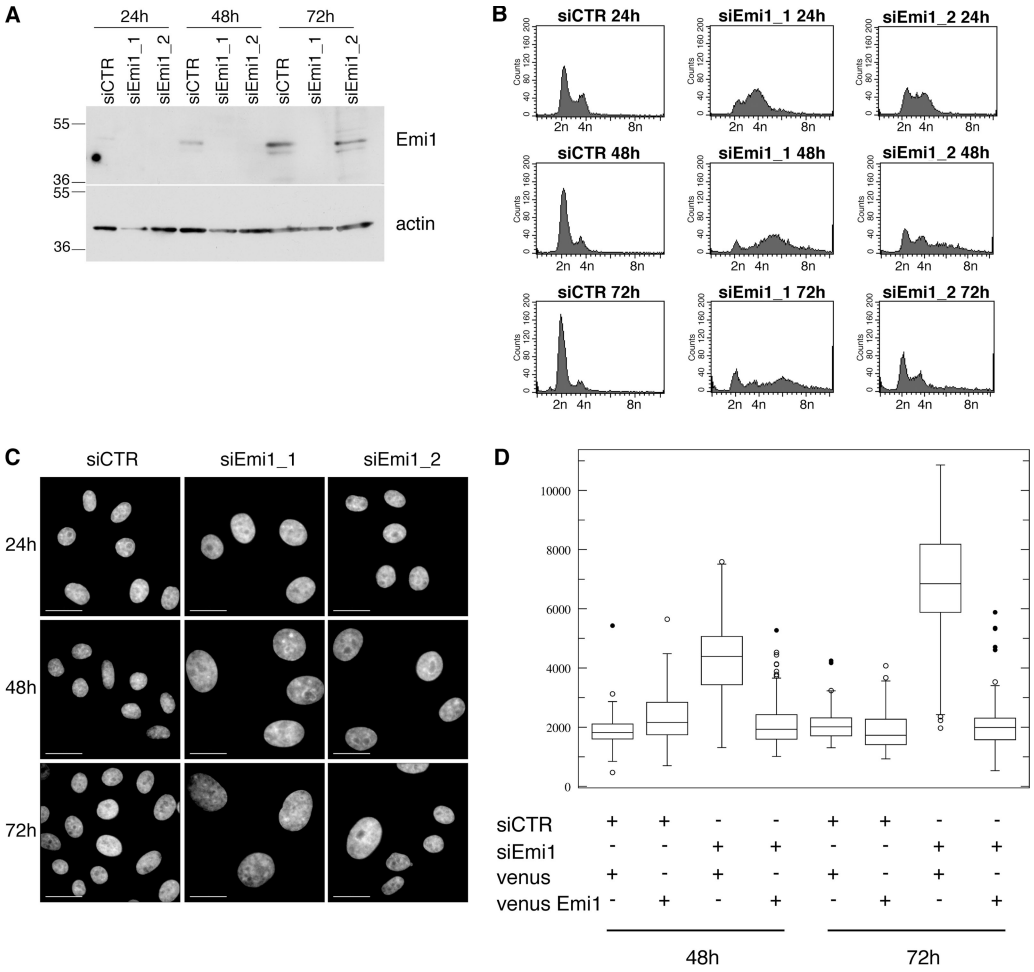

Figure 5. **Emi1 is required to prevent rereplication.** (A) Asynchronous HeLa cells were transfected with control or two different Emi1 siRNAs, and Emi1 levels were analyzed by Western blotting 24, 48, and 72 h after transfection. Actin is shown as a loading control. Results are representative of four experiments. (B) DNA content of cells transfected as in A and analyzed by flow cytometry after staining with propidium iodide. Results are representative of four experiments. (C) Cells transfected as in A were stained with Hoechst to visualize the nuclei. Bars, 30 μm. (D) Cells cotransfected with control and Emi1 siRNAs plus expression vectors encoding Venus (control) or Venus-Emi1 (as indicated) were fixed 48 and 72 h after transfection, and DNA was stained with Hoechst. The nuclear size (in pixels on the y axis) was measured using ImageJ software. The small bars in the dot plot show the minimum and maximum values, and the box shows the first and third quartiles. The bar in the box is the median value. Outliers (closed circles) and suspected outliers (open circles) as determined by statistical analysis are shown. Results are representative of two independent experiments. More than 100 nuclei were measured for each sample.

nuclei (Fig. S4 D). Together, these results indicated that Emi1-depleted cells undergo extra rounds of DNA replication.

To prove the specificity of this phenotype, we cotransfected siRNA oligonucleotides with an expression plasmid encoding Emi1. The siEmi1_1 siRNA oligonucleotide is directed against the STOP codon of Emi1 and its 3′ untranslated region; the Venus-Emi1 construct does not contain the 3′ untranslated region and is thus resistant to siRNA. Control and Emi1 siRNA duplexes were cotransfected with either a plasmid expressing Venus-Emi1 or an empty vector expressing Venus. Because the size of the nuclei correlates with the degree of ploidy (Fig. 5 C), we measured the area of the fluorescent nuclei when the Emi1 or control oligonucleotides were cotransfected with either pVenus or pVenus-Emi1. The nuclei of cells cotransfected with pVenus and the Emi1 siRNA oligonucleotides had clearly increased in size (Figs. 5 D and S4 E), which is similar to those in which the siRNA oligonucleotides alone were transfected (Fig. 5 C). In contrast, cotransfecting pVenus-Emi1 with the Emi1 siRNA oligonucleotides prevented the increase in nuclear size (Figs. 5 D and S4 E). Cotransfecting pVenus-Emi1 did not have a major effect on the size of nuclei in cells transfected with control siRNA oligonucleotides (Figs. 5 D and S4 E).

To rule out the possibility that cells cotransfected with pVenus-Emi1 and the Emi1 siRNA oligonucleotides did not re-replicate their DNA because the cells arrested in the cell cycle, we labeled cells with BrdU to measure DNA synthesis. In cells cotransfected with siRNA oligonucleotides against Emi1 and either the empty vector or Emi1-expressing plasmid, most of the cells were positive for BrdU, indicating that they were proliferating (unpublished data).

Rereplicating cells show increased levels of cyclin E and low levels of cyclins A and B1 and geminin

Previous studies had shown that both cyclins A and E are capable of stimulating DNA replication; therefore, we predicted that

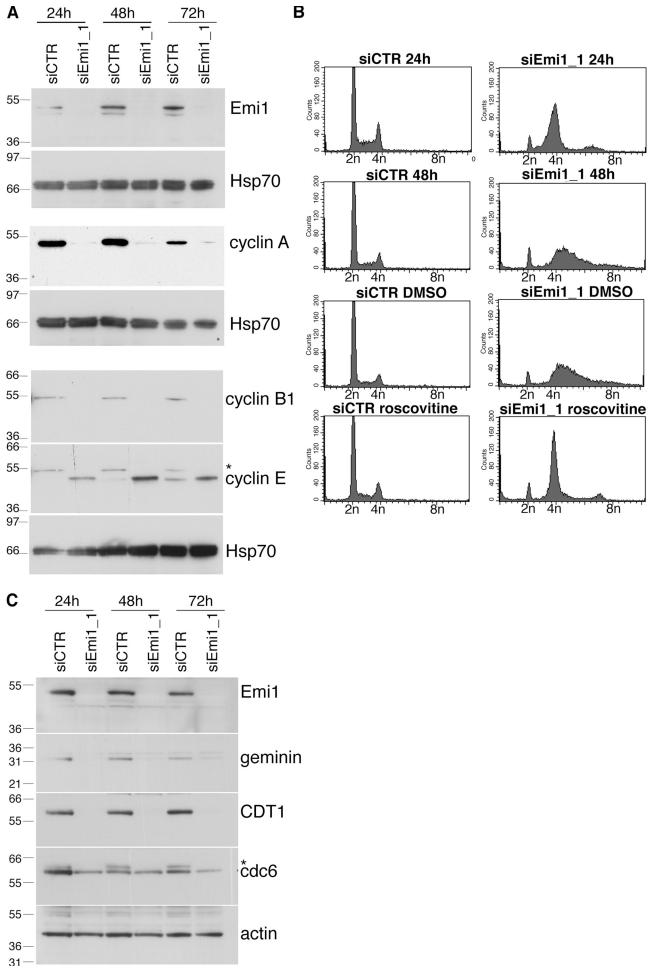

Figure 6. **Cyclins A and B1 and geminin levels are reduced, whereas cyclin E increases in rereplicating cells induced by Emi1 knockdown.** (A) Asynchronous HeLa cells were transfected with control (siCTR) or Emi1 (siEmi1_1) siRNA duplexes, and samples were collected 24, 48, or 72 h after transfection. Hsp70 is shown as a loading control. The asterisk denotes residual cyclin B1 signal. The same extracts were run on three different gels, and a loading control is shown for each gel. Results are representative of three independent experiments. (B) Asynchronous HeLa cells were transfected with either control (siCTR) or Emi1 (siEmi1_1) siRNA oligonucleotides, and cell ploidy was analyzed by flow cytometry. DMSO or roscovitine was added or not added 24 h after transfection, and cells were analyzed 24 h later. Results are representative of two independent experiments. (C) Cells were transfected as in A to analyze geminin, CDT1, and cdc6 levels. Actin is shown as a loading control. The asterisk denotes residual CDT1 signal. Results are representative of two independent experiments.

cells lacking cyclin A through the depletion of Emi1 might be able to rereplicate their DNA because they contained cyclin E. Therefore, we assayed siRNA-treated cells for the levels of cyclins A, B1, and E. We found that cells depleted of Emi1 had very low levels of cyclins A and B1 but higher than normal levels of cyclin E (Fig. 6 A), which is consistent with the idea that cyclin E–Cdk activity was driving the rounds of rereplication. To assay whether cyclin E–Cdk2 was responsible for driving DNA synthesis, we treated Emi1-depleted cells with roscovitine to inhibit Cdk2 and found that this blocked rereplication (Fig. 6 B), and the cells remained with a 4N DNA content.

As mentioned in the previous paragraph, another APC/C substrate, geminin, is required to prevent rereplication in several human cell lines. Geminin binds, inactivates, and apparently stabilizes Cdt1, which loads the minichromosome maintenance (MCM) protein complex onto DNA origins. We assayed the levels of geminin and Cdt1 on immunoblots and found that geminin and Cdt1 were substantially reduced in Emi1-depleted cells compared with control cells, whereas Cdc6 did not appear to change substantially (Fig. 6 C). Thus, Emi1 is required to stabilize the mitotic cyclins, geminin, and Cdt1 in interphase cells.

Emi1 is required to antagonize Cdh1

In *Drosophila*, Rca1 has been shown to be epistatic to fizzy related, the homologue of Cdh1. To determine whether APC/C^{Cdh1} was responsible for degrading cyclins A and B1 in cells depleted of Emi1, we cotransfected cells with siRNA duplexes targeting Emi1 and Cdh1 and assayed the effect on cell division and cyclin levels. Cells were transfected with siRNA duplexes against Emi1 together with either control oligonucleotides or in combination with siRNA oligonucleotides targeting Cdh1 or an equal amount of control duplex, and protein levels were assayed by immunoblotting (Fig. 7 A). This revealed that codepleting Cdh1 antagonized the effect of depleting Emi1 such that cyclin A could then accumulate (Fig. 7 A), as did cyclin B1 and geminin (Fig. 7 A). In agreement with this, in cells depleted of both Emi1 and Cdh1, nuclear size was very similar to control transfected cells and much smaller than Emi1-depleted cells (Fig. 7 B), indicating that codepleting Cdh1 stopped rereplication in cells lacking Emi1. Furthermore, the cell number of Emi1-depleted cells was much lower than control cells, as one would expect from rereplicating cells that stop dividing, whereas there were a similar number of control and Emi1/Cdh1-codepleted cells at 72 h after transfection, indicating that these cells were proliferating. In contrast, codepleting Cdc20 had no effect on the rereplication phenotype (unpublished data), which was to be expected because we found that Cdc20 was unstable in Emi1-depleted cells (unpublished data).

Flow cytometry analysis confirmed these conclusions: in contrast to cells depleted of Emi1 that arrested with a 4N DNA content, which increased overtime as the DNA was rereplicated, cells depleted of both Emi1 and Cdh1 entered and then exited mitosis (Fig. S5, available at http://www.jcb.org/cgi/content/full/jcb.200611166/DC1) with a similar time course to control cells. Assaying cell division by trapping mitotic cells with taxol and measuring the cumulative mitotic index confirmed that the bulk of the Emi1-depleted cells did not enter mitosis, whereas cells depleted of both Emi1 and Cdh1 accumulated in mitosis (Fig. 7 C). Therefore, in the absence of Cdh1, Emi1 does not appear to be required for cell division.

Discussion

In this study, we have shown that in contrast to the prevailing view, Emi1 does not regulate the APC/C at the beginning of mitosis but is essential for cells to enter mitosis by inhibiting the APC/C to allow mitotic cyclins to accumulate. In the absence of Emi1, APC/C^{Cdh1} remains active after mitosis, but cells are still able to begin the next S phase and eventually rereplicate their genomes because geminin continues to be degraded.

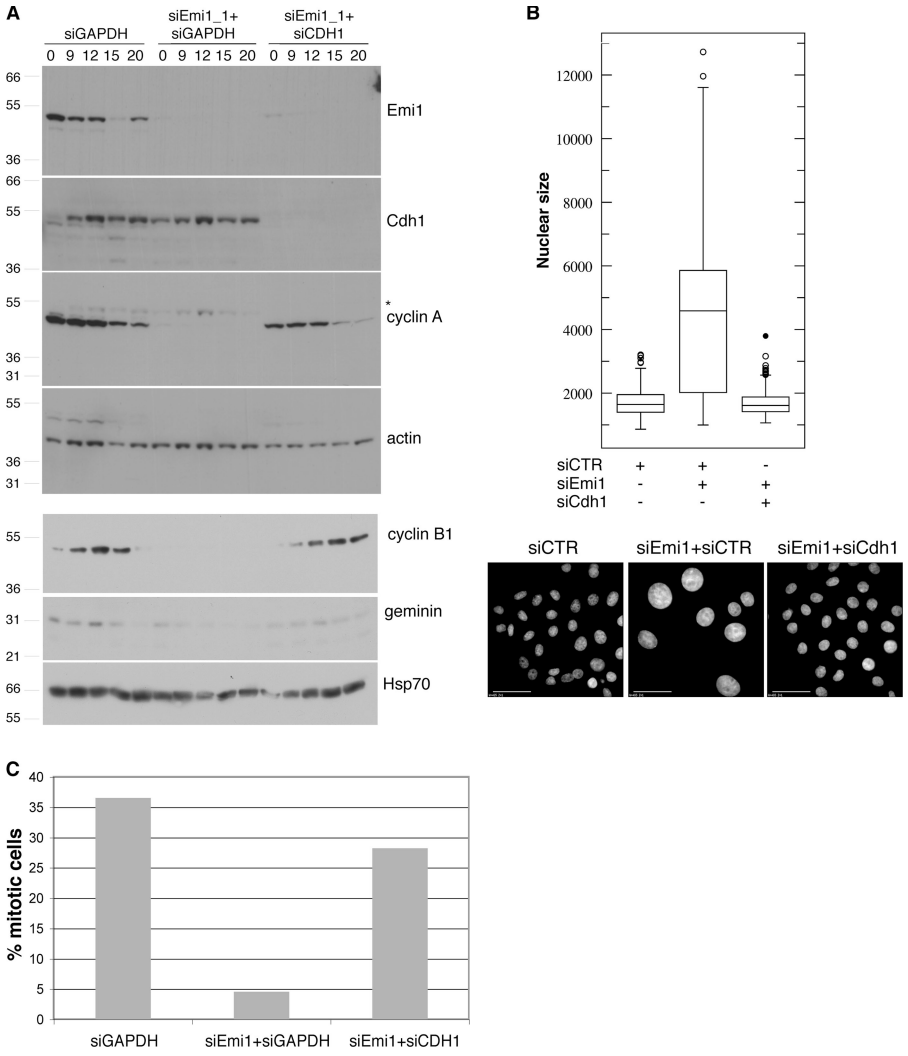

Figure 7. **Rereplication induced by Emi1 down-regulation depends on Cdh1 activity.** (A) Thymidine/aphidicolin-synchronized HeLa cells were transfected during release from the thymidine block with the indicated combinations of siRNA oligonucleotides directed against glyceraldehyde-3-phosphate dehydrogenase (negative control), Emi1, and Cdh1. Protein levels were analyzed at the indicated times after aphidicolin release. Actin is shown as a loading control. The asterisk denotes residual Cdh1 signal. (B) Asynchronous HeLa cells were transfected with siRNA oligonucleotides as indicated. 72 h after transfection, cells were fixed, and DNA was stained with Hoechst 33342. The nuclear size was measured and plotted as in Fig. 5 D. 200–300 nuclei were quantified for each sample. The small bars in the dot plot show the minimum and maximum values, and the box shows the first and third quartiles. The bar in the box is the median value. Outliers (closed circles) and suspected outliers (open circles) as determined by statistical analysis are shown. (C) Cells were synchronized and transfected as in A, taxol was added 10 h after release from aphidicolin, and mitotic figures were scored 10 h later as a measure of mitotic entry. About 1,500 cells were analyzed for each sample. (A–C) Representative of two experiments. Bars, 30 μm.

Therefore, Emi1 is essential to couple DNA replication and mitosis in the somatic cell cycle. Although Emi1 is degraded in prophase, this is not required to activate APC/C^{Cdc20}: in contrast to previous reports (Reimann et al., 2001a; Hsu et al., 2002), we find that cyclins A and B1 and securin all begin to be degraded at their normal times regardless of the amount of Emi1 remaining in cells. Moreover, our results also show that Emi1 does not have a special role in protecting cyclin A from early degradation by the APC/C^{Cdc20}. These findings have implications for how the cell cycle is regulated and question the general model for the regulation and role of the APC/C in interphase.

Emi1 degradation is not required to activate APC/C^{Cdc20}

Emi1 has been reported to be degraded just before the APC/C is activated in mitosis, and this constitutes an important control point because nondegradable Emi1 blocks cells in prometaphase by preventing the APC/C^{Cdc20} from being activated (Reimann et al., 2001a; Hsu et al., 2002). Thus, Emi1 has been proposed to play a role in inhibiting early mitotic APC/C^{Cdc20} activity before the spindle checkpoint becomes active and to prevent the premature degradation of cyclin A in early mitosis using a different mechanism from the spindle checkpoint (Reimann et al., 2001b; Miller et al., 2006). In contrast, we find that Emi1 begins to be degraded before NEBD, and, regardless of when this occurs, the timing of cyclin A degradation is unaffected. Furthermore, nondegradable forms of Emi1 delay cells in mitosis with no endogenous cyclin A remaining, and both cyclin B1 and securin degradation start on time. Thus, Emi1 destruction is not a prerequisite for APC/C^{Cdc20} activation, and Emi1 does not inhibit the early mitotic APC/C. Our results are consistent with results obtained in *Drosophila*, in which genetic analysis has shown that Rca1 does not act through Cdc20/fizzy (Grosskortenhaus and Sprenger, 2002).

Emi1 is targeted for destruction through phosphorylation by the Plk1 kinase, which creates a phosphodegron recognized by the $SCF^{\beta\text{-}TrCP}$ ubiquitin ligase (Hansen et al., 2004; Moshe et al., 2004), and our results are consistent with the phenotype of cells depleted of Plk1 by siRNA or treated with a Plk1 inhibitor in which Emi1 is stabilized but cyclin A is destroyed as normal in prometaphase (Kraft et al., 2003; van Vugt et al., 2004; Lénárt et al., 2007).

Our results contrast with another report that cyclin A degradation is delayed in Plk1 siRNA cells (Hansen et al., 2004),

but these data were obtained on a population of cells, which is less accurate than the single-cell analysis we performed in this study. Cyclin A and B1 levels have also been reported to be higher when β-TrCP is missing or depleted in β-TrCP$^{-/-}$ mouse embryonic fibroblasts or after siRNA treatment, which impairs the degradation of Emi1 (Guardavaccaro et al., 2003). However, this effect might be indirect.

When we injected nondegradable Emi1 closer to its endogenous level, about half of the cells progressed through and exited from mitosis with normal timing, indicating that Emi1 does not block APC/C^{Cdc20}. This is consistent with β-TrCP$^{-/-}$ mouse embryonic fibroblasts, where Emi1 degradation is prevented, which show only a minor delay in mitotic progression (Guardavaccaro et al., 2003), and with cells treated with the Plk1 inhibitor that exit mitosis when the spindle assembly checkpoint is abrogated (Lénárt et al., 2007). Although cyclins A and B1 and securin destruction begin on time in mammalian cells expressing supraphysiological levels of Emi1, the cells do delay in metaphase. However, transfecting ectopic Emi1 produces the protein at 10–30-fold over endogenous levels (Lehman et al., 2006; unpublished data), yet this only delays the destruction of 20% or less of cyclin B1 in the cell (Fig. 2 E). An active spindle checkpoint may not explain the delay in mitosis in cells expressing high levels of nondegradable Emi1 because Mad2 is no longer localized to the kinetochores (unpublished data).

Emi1 is required in G2 to accumulate mitotic cyclins and prevent rereplication

Our results show that Emi1 does not regulate the APC/C in early mitosis, but it is essential to inhibit the APC/C in interphase and, thereby, prevent rereplication. We find that depleting Emi1 by siRNA delays cells in G2 phase, specifically inhibiting mitotic entry, and this correlates with a failure to accumulate cyclins A and B1, as has been previously reported in *Drosophila* Rca1 mutants (Grosskortenhaus and Sprenger, 2002) and in *Xenopus* egg extracts depleted of Emi1 (Reimann et al., 2001a). Consistent with our observation that Emi1 is needed to prevent rereplication, in *Drosophila* Rca1 mutant cells, the nuclei were enlarged and had higher DNA levels (Grosskortenhaus and Sprenger, 2002), and cells from embryos expressing ectopic Cdh1/fizzy-related protein also endoreduplicate (Sigrist and Lehner, 1997). A requirement for Emi1 in normal proliferation but not in rereplicating cells is also consistent with the phenotype of Emi1$^{-/-}$ embryos, in which the inner cell mass cells die but the trophoblast giant cells (which undergo rereplication) are relatively unaffected (Lee et al., 2006). In contrast, two previous studies concluded that siRNA against Emi1 blocked DNA replication in mammalian cells, but one of these could not distinguish where in the cell cycle cells arrested (Hsu et al., 2002), and the other did not measure DNA replication directly (Rape and Kirschner, 2004).

In many organisms, mitotic cyclin–Cdk complexes prevent rereplication. In yeast, the loading and firing of DNA replication origins are inhibited by high cdk1 activity (Diffley, 2004). In fission yeast (Broek et al., 1991; Hayles et al., 1994) and *Drosophila* (Lehner, 1991; Sauer et al., 1995), mutations in mitotic cyclin–Cdk cause rereplication, and some mammalian cells also rereplicate after cdc2 is inactivated (Itzhaki et al., 1997). However, in other vertebrate cells, geminin has a more important role in inhibiting rereplication (Wohlschlegel et al., 2000). Regardless of the reliance placed by the cell on geminin or mitotic cyclin–Cdk complexes, Emi1 is required to prevent rereplication because it stabilizes both factors. During the preparation of this paper, a similar conclusion was reached by Machida and Dutta using human tissue culture cells (Dutta, A., personal communication).

Remarkably, we found that cells without Emi1 rereplicate with very low levels of Cdt1. This is consistent with the reduction in levels of geminin because Cdt1 stability depends on geminin. Cdt1 acts to load MCM complexes at DNA origins; therefore, it appears that the rereplication we observe has a reduced requirement for Cdt1 compared with normal replication, perhaps reflecting a difference in the behavior of the MCM complexes in the absence of cyclin A. In the absence of Emi1, there is also a substantial increase in the amount of cyclin E (which is not an APC/C substrate), and this correlates with rereplication that could be blocked by inhibiting Cdk2 activity with roscovitine. These results are consistent with the role for cyclin E–Cdk2 in endoreplication uncovered in both *Drosophila* (Knoblich et al., 1994) and mice (Geng et al., 2003; Parisi et al., 2003).

How is the APC/C regulated in interphase?

After mitosis, the APC/C remains active in G1 phase in both yeast and somatic animal cells. In budding yeast, the G1- (Amon et al., 1994) or S-phase (Yeong et al., 2001) cyclin–Cdks inactivate the APC/C by phosphorylating Cdh1, and this is maintained until anaphase (Amon et al., 1994; Zachariae et al., 1998; Jaspersen et al., 1999). In animal cells, the APC/C is inactivated against a mitotic cyclin reporter at the start of S phase (Brandeis and Hunt, 1996). Further studies led to the proposal that Cdh1 is phosphorylated and inactivated by cyclin A–Cdks (Lukas et al., 1999; Sorensen et al., 2001), but this led to a paradox because cyclin A is itself a substrate of the APC/C. Indeed, we show here that in the absence of Emi1, cyclin A is insufficient to inhibit Cdh1 in interphase.

Subsequently, Emi1 was proposed to inactivate the APC/C to allow cells to enter S phase and to accumulate cyclin A that would then keep Cdh1 inactive (Hsu et al., 2002). However, we have shown that cells enter and appear to progress through S phase in the absence of Emi1 with normal timing and patterns of origin firing, except that the origins continued to fire after cells reached a 4N DNA content. Thus, either the APC/C does not need to be inactivated for cells to replicate their DNA, which has implications for the current model that the APC/C targets several substrates involved in DNA replication (Diffley, 2004), or the APC/C can be inactivated in S phase by a mechanism independent of Emi1 and cyclin A such that Emi1 is only required to inactivate the G2-phase form of the APC/C. Clearly, Emi1 is important for inhibition of the APC/C in G2 phase to impose directionality on the cell cycle and couple S phase with mitosis, but our results indicate that it is important to reopen the question of how the APC/C is regulated in S phase.

Materials and methods

Cell culture and synchronization

HeLa cells were cultured in Advanced DME (Invitrogen). Cells were synchronized at the G1/S transition by a thymidine/aphidicolin block. The day after seeding, cells were blocked with 2.5 mM thymidine (Sigma-Aldrich) for 24 h, released for 12 h, and then blocked again with 5 μg/ml aphidicolin (Sigma-Aldrich) for 24 h. Cells were then released into fresh DME. hTert-RPE cells were cultured in 10% FBS + DME-F12 medium (Sigma-Aldrich). For synchronization, cells were serum starved for 24 h and re-fed with 25% FBS-containing medium. Cell cycle analyses were performed by flow cytometry using a cytometry system (FACSCalibur; Becton Dickinson).

Plasmids

Chimeric Emi1 proteins were produced by PCR from IMAGE clone ID3960052 using Advantage polymerase (CLONTECH Laboratories, Inc.). The fragment was cloned into the pT7-Venus-C1 vector. Emi1 mutants were generated by QuikChange Site-Directed Mutagenesis (Stratagene) using the following oligonucleotides: CCAGTAGACTTTATGAAGCCAGTGGCTATTCCTCA (forward) and TGAGGAATAGCCACTGGCTTCATAAAGTCTACTGG (reverse) for the D144A mutant; GACTTTATGAAGACAGTGTCTATTCCTCATTTTCTC (forward) and GAGAAAATGAGGAATAGACACTGTCTTCATAAAGTC (reverse) for the G146V mutant; and CCAGTAGACTTTATGAAGACGCTGGCTATTCCGCATTTTCTCTACAAAGTGG (forward) and CCACTTTGTAGAGAAAATGCGGAATAGCCAGCGTCTTCATAAAGTCTACTGG (reverse) for the S145A/S149A mutant. IRES constructs were generating by pasting the Emi1 D144A and Emi1 G146V sequences into pIRES2-EGFP (CLONTECH Laboratories, Inc.). All clones were confirmed by automated sequencing. Cyclins A2 and B1 and securin constructs have been described previously (den Elzen and Pines, 2001; Hagting et al., 2002).

Microinjection and time-lapse imaging and analysis

Either plasmid DNA or in vitro–transcribed mRNA was used for microinjections. mRNA was transcribed from the pT7-Venus-C1 constructs, starting from the T7 promoter, using the mMESSAGE mMACHINE T7 Ultra kit (Ambion).

For microinjection and time-lapse microscopy, the culture medium was replaced with Leibovitz's L-15 medium (Invitrogen). Plasmids or in vitro–transcribed mRNA were microinjected at a concentration of 5 ng/μl and 1 μg/μl, respectively, into G2-phase cells using a semiautomatic microinjector (Eppendorf) on a microscope (DMIRBE or DMIRE2; Leica). DIC and fluorescence images were captured every 3 min with a MicroMax (Princeton Instruments) or Quantix (Photometrics) cooled CCD camera using IP Lab software (Scanalytics) or a CoolSNAP HQ cooled CCD camera (Photometrics) and Slidebook software (Intelligent Imaging Innovations). Fluorescence could be detected ∼30 min after injecting mRNA and ∼90 min after injecting cDNA. A modified version of ImageJ software (National Institutes of Health) was used to quantify the amount of fluorescence after background subtraction. DIC microscopy was used to monitor mitosis, and the mitotic phases were determined by chromosome behavior and nuclear morphology. Images were converted to PICT format, exported to PhotoShop (Adobe), and converted from 16- to 8-bit images.

To estimate the levels of Emi1 overexpression in injected cells, Venus-Emi1 was transfected, and levels of the tagged protein were compared with the endogenous by quantitative immunoblotting using the Odyssey Infrared Imaging System (LI-COR) after normalizing for the transfection efficiency. The fluorescence was then quantified and compared in transfected versus injected cells.

RNAi

siRNA duplexes against Emi1 corresponding to the sequence GATTGTGATCTCTTATTAA (siEmi1_1, which starts at 1,337 bp and overlaps the STOP codon) and ACTTGCTGCCAGTTCTTCA (siEmi1_2, which starts at 569 bp) and against Cdh1 (Brummelkamp et al., 2002) were synthesized by Dharmacon. Nontargeting siRNA against luciferase (Dharmacon) or glyceraldehyde-3-phosphate dehydrogenase siRNA (Ambion) were used as negative control siRNAs. Oligonucleotides were transfected at a final concentration of 50 nM according to the manufacturer's instructions using Oligofectamine (Invitrogen). Asynchronously growing cells were transfected, and samples were taken 24, 48, and 72 h after transfection. 28 μM roscovitine (Sigma-Aldrich) was added 24 h after transfection, and DNA content was analyzed 24 h later. 50 μM MG132 (Calbiochem) was added 24 h after transfection for 6 h.

To analyze the effect of siRNAs on cell cycle progression, cells were transfected 5 h after release from thymidine, blocked in aphidicolin, and analyzed after the release from aphidicolin by flow cytometry and Western blotting. 10 μM taxol (Sigma-Aldrich) was added 10 h after release from aphidicolin, and cells were fixed 10 h later to quantify the mitotic index.

In the rescue experiments, 50 nM of oligonucleotide was cotransfected with pT7-Venus-Emi1 using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's protocol. The exogenous cDNA does not contain the 3′ untranslated region and is therefore not depleted by the siEmi1_1 oligonucleotide, which is directed against a region overlapping the STOP codon. In the double siRNA experiments, 50 nM siRNA against Emi1 was cotransfected with 50 nM of either control or Cdh1 duplexes. 100 nM of control oligonucleotides were transfected in the control sample.

Immunofluorescence

Cells were fixed either in 3% PFA and 2% sucrose for 5 min or in ice-cold methanol/acetone (1:1) for 2 min. After permeabilization (for PFA-fixed cells), cells were blocked in 3% BSA-PBS–Tween 20 and then incubated with antibodies. All antibodies were diluted in 3% BSA-PBS–Tween 20 (0.2%), and washes were performed with PBS–Tween 20 (0.2%). Anti–cyclin A antibodies AT10.3 (Cancer Research UK) and BF683 (Cell Signaling) were used at 1:5,000 or 1:200, respectively. Anti–cyclin B1 antibody (GNS-1; BD Biosciences) was used at 1:200. Anti–aurora B antibody (Abcam) was used at 1:1,000, and CREST serum (a gift from W. Earnshaw, University of Edinburgh, Edinburgh, UK) was used at 1:20,000. Secondary antibodies conjugated to AlexaFluor568 or -647 (Invitrogen) were diluted at 1:400. DNA was stained with Hoechst 33342.

For analysis of the different patterns of DNA replication, BrdU was added to a final concentration of 50 μM 30 min before fixation. In the rescue experiments, BrdU was added 4 h before fixation. Cells were then fixed and stained using the BrdU Detection kit (Roche). Anti-BrdU FITC-conjugated antibody (B44; Becton Dickinson) was used for BrdU detection by flow cytometry.

For quantification of endogenous cyclins, samples were analyzed with a deconvolution microscope (DeltaVision Spectris; Applied Biosystems) using a 60× NA 1.4 lens to collect a series of 0.2-μm z sections. Sections were projected on a single plane, and the total florescence was measured. Background fluorescence was subtracted. For BrdU and Hoechst staining, a single section image was collected. Nuclear size was automatically measured using a threshold function in ImageJ software.

Immunoblotting

Cells were lysed directly in SDS loading buffer. Samples were then syringed, boiled, and run on NuPAGE 4–12% Bis-Tris gels except where otherwise indicated. Proteins were transferred to a polyvinylidene difluoride membrane. Membrane saturation and all of the following incubation steps were performed in 5% low fat milk in PBS–Tween 20 (0.2%). Anti-Emi1 antibody (Zymed Laboratories) was used at 1:100. Anti–cyclin A (BF683; Cell Signaling), cyclin B1 (GNS-1; BD Biosciences), and cyclin E (HE12; Abcam) antibodies were used at 1:1,000. Antiphospho-S133 cyclin B1 (Jackman et al., 2003) was used at 1:500. Anti-Cdh1 antibody (a gift from T. Hunt and J. Gannon, Cancer Research UK, Cambridge, UK) was used at 1:50. Anti–geminin antibody (a gift from R. Laskey, Hutchison/Medical Research Council Research Centre, Cambridge, UK) was used at 1:500. Anti-Cdt1 and anti-cdc6 antibodies (gifts from K. Helin, Biotech Research and Innovation Centre, Copenhagen, Denmark) were used at 1:100 and 1:2,000, respectively. Anti-actin (AC-40; Sigma Aldrich) and anti-Hsp70 (H-5147; Sigma-Aldrich) antibodies were used at 1:1,000 and 1:5,000, respectively. HRP-conjugated secondary antibodies (DakoCytomation) were used at 1:5,000. The antibodies were detected using ECL-plus (GE Healthcare) or were analyzed with the Odyssey Infrared Imaging System (LI-COR) for quantitative immunoblotting. Only the output levels were adjusted to assemble the panels in Photoshop.

Online supplemental material

Fig. S1 shows the specificity of the Emi1 antibody and Venus Emi1 degradation in hTERT-RPE cells. Fig. S2 shows mitotic cyclin degradation in hTERT-RPE cells injected with Venus Emi1 and in HeLa cells after the injection of untagged Emi1. Fig. S3 shows the requirement for Emi1 for mitotic entry using an independent Emi1 siRNA oligonucleotide. Fig. S4 shows that hTERT-RPE cells have the same response as HeLa cells to depleting Emi1 by siRNA treatment and demonstrates the specificity of the phenotype by rescue with a plasmid expressing Emi1. Fig. S5 shows the rescue of mitotic entry in Emi1-depleted cells by codepleting Cdh1 as assayed by flow cytometry. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200611166/DC1.

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