# Cell cycle adaptations of embryonic stem cells

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**ES cells proliferate with very short gap phases yet maintain their capacity to differentiate. It had been thought that the levels of cyclins and other substrates of ubiquitin ligase APC/C remain nearly constant and Cdk activity remains constitutively high in mouse ES cells. Here we demonstrate that APC/C (anaphase-promoting complex/cyclosome) enzyme is active in ES cells but attenuated by high levels of the Emi1 (early mitotic inhibitor-1) protein. Despite the presence of high Cdk activity during the G1 phase, chromatin can be effectively licensed for DNA replication and fast entry into the S phase can still occur. High Cdk activity during S-G2-M phases produces high levels of the DNA replication factor Cdt1, and this leads to efficient Mcm proteins loading on chromatin after mitotic exit. Although disturbing the usual balance between Cdk activity and APC/C activity found in somatic cells, a few key adaptations allow normal progression of a very rapid cell cycle.**

pluripotency | differentiation | protein degradation

Embryonic stem cells show unusual cell-cycle features: the duration of the S phase is comparable to somatic cells but they have remarkably short G1 and G2 phases (1–3). In somatic cells, the duration of G1 and G2 is determined by relative levels of Cdk kinase activity and other cell cycle-related proteins (4). Many of these proteins, including Cyclin A, Cyclin B, Cdt1, Cdc6, and Geminin fluctuate along the cell cycle because of degradation mediated by E3 ubiquitin ligase APC/C (anaphase-promoting complex/ cyclosome) together with E2 enzymes, such as UbcH10 and UBE2S (5–8). APC/C is activated at the end of mitosis by interaction with Cdc20 and Cdh1 proteins and inactivated just before the S phase by the pseudosubstrate inhibitor Emi1 (early mitotic inhibitor-1) and by the phosphorylation and degradation of Cdh1 (6, 9, 10). Cdk kinases are activated by Cyclins and phosphorylate a number of cell-cycle proteins important for mitotic and S phase progression. Cdk activity is inhibited during G1 in somatic cells because of degradation of Cyclins and presence of inhibitor proteins, like p21 (11). Inhibition of Cdk activity in the G1 phase allows the replication factors Cdt1 and Cdc6 to recruit Mcm proteins on chromatin, form prereplicative complexes (pre-RCs), and license DNA for replication (12–14). Geminin protein inhibits Cdt1 during the S phase and promotes its stabilization during mitosis (3, 13, 15–20). A puzzling feature of ES cells is that APC/C substrates were shown to be constant and Cdk activity to be high throughout the ES cell cycle (1, 3, 21), raising the question of whether the APC/C complex is functional and how ES cells regulate pre-RC assembly at G1. Remarkably, APC/C substrates and other positive cell-cycle regulators decrease after differentiation (1, 3, 22). We carefully reinvestigated cell-cycle dynamics in ES cells. Contrary to previous conclusions, APC/C substrate levels and Cdk activity both oscillate, although in a more muted manner compared with most studied somatic models. A few key adaptations promote an abbreviated cell cycle and avoid the licensing problem.

## Results

**APC/C Is Functional in ES Cells.** It was previously reported that the levels of APC/C substrates in mouse ES cells remain nearly constant during the cell cycle (1, 3, 21). This unusual finding raised the question of how the cell can cycle in the absence of oscillation of Cdk activity and by what means APC/C is inhibited. To ask whether APC/C is active or whether, whatever low activity there is, it oscillates, we analyzed the levels of well-defined APC/C substrates at different phases of the cell cycle. We were able to create an effective M-phase synchronization protocol by treating ES cells sequentially with thymidine and Nocodazole (see *Materials and Methods*). The high quality synchronization during the G1 phase was revealed by FACS analysis (*SI Appendix*, Figs. S1 and S2). After immunoblotting for several APC/C substrates, including Cyclin A, Geminin, Cdt1, Securin, Cyclin B, Cdc20, Cdh1, Plk1, and Aurora A, we observed that protein levels of all of these substrates decrease markedly after mitotic exit (Fig. 1*A*), although degradation of APC/C substrates are not as striking as observed in somatic cells (13, 16). The discrepancy with published work is likely in part a result of the suboptimal synchrony previously achieved, exacerbated by the very short G1 phase in ES cells (3). To confirm that the drop in substrate levels is mediated by APC/C, we assayed substrate degradation in vitro with mitotic (i.e., Nocodazole-arrested) ES cell extracts by adapting protocols we had developed previously for somatic cell extracts (5). Exogenously added Securin was not degraded in mitotic extracts (Fig. 1*B*), in agreement with the expectation that APC/C is inactive during early mitosis, when the checkpoint is in force. As we had shown previously (5), addition of exogenous E2 enzymes UbcH10 (which is specific for APC/C) or UBE2S (which elongates ubiquitin chains with K-11–linked ubiquitin) overrides the mitotic checkpoint and promotes degradation of Securin, especially when the two enzymes are added together (Fig. 1*B*). To detect oscillation of APC/C activity with the cell cycle, we assayed degradation of substrates with cell extracts made from cells at different phases of the cell cycle. In somatic cells, in the presence of the exogenous E2 enzyme UbcH10, APC/C activity progressively drops during G1 (23). Similarly, we observed that APC/C activity in ES cells is high in mitosis, intermediate in the G1 phase, and low in the S phase. We tested Securin with the CCE cell line, which was used in the previous study and showed virtually no substrate oscillation (3), and Cyclin A with the J1 cell line. In both cases we observed the expected changes in APC/C activity (Fig. 1*C*). In contrast to somatic cells (10, 24), the APC/C coactivator Cdc20 is present in the G1 and S phases of ES cells (Fig. 1*A*). We suspected that, to allow

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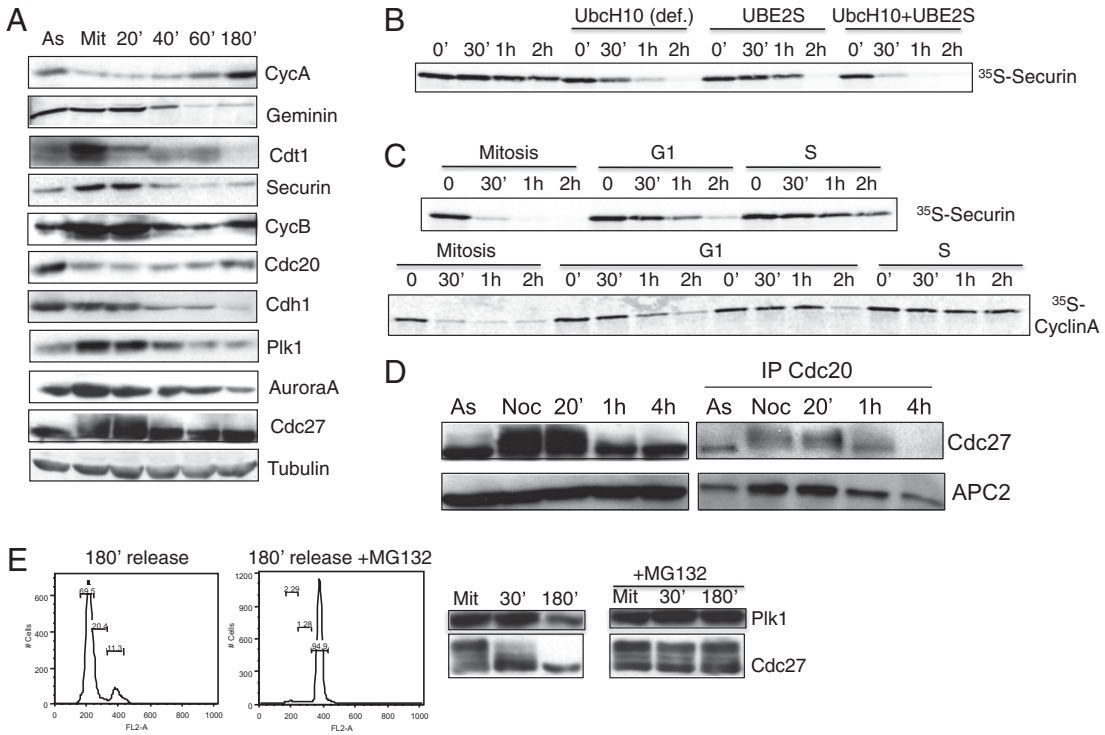


**Fig. 1.** APC/C activity oscillates in mouse embryonic stem cells. (*A*) Immunoblotting analysis for the indicated proteins from cells synchronized as shown in *SI Appendix*, Fig. S2. Cdt1 is partially degraded and migrating faster in the G1 phase; this pattern recapitulates somatic cells (13, 27) and the slower migration in SDS gel during mitosis is plausibly because of phosphorylation. In addition, Cdc27 and Cdh1 are phosphorylated during mitosis. (*B*) Degradation assay from mitotic ES cell extracts. Degradation assay is performed at 30 °C for the indicated times and in vitro-translated $^{35}$S-Securin is used as a substrate. E2 enzymes UbcH10 and UBE2S are purified as previously shown (5). Autoradiography for $^{35}$S-Securin is shown. (*C*) Degradation assays with extracts from cells synchronized at different phases of the cell cycle. (*Upper*) Degradation assay from CCE ES cells synchronized at mitosis, 1 h, and 4 h, released from mitosis with in vitro-translated $^{35}$S-Securin as a substrate. (*Lower*) Degradation assay from J1 ES cells synchronized at mitosis, 1 h, 1 h 30 min, and 4 h, released from mitosis with in vitro-translated $^{35}$S-Cyclin A as a substrate. Autoradiographies for $^{35}$S-Securin or $^{35}$S-Cyclin A are shown. (*D*) Cdc20 dissociates from the APC/C complex after mitotic exit. ES cells synchronized in mitosis and released for the indicated times are used to perform immunoprecipitation of Cdc20. Immunoblotting analysis for the indicated coimmunoprecipitated proteins is shown. (*E*) Proteasome inhibition blocks exit from mitosis and APC/C-dependent degradation. ES cells are released in presence or absence of proteasome inhibitor MG132 for 3 h. FACS analyses (*Left*) and immunoblotting (*Right*) for APC/C subunit Cdc27 and APC/C substrate Plk1.

for APC/C activity inhibition during DNA replication, Cdc20 dissociates from APC/C complex in the S phase. To test this theory, we immunoprecipitated Cdc20 from the M through S phase and observed that Cdc27 and APC2 associations are indeed significantly weakened during the S phase (Fig. 1*D*). Furthermore, in agreement with the idea that ubiquitination presumably by APC/C activity is required to exit mitosis, ES cells treated with the proteasome inhibitor MG132 fail to exit mitosis and Plk1, a classic APC/C substrate, is not degraded (Fig. 1*E*). Although Cdk2 activity was reported to be nearly constant in the cell cycle of ES cells (1, 3, 21), we clearly observed fluctuations of cyclin A protein levels. This finding led us to ask whether cyclin A-associated Cdk2 kinase activity also oscillates. As shown in *SI Appendix*, Fig. S3, similar to somatic cells, Cdk2 fluctuates in ES cells and Cdk kinase activity is necessary throughout the cell-cycle progression.

**ES Cells Have High Levels of the APC/C Inhibitor Emi1.** Compared with somatic cells, ES cells display reduced oscillations of APC/C substrates. For example, for Cyclin A we observe fluctuations of a two- to threefold range in ES cells and 10- to 30-fold in U2OS cells; for Cyclin B we observe fluctuations of three- to sevenfold range in ES cells and 30- to 50-fold in U2OS cells (*SI Appendix*, Fig. S4). The limited fluctuation of the substrates could reflect reduced APC/C activity because of high levels of Emi1 inhibitor (9, 23, 25). To show that this theory is true in mouse ES cells, we first made mitotic extracts of ES cells and looked at the response to Emi1 protein in degradation assays. As shown in Fig. 2*A*, Geminin degradation is inhibited by recombinant Emi1 protein. We then observed that Emi1 protein level is much higher in ES cells than in cells induced to differentiate with retinoic acid (RA) (Fig. 2*B*) and that the Emi1 protein level oscillates, as it does in somatic cells (9, 26). Accordingly, we also observed that mRNA for Emi1 is markedly decreased upon differentiation (Fig. 2*C*). We then tested if the levels of Emi1 protein associated with the APC/C complex are decreased during differentiation. We synchronized cells in mitosis to have matched synchronizations for optimal comparison between the two conditions and we isolated the APC/C complex by immunoprecipitation of Cdc20 protein or Oct4 (negative control). Although the fraction of pseudosubstrate Emi1 protein associated to APC/C is small because of a high dissociation rate (5, 9), we observed that in differentiated cells the levels of Emi1 protein associated to APC/C complex are dramatically decreased. Although ES cells have higher total Cdc20 protein, the levels of Cdc20 associated to the APC/C complex are similar in the two conditions, as shown by equal amounts of coimmunoprecipitated APC subunits APC2 and APC7 (Fig. 2*D*).

**APC/C Activity Is Attenuated in ES Cells.** The elevated levels of Emi1 would be expected to depress APC/C activity throughout the G1 phase. When we compared APC/C activity in degradation assays using exogenous Securin as a substrate, we found—as expected—that the rate of degradation is higher in extracts from mitotic

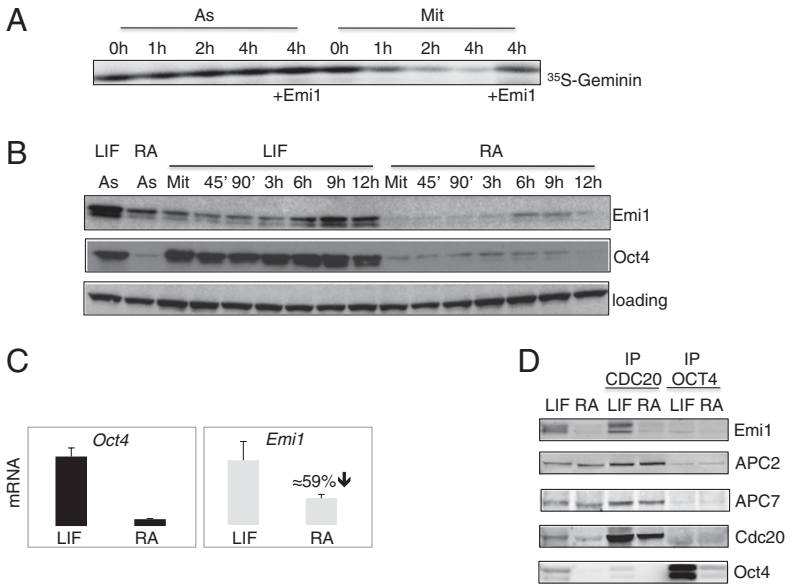


**Fig. 2.** Emi1 levels are high in ES cells. (*A*) Emi1 inhibits APC/C activity in ES cells. Degradation assay is performed with nonsynchronized or mitotic ES cells for the indicated times. In vitro translated $^{35}$S-Geminin is used as a substrate. Recombinant N-terminal–deleted Emi1 is used to inhibit the degradation. Autoradiography for $^{35}$S-Securin is shown. (*B*) Emi1 protein levels are decreased upon differentiation. ES cells are maintained in leukemia inhibitory factor (LIF) containing medium or treated with RA for 48 h to induce differentiation. Cells are synchronized in mitosis and released for the indicated times. Immunoblotting analysis for the indicated proteins is shown. Oct4 is used as a marker for pluripotency. (*C*) RNA levels for Emi1 are decreased during differentiation. RNA levels for Emi1 and Oct4 are determined by quantitative PCR (qPCR) performed on three independent biological samples synchronized at different time points during S-G2-M progression. Reduction in mRNA for Emi1 is shown. Samples are normalized to β-actin. (*D*) Emi1 levels associated to APC/C are decreased in differentiated cells. Control cells and cells treated with RA are synchronized in mitosis and immunoprecipitations of Cdc20 or Oct4 (negative control) are performed before immunoblotting analysis for the indicated proteins. Lanes 1 and 2 correspond to 2.5% input.

differentiated cells, where the Emi1 level is reduced, either in the presence of recombinant UbcH10 alone or together with recombinant UBE2S (Fig. 3*A*). We obtained similar results using exogenous Geminin protein and endogenous Cdt1 (27) (*SI Appendix*, Fig. S5). We then tested if depletion of Emi1 in ES cells increases APC/C activity by analyzing levels of APC/C substrates. We treated ES cells with siRNA for Emi1 and, as expected, we observed that Geminin and Cyclin A proteins levels are decreased (Fig. 3*B*). In contrast, when we expressed a version of Geminin mutated in the destruction box necessary for ubiquitin-mediated degradation, the mutant levels were unaffected. We also observed that the Cyclin A protein is less affected by siRNA treatment in presence of exogenous Geminin (Fig. 3*B*), possibly because of competition of substrates for APC/C. To test if the intrinsic activity of APC/C is reduced in ES cells, we assayed ubiquitination of Securin mediated by APC/C purified by immunoprecipitation under conditions where the Emi1 protein is not coimmunoprecipitated (5). We saw no differences between ES cells and differentiated cells (*SI Appendix*, Fig. S5). To investigate if change in APC/C activity upon differentiation is correlated to changes of levels of its subunits, we tested quantities of APC2 and APC7. We did not observe any change in subunits levels either along the cell cycle or after differentiation (*SI Appendix*, Fig. S5). Moreover, in agreement with the observation that Emi1 depletion reduces levels of APC/C substrates, we observed that ES cells treated with siRNA for Emi1 partially accumulate in the S-G2 phase and present some rereplication, similarly to cells treated with siRNA for Cyclin A and Geminin (15, 21), respectively, and this outcome was already detectable at 24 h (*SI Appendix*, Fig. S5). Overall, these results suggest that in ES cells APC/C activity is attenuated because of high Emi1 protein levels.

**ES Cells Have High Levels of the DNA Replication Factor Cdt1.** Mcm proteins are normally loaded onto chromatin after mitosis when Cdk activity has dropped to its lowest point in the cell cycle (13, 28, 29). However, as shown in *SI Appendix*, Fig. S3, Mcm proteins are loaded on chromatin during the G1 phase despite the persistence of Cdk activity. One explanation is that in ES cells during G1, the very high levels of licensing factors Cdt1 and Cdc6 compensate for the inhibitory effects of Cdk activity, and indeed a previous report

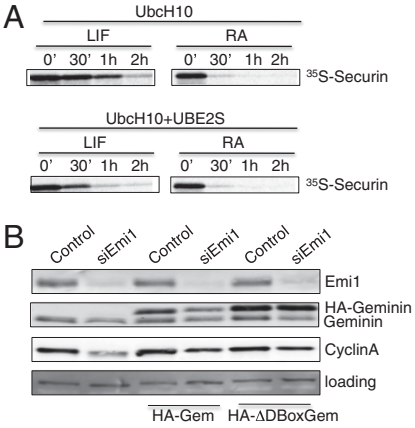


**Fig. 3.** APC/C activity is increased upon differentiation. (*A*) Degradation assay from undifferentiated ES cells or ES cells induced to differentiation for 48 h with RA is shown. In vitro translated $^{35}$S-Securin is used as a substrate. Recombinant UbcH10 alone or UbcH10 in combination with UBE2S were used for the comparisons. These data are from same experiment as shown in Fig. 1*B*. Autoradiography for $^{35}$S-Secruin is shown. (*B*) Emi1 depletion leads to decrease in APC/C substrates levels. Control or siRNA for Emi1 are transfected in ES cells using Lipofectamine 2000 transfection reagent (Invitrogen). Thirty hours after transfection, levels of the indicated proteins are evaluated by immunoblotting analysis. In lanes 3–6, the indicated HA-tagged versions of Geminin are transfected 6 h after siRNA transfection using FuGene 6 reagent (Promega).

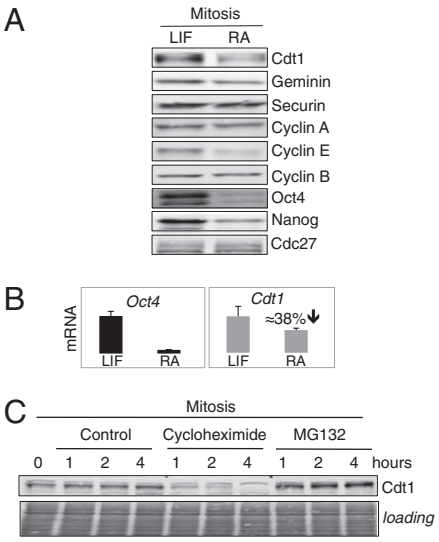


**Fig. 4.** Cdt1 is abundant in ES cells. (*A*) Cdt1 protein levels are higher in mitotic ES cells than in differentiated cells. Immunoblotting for the indicated proteins is shown. Oct4 and Nanog are used as markers of pluripotency. Cdc27 phosphorylation is used as a marker of equal synchronization in mitosis. Mitotic extract of ES cells and differentiated cells (after standard 48-h treatment with RA) are used. (*B*) RNA levels for Cdt1 are decreased during differentiation. RNA levels for Cdt1 and Oct4 are determined by qPCR performed on three independent biological samples synchronized at different time points during S-G2-M progression. Reduction in mRNA level for Cdt1 is shown. Samples are normalized to β-actin. (*C*) Cdt1 turnover in mitosis is fast and protein levels are maintained high. Mitotic ES cells are treated with protein-synthesis inhibitor cycloheximide or proteasome inhibitor MG132 for the indicated times. Immunoblotting for Cdt1 protein is shown and equal loading is evaluated by Ponceau staining.

showed that Cdc6 protein levels are high in ES cells (3). Furthermore, when we compared Cdt1 levels at mitosis (where level is correlated with licensing activity), we found that levels of Cdt1 protein are significantly higher in ES cells compared with differentiated cells (Fig. 4*A*). Cyclin A and B, the levels of which were previously reported to decrease upon differentiation (1, 3), are not significantly reduced. The discrepancy with previous reports is conceivably because of the short duration of RA treatment (48 h) associated to synchronization treatment (that leads to delay of cell-cycle progression). In contrast, after 48 h of RA treatment, we observed significant decrease of Cyclin E protein (Fig. 4*A*), the levels of which, differently from previously reported (3, 30), decrease markedly in the S phase; we think the discrepancy with previous reports is likely a result of the lack of specificity of antibodies used in those studies, as inferred from the tests we performed using Cyclin E knockout cells (*SI Appendix*, Fig. S6). In accordance with elevated E2F-dependent transcription in pluripotent cells (30, 31), we also observed that mRNA levels of Cdt1 are elevated in ES cells (Fig. 4*B*). To evaluate if Cdt1 turnover is high in ES cells similar to somatic cells (13, 32), we synchronized ES cells in mitosis and treated them either with protein synthesis inhibitor Cycloheximide or proteasome inhibitor MG132. Cdt1 levels rapidly decrease with Cycloheximide but increase when cells are exposed to MG132 (Fig. 4*C*), showing that Cdt1 levels are maintained at an elevated level both by high synthesis rate and by some degree of stabilization.

**Cdk Activity Before Cell Division Promotes Chromatin Licensing by Amplifying the Level of Cdt1 Protein.** Compared with somatic cells, in ES cells the level of Cdk activity in S-G2 and mitosis is high (3, 30). As Cdk activity is known to increase the binding between Cdt1 and Geminin before cell division in somatic cells (13), the cell accumulates high levels of inhibited Cdt1, which it then releases in response to the drop in Cdk activity at mitosis (13). In ES cells, upon mitotic release, the very high levels of Cdt1 should therefore promote efficient chromatin licensing in G1, despite persistence of Cdk activity. If this were indeed the case, high G2 phase Cdk activity of ES cells would be expected to promote the progression into subsequent S phase. First, to test if Cdt1 levels are regulated by Geminin as in somatic cells (13), we depleted Geminin by siRNA treatment during the G2 phase and we found that Cdt1 protein levels are decreased in mitosis (Fig. 5*A*). To test the hypothesis that high Cdk1 or Cdk2 levels result in increased levels of Cdt1, we treated ES cells released for 1 h from thymidine with Cdk1/2 inhibitor Roscovitine, starting at the time when they were in S-G2 phase. After incubation for 6 h, we observed decrease in Cdt1 protein levels upon Roscovitine treatment but also noted that the cells arrested at G2/M transition, as also confirmed by high levels of Cyclin A protein (Fig. 5*B*). Therefore, we decided to inhibit Cdk activity in a way that allows mitotic entry and cell-cycle progression by using a cell line with inducible p21, an inhibitor of Cdk activity. As expected, upon p21 expression cell cycle progression is delayed and only Cdk2 activity is repressed (*SI Appendix*, Fig. S7). We did not observe a permanent arrest, most likely because expression level of p21 is insufficient to induce complete Cdk inhibition. Confirming our hypothesis, we observed that cells expressing p21 protein have significantly lower levels of Cdt1 (Fig. 5*C*) and this result is conceivably because of time spent by cells in late S and G2 phases when Cdk2 activity is predominant. To confirm that Cdk2 activity is responsible for regulation of Cdt1 protein levels during mitosis and subsequent G1 phase, we depleted Cyclin A and Cyclin E proteins by siRNA treatment during late S and G2 phase, before synchronization in mitosis and release. Although the decrease of cyclin level was limited, we could clearly observe a decrease of Cdt1 levels concomitant with decrease of Cyclin A (but not with Cyclin E decrease) after release from mitosis (Fig. 5*D*). BrdU labeling confirmed delay in S phase entry because of cyclins or Cdt1 decrease (Fig. 5*D*). To test chromatin licensing, we treated cells with siRNA for Cyclin A for a longer time to increase the efficiency of silencing. We started synchronization only 24 h after siRNA transfection. Cells were then collected at different times during S-G2-M progression and after Nocodazole release. After immunoblotting we observed that Cdt1 protein levels are decreased and Mcm2 protein loading on chromatin is reduced upon depletion of Cyclin A. Even if we observed a delay in cell-cycle progression, we did not detect premature pre-RC loading, presumably because Cdk activity is not completely abolished under these conditions (*SI Appendix*, Fig. S8). To further corroborate our findings and to rule out that the previous result was caused by suboptimal synchronization during siRNA treatment (*SI Appendix*, Fig. S8), we inhibited Cdk activity by treatment with Roscovitine directly in the G1 phase. As expected from the notion that Cdk activity is inhibitory during the G1 phase, cells treated with Roscovitine displayed increased loading of pre-RC (Fig. 5*E*). Finally, to confirm that Cdt1 and Cdc6 proteins regulate S phase entry in ES cells, we depleted these proteins by siRNA treatment during the S-G2 phase after release from thymidine arrest. After siRNA transfection we synchronized the cells in mitosis, released them for 2 h 45 min with the final 15 min in the presence of BrdU, and we observed that decrease of Cdt1 or Cdc6 leads to elongated G1 phase. Simultaneous decrease of Cdt1 and Cdc6 has an additive inhibitory effect (Fig. 5*F*). These results show that along with Cdc6, Cdt1 protein is maintained at high levels in ES cells and that high levels of these proteins help override Cdk-dependent inhibition of chromatin licensing during G1. Cyclin A-dependent Cdk activity during late S-G2 phase assists efficient chromatin licensing by amplifying the levels of Cdt1 protein.

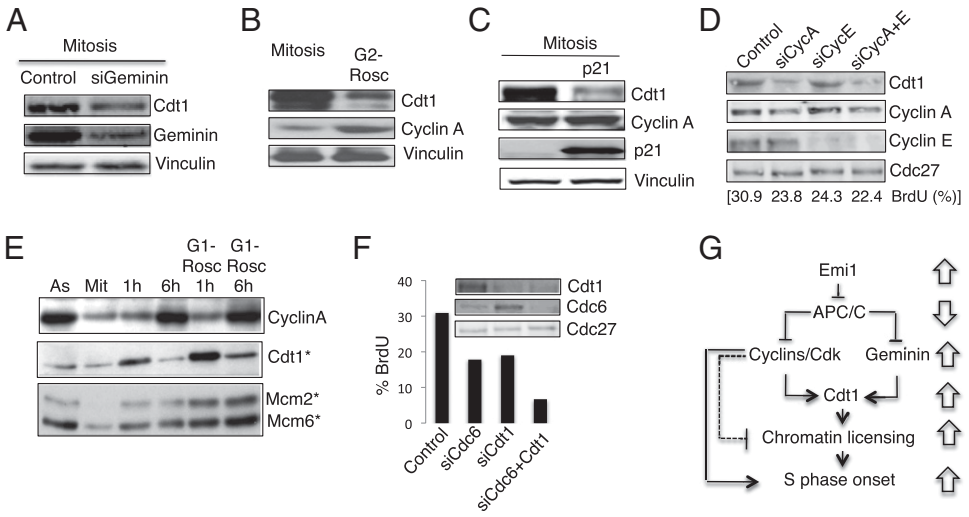


**Fig. 5.** Geminin and Cdk activity amplify Cdt1 protein levels. (*A*) siRNA for Geminin is transfected 1 h after release from thymidine arrest and in the presence of Nocodazole for synchronization in mitosis 7 h later. Immunoblotting analysis for the indicated proteins is shown. Vinculin is used as a loading control. (*B*) Roscovitine treatment in the G2 phase blocks cell-cycle progression at G2/M transition with lower levels of Cdt1 protein. ES cells released for 1 h after thymidine block are treated with Cdk1/Cdk2 inhibitor Roscovitine or DMSO (control) and incubated for other 6 h in the presence of Nocodazole. Immunoblotting for the indicated proteins is shown. Vinculin is used as a marker of equal loading. (*C*) Cdt1 is decreased in mitosis when Cdk inhibitor p21 is expressed. Cells expressing p21 by Doxycycline treatment are used together with control cells (i.e. not treated with Doxycycline) and synchronized in mitosis. Immunoblotting analysis for the indicated proteins is shown. (*D*) Cyclin A depletion reduces levels of Cdt1. ES cells released for 1 h from thymidine arrest are treated with the indicated siRNA for 7 h in the presence of Nocodazole. The cells are then released from Nocodazole block and immunoblotting for the indicated proteins is performed with lysates from cells released for 1 h. BrdU incorporation was evaluated 2 h 45 min after Nocodazole release (15-min labeling). (*E*) Cdk activity during the G1 phase inhibits pre-RC formation. ES cells are synchronized in mitosis before release and treatment with Roscovitine during G1 phase. Immunoblotting analysis for the level of indicated proteins is shown. The asterisk represents the chromatin-containing fraction. (*F*) Cdt1 and Cdc6 drive fast G1 progression in ES cells. ES cells are synchronized in the S phase by thymidine treatment and released in presence of Nocodazole and siRNA for Cdt1 and Cdc6. Cells are then released from mitosis for 2 h 45 min with the last 15 min labeling with BrdU. Quantification of BrdU-positive cells is shown. (*Inset*) Immunoblotting analysis for levels of the indicated proteins in the siCdc6 or/and siCdt1 treated cells. (*G*) Model for the regulation of fast S phase entry in ES cells. Block arrows represent comparison with somatic cells.

## Discussion

The ES cell cycle shows several features that contrast it to the cell cycle in somatic cells. Principally among them are a foreshortened G1 and a seeming failure of the major targets of the APC/C to oscillate (3, 21, 30). In the extreme, this finding has suggested that the basic Cyclin-based oscillators in G1/S and G2/M are either nonfunctional or under very different regulation in ES cells. As we show here, these conclusions are an exaggeration. The basic regulatory mechanisms in mammalian somatic cells are present and operating in ES cells but they operate in modified ways. To examine the cell cycle and its modifications in ES cells, we developed an improved synchronization protocol that allowed us to detect the muted fluctuations of APC/C substrates. We suggest that previous studies missed fluctuations of APC/C substrates because of suboptimal synchronization protocols, or underestimated the weak fluctuations that had been detected (3, 21, 30). Although ES cell lines appear to have distinct degrees of protein oscillations, we could detect fluctuations of APC/C activity and substrates in all cell lines tested (J1, CCE, and KH2) (Figs. 1, 2, and 5, and *SI Appendix*, Figs. S3, S7, S8, and S9). Actually, we observed that APC/C substrate Geminin oscillates also in human ES and induced pluripotent stem cell lines (*SI Appendix*, Fig. S9), suggesting that APC/C activity oscillation is a general feature of pluripotent cells. To explain the muted APC/C activity, we found that mouse ES cells, similar to human ES cells (21, 25), express high levels of the inhibitor Emi1. The combination of high E2F-dependent transcriptional activity and low APC/C ubiquitin ligase activity leads to high levels of the DNA replication inducers during the G1 phase, helping to explain the fast onset of the S phase after mitotic exit. Because high Cdk activity inhibits the formation of pre-replication complexes during G1, ES cells have high levels of replication factors Cdc6 (3) and Cdt1 to overcome the inhibition. It is the high Cdk activity before cell division that allows for the accumulation of high levels of Cdt1 protein during mitosis, thus ensuring efficient formation of pre-RC during the next cell cycle. In addition to promoting chromatin licensing, Cdt1 and Cdc6 could also assist fast onset of DNA replication, as previously proposed (32, 33). These results show that the basic APC/C Cdk oscillator operates in ES cells similar to somatic cells, and that the altered cell cycle of ES cells is because of altered regulation (Fig. 5*G*). In ES cells, APC/C activity is reduced and Cdk activity is increased, leading to a different balance and more limited oscillation. These imbalances are expected to alter cell-cycle events and, most likely, other aspects of stem-cell function, such as pluripotency, are entrained into the cell-cycle adaptations.

## Materials and Methods

Detailed materials and methods can be found in *SI Appendix*. Briefly, mitotic synchronization of ES cells was obtained with 1.25 mM thymidine for 14 h followed by treatment with 50 ng/mL Nocodazole for 7 h. ES cells were differentiated by withdrawal of leukemia inhibitory factor (LIF) and treatment with 1 μM retinoic acid (RA) for 48 h. Chromatin fractionation was performed as previously reported (32).

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