Geminin deploys multiple mechanisms to regulate Cdt1 before cell division thus ensuring the proper execution of DNA replication

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Cdc10-dependent transcript 1 (Cdt1) is an essential DNA replication protein whose accumulation at the end of the cell cycle promotes the formation of pre-replicative complexes and replication in the next cell cycle. Geminin is thought to be involved in licensing replication by promoting the accumulation of Cdt1 in mitosis, because decreasing the Geminin levels prevents Cdt1 accumulation and impairs DNA replication. Geminin is known to inhibit Cdt1 function; its depletion during G2 leads to DNA rereplication and checkpoint activation. Here we show that, despite rapid Cdt1 protein turnover in G2 phase, Geminin promotes Cdt1 accumulation by increasing its RNA and protein levels in the unperturbed cell cycle. Therefore, Geminin is a master regulator of cell-cycle progression that ensures the timely onset of DNA replication and prevents its rereplication.

In eukaryotic cells DNA replication occurs at a specific point of the cell cycle known as S phase, which is flanked by two periods, G1 and G2, during which there is no replication or cell division. The timing of S phase follows the formation of the pre-replicative complexes (pre-RCs) on chromatin during the preceding G1 phase and the activation of the cyclin-dependent kinase (CDK) and dumbbell forming 4 (Dbf4)-dependent kinase (DDK) in S phase (1). Cdc10-dependent transcript 1 (Cdt1) protein is indispensable for pre-RCs formation (2, 3); its levels fluctuate during the cell cycle, being high in G1 phase, allowing pre-RC formation, low in S phase, preventing pre-RC formation and immediate reinitiation, and high again in G2 and mitosis, presumably to prepare for G1 (3–5). Cdt1 activity is limited to G1 through the control of its synthesis, degradation, and activity. The low level in S phase is thought to result from targeted degradation (6–8), whereas its higher level in G2 is thought to result from its stabilization (9). However, the increase of Cdt1 in G2 poses a potential risk in allowing rereplication, which could occur if there were residual activity of the DNA-replicating enzymes in G2.

The control of Cdt1 levels also is a response to Geminin (4, 10), an unstable protein present only in metazoans, which is targeted for degradation by the anaphase-promoting complex (APC) (11). Geminin has two putative roles in the cell cycle: inhibiting Cdt1 and promoting the accumulation of Cdt1 during mitosis. Both Geminin and Cdt1 are expressed at high levels in G2, where Geminin binds Cdt1 and prevents DNA rereplication (12–14). A critical role of Geminin in regulating the accumulation of Cdt1 levels has been inferred by the observation that the depletion of Geminin leads to decreased Cdt1 protein levels in mitosis (4) and meiosis (10). However, it also has been suggested that Geminin actively inhibits Cdt1, because depletion of Geminin in G2 phase activates Cdt1 and causes DNA rereplication and, consequentially, DNA damage (12). Because Cdt1 and cell division cycle 6 (Cdc6) replication factors have been shown to be degraded after DNA damage (15–19), the Cdt1 decrease upon Geminin depletion simply may be an indirect consequence of DNA rereplication.

In this paper we clarify the role of Geminin in regulating Cdt1 and show more clearly how APC contributes to the regulation of the initiation of S phase and its duration. We show that although Cdt1 protein accumulates in G2 phase, it still turns over very quickly and that to produce high Cdt1 levels when cells exit mitosis into G1, the accumulation in G2 must overcome degradation. This regulation is a product of Geminin's positive regulation of Cdt1 protein and RNA in the preceding G2 phase. Degradation of Cdt1 is not a consequence of DNA damage, because Cdt1 levels decrease upon Geminin depletion even in presence of inhibitors of DNA synthesis. Metaphase unleashes a precipitous degradation of Geminin via APC, leading to the activation of Cdt1 in early G1 for pre-RC formation. Overall, these results show that Geminin is a master regulator of DNA replication in the cell cycle of metazoans, ensuring that each DNA segment of the chromosome is replicated on time and only once before each cell division.

Results

Cdt1 in G2 Phase Is Both Abundant and Unstable. It has been shown previously that Cdt1 levels increase after S phase and that Cdt1 protein is stabilized during G2 phase (4, 9). However, the levels of a protein are not necessarily related to its turnover rate. Therefore, we measured the turnover of Cdt1 protein in G2 phase by treatment with the protein synthesis inhibitor cycloheximide. We first synchronized U2OS cells in S phase by a double thymidine block. We then released the cells and started the cycloheximide treatment 8 h after the second thymidine release, when cells were still in S phase. We then collected cells for the next 4 h with time points at every hour (Fig. 1*A*). As shown by Western blot analysis, U2OS cells accumulate Cdt1 in G2, as they exit S phase, 10 h after release from thymidine. As expected, Cdt1 protein does not accumulate in G2 phase if the cells first are treated with cycloheximide during S phase. When cells were treated in parallel with a proteasome inhibitor, Cdt1 levels

Significance

The master cell-cycle processes governing DNA replication and mitosis in eukaryotic cells are regulated by cyclin/cyclin dependent kinase 1 and the anaphase-promoting complex, with checkpoint activity on these regulators. It is not these regulators but rather intermediaries that communicate to the processes. Here we show that the protein Geminin acts centrally in controlling DNA replication by ensuring that DNA is replicated during S phase and only once. This paper describes the Geminin "sub-master" regulatory circuit and the central role of Geminin in controlling events of the cell cycle.

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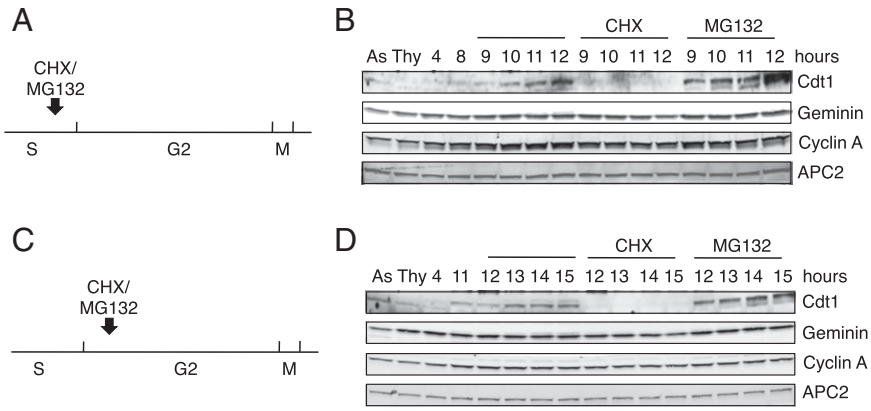

Fig. 1. Cdt1 is abundant in G2 phase but is highly unstable. (*A*) Schematic of the experiment. (*B*) U2OS cells were synchronized by double thymidine treatment and then were released for the indicated times. At 8 h after release (i.e., at the end of S phase) cells were left untreated or were treated with either cycloheximide (CHX) or carbobenzoxy-Leu-Leu-leucinal (MG132). Western blotting analysis for the indicated proteins is shown. APC2 protein was used as loading control. As, nonsynchronized cells; Thy, cells arrested in S phase. (*C*) Schematic of the experiment. (*D*) U2OS cells were synchronized by double thymidine treatment and then were released for the indicated times. At 11 h after release (i.e., in early G2 phase) cells were left untreated or were treated with either cycloheximide or MG132. Western blotting analysis for the indicated proteins is shown. APC2 protein was used as loading control.

increased progressively during S and G2 phases (Fig. 1*B*), indicating that Cdt1 protein is both synthesized and degraded. Turnover continues in G2. Repeating the experiment with cycloheximide and proteasome inhibitor treatments in G2 (Fig. 1*C*), we found that Cdt1 protein disappeared completely 1 h after the addition of cycloheximide (Fig. 1*D*) but accumulated when the proteasome is inhibited (Fig. 1*D*). In both experiments, Geminin and cyclin A remain stable. These experiments show that Cdt1 accumulates in the G2 phase of the cell cycle, but even in G2 it remains extremely unstable with a half-life of less than 30 min.

Geminin Acts Upstream of Cdt1, but Its Regulation Is Not Mediated by DNA Damage and Replication. It is known that depletion of Geminin blocks the accumulation of Cdt1 protein in mitosis (4) and meiosis (10); at the same time Geminin depletion leads to unscheduled DNA replication. Thus, it is a reasonable hypothesis that the Geminin effects on the synthesis or degradation of Cdt1 could be a consequence of DNA damage caused by rereplication induced by reactivation of Cdt1. To test whether rereplication is the proximate cause of a reduction in Cdt1, we depleted Geminin in G2 phase in the presence of the DNA synthesis inhibitor aphidicolin, a specific inhibitor of DNA polymerases α and δ. We synchronized U2OS cells with a double thymidine block and treated them during S phase with siRNA for Geminin, using an adaptation of a previously developed protocol (Fig. S1*A*) (4). The cells then were optionally treated at the S/G2 transition with aphidicolin and were arrested at end-G2/M with nocodazole. Both end-G2 and mitotic cells were collected, as previously described (4). After Western blot analysis, we observed that in both G2 and M phase Geminin depletion reduced Cdt1 levels to the same degree in the presence or absence of aphidicolin (Fig. 2 *A* and *B*). To confirm that the decrease of Cdt1 levels upon Geminin depletion is not caused by rereplication, we performed a similar experiment using thymidine (20) and assayed Cdt1 levels at mitosis. We again adapted a previous protocol (4) in which we synchronized U2OS cells with a double thymidine block and then released them for 10 h to let them enter the G2 phase. At this point, we treated cells with control siRNA or Geminin siRNA and in one case blocked replication with a third thymidine treatment. We then incubated the cells for 14 h to allow the depletion of Geminin before harvesting the mitotic cells by manual shake-off (Fig. S1*B*). There was no significant difference in the number of cells in mitosis, a sign that these treatments did not cause changes in cell-cycle progression. By Western blot the decrease of Cdt1 protein levels upon siRNA for Geminin was unchanged when DNA replication was inhibited (Fig. 2*C*). Furthermore the levels of Cdc6, which previously had been shown to decrease upon DNA damage (15, 16), and of Cdc7 remained unchanged upon Geminin depletion (Fig. 2*C*). These results suggest that depletion of Geminin leads to a decrease of Cdt1 independently of DNA rereplication. Moreover, the stability of Cdc6 and cell division cycle 7 (Cdc7) proteins suggests that any DNA rereplication in these experimental conditions is unlikely to have caused significant levels of DNA damage. These experiments therefore suggest that Geminin regulates Cdt1 in the normal cell cycle.

Geminin Is a Positive Regulator of Cdt1 mRNA. The most obvious way Geminin would regulate the increase in Cdt1 levels in G2 and mitosis is by stabilizing Cdt1 against degradation, considering that Geminin inhibits the polyubiquitination of Cdt1 (4). However, it also is possible that Geminin could achieve the same result by a positive action on Cdt1 synthesis. We therefore asked if Geminin expression affects the levels of Cdt1 RNA. We treated U2OS cells with siRNA for Geminin or, as a control, with Cdt1 for 48 h and evaluated Cdt1 RNA levels by quantitative RT-PCR. Upon depletion of Geminin, Cdt1 RNA decreased more than twofold (Fig. 3*A*). Given that the cell-cycle progression is altered when cells are treated with siRNA for Geminin for long periods (21, 22), we performed shorter siRNA treatments to avoid changes in the cell cycle. We used the protocol previously developed to treat cells with siRNA in S/G2 phase and to arrest them at the end of the G2 phase or mitosis of the same cell cycle (Fig. S2*A*) (4). U2OS cells were treated with siRNA for Geminin or with Cdt1, as a control, in S/G2 phase; then we measured the RNA levels of Cdt1 in G2 or mitosis (Fig. 3*B*). Again, we observed that Geminin depletion reduced Cdt1 RNA by approximately twofold in both G2 phase and mitotic cells and that these reductions correlated with similar reductions of the protein (Fig. 3*C*). To ask if an increase in Geminin levels would have the opposite effect, we used a cell line stably overexpressing full-length Geminin (4); we performed the same type of synchronization to harvest cells at the end of G2 phase or mitosis. As a control, we treated cells with siRNA for Geminin following the same protocol as before (Fig. S2*A*). We observed that when Geminin protein was overexpressed, Cdt1 RNA increased approximately twofold in G2 cells and 2.5-fold in mitotic cells (Fig. 3*D*). Interestingly, when a Geminin mutant unable to

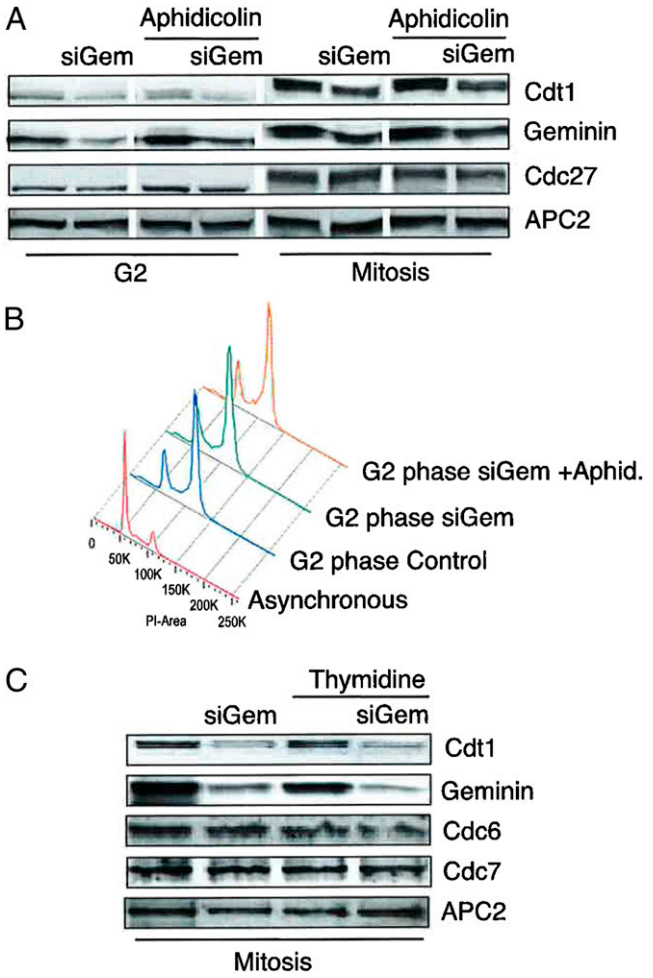

Fig. 2. Geminin depletion causes decrease of Cdt1 protein in the presence of DNA synthesis inhibitors. (*A*) U2OS cells were synchronized by double thymidine treatment and then were released from S-phase block for 2 h. Cells then were transfected with Geminin siRNA or mock-transfected before they were treated with nocodazole. Aphidicolin was added at the S/G2 transition where indicated. Cells then were incubated for another 9 h before the adherent cells (end-G2 cells) or the shake-off cells (mitosis) were harvested (see also Fig. S1*A*). Western blotting analysis for the indicated proteins is shown. APC2 protein was used as a loading control. The slower mobility of Cdc27 in mitosis is shown as a marker of good separation between G2 and mitotic cells (25). Cdt1 mobility decreases in mitosis, as previously reported (4). Spaces are intentionally left between pairs of bands to indicate that the images shown are taken from distant parts of the same gel. (*B*) FACS analyses were performed with the samples shown in *A* to control for good synchronization in G2 phase after depletion of Geminin protein. (*C*) U2OS cells were synchronized by double thymidine treatment and then were released from S-phase block. Ten hours later (at S/G2 transition), cells were treated with control siRNA or Geminin siRNA. Nocodazole and optional third thymidine were also added at this time. Cells then were incubated for an additional14 h before the mitotic cells were harvested by manual shake-off. Western blotting analysis for the indicated proteins is shown. APC2 protein was used as a loading control.

associate with itself and with Cdt1 (4, 23, 24) was stably overexpressed in place of the wild type, we detected no change in the RNA and protein levels of Cdt1 (Fig. 3*D*). As expected, cells treated with siRNA for Geminin had reduced levels of RNA for Cdt1 (Fig. 3*D*). We then measured Cdt1 RNA along the cell cycle. To this purpose, we synchronized U2OS cells in S and G2 phases as described in Fig. S2*B*. We noticed that, like two other replication factors, Cdc6 and mini chromosome maintenance 6 (Mcm6), Cdt1 RNA decreased approximately twofold in G2 phase (Fig. 3*E*). In the same experiment we also tested if depletion of Geminin also leads to Cdt1 RNA reduction in S-phase and in G2-phase cells that are not nocodazole-arrested. We indeed observed that Geminin depletion led to an almost twofold reduction of Cdt1 RNA levels in both S phase and G2 phase; Cdc6 RNA and Mcm6 RNA, on the contrary, did not decrease in S phase and decreased only slightly in G2 phase (Fig. 3*F*). In the same experiment we also tested if Geminin has an effect on the stability of Cdt1 RNA. To this purpose, we used the transcription inhibitor Actinomycin D. We treated cells at early S phase (Fig. S2*B*) with Actinomycin D and collected time points in mid-S phase and G2 phase. We noticed that Geminin depletion increased the rate of reduction of Cdt1 RNA, particularly after the inhibition of transcription (Fig. 3*G*). In particular, Cdt1 mRNA levels decreased less than 10% during the first 3 h of the time course with Actinomycin D but decreased nearly 45% when Geminin was depleted. Moreover, we noticed that the levels of Cdt1 RNA during S and G2 phases were little affected by Actinomycin D, suggesting that during S–G2 progression the synthesis of new Cdt1 RNA is modest. Overall these results show that although Geminin also may regulate RNA levels of other replication factors, it clearly has a more dramatic role in the regulation of RNA of Cdt1. Indeed, Geminin positively regulates the RNA levels of Cdt1 in S phase, G2 phase, and mitosis. These results also suggest that Geminin may need to bind to Cdt1 to regulate Cdt1 RNA. Furthermore, these results indicate that one of the mechanisms by which Geminin regulates Cdt1 RNA is by partial stabilization.

Geminin Affects Cdt1 Stability in G2 Phase and Mitosis. Although the effects of Geminin on the polyubiquitination of Cdt1 have been demonstrated (4), the effect of Geminin on Cdt1 stability had not been established directly. We first checked to see if there is a positive correlation between the levels of Geminin and the level of Cdt1 protein. Because we already knew that Cdt1 levels decrease when Geminin levels decrease, we asked if this correlation also holds when Geminin is overexpressed. Using the same protocol we used for the RNA (Fig. 3*D*), we observed that Cdt1 increased both in G2 phase and mitosis when Geminin protein levels increased (Fig. 4*A*). To confirm that Geminin increase amplifies Cdt1 protein levels, we also overexpressed a stable version of Geminin (4, 11) in HeLa cells. We then synchronized the cells in G1 phase (when Geminin normally is degraded) and measured the stability of Cdt1 protein during this phase. To this purpose, we treated the cells with cycloheximide for 30, 60, and 90 min. After Western blotting, we observed that in cells overexpressing Geminin the half-life of Cdt1 protein increased about twofold (Fig. 4*B*). To ask if Geminin also affects the turnover of Cdt1 protein, we measured the stability of Cdt1 protein after Geminin depletion. We synchronized U2OS cells in mitosis to compare cells at exactly the same phase of the cell cycle and treated the mitotic cells with cycloheximide. We noticed that the half-life of Cdt1 protein is increased in mitotic-arrested cells, as observed previously (25); Cdt1 has half-life of about 150 min in control cells, but when Geminin is depleted, Cdt1 has a half-life of less than 100 min. Mcm6 stability, on the contrary, was not affected by Geminin depletion (Fig. 4*C*). We then used another protein-synthesis inhibitor, puromycin, to confirm these results. This time we also collected G2 cells, and we extended the treatment with the protein-synthesis inhibitor. After Western blot analysis, we noticed again that Cdt1 is unstable during mitosis (with a half-life longer than 2 h) and that depletion of Geminin decreased the stability of Cdt1 (leading to a half-life shorter than 2 h). We also measured the protein levels of other cell-cycle regulators. Although Cdc6 and Mcm6 were stable during the mitotic time course, cyclin B was unstable. Most important, depletion of Geminin did not affect the stability of Cdc6 and Mcm6 and had only a small effect on the stability of cyclin B (and did not affect its

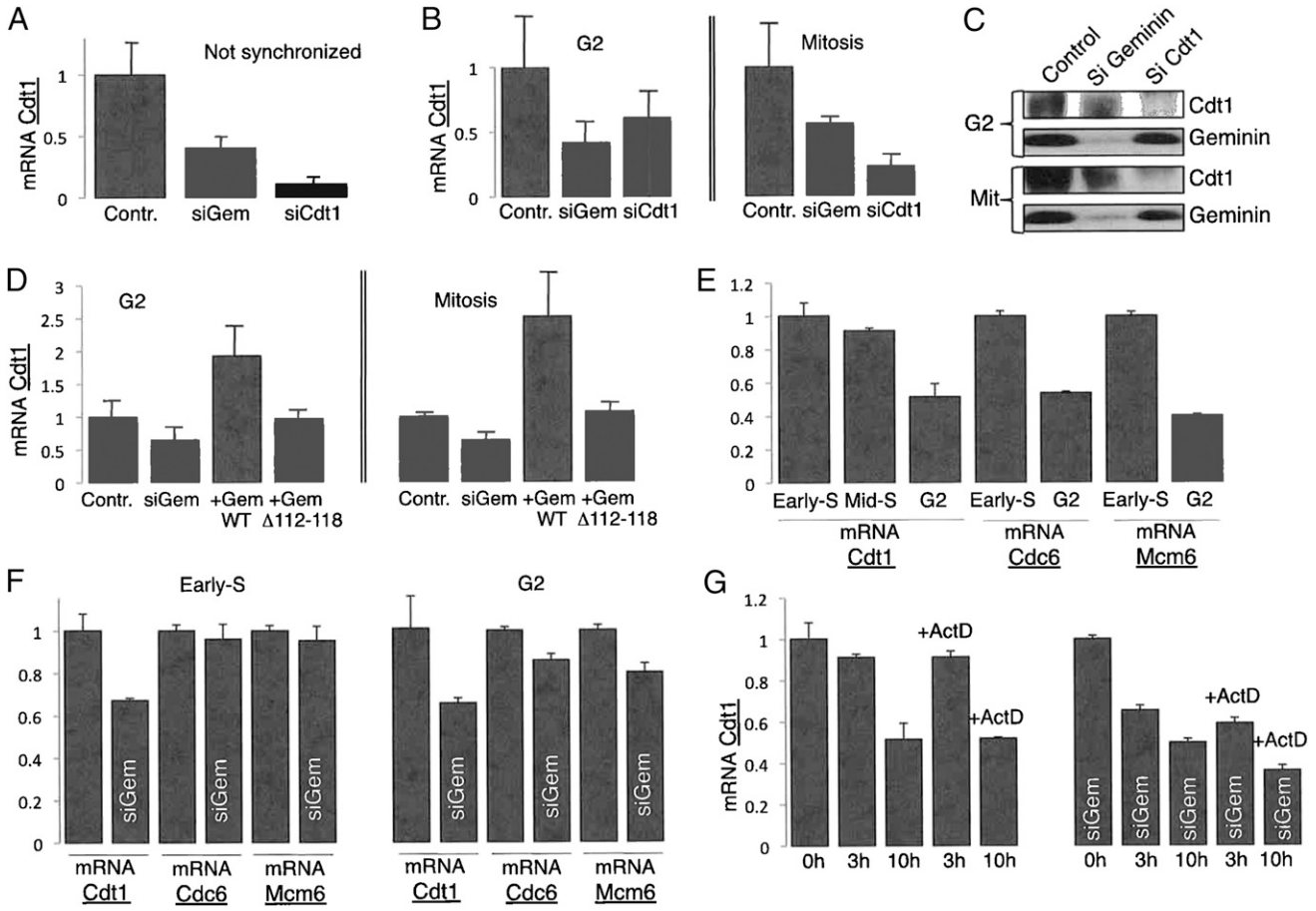

Fig. 3. Geminin positively regulates the RNA levels of Cdt1. (*A*) Geminin depletion decreases Cdt1 mRNA. U2OS cells were transfected with siRNA for Geminin or Cdt1 or were mock-transfected (Contr.) and incubated for 48 h. After cell lysis, mRNA for Cdt1 was quantified by quantitative RT-PCR, and quantifications were represented in a bar chart. Samples were normalized to β-actin. The control (mock-transfected) sample was arbitrarily given a value of 1. SDs are shown. (*B*) Geminin depletion decreases Cdt1 mRNA in G2 phase and mitosis. U2OS cells were treated with thymidine and released for 5 h. Cells then were transfected with siRNA for Geminin or Cdt1 or were mock-transfected, as previously reported (4) (see also Fig. S2*A*). Nocodazole was added 4 h after transfections, and cells were incubated for another 16 h before the adherent cells (end-G2 cells) or the shake-off cells (mitotic cells) were harvested. Cdt1 mRNA levels were evaluated by quantitative RT-PCR, and quantification is shown as bar charts. Control (mock-transfected) samples for G2 and mitotic cells were arbitrarily assigned a value of 1. SDs are shown. Samples were normalized to β-actin. (*C*) Western blotting analysis for the samples in *B* is shown to evaluate protein levels of Cdt1 and Geminin. (*D*) Geminin regulates Cdt1 RNA levels. Normal U2OS cells or U2OS cells stably overexpressing full-length WT Geminin or a form of Geminin deleted of amino acids 112–118 (4) were used for this experiment. Cells were synchronized by thymidine treatment and were released for 5 h before transfection with Geminin siRNA or mock-transfection as indicated. Nocodazole was added 4 h after transfections, and cells were incubated for another 12 h before the adherent cells (end-G2 cells) or the shake-off cells (mitotic cells) were harvested. Cdt1 mRNA levels were evaluated by quantitative RT-PCR, and quantification is shown as bar charts. Control (mock-transfected) samples for G2 and mitotic cells are arbitrarily assigned a value of 1. SDs are shown. Samples are normalized to β-actin. (*E*) Cdt1 mRNA decreases in G2 phase. U2OS cells were synchronized as described in Fig. S2*B*. Time points were taken at early S phase, mid S phase, and G2 phase. Cdt1, Cdc6, and Mcm6 mRNA levels were evaluated by quantitative RT-PCR, and quantifications are shown as bar charts. The first time points (early S phase) are arbitrarily assigned a value of 1. SDs are shown. Samples are normalized to β-actin. (*F*) Geminin depletion decreases Cdt1 mRNA in S and G2 phases. Some plates of U2OS cells were treated in parallel with siRNA for Geminin in the experiment shown in *E* (see also Fig. S2*B*). Cells were taken at early S phase and G2 phase, and Cdt1, Cdc6, and Mcm6 mRNA levels then were evaluated by quantitative RT-PCR. The first time points (in early S phase) are arbitrarily assigned a value of 1. SDs are shown. Samples are normalized to β-actin. (*G*) Geminin depletion has a little effect on stability of Cdt1 RNA. Some plates of U2OS cells also were treated in parallel with Actinomycin D in early S phase in the experiment shown in *E* and *F* (see also Fig. S2*B*). The treatment with the transcription inhibitor Actinomycin D was as long as 10 h. Cells were taken at early S phase, mid S phase, and G2 phase. Cdt1 mRNA levels then were evaluated by quantitative RT-PCR. The first time points (in early S phase) are arbitrarily assigned a value of 1. SDs are shown. Samples are normalized to β-actin.

mitotic levels) (Fig. 4*D*). In the same experiment, we also confirmed that Cdt1 is very unstable (more than in mitosis) during G2 phase. In contrast, we observed that Geminin, Cdc6, cyclin A, cyclin B, and Mcm6 were much more stable (Fig. 4*D*). The observation that Cdc6 and Mcm6 protein levels were not affected by Geminin depletion suggests that the small reductions of their RNA levels during G2 phase (Fig. 3*F*) are not sufficient to affect their protein levels. These results show that Cdt1 is an unstable protein before cell division and that Geminin regulates its stability. When Geminin levels are reduced, Cdt1 stability decreases further, and its levels drop dramatically.

Discussion

Assurance that DNA replication is limited to making a complete and single copy of the genome in each cell cycle is an important requirement of the cell cycle. Cdt1 plays a central role by restricting DNA replication to S phase and establishing the requirements for DNA replication in G1. To achieve its cell-cycle specificity, the levels and activity of Cdt1 are regulated by multiple mechanisms along the cell cycle. During mitosis Cdt1 levels are high to guarantee efficient pre-RC formations in the next cell cycle (4). The high level of Cdt1 also may reflect its involvement in kinetochore–microtubule attachments (26). During

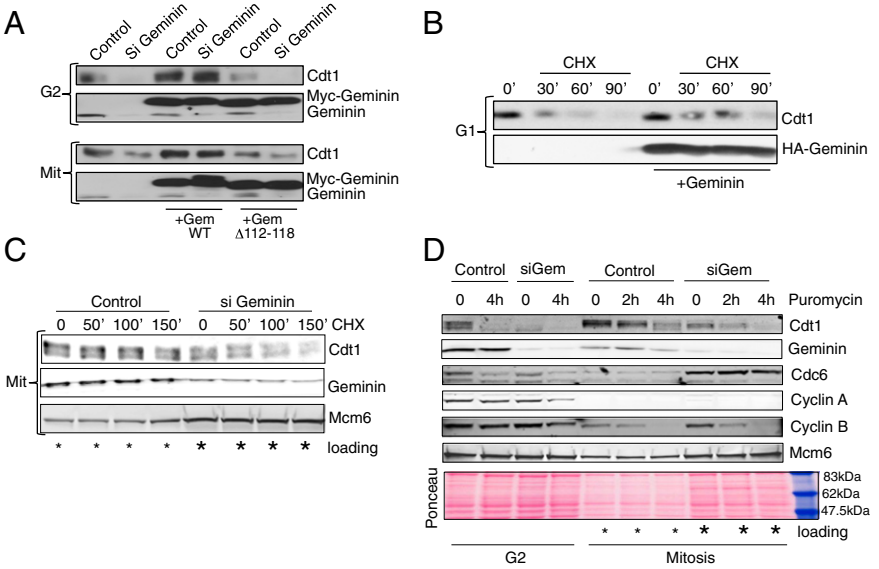

Fig. 4. Geminin positively regulates the stability of Cdt1 protein. (*A*) Geminin increase amplifies Cdt1 protein levels. Western blotting analysis for samples of cells treated as in Fig. 3*D* is shown to evaluate a positive correlation between Geminin and Cdt1 protein levels. Note that both the WT and mutant Geminin are Myc-tagged and mutated in the target sequence for siRNA, and for this reason the exogenous proteins are not affected by siRNA. (*B*) Geminin increase amplifies Cdt1 protein levels. HeLa cells were transfected with a vector expressing a stable version of Geminin mutated in the destruction box (4) or control. After synchronization in mitosis, cells were released in G1 phase for 4 h before treatment with cycloheximide (CHX) for the indicated times. Western blotting analysis for the indicated proteins is shown. (*C*) Geminin depletion decreases the stability of Cdt1 protein. U2OS cells treated with siRNA for Geminin or control were synchronized in mitosis and treated with cycloheximide for the indicated times. Western blotting analyses for Cdt1, Geminin, and Mcm6 are shown. To facilitate the comparison of Cdt1 half-lives in control cells and cells treated with siRNA for Geminin, the different amounts of total protein loaded on gel are represented by asterisk size; that is, the total protein loaded on gel in lanes marked by large asterisks is 3.5-fold the total protein in the lanes marked by small asterisks. (*D*) Geminin depletion decreases the stability of Cdt1 protein. U2OS cells treated with siRNA for Geminin and control cells were synchronized in mitosis or end-G2 phase and treated with puromycin (a protein-synthesis inhibitor) for the indicated times. Western blotting analyses for the indicated proteins are shown. To facilitate the comparison of Cdt1 half-lives in control cells and cells treated with siRNA for Geminin, the different amounts of total protein loaded on gel are represented by asterisk size; that is, total protein loaded on gel in lanes marked by large asterisks is 3.5-fold the total protein in the lanes marked by small asterisks. Ponceau staining of the nitrocellulose membrane used for the Western blot analysis is shown to control for total protein loaded in each lane of the gel.

mitosis CDKs inhibit Cdt1 activity (4, 12, 27); CDK activity may increase Cdt1 levels further by strengthening the binding between Cdt1 and Geminin (4, 25) (Fig. S3). Just after mitotic exit, Cdt1 is high in G1 phase, but Geminin also is quickly degraded. The degradation of Geminin activates Cdt1, and Cdt1 associates with chromatin to form the pre-RCs (2, 3, 28–30). During this phase, Cdt1 continues to be synthesized but is not stable (Fig. 4*B*). Its turnover is regulated by Cdh1-APC–mediated degradation (31). At S phase onset, Cdt1 is degraded more efficiently by the activation of the ubiquitin ligase activity mediated by Cul4-DDB1-Cdt2 (32), and its level decreases dramatically; only low levels of Cdt1 remain. This residual Cdt1, possibly associated with chromatin, could have some role in the efficient execution of DNA replication through interactions with DDK complex and cell division cycle 45 (Cdc45) (28, 30, 33, 34), but it no longer can form pre-RCs. It also is possible that the low S-phase levels of Cdt1 may be completely inactive by binding to Geminin, CDK and DDK. After S phase, Cdt1 needs to accumulate for its mitotic function and for pre-RC formation at the beginning of G1. In many cells G2 is very short. Three mechanisms contribute to Cdt1 accumulation in G2: the inactivation of the ubiquitin ligase activity associated with Cul4-DDB1-Cdt2 (7), the partial stabilization of Cdt1 caused by phosphorylation by the MAP kinases p38 and JNK (9), and the regulation by Geminin. These mechanisms are particularly important because they must counteract the decrease of Cdt1 RNA during G2 phase (Fig. 3*E*). The regulation of Cdt1 along the cell cycle is recapitulated in Fig. S4.

Geminin is essential for the accumulation of sufficient Cdt1 during mitosis (4) and meiosis (10). In particular, depletion of Geminin was shown previously to prevent the increase of Cdt1 at mitosis, and overexpression of Geminin protected Cdt1 from polyubiquitylation (4). These observations led to a model in which Geminin's role is to permit Cdt1 buildup in mitosis, providing enough Cdt1 for pre-RC formation in G1 of the following cell cycle. In this model its buildup in S phase was to prevent reinitiation of replication. However, more recently it was proposed that the real role of Geminin is not to prevent DNA rereplication in S phase, as previously hypothesized (21, 22), but rather to prevent rereplication in the G2 phase (12). We there-

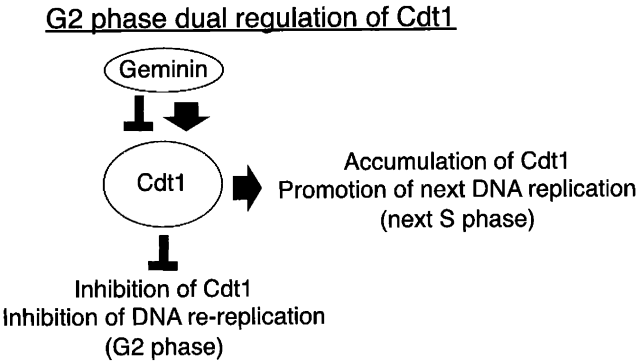

Fig. 5. Dual regulation of Cdt1 in G2 phase. Geminin inhibits and amplifies Cdt1 protein during the G2 phase of the cell cycle. In this way Geminin prevents DNA rereplication during the same G2 phase and promotes the timely onset of DNA replication in the S phase of the next cell cycle.

fore tested the hypothesis that Cdt1 levels respond to DNA replication in G2 phase and not as a result of the direct inhibition by Geminin. Using two different inhibitors of the DNA synthesis, we observed the same reduction of Cdt1 levels as in the control, whether or not Geminin was depleted. Cdc6, another replication factor that also is degraded after DNA damage (16), is not affected by Geminin depletion. Therefore under these experimental conditions, depletion of Geminin in G2 does not seem to induce DNA damage, or, if there is some DNA damage, this damage must be insufficient to stimulate degradation of Cdc6. Also, in agreement with this observation, the cell cycle is not arrested under these experimental conditions (4), and overexpression of Geminin can increase the levels of Cdt1 protein in G2 phase and mitosis (Fig. 4*A*). Overall, these results establish that Geminin is abundant in G2 phase, not only preventing DNA rereplication (12) but also increasing Cdt1 protein levels by stabilizing and increasing its RNA to guarantee the timely onset of DNA replication in the next cell cycle (Fig. 5).

A strategy of efficient and precise DNA replication arises from the concept of licensing that emanated from the earlier work (35–37). From these studies it became clear that the eukaryotic cell is constrained to pass through the cell cycle to initiate a new round of replication. The simultaneous capacity to form a preinitiation complex and the capacity actually to replicate DNA had to be suppressed sufficiently. Furthermore it is obvious that these separate events must be coordinated with mitosis, because neither replication nor pre-RC formation should take place while the cell is dividing and the chromatin is condensed. Geminin emerges more clearly from our present current study as a master regulator of this process, coordinating replication under the regulation of APC. Degradation of Geminin at the end of mitosis via APC allows pre-RC formation in G1, but Geminin is both an inhibitor of Cdt1 and a protector against Cdt1 degradation. A large pool of Cdt1 must accumulate before G1 because both Geminin and Cdt1 are susceptible to Cdh1-APC ubiquitination and degradation. Cdt1's pre-RC forming activity must be absent in S phase, and here the degradation of Cdt1 is augmented significantly by a Cul4-DDB1 E3 enzyme. Most importantly Cdt1 must accumulate rapidly in G2. The G2 phase is characterized by the loss APC activity; hence, Geminin can accumulate. The various means by which Geminin and Cdt1 are accumulated in G2 are central to this process. In this way Geminin can act as a master regulator, with its rapid degradation at mitosis helping initiate the replication of DNA. The subsequent increase in Geminin when APC is completely inactivated sets the stage for the accumulation of Cdt1 for the next S phase.

Materials and Methods

Detailed materials and methods are reported in *SI Materials and Methods*. Concisely, U2OS or HeLa human cell lines were synchronized in S phase with single or double 2.5-mM thymidine treatments. Cells released from S phase were synchronized in G2 phase either through arrest with 50 ng/mL nocodazole and harvesting of the adherent cells ("end-G2" cells) or through release for 10 h. Cells released from S phase were synchronized in mitosis through arrest with 50 ng/mL nocodazole and harvesting of the shake-off cells. siRNA were transfected transiently. Plasmids expressing Cdt1 or Geminin were expressed either transiently or permanently, depending on the case. Quantification of RNA was performed by quantitative RT-PCR.

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1. Symeonidou IE, Taraviras S, Lygerou Z (2012) Control over DNA replication in time and space. *FEBS Lett* 586(18):2803–2812.
2. Maiorano D, Moreau J, Méchali M (2000) XCDT1 is required for the assembly of pre-replicative complexes in Xenopus laevis. *Nature* 404(6778):622–625.
3. Nishitani H, Lygerou Z, Nishimoto T, Nurse P (2000) The Cdt1 protein is required to license DNA for replication in fission yeast. *Nature* 404(6778):625–628.
4. Ballabeni A, et al. (2004) Human geminin promotes pre-RC formation and DNA replication by stabilizing CDT1 in mitosis. *EMBO J* 23(15):3122–3132.
5. Nishitani H, Taraviras S, Lygerou Z, Nishimoto T (2001) The human licensing factor for DNA replication Cdt1 accumulates in G1 and is destabilized after initiation of S-phase. *J Biol Chem* 276(48):44905–44911.
6. Arias EE, Walter JC (2006) PCNA functions as a molecular platform to trigger Cdt1 destruction and prevent re-replication. *Nat Cell Biol* 8(1):84–90.
7. Nishitani H, et al. (2006) Two E3 ubiquitin ligases, SCF-Skp2 and DDB1-Cul4, target human Cdt1 for proteolysis. *EMBO J* 25(5):1126–1136.
8. Senga T, et al. (2006) PCNA is a cofactor for Cdt1 degradation by CUL4/DDB1-mediated N-terminal ubiquitination. *J Biol Chem* 281(10):6246–6252.
9. Chandrasekaran S, Tan TX, Hall JR, Cook JG (2011) Stress-stimulated mitogen-activated protein kinases control the stability and activity of the Cdt1 DNA replication licensing factor. *Mol Cell Biol* 31(22):4405–4416.
10. Narasimhachar Y, Coué M (2009) Geminin stabilizes Cdt1 during meiosis in Xenopus oocytes. *J Biol Chem* 284(40):27235–27242.
11. McGarry TJ, Kirschner MW (1998) Geminin, an inhibitor of DNA replication, is degraded during mitosis. *Cell* 93(6):1043–1053.
12. Klotz-Noack K, McIntosh D, Schurch N, Pratt N, Blow JJ (2012) Re-replication induced by geminin depletion occurs from G2 and is enhanced by checkpoint activation. *J Cell Sci* 125(Pt 10):2436–2445.
13. Tada S, Li A, Maiorano D, Méchali M, Blow JJ (2001) Repression of origin assembly in metaphase depends on inhibition of RLF-B/Cdt1 by geminin. *Nat Cell Biol* 3(2):107–113.
14. Wohlschlegel JA, et al. (2000) Inhibition of eukaryotic DNA replication by geminin binding to Cdt1. *Science* 290(5500):2309–2312.
15. Hall JR, et al. (2007) Cdc6 stability is regulated by the Huwe1 ubiquitin ligase after DNA damage. *Mol Biol Cell* 18(9):3340–3350.
16. Hall JR, et al. (2008) Cdt1 and Cdc6 are destabilized by rereplication-induced DNA damage. *J Biol Chem* 283(37):25356–25363.
17. Milhollen MA, et al. (2011) Inhibition of NEDD8-activating enzyme induces rereplication and apoptosis in human tumor cells consistent with deregulating CDT1 turnover. *Cancer Res* 71(8):3042–3051.
18. Pelizon C, d'Adda di Fagagna F, Farrace L, Laskey RA (2002) Human replication protein Cdc6 is selectively cleaved by caspase 3 during apoptosis. *EMBO Rep* 3(8):780–784.
19. Ralph E, Boye E, Kearsey SE (2006) DNA damage induces Cdt1 proteolysis in fission yeast through a pathway dependent on Cdt2 and Ddb1. *EMBO Rep* 7(11):1134–1139.
20. Bjursell G, Reichard P (1973) Effects of thymidine on deoxyribonucleoside triphosphate pools and deoxyribonucleic acid synthesis in Chinese hamster ovary cells. *J Biol Chem* 248(11):3904–3909.
21. Melixetian M, et al. (2004) Loss of Geminin induces rereplication in the presence of functional p53. *J Cell Biol* 165(4):473–482.
22. Mihaylov IS, et al. (2002) Control of DNA replication and chromosome ploidy by geminin and cyclin A. *Mol Cell Biol* 22(6):1868–1880.
23. De Marco V, et al. (2009) Quaternary structure of the human Cdt1-Geminin complex regulates DNA replication licensing. *Proc Natl Acad Sci USA* 106(47):19807–19812.
24. Saxena S, et al. (2004) A dimerized coiled-coil domain and an adjoining part of geminin interact with two sites on Cdt1 for replication inhibition. *Mol Cell* 15(2):245–258.
25. Ballabeni A, et al. (2011) Cell cycle adaptations of embryonic stem cells. *Proc Natl Acad Sci USA* 108(48):19252–19257.
26. Varma D, et al. (2012) Recruitment of the human Cdt1 replication licensing protein by the loop domain of Hec1 is required for stable kinetochore-microtubule attachment. *Nat Cell Biol* 14(6):593–603.
27. Chen S, Bell SP (2011) CDK prevents Mcm2-7 helicase loading by inhibiting Cdt1 interaction with Orc6. *Genes Dev* 25(4):363–372.
28. Xouri G, Dimaki M, Bastiaens PI, Lygerou Z (2007) Cdt1 interactions in the licensing process: A model for dynamic spatiotemporal control of licensing. *Cell Cycle* 6(13):1549–1552.
29. Xouri G, et al. (2007) Cdt1 associates dynamically with chromatin throughout G1 and recruits Geminin onto chromatin. *EMBO J* 26(5):1303–1314.
30. Lutzmann M, Maiorano D, Méchali M (2006) A Cdt1-geminin complex licenses chromatin for DNA replication and prevents rereplication during S phase in Xenopus. *EMBO J* 25(24):5764–5774.
31. Sugimoto N, et al. (2008) Identification of novel human Cdt1-binding proteins by a proteomics approach: Proteolytic regulation by APC/CCdh1. *Mol Biol Cell* 19(3):1007–1021.
32. Jin J, Arias EE, Chen J, Harper JW, Walter JC (2006) A family of diverse Cul4-Ddb1-interacting proteins includes Cdt2, which is required for S phase destruction of the replication factor Cdt1. *Mol Cell* 23(5):709–721.
33. Ballabeni A, et al. (2009) Human CDT1 associates with CDC7 and recruits CDC45 to chromatin during S phase. *J Biol Chem* 284(5):3028–3036.
34. Lydeard JR, et al. (2010) Break-induced replication requires all essential DNA replication factors except those specific for pre-RC assembly. *Genes Dev* 24(11):1133–1144.
35. Johnson RT, Rao PN (1970) Mammalian cell fusion: Induction of premature chromosome condensation in interphase nuclei. *Nature* 226(5247):717–722.
36. Harland RM, Laskey RA (1980) Regulated replication of DNA microinjected into eggs of Xenopus laevis. *Cell* 21(3):761–771.
37. Blow JJ, Laskey RA (1986) Initiation of DNA replication in nuclei and purified DNA by a cell-free extract of Xenopus eggs. *Cell* 47(4):577–587.