Human Geminin promotes pre-RC formation and DNA replication by stabilizing CDT1 in mitosis

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Geminin is an unstable inhibitor of DNA replication that negatively regulates the licensing factor CDT1 and inhibits pre-replicative complex (pre-RC) formation in *Xenopus* egg extracts. Here we describe a novel function of Geminin. We demonstrate that human Geminin protects CDT1 from proteasome-mediated degradation by inhibiting its ubiquitination. In particular, Geminin ensures basal levels of CDT1 during S phase and its accumulation during mitosis. Consistently, inhibition of Geminin synthesis during M phase leads to impairment of pre-RC formation and DNA replication during the following cell cycle. Moreover, we show that inhibition of CDK1 during mitosis, and not Geminin depletion, is sufficient for premature formation of pre-RCs, indicating that CDK activity is the major mitotic inhibitor of licensing in human cells. Taken together with recent data from our laboratory, our results demonstrate that Geminin is both a negative and positive regulator of pre-RC formation in human cells, playing a positive role in allowing CDT1 accumulation in G2–M, and preventing relicensing of origins in S–G2.

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Introduction

DNA replication initiates in eukaryotes from thousands of replication origins. Each origin needs to be licensed in order to be competent for DNA replication (Diffley, 1996; Gilbert, 2001; Bielsky, 2003). The licensing occurs in late mitosis and early G1, through the orderly assembly of a multiprotein complex, the pre-replicative complex (pre-RC), in the proximity of the origin of DNA replication (Wyrick *et al*, 2001). Upon firing of the origins, the pre-RC undergoes extensive modifications and rearrangements and the remaining proteins constitute the post-replicative complex (post-RC), which lacks the capability of initiating DNA replication, but which

may form a docking site essential for assembly of a new pre-RC during the subsequent telophase and G1 (Lei and Tye, 2001; Okuno *et al*, 2001; Stoeber *et al*, 2001; Dimitrova *et al*, 2002). The pre-RC indeed takes shape while assembling on the core of the post-RC, a hexamer called origin recognition complex (ORC1–6) (Rowley *et al*, 1995; Wyrick *et al*, 2001; DePamphilis, 2003). The pre-RC is formed by the recruitment of CDC6 and CDT1 to ORC during telophase and G1 and the subsequent loading of the minichromosome maintenance (MCM) proteins, consisting of MCM2–7 (Cocker *et al*, 1996; Maiorano *et al*, 2000; Nishitani *et al*, 2000; Lei and Tye, 2001; Okuno *et al*, 2001). Building of pre-RCs commences therefore immediately after anaphase exit transition and terminates during G1. While cyclin-dependent kinase (CDK) activity is then required for the onset of S phase (Jallepalli and Kelly, 1997; Krude *et al*, 1997; Zou and Stillman, 1998), DBF4/CDC7 (DDK) activity seems to regulate the timing of firing of each individual replication origin during S phase (Bousset and Diffley, 1998; Donaldson *et al*, 1998). Both CDKs and, more recently, DDK have been shown to phosphorylate subunits of the pre-RC and other replication proteins (Jiang *et al*, 1999; Nougarede *et al*, 2000; Frouin *et al*, 2002; Woo and Poon, 2003). After firing, origins are kept in an unlicensed state until the completion of the cell cycle. Whereas unicellular eukaryotes use CDK activity to inhibit pre-RC assembly and licensing during S–G2 (Dahmann *et al*, 1995; Petersen *et al*, 2000; Weinreich *et al*, 2001), metazoans, in addition, employ Geminin, a recently identified 25 kDa protein.

Geminin is an unstable protein that is present during S–G2–M phases and gets destroyed by APC/CDH1 at the metaphase/anaphase transition (McGarry and Kirschner, 1998). It binds and inhibits CDT1 whose levels are low in S due to proteasome degradation and high in G1 and M (Maiorano *et al*, 2000; Nishitani *et al*, 2000, 2001; Wohlschlegel *et al*, 2000; Tada *et al*, 2001). The depletion of Geminin in *Xenopus* metaphase egg extracts is sufficient for pre-RC formation (Tada *et al*, 2001), whereas depletion in *Drosophila* and human cells leads to over-replication of the genome and Chk1-dependent checkpoint activation (Mihaylov *et al*, 2002; Melixetian *et al*, 2004). It is therefore believed that Geminin has a prominent role in preventing rereplication in S–G2 in multicellular eukaryotes. Failure to apply this control results in genomic instability and checkpoint activation. Moreover, recently Geminin was shown to have a polycomb-like function and to be able to regulate both transcription and development (Del Bene *et al*, 2004; Luo *et al*, 2004).

Here we demonstrate a novel function of human Geminin. We show that human Geminin by stabilizing CDT1 during mitosis has a positive role in pre-RC formation. We demonstrate that Geminin protects CDT1 from ubiquitination and subsequent proteasome-dependent degradation during S and M phases and that depletion of Geminin during mitosis leads to CDT1 degradation, impairment of pre-RC formation and

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inhibition of DNA replication during the following cell cycle. We have mapped the domains of Geminin and CDT1 involved in the binding and show that the stabilization of CDT1 occurs by direct physical interaction with Geminin. Moreover, we show that inhibition of CDK1, and not Geminin depletion, is sufficient for the premature formation of pre-RCs in mitotic human cells, suggesting that the inhibition of pre-RC formation in mitosis is executed by CDK activity. Taken together with previously published data, our results therefore show that Geminin is both a negative and positive regulator of pre-RC formation in human cells.

Results

Geminin regulates CDT1 protein levels

To investigate the role of Geminin in mammalian cells, we inhibited Geminin expression in U2OS osteosarcoma cells

using small interfering RNAs (siRNAs). We noticed that the depletion of Geminin was associated with a decrease in the levels of CDT1 (Figure 1A). We observed that CDT1 expression was reduced to a similar extent using siRNA oligos specific for two different RNA target sequences of Geminin (Figure 1B). As a further control for the specificity of the siRNA oligos, we tested the effect of the siRNA (sequence B) in U2OS cells stably overexpressing a form of Geminin (Δ Gem) mutated in the target sequence for interference (Figure 1C). In these cells, endogenous Geminin was degraded; however, the overexpressed Geminin prevented the degradation of CDT1, demonstrating the specificity of the siRNA oligos to Geminin. All subsequent experiments have therefore been performed using oligos specific for target sequence B. We also observed reduction in CDT1 protein levels after Geminin depletion in other cell lines such as A549 (human lung carcinoma), RKO (human colon carcinoma), COS (monkey kidney carcinoma) and TIG3 (human diploid fibroblasts) (data not shown).

Our laboratory recently reported that constitutive overexpression of Δ Gem does not alter cell cycle progression but also that depletion of endogenous Geminin leads to S-phase accumulation (Melixetian *et al*, 2004). To rule out the possibility that the decreases in CDT1 protein levels are merely due to alterations of the cell cycle, we decided to synchronize the cells and perform siRNA treatment for shorter times in order to avoid cell cycle effects.

Geminin regulates basal levels of CDT1 during S phase

To understand if Geminin could stabilize CDT1 in S phase where the protein is known to be unstable (Maiorano *et al*, 2000; Wohlschlegel *et al*, 2000; Nishitani *et al*, 2001; Tada *et al*, 2001), we synchronized U2OS and HeLa cells in mitosis and released the cells in the presence of siRNA to Geminin or control and collected them in S phase (Figure 2A). Even though the levels of CDT1 are very low in S, we noticed that both U2OS and HeLa cells have lower levels of CDT1 protein upon Geminin depletion (Figure 2B and C). The decreases were partially rescued by treatment for 4 h with the proteasome inhibitor MG132, suggesting that the decreases were at least partially due to protein degradation. The levels of cyclin A indicate that the cells are at the same stage of S phase in the different samples.

To confirm that Geminin is capable of stabilizing unstable CDT1 during S phase, we overexpressed Geminin. Since we wanted to use a negative control in these experiments, we first mapped the region of CDT1 required for Geminin binding *in vitro* and *in vivo*. Mutants with deletions in the region of amino acids 150–190 were not able to bind to Geminin (Supplementary Figure 1). We called this region Geminin-binding domain (GBD) (Figure 2D). We therefore used a mutant of CDT1 lacking amino acids 170–190 (indicated as Δ GBD) as a negative control for testing the ability of Geminin to stabilize CDT1. HeLa cells were synchronized in S phase by a double-thymidine block, followed by a 1 h release, and they were subsequently co-microinjected with plasmids expressing wild type (WT) or Δ GBD HA-tagged CDT1 and Geminin. IgG was co-injected in all experiments to mark positively injected cells. After 3 h, we fixed the cells and stained for CDT1 expression using an anti-HA antibody (Figure 2E and F). We observed that Geminin specifically stabilized WT but not the Δ GBD mutant of CDT1. As a control for the

Figure 1 Human Geminin regulates CDT1 protein levels. (A) Decrease in CDT1 protein levels upon interference with Geminin expression. U2OS cells were either nontransfected or transfected for 60 h with siRNAs to Geminin, CDT1 or unrelated control. Expression of CDT1 and Geminin was determined by immunoblotting together with vinculin as loading control. (B) Two different siRNA target sequences on the gene for human Geminin were tested as in (A) and the expression of the indicated proteins was determined by Western blotting. (C) Expression of a form of Geminin mutated in the target sequence rescues CDT1 protein levels. The decrease in CDT1 levels is specifically due to the loss of Geminin expression. Cells were treated and analyzed as in (A). Control U2OS cells are indicated as '–'. HA-Geminin migrates slower than endogenous Geminin.

Figure 2 Human Geminin stabilizes basal levels of CDT1 in S phase. (A) Experimental outline. (B, C) Geminin depletion in S phase leads to a decrease in CDT1 protein levels. (B) U2OS and (C) HeLa were treated during G1 with siRNAs for the indicated genes and collected during S phase 14 and 12 h after nocodazole release, respectively, and treated with DMSO or MG132 for the last 4 h of incubation. The cell lysates were immunoblotted for the indicated proteins. (D) GBD is located between residues 150 and 190 of human CDT1 protein. (E, F) Overexpression of Geminin in S phase stabilizes CDT1 protein. (E) Plasmids expressing HA-CDT1 (WT and Δ GBD) were injected, with or without an expression plasmid for Geminin, in S phase-synchronized HeLa cells and 3 h later fixed and stained for HA or DAPI. IgG were co-injected and counterstained to identify the injected cells. MG132 was used in parallel as control for protein expression. Representative fields are shown. (F) Histogram representing the percentage of cells positive for CDT1 staining in the injected population of cells for each of the treatment shown in (E).

expression and proteasome-mediated degradation of CDT1, we treated cells with the proteasome inhibitor MG132. As shown in Figure 2E and F, proteasome inhibition led to a strong expression of both mutant and WT CDT1. To strengthen the point that the lack of stabilization of the Δ GBD CDT1 mutant upon Geminin overexpression was due to lack of stabilization and not to misfolding of the protein, we analyzed other CDT1 deletion mutants. We observed that mutants with deletions outside GBD were stabilized by Geminin overexpression, whereas a mutant lacking residues 150–170 of the GBD was not (Supplementary Figure 1D). From these experiments, we conclude that Geminin can specifically stabilize the basal levels of CDT1 in S phase, and that this stabilization is dependent on the physical association between the two proteins.

CDT1 accumulation during mitosis is mediated by Geminin

Mitosis is the only phase of the cell cycle where both CDT1 and Geminin protein levels are high (Nishitani *et al*, 2001). We therefore hypothesized that one of the physiological roles of Geminin could be to ensure the accumulation of CDT1 during mitosis. To test this, U2OS cells were synchronized in S phase by a thymidine block and released for 6 h before transfecting the cells with siRNA to Geminin or control (Figure 3A). At 4 h after transfection, cells were treated with nocodazole for an additional 8 h to obtain mitotic cells after manual shake-off. Excellent synchronization in meta-phase was obtained as indicated by the absence of cyclin A

and tyrosine 15-phosphorylated CDK1, and the presence of cyclin B1 (Figure 3B). We found that CDT1 is abundant and, as described by others (Maiorano *et al*, 2000; Nishitani *et al*, 2001), migrates slowly during mitosis due to phosphorylation as confirmed also by change in gel mobility after phosphatase treatment (Figure 3B). Shifted CDT1 appears as a doublet and this suggests that two differently phosphorylated forms of CDT1 exist during mitosis. We also observed that mitotic Geminin migrated slightly slower than Geminin extracted from asynchronously growing cells. We noticed that CDT1 levels were dramatically decreased in Geminin-depleted cells, as observed by immunoblotting and immunofluorescence analysis (Figure 3B and C, respectively). The decrease was more prominent in mitotic cells, because we observed minor reductions in CDT1 levels in the population of adherent cells that were not detached after manual shake-off and that represent bona fide G2-synchronized cells (Supplementary Figure 2A), as confirmed by immunoblot and FACS analysis (data not shown). Mitotic CDT1 was observed to be destabilized also in other cell lines, such as A549, HCT116 and RKO (see Supplementary Figure 2B). However, in G2 of these cell lines, we found that Geminin was important for the stability of CDT1 to a higher extent than in U2OS cells. Thus, we conclude that Geminin is involved in the regulation of CDT1 levels in S–G2–M, but that Geminin is only sufficient for allowing CDT1 accumulation in G2–M.

To test if the decreases in CDT1 levels were due to the lack of physical association between CDT1 and Geminin, we first mapped the region of Geminin necessary for the interaction

Figure 3 CDT1 accumulation during mitosis is mediated by interaction with Geminin. (A) Outline of the experimental protocol used to transfect G2/M cells with siRNAs (see text for details). (B) Depletion of Geminin in mitosis destabilizes CDT1. U2OS cells were transfected with siRNAs for the indicated genes and immunoblotted for the indicated proteins. 'As' represents asynchronous cells. The inset shows immunoprecipitated CDT1 incubated with or without alkaline phosphatase (AP) to evaluate the change in electrophoretic mobility. Immunoblotting for Geminin was performed using a 12% acrylamide gel. (C) Indirect immunofluorescence of U2OS cells treated as in (B) and stained for the indicated proteins or DAPI. (D) The CBD is located between residues 112 and 118 of human Geminin, in the first heptad repeat of the coiled-coil (c.c.) domain. (E) Overexpression of a form of myc-Geminin mutated in the target sequence rescues CDT1 protein levels. Depletion of endogenous Geminin in U2OS-overexpressing myc-tagged Δ iGeminin or Δ iGeminin Δ CBD treated as in (B) and immunoblotted for the indicated proteins is shown. (F) MG132 treatment rescues CDT1 protein levels. U2OS cells treated as in (B) to which MG132 was added 4 h before harvesting and immunoblotted for the indicated proteins are shown.

(see Supplementary Figure 3A–D). We called this region (corresponding to residues 112–118 of human Geminin) CDT1-binding domain (CBD) (Figure 3D). CBD corresponds to the first heptad repeat of the putative coiled-coil region identified using the PARCOIL algorithm (Lupas *et al*, 1991; McGarry and Kirschner, 1998). We observed that CDT1 was stable after depletion of endogenous Geminin in cells stably overexpressing myc-tagged Δ iGeminin but not in cells stably overexpressing a version of Δ iGeminin deleted in CBD (Δ CBD) (Figure 3E). Treatment of Geminin-depleted cells with a proteasome inhibitor restored the levels of CDT1 (Figure 3F). We confirmed these observations using U2OS cells stably expressing other deletion mutants of Geminin (see Supplementary Figure 3E). Taken together, these results show that Geminin by direct binding to CDT1 protects it from proteasome-dependent degradation and ensures the accumulation of CDT1 during G2 and M.

Geminin inhibits CDT1 ubiquitination

To understand the mechanism by which Geminin stabilizes CDT1 degradation, we tested if Geminin inhibits CDT1 ubi-

quitination (Li *et al*, 2003). We synchronized 293T cells in S phase with a thymidine block after transfection with plasmids expressing HA-ubiquitin and CDT1 together with mock plasmid or plasmids overexpressing myc-Geminin or Δ CBD mutant. To detect better the ubiquitinated forms of CDT1, we released the cells from the thymidine block in the presence of the proteasome inhibitor MG132 for 6 h. Cells were lysed and ubiquitin and associated proteins were immunoprecipitated with an antibody to the HA epitope. As shown in Figure 4A, CDT1 ubiquitination was reduced upon overexpression of Geminin but not of Δ CBD mutant as deduced by the decrease of slower migrating bands in the immunoprecipitated samples. These slow-mobility bands represent bona fide ubiquitinated forms of CDT1 as deduced by their absence in control immunoprecipitations and in cells not expressing HA-ubiquitin and by their increased abundance in cells treated with MG132. The levels of SKP2, a component of the E3 ubiquitin ligase that was recently shown to have specificity for CDT1 (Li *et al*, 2003, 2004; Kondo *et al*, 2004; Sugimoto *et al*, 2004), were unchanged in all conditions tested, suggesting that the decrease in CDT1 ubiquitination

Figure 4 Geminin inhibits CDT1 ubiquitination. (A) myc-Geminin overexpression decreases CDT1 ubiquitination. 293T cells were transfected and synchronized in S phase to detect CDT1 ubiquitination. The upper part of the panel shows the overexpressed proteins for each type of treatment and the use of MG132 for the last 6 h of incubation after release from the thymidine block. In the lower part, immunoblottings for the indicated proteins are shown both for cell lysates and immunoprecipitated samples. Mock immunoprecipitation was performed with an unrelated antibody and is indicated. Myc-Geminin forms (WT and ΔCBD) have lower mobility in comparison to endogenous Geminin. Ubiquitinated forms of CDT1 appear as smeared bands with lower mobility on gel in comparison to endogenous CDT1. (B) myc-Geminin overexpression does not decrease general ubiquitination. Immunoblotting for HA of the same lysates and immunoprecipitated samples indicated in (A) and run on a 17% polyacrylamide gel is shown. The position of the monomeric form of HA-ubiquitin in cell lysates is indicated as well as the smears representing total protein ubiquitination. (C) The inhibition of ubiquitination is not due to secondary cell cycle effects. Immunoblotting for the indicated proteins of the lysates shown in (A) and (B) is represented. 'As' represents asynchronous and untreated sample.

was not due to a decrease in the levels of the E3 ubiquitin ligase SKP2. Presently, we do not know if Geminin is affecting SKP2 E3 ligase activity or the interaction between SKP2 and

CDT1. In the condition used for the ubiquitination assay, we were not able to detect endogenous SKP2 and Geminin after immunoprecipitation. In contrast, we were able to detect with the same intensity the two forms of overexpressed myc-Geminin, suggesting that both full-length Geminin and ΔCBD mutant are equally ubiquitinated *in vivo* (Figure 4A). The same lysates and immunoprecipitated samples blotted for HA on a 17% gel revealed that expression of HA-ubiquitin as well as the general cellular ubiquitination was not affected by the overexpression of Geminin (Figure 4B), suggesting that Geminin is not a general inhibitor of ubiquitination. The levels of the cyclin A were identical in all conditions tested, suggesting that the inhibition of ubiquitination was not due to secondary cell cycle effects (Figure 4C). Based on these results, we suggest that Geminin stabilizes CDT1 levels by preventing its ubiquitination. Future experiments are required to determine the precise mechanism by which this occurs.

CDK inactivation, and not Geminin depletion, is sufficient for pre-RC formation during mitosis

Although inhibition of Geminin synthesis resulted in substantial reduction in the amounts of CDT1, the absence of Geminin might allow nondegraded CDT1 to load onto chromatin. In fact it has been previously observed that depletion of Geminin in *Xenopus* metaphase extracts led to loading of MCM proteins on chromatin and to licensing (Tada *et al*, 2001). Moreover, it was recently described that mouse Geminin can inhibit DNA-binding activity of CDT1 *in vitro* (Yanagi *et al*, 2002). To investigate if depletion of Geminin could allow premature CDT1 loading on chromatin during mitosis, we separated the chromatin fraction from the soluble fraction. Interestingly, we did not observe any CDT1 and MCM proteins (indicative of pre-RC formation) bound to chromatin in Geminin-depleted cells, whereas block of CDK1 activity, which can drive origin resetting in yeast (Noton and Diffley, 2000), led to the loading of CDT1 and MCM on chromatin both in the presence and absence of Geminin (Figure 5A). As control for the CDT1 dependence of MCM loading on chromatin, we showed that inhibition of CDT1 expression by siRNA abolished the association of MCM with chromatin (Figure 5A). Only unphosphorylated CDT1 bound to chromatin (data not shown). Almost identical results were obtained in A549 and RKO cells (data not shown). We conclude that CDK1 inhibition is sufficient for the recruitment of CDT1 on chromatin, whereas inhibition of Geminin synthesis is not.

Geminin depletion did not lead to pre-RC formation also in the presence of proteasome inhibitor MG132, indicating that the lack of licensing was not due to the lower levels of CDT1 (Figure 5B). The observation that treatment of cells, containing physiological levels of Geminin, with roscovitine is sufficient for CDT1 and MCM recruitment to chromatin implies that CDK1 is the main mitotic regulator of CDT1 loading on chromatin in human cells. We also noticed that after CDK1 inhibition the unphosphorylated form of CDT1 is much less destabilized in the absence of Geminin. This suggests that Geminin stabilizes more efficiently the phosphorylated forms of CDT1 and, as observed in Figures 3B, E, F and 5A, it preferentially stabilizes the hyperphosphorylated form. One possibility is that Geminin binds with different affinities the different forms of CDT1. To test this, we

Figure 5 CDK inactivation and not Geminin depletion leads to premature pre-RC formation during mitosis. **(A)** CDK1 inhibition and not Geminin depletion causes premature MCM loading. U2OS cells treated as in Figure 3B were in parallel treated with roscovitine for the last 3 h of nocodazole incubation and treated in order to separate soluble fraction from chromatin fraction (see Materials and methods for details). The different samples were immunoblotted with antibodies specific for the indicated proteins. 'sol' indicates the soluble fraction, whereas 'chrom' indicates the DNaseI-released chromatin fraction. **(B)** The lack of MCM loading upon Geminin depletion is not due to decrease in CDT1 protein levels. Immunoblotting for the indicated proteins of the same samples shown in (A) or treated with MG132 for the last 3 h of incubation is shown. **(C)** Geminin binds to both phosphorylated and nonphosphorylated forms of CDT1. Immunoblotting for the indicated proteins (indicated as 'Input') treated or not with roscovitine, or samples immunoprecipitated with an unrelated antibody or an antibody for Geminin is shown. The differences in mobility on gel are due to roscovitine treatment. MCM2 (in soluble and chromatin fractions) and MCM4 (chromatin fraction) appear as doublets.

immunoprecipitated Geminin from mitotic cells previously treated with roscovitine for 3 h. We observed that both forms of CDT1 are co-immunoprecipitated with Geminin although

we noticed a slight decrease in the levels of hypophosphorylated CDT1 form (Figure 5C). This suggests that Geminin has higher affinity for the hyperphosphorylated form of CDT1.

CDT1 and pre-RC levels are decreased in G1 after mitotic depletion of Geminin

To understand if pre-RC formation and DNA replication were affected in Geminin-depleted cells, we released nocodazole-treated cells into the cell cycle (Figure 6A). Geminin is rapidly degraded in G1 and for this reason any possible effect observed in G1 should be a consequence of the lack of Geminin during the preceding mitosis. When we released cells depleted in mitosis for CDT1 and Geminin, we did not observe any alteration in spindle formation (by immunofluorescence analysis, data not shown). However, the levels of CDT1 strongly decreased in G1 after mitotic depletion of Geminin (Figure 6B). This result explains the strong reduction of CDT1 levels even in asynchronously growing cells depleted for Geminin (Figure 1), and the levels of CDT1 during G1 therefore strictly depend on its accumulation during mitosis. The levels of CDT1 in G1 were on the other hand unchanged in cells stably overexpressing Δ Geminin but not in cells stably overexpressing Δ Geminin Δ CBD. Consistent with the lower levels of chromatin-associated CDT1, also the amounts of MCM proteins associated with chromatin are significantly reduced in G1 after mitotic depletion of Geminin (Figure 6C). In contrast, we did not detect any difference in the amounts of chromatin-associated ORC2 and CDC6 following Geminin depletion. This result is consistent with the notion that these proteins are loaded to chromatin by a CDT1-independent mechanism (Diffley *et al*, 1995; Donovan *et al*, 1997; Seki and Diffley, 2000; Labib *et al*, 2001; Lei and Tye, 2001; Okuno *et al*, 2001). To understand if the differences in MCMs loading observed were due to differences in mitotic exit rates between the different treatments, we performed FACS analysis 1 and 5 h after nocodazole release. We observed that the rate of mitotic exit did not differ significantly between the different treatments (Figure 6D). To test if the decrease in CDT1 protein levels and MCMs loading onto chromatin could be due to the use of nocodazole and, as a consequence, to activation of the spindle checkpoint, we treated U2OS cells with siRNAs in G2 as shown in Figures 3A and 6A. After siRNA treatment, cells were not synchronized in mitosis with nocodazole but collected 14 h after transfection, when the majority of the cells had entered the next cell cycle. Western blotting showed that also in the absence of nocodazole CDT1 levels are decreased as a consequence of Geminin depletion. Moreover, significant lower amounts of MCM6 were recruited to chromatin (Figure 6E). FACS analysis of the same samples showed that the siRNA treatment did not lead to significant alterations in the cell cycle (Figure 6F). Based on these results, we conclude that Geminin is required for stabilizing CDT1 in mitosis and the subsequent efficient formation of pre-RCs.

DNA replication is impaired in cells depleted for Geminin during mitosis

To evaluate if DNA replication is affected under these conditions, we released the siRNA-treated mitotic cells from nocodazole (see Figure 6A) in the presence of [3 H]methylthymidine and measured DNA replication at different times

Figure 6 Geminin depletion impairs pre-RC formation in the following cell cycle. **(A)** Outline of the experimental protocol (see text for details). **(B)** The CDT1 levels in G1 phase depend on the Geminin-mediated mitotic accumulation. The indicated cell lines were treated as in Figure 2B, released from the nocodazole block for 6 h and immunoblotted for the indicated proteins after separation on a 12% polyacrylamide gel. M represents a mitotic sample not released from the nocodazole block. **(C)** Geminin depletion during mitosis impairs licensing at mitotic exit. U2OS cells treated as in (B) and released into G1 for 6 h were treated in order to separate soluble fraction from chromatin fraction and immunoblotted for the indicated proteins. MCM2 (soluble fraction) appears as a doublet. **(D)** Geminin and CDT1 depletions during mitosis do not alter mitotic exit rates. FACS analysis profiles of the cells treated as in (B) and (C) and released from the nocodazole block for 1 and 5 h to show the same rate of mitotic exit between the different treatments are shown. **(E)** Impairment of licensing upon Geminin depletion does not depend on the use of the microtubule-destroying drug nocodazole. Immunoblotting for the indicated proteins of cells treated with the indicated siRNAs during G2 and collected 14 h later without the use of nocodazole (see Materials and methods for details) is shown. **(F)** Geminin and CDT1 depletions during G2 do not alter cell cycle progression during G2–M–G1 transition. FACS analysis profiles of the samples shown in (E) are represented. The percentages of cells in G1 phase as evaluated by FACS analysis are shown.

after mitotic exit. As shown in Figure 7A, DNA replication was significantly decreased in cells depleted for Geminin or CDT1 during the previous mitosis. At later time points, Geminin-depleted cells started to re-replicate their DNA (Melixetian *et al*, 2004). Recent results from our laboratory have shown that human Geminin during this phase of the cell cycle is required for preventing multiple rounds of replication and thus genomic stability (Melixetian *et al*, 2004). To confirm that the decreased DNA replication is specific to loss of Geminin, we used Δ Geminin-overexpressing cell lines to perform similar experiments as described above. As shown in Figure 7B, DNA replication in these cells was not significantly inhibited in the presence of siRNA to Geminin. In agreement with this, the loading of MCM proteins to chromatin during G1 was very similar to nontreated WT cells (Figure 7B). Thus, we conclude that Geminin promotes the formation of preRCs in mitosis by stabilizing CDT1.

Discussion

Our data suggest that Geminin promotes pre-RC formation and DNA replication through stabilization of CDT1 during mitosis. Geminin is involved in regulating CDT1 stability in S, G2 and M, but only allows the accumulation of CDT1 in late

G2 and mitosis. CDT1 levels in G1 are dependent on this accumulation. We have shown that the turnover of CDT1 during G1 (data not shown) and the decrease in CDT1 protein levels at mitotic exit cannot be recovered later by *de novo* protein synthesis. For this reason, the Geminin-dependent accumulation of CDT1 during mitosis is essential for pre-RC formation and DNA synthesis in the following cell cycle. The DNA replication occurring in cells treated with siRNAs to Geminin or CDT1 is most likely due to the incomplete abrogation of the expression of the two proteins.

In this paper, we have focused on the role of Geminin in stabilizing CDT1 protein levels. In addition to this, Geminin may have a role in regulating CDT1 transcription as suggested by results obtained in *Drosophila* (Mihaylov *et al*, 2002) and in human cells (our preliminary data). These results are in agreement with the recent findings that Geminin has a role in transcriptional regulation inhibiting Hox function (Del Bene *et al*, 2004; Luo *et al*, 2004). However, our data suggest that Geminin primarily influences CDT1 levels directly by binding to the protein. This binding leads to a decrease in ubiquitination of CDT1, suggesting a mechanism by which Geminin controls CDT1 levels. Global ubiquitination appears unaffected by the presence of high levels of Geminin, indicating that Geminin specifically inhibits the ubiquitination of CDT1.

Figure 7 Geminin depletion impairs DNA replication in the following cell cycle. **(A)** Geminin depletion during mitosis impairs DNA replication during the subsequent cell cycle similar to CDT1 depletion. U2OS cells, treated as in Figure 4B, were released from the nocodazole block in the presence of [³H]thymidine and harvested at the indicated time points. The counts per minute (cpm), indicative of [³H]thymidine incorporation, were plotted as mean $\pm$ s.d. **(B)** The overexpression of a form of Geminin mutated in the target sequence partially rescues DNA replication. The same protocol as described in (A) was used also for Δ iGeminin-overexpressing cells in parallel with control U2OS (indicated as WT). [³H]thymidine incorporation was evaluated as before 10 h after nocodazole release. The solid bars indicate control treatment and siRNA treatment for Geminin, respectively. The insets show immunoblots for chromatin-associated MCM2 and ORC2 in samples prepared 6 h after the nocodazole release.

It is not clear whether Geminin affects the ubiquitination of substrates other than CDT1, and the mechanism by which Geminin inhibits ubiquitination.

Recent data have shown that CDT1 degradation can be mediated by SKP2, and that the interaction between CDT1 and SKP2 is dependent on CDT1 phosphorylation by CDK activity (Li *et al*, 2003, 2004; Kondo *et al*, 2004; Nishitani *et al*, 2004; Sugimoto *et al*, 2004). In addition, proteasome inhibitors were shown to stabilize a slow-migrating form of CDT1 during S phase (Nishitani *et al*, 2001). Thus, we suggest that CDKs are the main regulators of CDT1 stability in G1 and S. CDT1 is stable in G1 due to the absence of CDK activity, whereas it is targeted for degradation by CDKs at the G1/S transition (Figure 8A), anticipating the synthesis of Geminin. At this point of the cell cycle, Geminin is allowed to accumulate due to the inactivation of the anaphase-promoting complex (APC/C-CDH1) (McGarry and Kirschner, 1998; Bastians *et al*, 1999). Geminin is accumulating in S phase and stabilizes the levels of newly synthesized CDT1; however, it is not sufficient for its accumulation (Nishitani *et al*, 2001; Li *et al*, 2003). Rather, the main role of Geminin in S is to prevent relicensing of origins and therefore rereplication (Mihaylov *et al*, 2002; Melixetian *et al*, 2004). During mitosis, in contrast, Geminin stabilizes CDT1 and works as a licensing cofactor boosting pre-RC formation (Figure 8A).

One question remaining to be addressed is why Geminin only allows CDT1 accumulation during G2 and M, considering that the levels of Geminin are similar in S and M phases.

Figure 8 Model for the role of human Geminin in regulating DNA replication: Geminin promotes licensing at mitotic exit and prevents rereplication during S phase. **(A)** Human Geminin depletion has different consequences in S and M phases (see Discussion for details). The diagram represents the levels of CDT1 and Geminin during the cell cycle. The two lines are independent lines that do not refer to each other. In the lower part of the panel, U2OS cells synchronized in different ways (see below) were collected at different times during cell cycle and analyzed by FACS analysis or immunoblotted for the indicated proteins. The cells were collected (left to right) 6 h after nocodazole release (lane 1), 4 h (lane 2) and 8 h (lane 3) after thymidine release or taken from the attached (lane 4) or detached (lane 5) populations of nocodazole-treated cells. **(B)** Human Geminin stabilizes CDT1 during mitosis and appears not to restrict pre-RC formation. In contrast, Geminin is required for the accumulation of CDT1. The main regulator of origin licensing is instead CDK1, which inhibits pre-RC formation by interfering with CDT1 and MCM binding to chromatin.

A possible answer to this question is provided by the fact that CDT1 is degraded at the G1/S transition before Geminin is allowed to accumulate. *De novo* synthesis of CDT1 is there-

fore necessary before it can accumulate and transcription/translation of CDT1 may vary along the cell cycle. Alternatively, our data suggest that Geminin has a slight increase in affinity for mitotic hyperphosphorylated CDT1. Similarly, previous data have shown that the affinity of Geminin for CDT1 is higher in *Xenopus* metaphase egg extracts (Hodgson *et al*, 2002). Therefore, we would like to speculate that the high affinity of Geminin for CDT1 during mitosis is a prerequisite for the accumulation of CDT1. In contrast, the weaker binding between the two proteins in S phase may not allow the accumulation of CDT1, but it is sufficient for preventing relicensing of origins. Moreover in mitosis, and differently from S phase, we have not been able to find a role for human Geminin in the prevention of pre-RC formation. Instead, the regulation of pre-RC formation in mitosis appears entirely to be regulated by CDK activity (Figure 8B).

Future work will be aimed at testing if CDT1 and the MCM proteins are among the essential substrates for CDK1 and how Geminin inhibits the ubiquitination process.

Materials and methods

Cell lines and drugs

Human U2OS osteosarcoma, A549 lung carcinoma, RKO colon carcinoma, HeLa cervix carcinoma and monkey COS kidney carcinoma cell lines were grown in DMEM medium containing 10% fetal bovine serum. TIG3 diploid fibroblasts, HCT116 colon carcinoma and 293T kidney transformed cell lines were grown in DMEM containing 10% North American serum. The following were used: 2.5 mM thymidine (Sigma), 50 ng/ml nocodazole (Sigma), 20 μ M MG132 (BioMol), 25 μ M roscovitine (BioMol) and 5 mM NEM (Sigma). The synchronization for microinjection was performed with a double-thymidine block. The synchronization for siRNA treatment during G1 was performed with a double-thymidine block followed by a nocodazole block. The synchronization for siRNA treatment during G2 was performed with a single-thymidine block before nocodazole treatment. The synchronization for siRNA during G2 and analysis without nocodazole was performed with a double-thymidine block before release for 6 h, siRNA treatment in serum-free medium for 4 h and other 14 h of incubation with 10% serum.

Plasmids and siRNAs

siRNA oligonucleotides (Dharmacon) were made to the following sequences (sense-strand): Geminin (A) (5'-AAUGAGCU GUCCGAGGCUUG-3') or (B) (5'-AACUCCAGCCUGGGGUUUAU-3'), CDT1 (5'-AACGUGGAUGAAGUACCCGAC). Control unrelated oligo was AGACGAACAAGUCACCGACUU. Transfections were performed with 40 nM of double-stranded siRNA oligonucleotides using OligofectamineTM (Invitrogen), according to the instructions of the manufacturer. Human CDT1 was cloned by reverse transcription-PCR. A fragment of 820 base pairs (primers: forward 5'-CTA GTC GAC GAG AAG GCG CCC GCC TAC CAG CGC TTC-3'; reverse 5'-CTA GTC GAC TCA CAT CTG TGC CAG CTG CTT CTG TGC-3') was subcloned into the TA vector pCR2.1. This clone was used to screen a lambda M426 human cDNA library and to isolate a phage encoding the full-length cDNA (1.9 kb) of human CDT1. Full-length CDT1 was cloned in pGEX for GST fusion protein production. The *Sall*-cut fragment was instead cloned into pCMV HA-tagged and pCDNA3 plasmids. Progressive deletion mutants were constructed by PCR. The full-length open reading frame of Geminin was cloned in pCMVneoBam modified for Gateway system and tagged with HA or myc epitopes. Progressive deletion mutants of Geminin were constructed by PCR. The silent mutations (Δ i) in the target sequence were constructed in order to change two nucleotides without changing the amino-acid sequence. The plasmid expressing HA-ubiquitin has been described before (Petersen *et al*, 2000).

Antibodies

To generate CDT1 antibodies, a GST fusion protein containing a C-terminal portion of CDT1 was used to immunize Balb/c mice, and monoclonal antibodies were made using standard protocols (Harlow and Lane, 1988). Purified antibody (clone P26A6) was used for Western blotting and immunofluorescence analysis at 2 μ g/ml. The ORC2 monoclonal antibody (10E5) was generated using standard protocols with a mix of GST-ORC2 aa 8–221 and 471–566. Other antibodies used were as follows: Geminin (Santa Cruz FL-209, cat. no. 13015), cyclin A2 (Santa Cruz, cat. no. 596), cyclin B1 (Santa Cruz GNS1, cat. no. 245), Y15-CDK1 (Cell Signalling, cat. no. 9111), MCM2 (Santa Cruz, cat. no. 9839), MCM4 (a kind gift of Rolf Knippers, polyclonal rabbit), MCM6 (Santa Cruz, cat. no. 9843), CDC6 (DCS 180, mouse monoclonal) (Petersen *et al*, 1999), SKP2 (Santa Cruz, cat. no. 7164), HA (12CA5) and Myc (9E10).

Immunoprecipitation and GST *in vitro* binding experiments

To immunoprecipitate Geminin during mitosis, we used goat polyclonal antibody (Santa Cruz, cat. no. 8448) at 3 μ g/mg total protein. HA-ubiquitin was immunoprecipitated using HA antibody at 5 μ g/mg total protein. GST *in vitro* binding experiments were performed using 1 μ g of GST-fused proteins incubated with 20 μ l of lysates of ³⁵S-labeled *in vitro*-translated Geminin or 300 μ g of total protein lysates of cells overexpressing DNA for Geminin.

In vivo ubiquitination assay

Ubiquitinated CDT1 was detected after transfecting 293T cells by the Ca-phosphate method with plasmids expressing CDT1, HA-ubiquitin and mock plasmid or plasmid for myc-Geminin (WT or Δ CBD mutant). The Ca-phosphate precipitates were left on the cells for 16 h before washing, replacement of the medium and adding of thymidine for 24 h. S-phase cells were then released from thymidine block for 6 h in the presence of MG132. Cells were then lysed in modified RIPA buffer (Li *et al*, 2003) (plus 1 mM aprotinin and leupeptin, 10 mM PMSF and NaF and 5 mM NEM). Lysates were kept on ice for 20 min before sonication, centrifugation (10 min at 12 000 rpm in a microcentrifuge) and immunoprecipitation.

Microinjection and immunofluorescence

HeLa cells were microinjected in the nucleus 1 h after thymidine release with plasmids for CDT1 (WT and deletion mutants) (5 ng/ml) or Geminin (40 ng/ml). Rabbit IgG (Jackson laboratories) were co-injected at 1 mg/ml concentration. MG132 was added 10 min after microinjection. The cells were fixed with paraformaldehyde 3 h later, permeabilized and stained with the indicated antibody or DAPI stain. The injection was performed using a Zeiss automatic injection system. For immunofluorescence of nocodazole-treated mitotic cells, the coverslips were treated with poly-D-lysine before seeding. After incubation with nocodazole, the cells were fixed with paraformaldehyde and incubated with antibodies for CDT1 (2 μ g/ml) and Geminin (200 ng/ml). Cy3- and FITC-conjugated secondary antibodies were used. Cells were stained with DAPI before mounting on microscope slides.

Chromatin fractionation

Cells were lysed in cytoskeleton (CSK) buffer containing 0.5% Triton X-100, 10 mM Pipes pH 6.8, 100 mM NaCl, 1.5 mM MgCl₂, 300 mM sucrose, 1 mM each of aprotinin, leupeptin and PMSF, 10 mM NaF and 1 mM ATP, for 20 min on ice. Lysed cells were sonicated and centrifuged for 10 min at 10 000 rpm to obtain total lysates. For the subcellular fractionation, lysed cells were instead centrifuged for 4 min at 3200 rpm and the obtained supernatant represented the soluble fraction. To obtain DNaseI-released chromatin fraction, the pellet obtained in the previous step was treated with DNaseI and 300 mM NaCl for 45 min at 33°C. After centrifugation for 15 min at 10 000 rpm, the supernatant was recovered. Western blotting analysis was performed according to standard procedures quantifying the protein concentration with Bradford analysis. Separation of the proteins by electrophoresis was performed using 10% polyacrylamide gels (unless differently specified).

FACS analysis

Synchronized cells were analyzed by FACS analysis after propidium iodide staining using a Becton Dickinson Flow Cytometer and a Cell Quest software.

DNA synthesis measurements

Cells synchronized in mitosis and treated with different siRNAs were released into G1 by plating the same number of cells in the presence of 5 μ Ci/ml [3 H]methyl-thymidine (Amersham Biosciences) and incubating for the indicated times at 37°C in a 5% CO₂ incubator. The cells were subsequently washed with PBS, treated with 5% TCA and washed twice with EtOH. Dried cells were lysed with 1% SDS and 10 mM NaOH for 30 min. The measurement of [3 H]methyl-thymidine incorporation was performed using a Packard β -counter for liquid scintillation.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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