Article

Loss of Geminin induces rereplication in the presence of functional p53

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Strict regulation of DNA replication is essential to ensure proper duplication and segregation of chromosomes during the cell cycle, as its deregulation can lead to genomic instability and cancer. Thus, eukaryotic organisms have evolved multiple mechanisms to restrict DNA replication to once per cell cycle. Here, we show that inactivation of Geminin, an inhibitor of origin licensing, leads to rereplication in human normal and tumor cells within the same cell cycle. We found a CHK1-dependent checkpoint to be activated in rereplicating cells accompanied by formation of γH2AX and RAD51 nuclear foci. Abrogation of the checkpoint leads to abortive mitosis and death of rereplicated cells. In addition, we demonstrate that the induction of rereplication is dependent on the replication initiation factors CDT1 and CDC6, and independent of the functional status of p53. These data show that Geminin is required for maintaining genomic stability in human cells.

Introduction

Licensing of DNA replication origins occurs by the assembly of a prereplicative complex of initiation proteins during late mitosis and early G1. Phosphorylation, nuclear exclusion, and degradation of initiation proteins have been described as important mechanisms preventing rereplication of origins during the same cell cycle (Kelly and Brown, 2000; Bell and Dutta, 2002). In common for several of these mechanisms is that they are regulated by Cdks, and in fact, the inactivation of the B-type cyclins in yeast is sufficient for the induction of rereplication (Kelly and Brown, 2000; Bell and Dutta, 2002). An additional level of control has evolved in higher eukaryotes and is presented by Geminin. Geminin was originally discovered in *Xenopus* as a protein degraded by the anaphase-promoting complex at the end of mitosis (McGarry and Kirschner, 1998). Geminin orthologues were subsequently found in other higher eukaryotes, including *Drosophila*, mouse, and human, and it was found to inhibit the activity of CDT1 (Wohlschlegel et al., 2000;

Quinn et al., 2001; Tada et al., 2001). Geminin has been shown to be a main inhibitor of origin licensing in mitotic *Xenopus* extracts; however, depletion of Geminin in *Xenopus* is not sufficient for induction of rereplication (McGarry and Kirschner, 1998; Tada et al., 2001; McGarry, 2002). In contrast, Geminin deficiency in *Drosophila* leads to partial overreplication of the genome (Quinn et al., 2001; Mihaylov et al., 2002). Although these data demonstrate that Geminin is an important regulator of origin licensing in both organisms, they also suggest that some organisms have evolved additional levels of checkpoint controls to prevent rereplication in addition to Geminin. Consistent with this suggestion is recent results, obtained in human cell lines, in which inactivation of p53 was required for inducing rereplication as a consequence of CDT1 and CDC6 overexpression (Vaziri et al., 2003). Here, we have addressed the functional role of Geminin in human cells by inhibiting its expression using small interfering RNA (siRNA). We show that inhibition of Geminin expression is sufficient to induce rereplication in the presence of functional p53. These results demonstrate that Geminin is essential in human cells to prevent rereplication, and loss of

Figure 1. **Depletion of Geminin by siRNA induces rereplication.** Western blot (A) and phase-contrast microscopic images (B) of HCT116 cells treated with Geminin or control (GL2) siRNA for 48 h. Western blots were probed with antibodies specific for Geminin and Vinculin (loading control). (C) Flow cytometric profiles of HCT116 treated with Geminin siRNA for 48 h. Before harvesting, the cells were pulsed with BrdU for 2 h and processed for combined propidium iodide staining together with cyclin B1– and BrdU-specific antibodies. The black bars in the phase-contrast pictures correspond to 10 μm. (D) Summary of the protocol used in Geminin siRNA–treated U2OS cells to measure DNA rereplication within a single cell cycle. (E) Geminin expression levels in U2OS cells released from nocodazole block and treated with GL2 (control) or Geminin siRNA. (F) Distribution of [^{3}H]thymidine-labeled DNA in the CsCl gradient fractions. U2OS cells were processed according to the protocol described in A. DNA was prepared and subjected to CsCl density gradient centrifugation as described in Materials and methods. Y axes: [^{3}H]thymidine radioactivity in cpm; x axes: number of gradient fraction collected from the bottom. The fractions containing LL (light-light), HL (heavy-light), and HH (heavy-heavy) DNA were determined by a series of control experiments (not depicted).
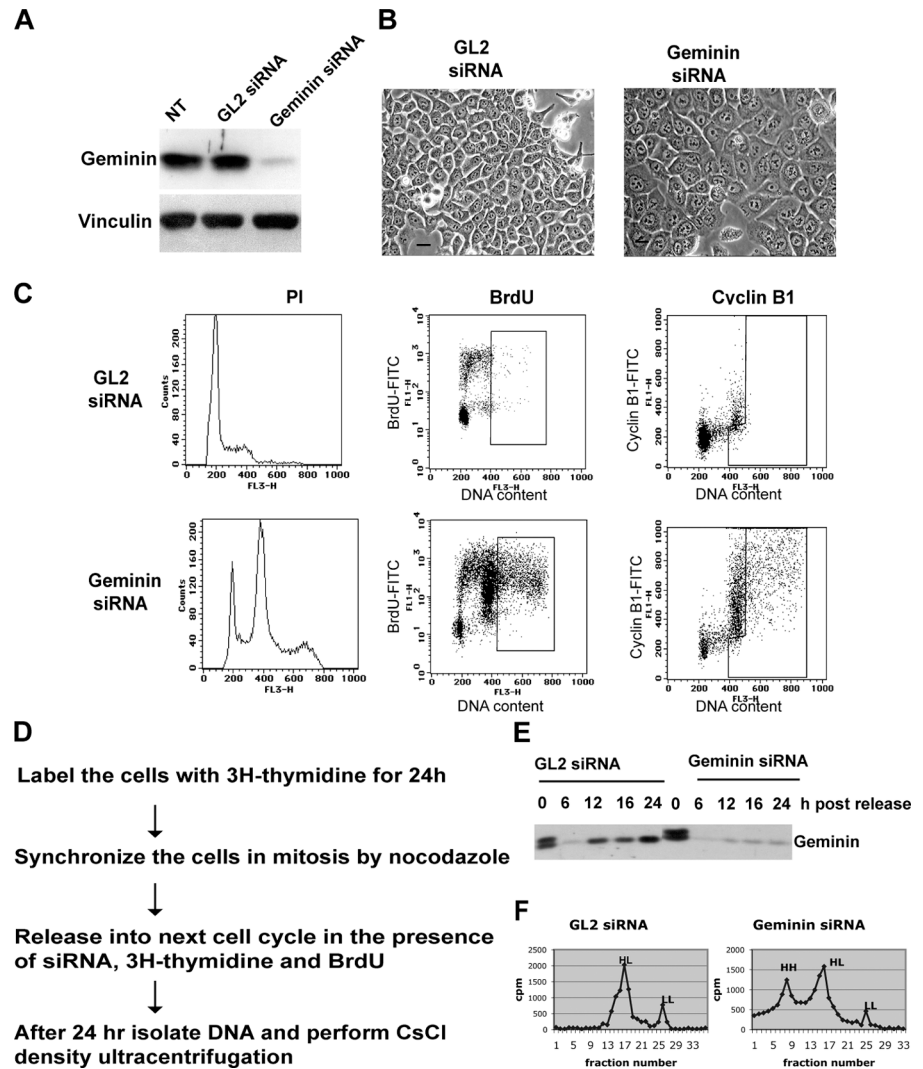
Geminin is sufficient to induce genomic stability. Because genomic stability has been suggested to be one of the key features of human cancer, our results suggest that Geminin is a putative tumor suppressor gene.

Results

Geminin is essential for preventing rereplication in human cells

To examine the role of Geminin in mammalian cells, we abolished its expression in HCT116 colon carcinoma cells using siRNA. Treatment of the cells with Geminin siRNA (but not control) led to a rapid drop of Geminin protein levels (Fig. 1 A). After 48 h of siRNA, most Geminin-depleted cells were enlarged and contained a single giant nucleus (Fig. 1 B). FACS® analysis showed a significant proportion of Geminin-depleted cells with 4N and greater DNA content (Fig. 1 C). These cells incorporated BrdU during a 2-h pulse period and expressed high levels of cyclin B1 (Fig. 1 C). These data suggest that Geminin-depleted cells accumulate in S phase with rereplicated DNA. Similar results were obtained in U2OS osteosarcoma cells and TIG3 human diploid fibroblasts (see Figs. 2, 3, and 5).

To examine if rereplication occurs during a single cell cycle, we separated BrdU-labeled DNA according to mass by CsCl density ultracentrifugation. This method allows the separation of DNA labeled with BrdU on both strands (rereplicated; heavy-heavy), on one strand (replicated only once; heavy-light), and on no strands (unreplicated DNA, light-light; Blow and Laskey, 1988; Yamaguchi and Dutta, 2000). We developed a protocol in which Geminin expression was inhibited after exit of mitosis (Fig. 1, D and E). Cells were released from mitosis, transfected with Geminin siRNA, and labeled with BrdU for a single cell cycle (24 h). DNA was isolated and separated by CsCl density centrifugation. Analysis of CsCl gradient fractions from Geminin-depleted cells revealed three separate peaks of DNA corresponding to rereplicated (heavy-heavy), replicated (heavy-light), and unreplicated (light-light) DNA, whereas control cells did not contain rereplicated DNA (Fig. 1 F). The authenticity of the heavy-heavy fraction was shown in control experiments in which cells were labeled for two consecutive cycles with BrdU (unpublished data). Furthermore, we performed parallel analysis to measure BrdU incorporation and mitotic histone H3 phosphorylation throughout the 48-h time course. The results from these
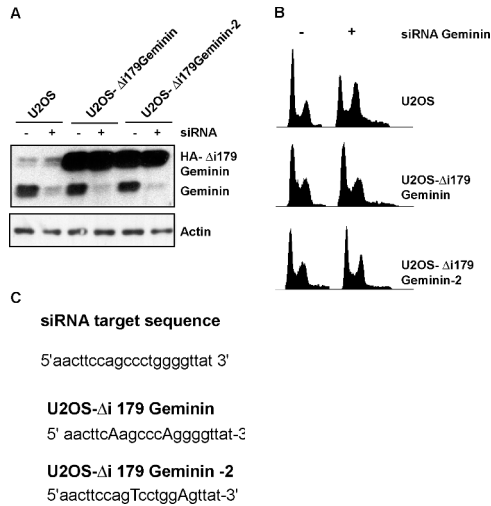
Figure 2.   **Rescue of Geminin siRNA phenotype in cells stably expressing HA-tagged RNAi mutants of Geminin.** U2OS cell lines expressing two different mutant HA-tagged versions of Geminin were generated (Geminin-Δi179 and Geminin-Δi179-2). In the cDNA for Geminin, two residues were changed in the siRNA target sequence in a way that the nucleotide sequence, but not the amino acid sequence, was altered (C). The cell lines were treated with Geminin siRNA for 48 h and analyzed by Western blot with antibodies specific for the indicated proteins (A) and by FACS® analysis for DNA content (B). Note the decrease in cells with DNA content greater than and equal to 4N.

experiments showed that rereplication occurs in Geminin-depleted cells within one single S phase without intervening mitosis (see Fig. 5).

As a control for the specificity of the siRNA oligonucleotides, we reconstituted Geminin expression by creating two different U2OS cells lines expressing HA-tagged Geminin in which two residues in the siRNA target sequence were changed in a way that the nucleotide sequence, but not the amino acid, was altered (Fig. 2). Treatment of these cell lines with siRNA to Geminin led to specific decrease of endogenous Geminin levels, and a strong reduction in the number of cells with 4N and greater DNA content (Fig. 2), demonstrating that rereplication is a consequence of Geminin loss.

Geminin depletion leads to rereplication in normal diploid human cells

Next, we wished to investigate on a single-cell basis whether rereplicated DNA originated from early- or late-firing replication origins. To examine this, we performed FISH using as probes bacterial artificial chromosome (BAC) clones containing the chromosomal regions of either the lamin B2 (early firing) or the β-globin (late firing) replication origins (Kitsberg et al., 1993; Abdurashidova et al., 2000; Avni et al., 2003). The specific hybridization of the BAC clones to their relevant chromosomal loci was
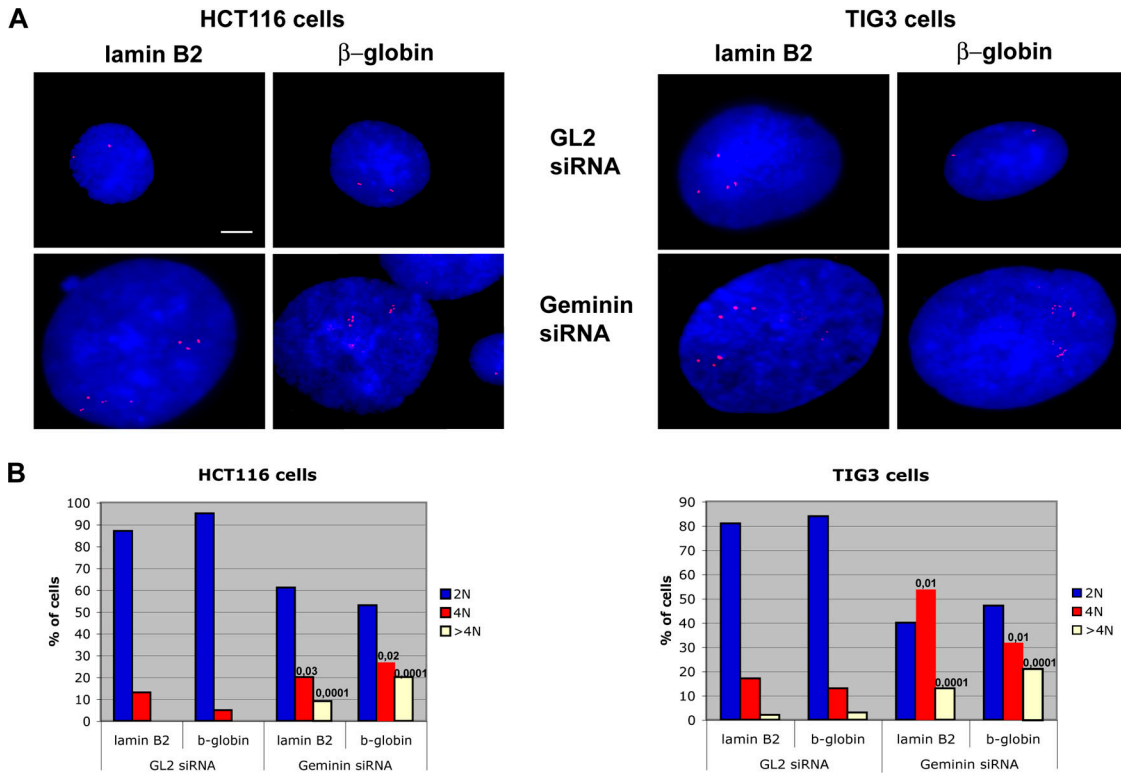

Figure 3.   **Loss of Geminin leads to rereplication of DNA both from early- and late-firing origins.** (A) Representative FISH experiment performed on HCT116 colon carcinoma cells and TIG3 human diploid fibroblasts. HCT116 cells were treated with siRNA for 48 h, and TIG3 cells for 96 h. BAC probes corresponding to lamin B2 and β-globin replication origin containing chromosome segments were labeled with Cy3-dUTP (red) and used for hybridization. Chromosomal DNA was stained with DAPI. The BAC clones hybridize exclusively with relevant single chromosomal loci at 19p13.3 (lamin B2) and 11p15.4 (β-globin) (not depicted). (B) Histograms summarizing results obtained from examination of at least 200 nuclei of each culture, hybridized with the indicated FISH probe. For statistically significant FISH phenotypes (Geminin vs. GL2 siRNA), P values calculated from a two-tailed test are indicated. Bar, 3 μm.

Figure 4. **Rereplication induced by Geminin depletion activates CHK1 and a DNA damage response.** (A) Western blot analysis of cellular extracts prepared at the indicated time points from HCT116 cells treated with Geminin or control (GL2) siRNAs. S317CHK1, Y15CDC2, and S15p53 indicate the use of antibodies specifically recognizing the phosphorylated amino acid of the proteins. (B) Rereplicated cells contain H2AX and Rad51 nuclear foci. U2OS cells treated with control (GL2) or Geminin siRNA for 48 h were immunostained with rabbit polyclonal H2AX and RAD51 antibodies. (C) Rereplicated cells contain ssDNA coated by RPA70. HCT116 cells were prelabeled with 10 μM BrdU for 24 h, incubated with Geminin or control (GL2) siRNA for 48 h, fixed with methanol, and immunostained with a BrdU-specific antibody without denaturation of DNA. BrdU foci (green) correspond to the sites of ssDNA breaks. RPA immunostaining is shown in red. (D) Formation of giant nuclei and CHK1 activation in TIG3 human diploid fibroblasts treated with Geminin siRNA. TIG3 cells were transfected twice with GL2 (control) or Geminin siRNA, fixed, and stained 96 h after the first transfection.
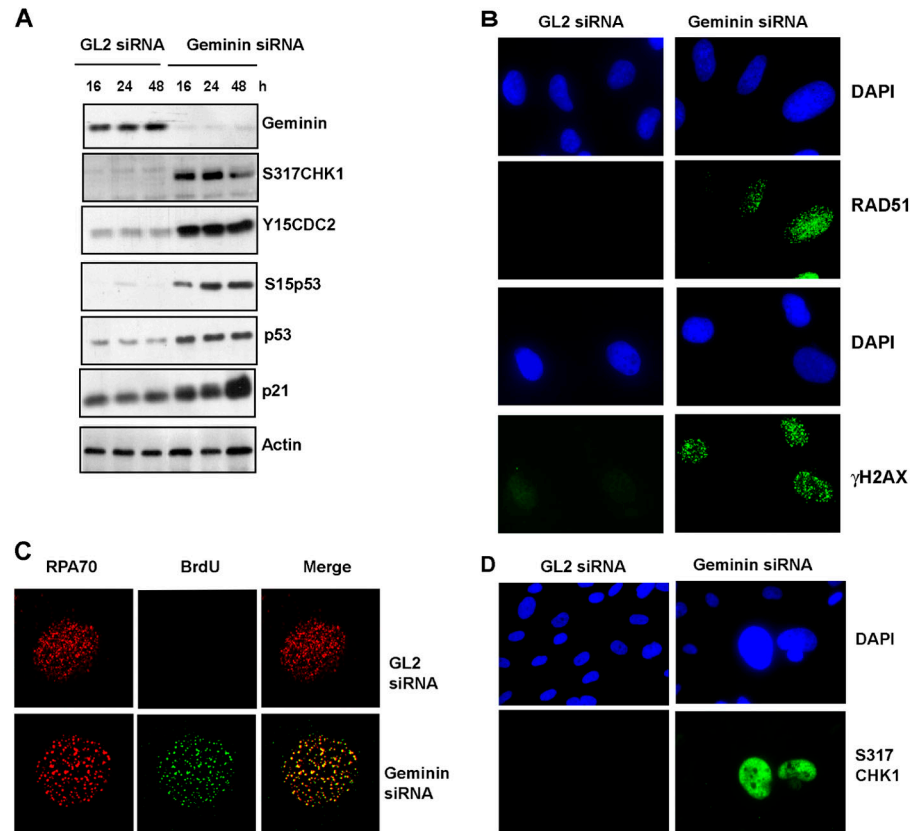
confirmed in separate experiments (unpublished data). HCT116 colon carcinoma cells and TIG3 human diploid fibroblasts were treated with Geminin or control siRNA and FISH analysis was performed. As demonstrated in Fig. 3, Geminin depletion both in HCT116 and TIG3 cells lead to accumulation of cells containing more than four foci for each replication origin region, demonstrating that rereplication affects both early and late origins. Interestingly, we noted that hybridization foci in both HCT116 and TIG3 cells corresponding to the rereplicated lamin B2 origin region were efficiently separated within nucleus, whereas spots corresponding to the rereplicated β-globin region were found to be clustered in two nuclear regions, which could correspond to each chromosome (Fig. 3 A). These data are consistent with the fact that newly synthesized DNA at heritable silenced genes and heterochromatic domains separate poorly due to enhanced sister chromatid cohesion (Hansen et al., 1995; Gartler et al., 1999; Azuara et al., 2003). Based on these results, we conclude that Geminin is required for preventing rereplication both in transformed and primary cells.

Inhibition of Geminin expression activates CHK1 and a DNA damage response

We found that proliferation is inhibited in Geminin-depleted cells (unpublished data; Fig. 8), suggesting the possible activation of a checkpoint in cells undergoing rereplication. To investigate the mechanism leading to cessation of proliferation, we analyzed if DNA damage checkpoint pathways were activated in Geminin-depleted cells. As shown in Fig. 4 A, depletion of Geminin led to a dramatic increase in phosphorylation of CHK1 on serine (S) 317, inhibitory phosphorylation of CDC2 on tyrosine (Y) 15, and phosphorylation of p53 on S15. These are the hallmarks of ATR/ATM-dependent DNA damage checkpoint activation (Bartek and Lukas, 2001; Shiloh, 2003). Consistent with increased p53 S15 phosphorylation, p53 levels were increased in Geminin-depleted cells; however, the expression of p21, a transcriptional target of p53, was induced only at later time points (48 h), suggesting that p21 activation is a secondary response to rereplication. To confirm that rereplication activates the DNA damage checkpoint, we analyzed if rereplicating cells contained DNA damage–induced nuclear foci. In fact, we observed H2AX and RAD51 nuclear foci formation in rereplicating cells (Fig. 4 B). H2AX phosphorylation is an early mark of double-stranded DNA breaks (DSBs) and is essential for a proper checkpoint response, whereas RAD51 is involved in homologous recombination (Shiloh, 2003). These data suggest that rereplication leads to accumulation of DSBs.

An important intermediate for activation of the checkpoint in response to DNA damage and stalled replication forks is single-stranded DNA (ssDNA; Zou and Elledge, 2003). Therefore, we examined if ssDNA is present in rereplicating cells. To do this, we measured the incorporation of BrdU without prior DNA denaturation (Raderschall et al., 1999). Geminin-depleted cells (but not controls) contained numerous BrdU foci colocalizing with the ssDNA-binding protein RPA70 (Fig. 4 C). These observations suggest that rereplication leads to formation of DNA strand breaks. The mechanism by which DNA strand breaks are generated is unclear; however, one possible scenario is that unscheduled
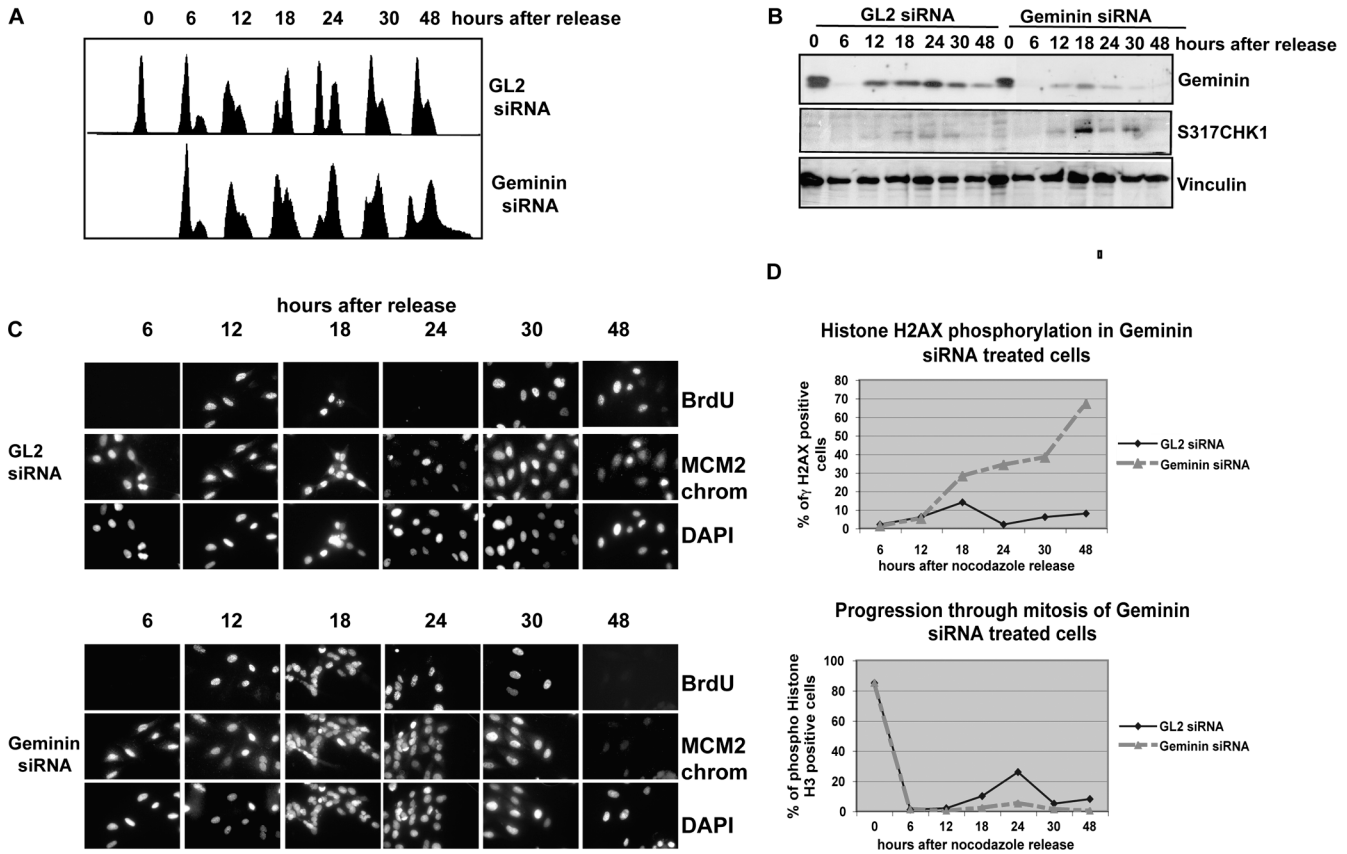
Figure 5. **Geminin depletion activates the checkpoint response during S phase.** U2OS cells were transfected with siRNA to Geminin or control and synchronized in mitosis by nocodazole treatment for 16 h. The cells were subsequently released into the next cell cycle and retransfected with Geminin or control siRNA. The cells were harvested at the indicated time points and analyzed by Western blot using the indicated antibodies (A), and for DNA content by FACS® analysis (B). (C) Cells grown on the coverslips were labeled with BrdU for 10 min at each time point, pre-extracted with 0.5% Triton X-100 in cytoskeleton buffer, fixed with PFA, and immunostained for BrdU and chromatin-bound MCM2. (D) Kinetics of DNA damage checkpoint induction and progression through mitosis of Geminin- or control siRNA–treated cells. The cells were plated on poly-D-lysine–coated coverslips, fixed, and stained with phosphohistone H3 and γH2AX antibodies. 70 cells were counted for each time point.

reinitiation of DNA replication could lead to replication fork collision and the generation of single-stranded DNA breaks, which subsequently could lead to replication fork collapse and DSB (Kuzminov, 2001).

To investigate if inhibition of Geminin expression also results in checkpoint activation in normal human diploid fibroblasts, we transfected TIG3 fibroblasts with control (GL2) or Geminin siRNA. As shown in Fig. 4 D, the inhibition of Geminin expression in TIG3 cells also leads to the strong activation of CHK1 and the formation of giant cells. These results are all consistent with the notion that loss of Geminin is sufficient for the induction of rereplication in diploid cells.

Next, we investigated at which stage of the cell cycle Geminin depletion leads to checkpoint activation. We analyzed cell cycle progression of cells synchronized in mitosis by nocodazole treatment and released into the next cell cycle in the presence of Geminin or control siRNA (Fig. 5 A). Geminin-depleted cells progress normally into S phase (Fig. 6 B). In contrast to control-treated cells, Geminin-depleted cells continue to incorporate BrdU and show constant association of MCM2 with chromatin throughout the 30-h period after exiting mitosis (Fig. 5 C). Moreover, Geminin-depleted cells

(but not controls) appear to arrest in S phase because the mitotic indices of Geminin-depleted cells, as measured by phopshohistone H3 staining, remained low throughout the period (Fig. 5 D; see also Fig. 5 A). The checkpoint response was activated during the first hours of S phase as monitored by the activatory phosphorylation of CHK1 and histone H2AX (Fig. 5, A and D). Together, our results show that inhibition of Geminin expression leads to rereplication within the same cycle, checkpoint activation, and subsequent block in entry into mitosis.

Abrogation of the CHK1-dependent checkpoint leads to abortive mitosis and death of rereplicated cells

To understand the functional significance of CHK1-dependent checkpoint activation in response to rereplication, we treated rereplicated cells with caffeine and UCN01. Caffeine inhibits both ATM and ATR kinases (Sarkaria et al., 1999), whereas UCN01 is an inhibitor of CHK1 kinase (Busby et al., 2000). HCT116 cells treated with Geminin siRNA for 48 h were incubated with caffeine or UCN01 (Fig. 6). Already after 1 h of drug treatment, rereplicated cells started to enter mitosis as monitored by accumulation of cells staining positive for phosphohistone H3 (Fig. 6 A). However, the
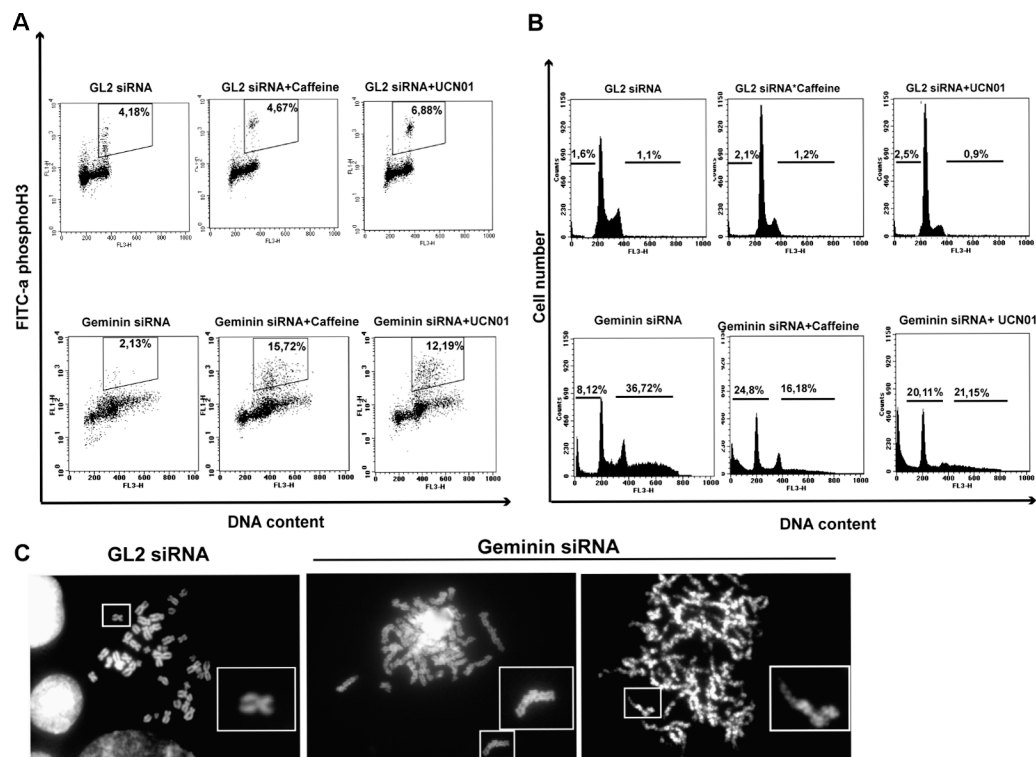
Figure 6.   **Inhibition of CHK1-dependent checkpoint leads to abortive mitosis and death of rereplicated cells.** (A) Rereplicated cells enter mitosis when treated with caffeine or UCN01. HCT116 cells were incubated with Geminin-specific or control (GL2) siRNA for 48 h, and subsequently treated with 5 mM caffeine or 300 nM UCN01 for 2 h. Entry into mitosis was measured with phosphohistone H3 staining by FACS[®]. (B) Death of rereplicated cells upon prolonged treatment with checkpoint inhibitors. HCT116 cells were treated as described in A, but the incubation period with caffeine and UCN01 was prolonged to 24 h. The percentage of dead (subG1) and polyploid cells (>4N) was determined by propidium iodide staining and FACS[®] analysis. (C) Extensive chromosomal breakage in rereplicated cells treated with UCN01. The cells were treated with UCN01 as in A and then incubated in the presence of colcemid for 30 min. Metaphase spreads were prepared as described in the Materials and methods. Two cells with different extent of chromosome breaks are shown. We never observed any chromosome breaks in cells treated with UCN01, nor in cells treated with GL2 siRNA. More than 50% of the Geminin siRNA–transfected and UCN01-treated cells showed extensive chromosomal breakage, which in this experiment were corresponding to the transfection efficiency of the siRNA oligonucleotide to Geminin. Insets show magnified views of chromosomes marked with white boxes.

treated cells did not complete mitosis, and 24 h after incubation with drugs we observed reduction in number of cells with DNA content >4N and accumulation of dead cells (Fig. 6 B). In contrast, control cells showed only modest accumulation of mitotic cells upon 1 h of UCN01 treatment (Fig. 6 A) and did not undergo apoptosis after 24 h of treatment (Fig. 6 B). Analysis of mitotic spreads from Geminin-depleted cells treated with UCN01 revealed extensive chromosomal breakage and, in some cells, chromosome fragmentation (Fig. 6 C). These data indicate that rereplicated cells undergo "mitotic catastrophe" in the presence of checkpoint inhibitors. Thus, checkpoint inhibition in rereplicating cells does not lead to further rereplication, but induces abortive mitosis and cell death.

Rereplication is induced in the presence of functional p53

p53 plays an important role in the DNA damage response, and it was recently reported to prevent rereplication induced by CDT1 and CDC6 overexpression in human cells (Vaziri et al., 2003). Because we observed rereplication in human diploid fibroblasts as well as in transformed cell lines HCT116 and U2OS that have often been used as model cell lines for monitoring functional p53 (Heise et

al., 1997; Bunz et al., 1998), our data demonstrate that the presence of functional p53 is not sufficient for preventing rereplication. However, p53 could be involved in regulating the DNA damage checkpoint induced by Geminin depletion. To analyze this, we used cells in which p53 expression was abolished by a retrovirus expression, a short hairpin RNA against p53 (U2OS), or by overexpression of human papilloma virus E6 (HCT116). Ablation of p53 abrogated p21 induction in HCT116 cells, but did not promote further rereplication and did not affect CHK1 activation in both cell lines (Fig. 7). These data suggest that inactivation of p53 is not required for the induction of rereplication or the rereplication checkpoint. Moreover, inactivation of p53 did not affect death of rereplicated cells upon caffeine and UCN01 treatment (unpublished data), consistent with the notion that p53 is not involved in cell death as a consequence of mitotic catastrophe (Roninson et al., 2001).

CDT1 and CDC6 are required for rereplicaton induced by Geminin deficiency

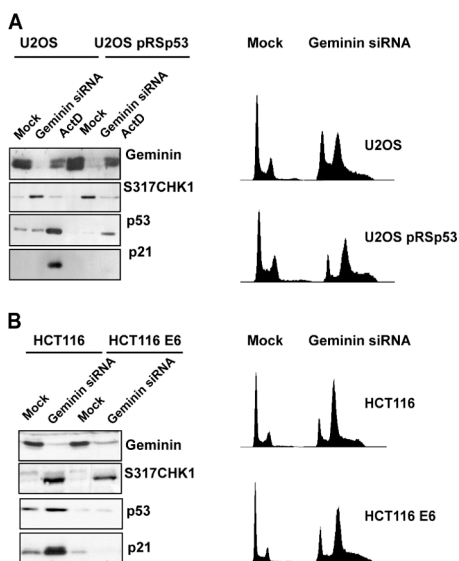To investigate the mechanism by which rereplication is induced by loss of Geminin expression, we abolished the ex-

Figure 7. **Loss of p53 is not required for rereplication or DNA damage response.** (A) U2OS cells were infected with virus generated by pRetroSuper (pRS) p53 producing short hairpin RNA against p53 or with empty virus. Cells were subsequently treated with Geminin-specific siRNA for 48 h and analyzed by Western blotting and FACS® analysis. Western blots were probed with antibodies specific for the indicated proteins. Actinomycin D treatment (1 nM) was used as a positive control for p53 activation. (B) HCT116 cells infected with empty virus or E6-expressing virus were treated and analyzed as in A.

pression of CDT1 and CDC6, two proteins required for the licensing of origins. Co-silencing of CDT1 or CDC6 in combination with Geminin prevented formation of giant nuclei and accumulation of rereplicated cells (Fig. 8, A–C; unpublished data), suggesting that rereplication is indeed induced by relicensing of origins. In addition, we found that co-silencing of Geminin in part rescues proliferation of CDT1-depleted cells (Fig. 8, C and D). This finding suggests that a quantitative balance between CDT1 and Geminin is essential for cell proliferation.

Discussion

In this manuscript, we demonstrate that Geminin is required for maintaining genomic stability in S phase, and that loss of Geminin expression causes rereplication in human cells (Fig. 8 E). In addition to providing an essential function for Geminin in regulating DNA replication in mammalian cells, our data are the first to demonstrate a function for Geminin in S phase. Previously published data using *Xenopus* extracts have pointed to a role of Geminin in regulating origin licensing in mitosis, but not in S phase (see discussion in Madine and Laskey, 2001), and the specific requirement of Geminin in the various phases of the cell cycle has not been addressed in other organisms. Our finding that rereplication induced by Geminin loss is prevented by simultaneous loss of CDT1 or CDC6 suggests that rereplication is induced by relicensing of already-fired origins.

We found that rereplication activates a CHK1-dependent checkpoint that provides a classical mechanism for preventing cells with damaged or unreplicated DNA to proceed into mitosis. In this manuscript, we have not investigated the

mechanism by which rereplication can lead to activation of CHK1; however, it is widely believed that checkpoint proteins are activated on the detection of DNA damage or defects occurring during replication fork progression (Zhou and Elledge, 2000). Importantly, in the Geminin-depleted cells we have not detected cells with fully rereplicated DNA, suggesting that replication forks are stalled during the process of rereplicaton. Several potential mechanisms could explain the presence of stalled replication forks (which could be a consequence of the activation of CHK1), including collision of excessive replication forks due to the refiring of origins, imbalance of origin-firing activities such as the cyclins, or imbalance between replicated DNA and the histone pools (Coverley et al., 2002; Gunjan and Verreault, 2003). We observed rereplication of both early- and late-replicating chromosomal domains in Geminin-depleted cells. This observation distinguishes the rereplication checkpoint described here from the previously described replication checkpoint triggered by DNA synthesis inhibitors in which checkpoint activation prevents firing of late origins in response to replication stalling at early origins (Santocanale and Diffley, 1998; Feijoo et al., 2001). These data suggest that the CHK1-dependent rereplication checkpoint prevents entry into mitosis rather than S phase progression. Abrogation of this checkpoint in rereplicated cells results in entry into mitosis and subsequent cell death, but does not promote further rereplication. Consistent with our data, previous results using *Xenopus* embryos (McGarry, 2002) and *Drosophila* cells (Mihaylov et al., 2002) have shown that CHK1 is activated upon depletion of Geminin. However, in contrast to the previously published results we provide a mechanism for the activation of CHK1 and more importantly, also in contrast to the data obtained in *Xenopus* and *Drosophila*, we show that abrogation of the checkpoint does not impair rereplication, but results in mitotic catastrophe. We believe that the discrepancy between our data and those obtained in *Xenopus* and *Drosophila* most likely are due to the experimental approach. In particular, it has been shown that CHK1 is required for normal proliferation (Liu et al., 2000; Takai et al., 2000), and it would therefore be expected that siRNA to CHK1 leads to inhibition of cell proliferation and impairment of cell growth. In agreement with this, we have observed that siRNA to CHK1 in human cells leads to accumulation of cells in G1 and apparently rescues rereplication induced by Geminin depletion (unpublished data). However, if CHK1 is inhibited after rereplication has occurred (as shown here using the CHK1 inhibitor UCN01), cells undergo mitotic catastrophe. We believe that this result makes biological sense because it provides a classical example for how a DNA damage response (rereplication) leads to activation of a checkpoint, and that abrogation of the checkpoint does not rescue the DNA damage response, but in contrast results in cell death.

We also demonstrate that p53 is not sufficient to prevent rereplication in Geminin-depleted cells and that ablation of p53 does not promote further rereplication. This result appears not to be surprising because p53 primarily controls G1 checkpoint in response to DNA damage, whereas rereplication checkpoint is induced in S phase beyond G1–S transition (Zhou and Elledge, 2000; Bartek and Lukas, 2001). Thus, the rereplication checkpoint pathway we describe is

Figure 8. **Rereplication induced by Geminin depletion is dependent on CDT1 and CDC6.** HCT116 cells were treated with control (GL2), Geminin, CDT1, and CDC6 siRNA at the indicated combinations for 48 h. Cells were harvested and analyzed for protein expression (A), DNA content (B), and cell proliferation (D). (C) Phase-contrast microscopic images taken of cells treated for 48 h with the siRNA for the indicated transcripts. Note the rescue of giant nuclei formation formed in Geminin siRNA–treated cells when cotransfected with CDC6 or CDT1 siRNAs. (E) Model for the role of Geminin in controlling origin firing in S phase. Rereplication results in the activation of an ATR-CHK1–dependent checkpoint that results in block of entry into mitosis. Even though rereplication leads to DNA strand breaks (e.g., phosphorylation of H2AX and formation of RAD51 nuclear foci) and subsequent p53 activation, functional p53 does not appear to be involved in either regulation of rereplication or the DNA damage checkpoint response.
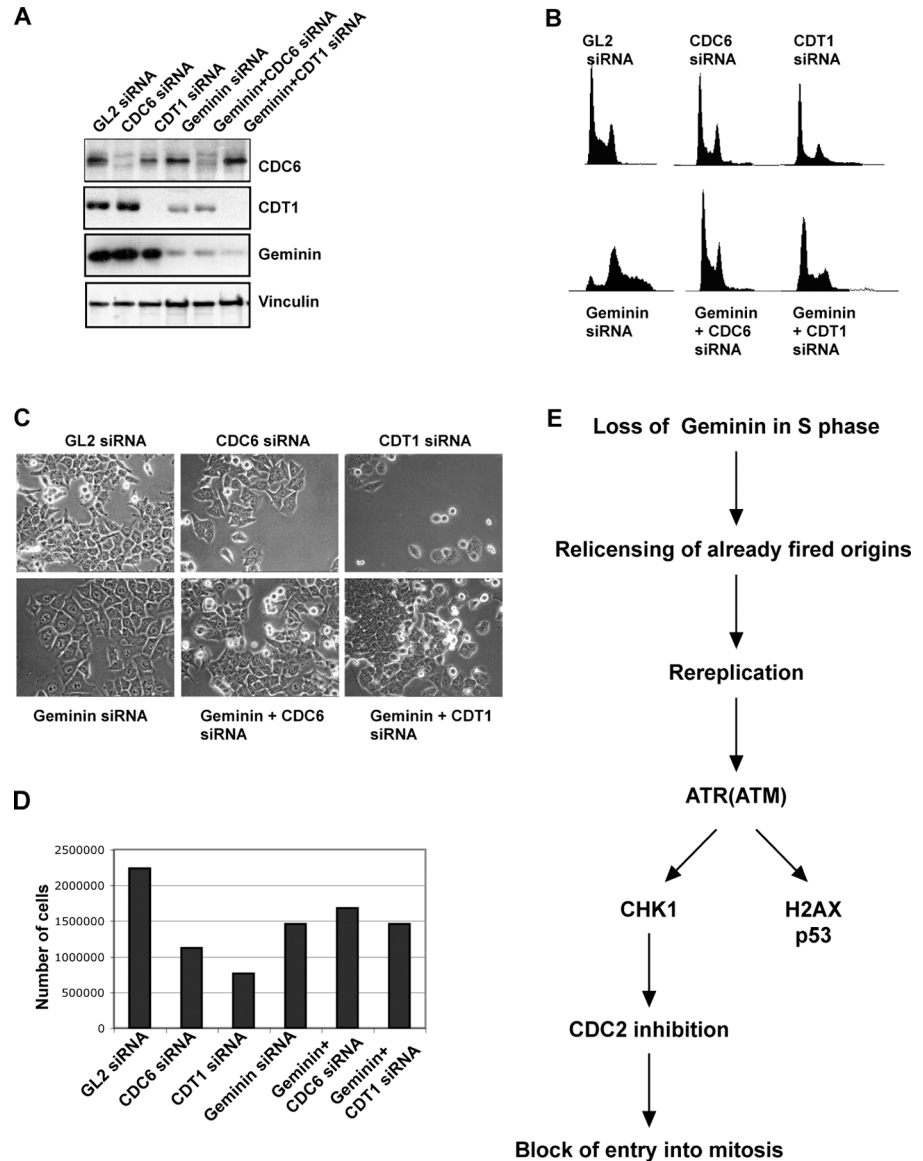
different from the p53-dependent rereplication checkpoint observed upon overexpression of CDT1 and CDC6 reported by Vaziri et al. (2003). We believe that these differences most likely are due to the different experiment conditions used. In particular, the overexpression of CDT1 and CDC6 could result in nonphysiological activation of the p53 pathway. In contrast, we show that inhibition of Geminin expression by siRNA is specific because we can rescue the phenotype by overexpressing mutants of Geminin in which the target sequence for the siRNA has been mutated.

Although several reports (for review see Bartek and Lukas, 2003; Shiloh, 2003) have shown a connection between defects in DNA damage checkpoint genes and predisposition to cancer, few have connected deregulation of DNA replication control with the development of cancer. The demonstration that loss of Geminin can result in rereplication shows that this gene is required for maintaining genomic stability. Together with the recent finding that *CDT1* can function as an oncogene in mammalian cells (Arentson et al., 2002), our data suggest that Geminin is a putative tumor suppressor.

Materials and methods

Cell lines, siRNA, and drug treatment

Human U2OS osteosarcoma, HCT116 colon carcinoma cell lines, and TIG3 human diploid fibroblasts were grown in DME containing 10% FBS. siRNA oligonucleotides (Dharmacon) were made to the following sequences (sense-strand): Geminin, 5′-AACUUCCAGCCCUGGGGUUAU-3′ or 5′-AAUGAGCUGUCCGCAGGCUUC-3′; CDC6, 5′-AAGAAUCUGCAUGU-GUGAGAC-3′; CDT1, 5′-AACGUGGAAGUACCCGAC-3′; GL2, 5′-CGUACGCGGAAUACUUCGA-3′.

Transfections were performed with 40 nM of each RNA oligonucleotide using Oligofectamine™ (Invitrogen) according to the instructions of the manufacturer. TIG3 cells were treated with siRNA twice within 96 h. In checkpoint inhibition experiments, caffeine (Sigma-Aldrich) was used at a concentration of 5 mM. UCN01 was a gift from R.J. Shultz (National Cancer Institute, Bethesda, MD) and was used at a concentration of 300 nM.

Protein expression and antibodies

Cells were extracted in cytoskeleton buffer containing 0.5% Triton X-100, 10 mM Pipes, pH 6.8, 100 mM NaCl, 1.5 mM $MgCl_2$, 300 mM sucrose, 1 mM each of aprotinin, leupeptin, and sodium fluoride, and 2.5 mM ortho-vanadate. Western blotting was performed according to standard procedures, and proteins were detected using the following antibodies: Geminin (FL-209, Cat. No. 13015; Santa Cruz Biotechnology, Inc.), CDC6 (DCS 181 [Petersen et al., 1999]), CDT1 (mouse mAb raised against human CDT1), RAD51 (Cat. No. 8349; Santa Cruz Biotechnology, Inc.), and

RPA70 (a gift of Jerard Hurwitz, Memorial Sloan-Kettering Cancer Center, New York, NY). Moreover, phosphospecific antibodies to S317CHK1 (Cat. No. 2344; Cell Signaling Technology), S15p53 (Cat. No. 9284; Cell Signaling Technology), Y15CDC2 (Cat. No. 9111; Cell Signaling Technology), H2AX (Cat. No. 07-164; UBI), and H3 (Cat. No. 06570; UBI) were used.

FACS® analysis

To measure BrdU incorporation, cells were incubated with 33 μM BrdU (Sigma-Aldrich) for 2 h. BrdU and cyclin B1 were detected using mouse mAbs against BrdU (Cat. No. 347580; Becton Dickinson) and cyclin B1 (Cat. No. 554176; BD Biosciences) according to previously described procedures (Faretta et al., 1997). Imunofluorescent detection of phosphorylated histone H3 was performed as described previously (Xu et al., 2001). The cells were analyzed on a Becton Dickinson Flow Cytometer using CellQuest™ software.

Immunofluorescence

To detect H2AX and RAD51 nuclear foci, cells were preextracted with cytoskeleton buffer containing 0.5% Triton X-100 with protease and phosphatase inhibitors and were then fixed with PFA. To detect ssDNA, cells were prelabeled with 10 μM BrdU for 24 h, treated with siRNA in the presence 10 μM BrdU for 48h, and then fixed with methanol for 5 min at −20°C. Staining with BrdU antibody was performed without prior denaturation of DNA. Microscopic images were acquired with a fluorescence microscope (model BX61; Olympus) equipped with a cooled CCD camera (model C5985; Hamamatsu Corporation) or by a confocal microscope (model 1024; Bio-Rad Laboratories) equipped with a 20-mW argon/krypton laser. The images were acquired and analyzed using Adobe Photoshop® 5.0 software.

FISH

Mitotic spreads were prepared as described previously (Specchia et al., 1999). BAC clones were obtained from the Children's Oakland Research Institute (Oakland, CA). They included BAC clone number RP11-211I3 (containing the lamin B2 replication origin) and BAC clone number RP11-645I8 (containing the β-globin replication origin). The BAC clones were directly labeled with CY3-dUTP (Amersham Biosciences) by nick translation as described previously (Specchia et al., 1999). The labeled probes and the cell spreads were then cohybridized with HYBrite™ (Vysis) at a melting temperature of 69°C for 2 min and then at 37°C overnight. Posthybridization washes were performed at 60°C in 0.1×SSC (×3) followed by DAPI counterstain. FISH analysis was performed using a fluorescent microscope (DMRXA; Leica), and images were acquired and analyzed with FISHView 2.0 software (Applied Spectral Imaging).

Synchronization of the cells and CsCl density gradient ultracentrifugation

To measure rereplication of DNA in U2OS cells, exponentially growing cells were incubated with 100 nCi/ml [³H]-methyl-thymidine (Amersham Biosciences) for 24 h, and then treated with 100 ng/ml nocodazole (Sigma-Aldrich) for 16 h. Nocodazole-arrested cells were collected by mitotic shake-off and were released from mitotic block by incubating them in nocodazole-free medium in the presence of 100 μM BrdU and 100 nCi/ml [³H]-methyl-thymidine. 1 h after the release, cells were treated with Geminin or control siRNA oligonucleotides and incubated for 24 h. The DNA was prepared and analyzed by CsCl density gradient ultracentrifugation as described previously (Yamaguchi and Dutta, 2000).

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