

# Selective Killing of Cancer Cells by Suppression of Geminin Activity

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## Abstract

**Eukaryotic cells normally restrict genome duplication to once per cell division. In metazoa, re-replication of DNA during a single S phase seems to be prevented solely by suppressing CDT1 activity, a protein required for loading the replicative MCM DNA helicase. However, siRNA suppression of geminin (a specific inhibitor of CDT1) arrested proliferation only of cells derived from cancers by inducing DNA re-replication and DNA damage that spontaneously triggered apoptosis. None of these effects were detected either in cells derived from normal human tissues or in cells immortalized by a viral oncogene. To induce these effects in noncancer cells required suppression of both geminin and cyclin A, another cell cycle regulator. Therefore, initiating DNA replication in some cancer cells is limited solely by regulating the level of CDT1 activity with geminin, whereas noncancer cells contain additional safeguards that prevent DNA re-replication. These results show that inhibition of geminin activity could be used to selectively kill cancer cells without harming other cells.** [Cancer Res 2009;69(11):4870–7]

## Introduction

DNA replication begins with assembly of prereplication complexes (preRC) at multiple sites throughout the genome as cells exit metaphase (1). PreRCs are then assembled into preinitiation complexes that are subsequently activated by protein kinases to begin DNA synthesis (S phase; ref. 2). Once S phase begins, further assembly of preRCs is prevented by phosphorylation, ubiquitination, and degradation of preRC proteins ORC1, CDC6, and CDT1, and by geminin, a specific protein inhibitor of CDT1 activity unique to metazoa (3, 4).

Eukaryotic cell division is regulated through multiple convergent pathways to restrict genome duplication to once and only once each time a cell divides (3, 4). When these pathways are subverted, random reinitiation of DNA replication occurs throughout the genome before mitosis, an event called DNA re-replication. This results in cells with greater than 4N DNA content that are sensitive to drugs that inactivate DNA damage response pathways (5–7). Therefore, if DNA re-replication could be induced selectively in cancer cells, cancer cells could be killed without harming normal cells.

An effective cancer therapy must target cancer cells without harming noncancer cells. Over expression of geminin in cancer cells does not prevent their proliferation (8–10), but inhibition of

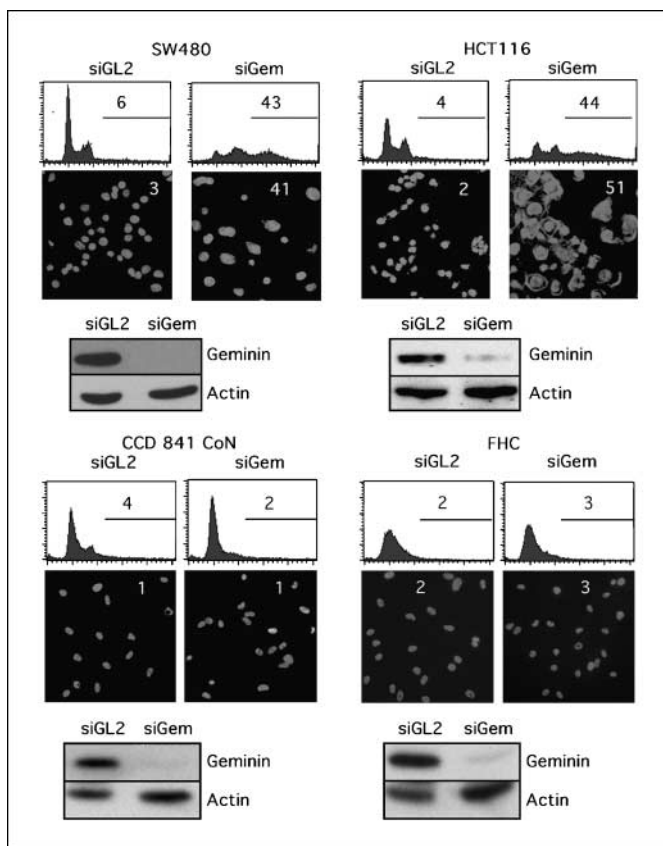
preRC assembly either by overexpressing a nondegradable form of geminin (9, 10), or by suppressing expression of Orc2, Cdc6, or Mcm2 genes induces apoptosis. However, these treatments also arrest noncancer cells in G<sub>1</sub> phase (10–12). Cancer cells induce apoptosis under these conditions by entering S phase with too few licensed replication origins, a situation that increases the frequency of stalled replication forks and thereby triggers the DNA damage response of the cell (13). Unfortunately, noncancer cells that are arrested in G<sub>1</sub> phase by preventing preRC assembly will also eventually undergo apoptosis because only cells arrested in a quiescent G<sub>0</sub> state are stable for prolonged periods of time. PreRC assembly occurs during the mitotic anaphase to G<sub>1</sub> phase transition (1, 14). When the cell cycle is arrested before phosphorylation of the retinoblastoma tumor suppressor protein (Rb) and before the “restriction point” at which entry into S phase becomes independent of serum mitogens, they undergo apoptosis, although perhaps not as quickly as cancer cells (15, 16). This difference between cancer and noncancer cells arises from the fact that cancer cells can pass through the restriction point (G<sub>1</sub>-S phase checkpoint) under conditions where noncancer cells cannot (17). Thus, inhibition of origin licensing simply induces apoptosis more quickly in cancer cells than in noncancer cells.

Here, we took the opposite tack in an effort to determine the feasibility of a cancer therapy based on the spontaneous induction of apoptosis in response to relicensing replication origins during S phase. From yeast to mammals, relicensing of replication origins during cell proliferation is prevented either by modification or degradation of various preRC proteins (3, 13, 18). However, numerous results suggest that the primary regulatory target in most, perhaps all, metazoan cells is CDT1, a preRC protein that is required for loading the MCM DNA helicase onto the replication origin. Overexpression of CDT1 induces DNA re-replication in cells from mammals (19, 20), flies (21), frogs (22–25), and plants (26), and siRNA depletion of geminin induces DNA re-replication in cells from flies (27) and mammals (5–7, 28). Moreover, deletion of the geminin gene results in DNA re-replication in preimplantation mouse embryos (29, 30). These results make a compelling case that CDT1 activity is the rate-limiting component for initiation of DNA replication in metazoa.

There are two ways to increase CDT1 activity in mammalian cells: increase the amount of CDT1 protein or inactivate geminin. Overexpression of CDT1 in mammalian cells has been reported to induce DNA re-replication only in cells that lack p53 activity (19, 20), but other studies conclude that overexpression of CDT1 does not induce significant DNA re-replication in mammalian cells as long as the ATR checkpoint is intact (31). Moreover, DNA lesions accumulate in primary cells even when CDT1 overexpression failed to induce re-replication, making all cells with excess CDT1 sensitive to genotoxic stress (31). Thus, overexpression of CDT1 does not seem to be a useful approach to cancer therapy. Therefore, we considered the alternative approach of increasing CDT1 activity by reducing geminin levels.

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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**Figure 1.** Depletion of geminin induced DNA re-replication in colorectal cancer cells (SW480 and HCT116) but not in normal colon cells (CCD 841 CoN and FHC). The indicated cells were transfected with siRNA against either firefly luciferase (siGL2) or human geminin (siGem). At 48 h posttransfection, cells were harvested and stained either with propidium iodide to quantify their DNA content by FACS analysis, or with DAPI to visualize their nuclei by fluorescence microscope. The percentage of cells is given with greater than 4N DNA content (FACS profiles) or with nuclei whose diameter is greater than twice that of nuclei in siGL2 treated cells. Geminin and actin proteins were detected by Western immunoblotting.

Remarkably, the response of mammalian cells to geminin depletion was entirely cell type dependent. Depletion of geminin in cells derived from cancers resulted in arrest of cell proliferation, DNA re-replication, and apoptosis without addition of inhibitors of the DNA damage response pathway. Depletion of geminin in immortalized cells or cells derived from normal tissues did not arrest their proliferation and did not induce DNA re-replication. Moreover, normal cells neither accumulated DNA damage nor expressed DNA damage control genes. Normal cells responded to geminin depletion such as cancer cells only when they were depleted of both geminin and cyclin A, a protein required for cell cycle-dependent CDK phosphorylation of preRC proteins. These and other results show that regulation of CDT1 activity by geminin is rate limiting for initiation of DNA replication only in certain cancer cells, and provide clear evidence for the supposition that suppression of geminin activity could be used to selectively kill cancer cells under conditions that do not affect growth and proliferation of normal cells.

## Materials and Methods

**Cells and cell culture.** The human cells listed in Supplementary Table S1 were grown in media recommended by the providers. Primary

dermal fibroblasts D-1 and primary epidermal keratinocytes K-1 were provided by Dr. Jonathon Vogel (National Cancer Institute/NIH, Bethesda, MD). 786-O was provided by Dr. Marston Linehan (National Cancer Institute/NIH, Bethesda, MD). D-1 cells were maintained in DMEM supplemented with 10% fetal bovine serum. K-1 were maintained in Keratinocyte Medium (ScienCell). MCF10A and H1299 cells were kindly provided by Dr. Z. Sheng Guo (University of Pittsburgh Cancer Institute, Pittsburgh, PA). H1299 cells were maintained in DMEM supplemented with 10% fetal bovine serum, and MCF10A cells were maintained MCF10A breast cells were grown in a serum-free MEGM (Mammary Epithelial Growth Medium) supplemented with a MEGM bullet kit (Lonza, cc3150).

**siRNA.** Short interfering oligoribonucleotides (Dharmacon) for luciferase (siGL2) and cyclin A (siCCNA) were as previously described (6, 7). Three siRNAs targeted against geminin RNA were siGEM (UGCCAACUCUGGAAU-CAA; ref. 7), siGEM2 (AACUCCAGCCUGGGGUUUAU; ref. 28), and siGEM3 (AAUCACUGGAUAUCAGGAAU). Transfections were performed with 20 to 50 nmol/L siRNA oligonucleotide duplexes using Oligofectamine (Invitrogen) according to the manufacturer's instructions. In brief, cells 50% to 60% confluent were transfected twice (0 and 24 h later) for 4 h per transfection before adding fresh medium and then harvested 48 h after the first transfection, unless otherwise indicated.

**Fluorescence-activated cell sorting analysis.** Fluorescence-activated cell sorting was done on cells that were collected by trypsinization, fixed with 70% ethanol overnight at 4°C, then collected by centrifugation, stained in 50 ng propidium iodide/mL, 0.05% NP40, and 10 µg RNase A/mL, and then analyzed with a Becton Dickinson FACSCalibur using Cellquest software.

**Protein detection.** Western immunoblotting was done using Rabbit antigeminin Geminin (FL-209; Santa Cruz Biotechnology), rabbit anti-actin (A2066; Sigma), rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (G9545; Sigma). Mouse anti-p53 (Sigma), rabbit anti-p53 phosphorylated at serine15 (Cell Signaling), rabbit anti-p21 (C-19; Santa Cruz), and rabbit anti-cyclin A (H432; Santa Cruz) were used for immunoblotting. Rabbit anti-HsCDT1 antibody was a gift from Dr. Anindya Dutta (University of Virginia Medical School, Charlottesville, VA). Cells were washed twice in PBS and then sonicated in radioimmunoprecipitation assay buffer. Proteins were fractionated by SDS-PAGE, transferred to nitrocellulose, and incubated with the indicated antibodies. For immunofluorescence cells were fixed with 2% paraformaldehyde in PBS for 10 min, permeabilized with 0.2% Triton X-100 for 10 min at room temperature, and then washed and mounted with solution containing 4',6'-diamidino-2-phenylindole (DAPI; Vector Laboratories).

**Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay.** Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assays were performed according to manufacturer's instructions (Roche manual). Briefly, cells were washed thrice in PBS, and fixed in freshly prepared 4% paraformaldehyde in PBS for 1 h at room temperature, and then washed with PBS and permeabilized in 0.1% Triton X-100 in 0.1% sodium citrate. Cells were then incubated with TUNEL reaction mixture (Roche) for 60 min at 37°C in a humidified atmosphere in the dark. After washing twice with PBS, cells were mounted in a DAPI solution (Vector Laboratories). A positive control run in parallel consisted of permeabilized cells that had been incubated with DNase I (1 U/µL) for 10 min at room temperature to introduce DNA strand breaks before the TUNEL assay.

## Results

**Geminin depletion selectively induced DNA re-replication in cancer cells.** Previous studies (7) have shown that siRNA targeted against geminin (siGem) can induce DNA re-replication in HCT116 cells derived from a colorectal carcinoma, as evidenced by increased DNA content and the appearance of giant nuclei. To determine whether or not this result was unique to these cells, their response to siGem was compared with the response of three other colon cancer cells (SW480, COLO 320DM, and DLD-1; Supplementary Table S1). In each case, geminin was reduced at least 10-fold by siGem (Supplementary Fig. S3; Fig. 1). The fraction of cells with greater than 4N DNA increased 7 to 11-fold within 2 days of

siGem transfection, as judged by FACS analysis, and the number of giant nuclei detected by microscopy increased 14- to 25-fold (Supplementary Fig. S3; Fig. 1). In total, from 30% to 50% the cells in each population underwent DNA re-replication, a proportion equivalent to the population of cells in S and G<sub>2</sub>-M phases of the cell cycle (those phases in which geminin is present). In contrast, transfection with siRNA targeted against firefly luciferase (siGL2), a gene not encoded by mammals, did not affect these parameters.

To determine whether or not siGem had the same effect on noncarcinogenic colon cells, the experiment was repeated with cells derived from normal fetal colon tissue (CCD841 CoN and FHC cells; Supplementary Table S1). Again, siGem reduced the level of geminin protein in these cells at least 10-fold (Fig. 1). However, no change was detected either in the fraction of normal colon cells with greater than 4N DNA content, or in the fraction of normal colon cells with giant nuclei (Fig. 1). Thus, geminin depletion induced DNA re-replication in colorectal cancer cells but not in their normal epithelial counterparts (summarized in Table 1).

To determine whether or not similar results would be obtained with other tissues, the same experiment was carried out with epithelial and fibroblast cells from breast, lung, kidney, bone, brain, skin, and cervix (Supplementary Table S1). In each case, siGem reduced geminin levels by ~10-fold. Nevertheless, of the 10 cell types derived from normal tissues, and the 13 cell types derived from cancer tissues, only those cells derived from cancers were induced to re-replicate their DNA upon geminin depletion

(Supplementary Figs. S1–S5; Table 1). Otherwise, no change was detected either in the fraction of cells with greater than 4N DNA, or in the fraction of giant nuclei. Surprisingly, two cells derived from normal tissues but later immortalized by constitutive expression of SV40 large tumor antigen (293T and hGOB 1.19) also were not induced to re-replicate their DNA in response to geminin depletion. Because 293T cells are tumorigenic but nonmetastatic (32), only cancer cells seem to be sensitive to geminin depletion. However, two skin melanoma cells (WM-266-4 and A375) as well as the frequently studied cervix adenocarcinoma HeLa cells also were resistant to induction of DNA re-replication in response to geminin depletion. In contrast, cells derived from breast adenocarcinoma (MCF7), lung carcinoma (H1299), kidney adenocarcinoma (786-O), osteosarcoma (U2OS), and brain glioblastoma (U87 MG; Y98G) were as sensitive to geminin depletion as were cells derived from colon cancers.

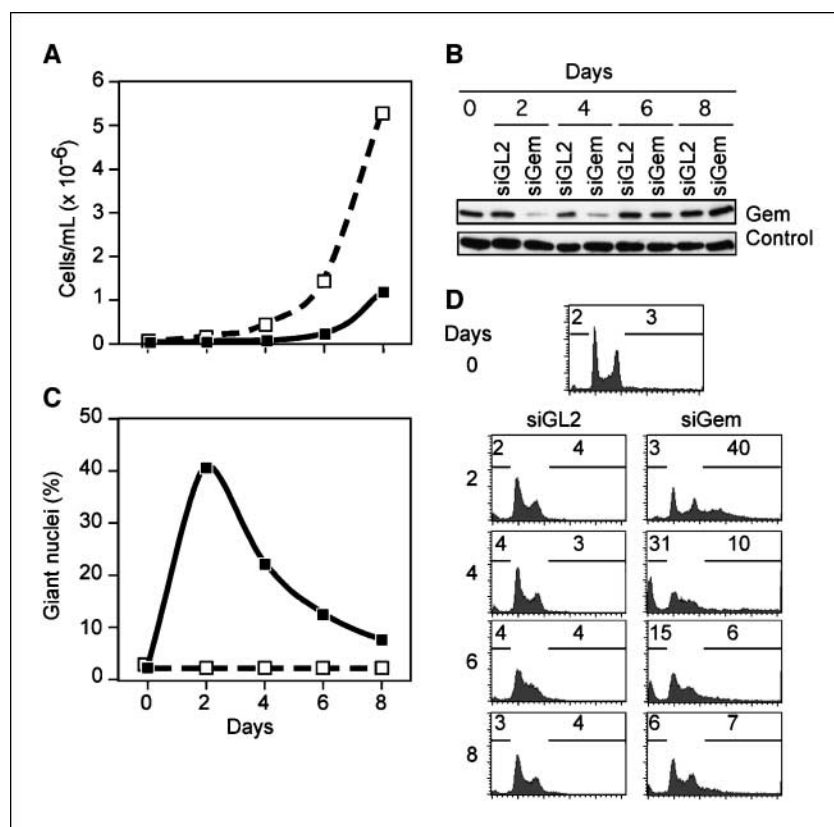
Simply stated, cells derived from normal tissues, including those that were subsequently immortalized by a viral oncogene, seemed unaffected by geminin depletion, whereas cells derived from cancers underwent DNA re-replication. Moreover, not all cells derived from cancers were sensitive to siGem; cervix adenocarcinoma and skin melanoma cells were as insensitive to geminin depletion as were normal and immortalized cells. These results were obtained with three different siRNAs directed against different sites within the geminin structural gene (Supplementary Fig. S7). Thus, DNA re-replication in these cells resulted from increased CDT1 activity as a consequence of reduced geminin activity.

**Table 1.** Effect of siRNA against geminin on DNA re-replication in human cells

Tissue	Cell type	Cell name	DNA re-replication (%)		
			siGL2	siGem	siGem+siCcnA
Colon	Normal epithelial	CCD-841 CoN	4	2	29
	Normal epithelial	FHC	2	3	
	Carcinoma	HCT-116	4	44	50
	Adenocarcinoma	SW480	6	43	
	Adenocarcinoma	COLO 320DM	4	30	
Breast	Adenocarcinoma	DLD-1	3	28	
	Normal epithelial	AG11132	1	2	30
	Normal epithelial	AG11134	6	5	
	Normal epithelial	MCF-10A	4	4	35
Lung	Adenocarcinoma	MCF7	6	36	
	Normal fibroblast	WI-38	4	3	
Kidney	Carcinoma	H1299	2	33	
	Immortalized epithelial	293T	3	4	
Bone	Adenocarcinoma	786-O	1	28	
	Immortalized osteoblast	hFOB 1.19	5	3	
Brain	Osteosarcoma	U-2OS	2	42	
	Glioblastoma	U-87 MG	4	30	
Skin	Glioblastoma	T98G	2	39	
	Normal fibroblast	D-1	1	1	44
	Normal fibroblast	K-1	3	4	
Cervix	Melanoma	WM-266-4	2	2	
	Melanoma	A375	1	2	
	Adenocarcinoma	HeLa	2	2	

NOTE: Shaded boxes are cells derived from cancers. Extent of DNA re-replication was calculated from FACS analyses (Supplementary Figs. S1–S5; Figs. 1 and 5).

**Figure 2.** Geminin depletion suppressed cancer cell proliferation and induced apoptosis. *A*, colon carcinoma HCT116 cells were transfected with either siGem (■) or siGL2 (□), harvested at indicated times posttransfection, and the total number of cells was recorded. *B*, cells treated as in *A* were subjected to Western immunoblotting for geminin. *C*, the fraction of cells in *A* that remained attached to the dish with giant nuclei was determined by fluorescent microscopy. *D*, total cells (attached plus unattached) in *A* were analyzed by FACS. Percentage with less than 2N DNA content, and the percentage with greater than 4N DNA content are indicated.



**DNA re-replication suppressed cell proliferation by inducing apoptosis.** To determine the effect of DNA re-replication on cell proliferation, HCT116 cells were transfected either with siGL2 or with siGem and then harvested at various times posttransfection. The population of cells transfected with siGL2 increased ~350-fold by 8 days with a doubling time of 18 to 20 hours, and geminin levels in these cells remained constant (Fig. 2*A* and *B*). In contrast, geminin levels were suppressed within 2 days of the first transfection with siGem (Fig. 2*B*), and they did not begin to proliferate (Fig. 2*A*) until their geminin levels were restored to normal ~6 days after the first transfection. Similarly, depletion of geminin suppressed cell proliferation in cells derived from colon adenocarcinoma (DLD-1), non-small lung carcinoma (H1299), and mammary gland adenocarcinoma (MCF7), but not in five normal cells (Fig. 4*D*).

The failure of geminin-depleted cancer cells to proliferate resulted from induction of apoptosis. Two characteristics of apoptosis are loss of cell adhesion and reduction in DNA content. Cells with giant nuclei appeared within 2 days of siGem transfection and then detached from the dish and disintegrated (Fig. 2*C*). Because cells were cultured in the absence of siGem, their place was taken by new cells that expressed geminin. FACS analysis confirmed that geminin depletion induced DNA re-replication in cancer cells within 2 days, but within 4 days, cells with >4N DNA content disappeared, and cells with <2N DNA content took their place (Fig. 2*D*). In contrast, cells transfected with siGL2 remained typical of proliferating cell populations (Fig. 2*D*) and did not accumulate cells with giant nuclei (Fig. 2*C*).

To confirm that siGem induced apoptosis in cancer cells, geminin-depleted HCT116 cells were assayed both for nuclear DNA

fragmentation and for a rounded cell morphology with blebbing, two additional characteristics of apoptosis (33). Nuclear DNA fragmentation was detected with the TUNEL assay in which terminal deoxyribonucleotidyl transferase is used to label free 3'-OH-terminated DNA fragments with fluorescein-conjugated dUTP. HCT116 cells with giant nuclei were TUNEL positive, whereas HCT116 cells with normal nuclei were not (Fig. 3*A*). This was consistent with previous studies in which DNA strand breaks were detected in cells undergoing DNA re-replication (6). TUNEL-positive nuclei were not detected in HCT116 cells treated with siGL2 (Fig. 3*A*). Cells with giant nuclei exhibited a rounded cell morphology with multiple blebs, whereas cells with normal nuclei did not (Supplementary Fig. S6).

Geminin depletion induces DNA re-replication in cancer cells, regardless of the presence or absence of the tumor suppressor protein p53, but p53<sup>+/+</sup> cancer cells induce expression and phosphorylation of p53, as well as expression of the CDK-specific inhibitor p21 whose transcription is p53 dependent (Fig. 3*B*; ref. 7). These events are part of the DNA damage response in mammalian cells, as shown by treatment of cells with etoposide, a specific inhibitor of topoisomerase II that induces DNA breaks (Fig. 3*B*). Thus, induction of DNA re-replication by siGem in cells from cancers resulted in apoptosis within 4 to 8 days posttransfection.

**Geminin depletion did not affect normal cell proliferation.** Normal somatic cells typically proliferate slowly compared with cancer cells. For example, normal breast AG11132 cells doubled in 38 to 40 hours, whereas breast adenocarcinoma MCF7 cells doubled in 30 to 32 hours. Normal colon FHC cells doubled in 39 hours, whereas colon carcinoma HCT116 cells required only 18 to 20 hours. Because differences in the time required for cell

division in mammalian cells reflect differences in the length of their G<sub>1</sub> phase, the absence of DNA re-replication in normal cells treated with siGem may result simply from fewer S, G<sub>2</sub>, and M phase cells, the only cells expressing geminin protein. In that case, normal cells may simply require more time to respond to geminin depletion. However, this was not the case.

The rate of proliferation for normal primary skin D1 fibroblasts was not affected significantly by siGem relative to siGL2 (Fig. 4A), despite the fact that geminin levels were reduced markedly in cells transfected with siGem but not in cells transfected with siGL2 (Fig. 4B). Furthermore, recovery of geminin expression in D1 cells transfected with siGem (Fig. 4B) followed the same time course as observed with HCT116 cells (Fig. 2B). FACS profiles for both siGL2 and siGem-treated D1 cells were characteristic of proliferating cell populations, and they exhibited no significant change in the fraction of cells containing >4N DNA (Fig. 4C).

To determine whether or not normal cells accumulated DNA damage in response to geminin depletion, each cell type was treated with either siGL2 or siGem and then assayed for nuclear DNA fragmentation. Under conditions that readily detected TUNEL-positive cells in cancer cells treated with siGem, no TUNEL-positive cells were detected in normal cells such as D1 (Fig. 3A), and other noncarcinogenic cells such as colon CCD841 CoN, lung WI-38, breast AG11132, and MCF10A (data not shown; Fig. 4D). Moreover, the amount of p53 in normal cells was not increased by geminin depletion and p53 was not phosphorylated. Moreover, siGem did not induce expression of p21 (Fig. 3B). Therefore, siGem did not induce genotoxic stress in normal cells under conditions where it did so in cancer cells.

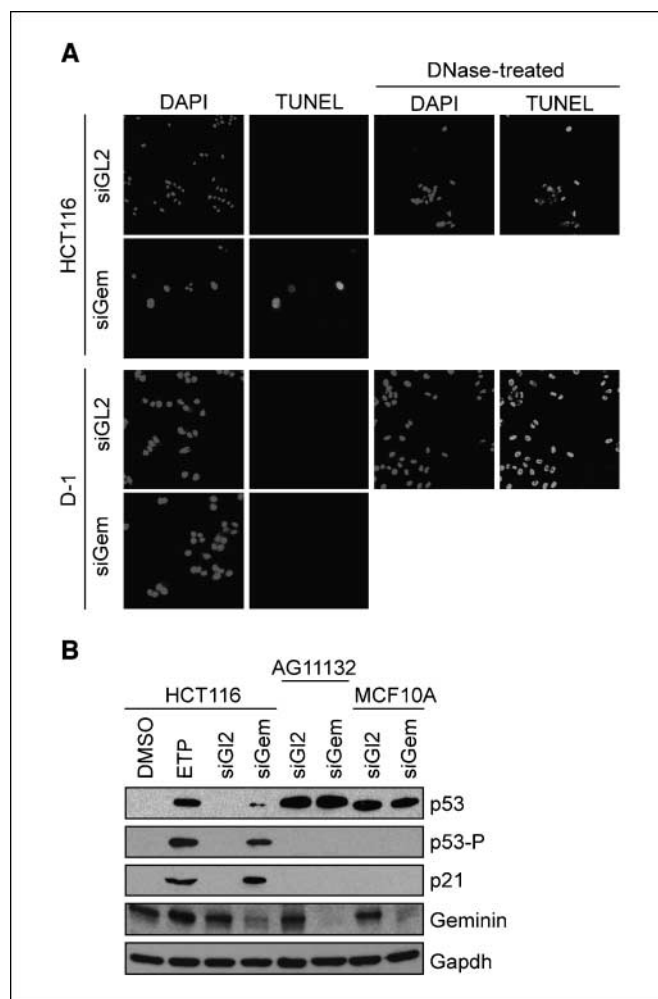
**Sensitivity to siGem did not correlate with cellular levels of either geminin or CDT1.** The simplest explanation for why geminin depletion induces DNA re-replication in cancer cells but not in normal cells is that the ratio of CDT1 to geminin is increased to a greater extent in cancer cells than in normal cells. To assess this possibility, geminin and CDT1 protein levels were assayed before and after geminin depletion in normal skin D1 cells and in colon carcinoma HCT116 cells (Fig. 5). Geminin-depleted D1 cells experienced a 3- to 4-fold decrease in CDT1, although the ratio of CDT1 to geminin actually increased 5-fold. Similarly, geminin-depleted HCT116 cells experienced at least a 10-fold decrease in CDT1 (as previously reported; ref. 7), although with little, if any, change in the CDT1/geminin ratio. Therefore, the ability of geminin depletion to induce DNA re-replication in HCT116 cells, but not in D1 cells, did not correlate with increased CDT1 protein levels.

The levels of geminin and CDT1 protein were compared in other cells as well. As expected, the levels of both geminin and CDT1 were ~5-fold greater in colon cancer cells (HCT116, DLD-1, SW480, and COLO320DM) than in normal colon cells (CCD841CoN and FHC), and the ratios of geminin to CDT1 in each of these cells was essentially unchanged (Supplementary Fig. S8A). As expected, cell populations with high geminin levels contained a greater proportion of cells in S and G<sub>2</sub>-M phases than cell populations with lower geminin levels (Supplementary Fig. S8B). Normal skin D1 cells were similar to normal colon cells. Therefore, one would not expect depletion of geminin to be more injurious to cancer cells than to normal cells.

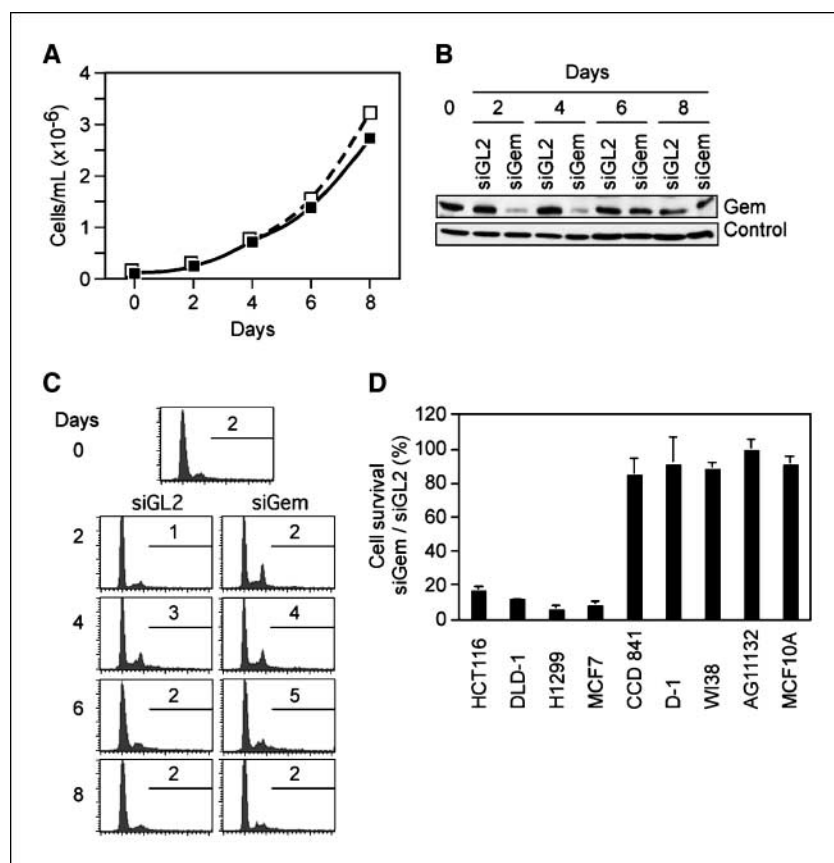
On the other hand, normal breast cells (AG11132, AG11134, and MCF10A) exhibited higher geminin levels but lower CDT1 levels than breast adenocarcinoma MCF7 cells (Supplementary Fig. S8A). Again, these results were consistent with the relative fraction of

cells in S and G<sub>2</sub>-M phases (Supplementary Fig. S8B). Thus, a simple correlation between geminin levels, geminin to CDT1 ratios, and sensitivity to geminin depletion was not apparent. Normal colon cells contained low levels of geminin, because they consisted predominantly of G1 phase cells, and normal breast cells contained high levels of geminin, because they contained a higher proportion of proliferating cells, but neither cell type underwent DNA re-replication when transfected with siGem.

**Induction of DNA re-replication in normal cells required suppression of multiple regulatory pathways.** Several different but convergent pathways have been described that contribute to restricting genome duplication to once each time a cell divides (3, 4, 13). At least three of them involve CDK-dependent phosphorylation of preRC proteins, and in metazoa, cyclin A is



**Figure 3.** Only cells that re-replicated their DNA experienced genotoxic stress and apoptosis. **A**, colon carcinoma HCT116 cells were transfected with either siGL2 or siGem. At 4 d posttransfection, cells not attached to the dish were isolated by centrifugation and subjected to the TUNEL assay. HCT116 cells treated with DNase I provided a TUNEL-positive control. Normal skin D1 cells were treated likewise. **B**, the indicated cells were treated with either siGL2 or siGem as in Fig. 1, and then subjected to Western immunoblotting for the indicated protein (p53-P is p53 phosphorylated on serine 15). To show the effects of genotoxic stress, p53<sup>+/+</sup> HCT116 cells were treated with 3  $\mu$ mol/L etoposide (ETP) for 16 h in parallel with HCT116 cells treated with the same buffered solvent (DMSO). Longer exposures revealed p53 in HCT116 cells, and shorter exposures confirmed that p53 levels in AG11132 and MCF10A cells remained unchanged.



**Figure 4.** Depletion of geminin did not suppress proliferation of normal cells and did not induce apoptosis. **A**, the total number of normal skin D1 cells transfected with either siGL2 (□) or siGem (■) at the indicated times posttransfection. **B**, cells treated as in **A** were subjected to Western immunoblotting for geminin and actin. *Control*, an unidentified protein that cross-reacted with anti-geminin antibody. **C**, cells in **A** were analyzed by FACS, as in Fig. 1. Percentage of cells with >4N DNA is shown. **D**, the indicated cells were transfected with either siGem or siGL2. Six days later, the total number of cells was counted, and the ratio of cells surviving siGem transfection relative to those surviving siGL2 transfection was determined.

the principle activator of CDK activities that suppress DNA re-replication. To determine whether or not suppression of multiple pathways induces DNA re-replication in normal cells, D1 cells were treated with both siGem and siRNA-targeted against cyclin A (siCcnA). Remarkably, this combination induced DNA re-replication in normal cells to an extent similar to that observed in geminin-depleted HCT116 cells (Fig. 5). The response to depletion of cyclin A alone in D1 cells was detectable but modest compared with depletion of both cyclin A and geminin. Similar results were obtained with normal breast (AG11132 and MCF10A) and colon (CCD841 CoN) cells (Table 1). In contrast, the response to depletion of both cyclin A and geminin in HCT116 cells was essentially the same as with depletion of geminin alone. Therefore, DNA re-replication is prevented primarily by the ratio of geminin to CDT1 in HCT116 cells, whereas DNA re-replication in D1 cells is prevented by cyclin A-dependent pathways as well as by geminin.

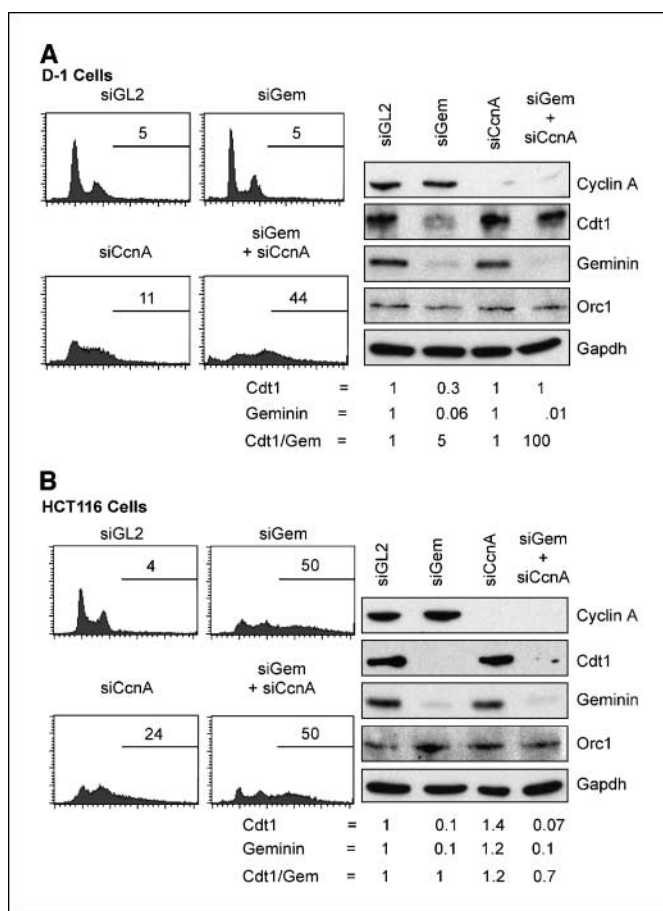
This conclusion was confirmed by changes in the levels of CDT1 and geminin in these experiments (Fig. 5). Depletion of cyclin A in D1 cells did not affect the levels of either CDT1 or geminin, whereas depletion of both cyclin A and geminin prevented loss of CDT1 (Fig. 5), consistent with a critical role for CDT1 in DNA re-replication. However, although depletion of cyclin A did not affect CDT1 and geminin levels in HCT116 cells, neither did it prevent loss of CDT1 in geminin-depleted cells. Because neither siGem nor siGem plus siCcnA increased the ratio of CDT1 to geminin in HCT116 cells, elevation of CDT1 activity does not account for DNA re-replication under these conditions. Both CDK and geminin-dependent pathways prevent DNA re-replication in normal cells, whereas cancer cells rely primarily, if not exclusively, on geminin.

## Discussion

Studies on cells from flies, frogs, plants, and mammals suggest that CDT1 activity is the rate-limiting step in preRC assembly in metazoa, and that geminin is the critical regulator of CDT1 activity (reviewed in refs. 3, 18, 29, 30, 34–38). Nevertheless, exceptions have been reported. Whereas geminin depletion induced DNA re-replication in some cancer cells (5–7), it did not do so in HeLa (20, 39, 40), MCF10A (40), and 293T (41) cells. Moreover, overexpression of both CDT1 and Cdc6 induced DNA re-replication in some, but not all, lung carcinoma cells, and it did not induce DNA re-replication in primary human and rodent cells, 293T cells, HeLa cells, and other cervical carcinoma cell lines (19, 42). These results suggested to us that CDT1 activity may be the rate limiting factor for preRC assembly in rapidly proliferating cells such as cleavage stage embryos, preimplantation embryos, and cancer cells but not in normal cells.

To test this hypothesis, siRNA targeted against the human geminin gene was transfected into 23 different cell lines derived from eight different human tissues (Supplementary Table S1; Table 1). In six of these tissues, cells were derived from biopsies of both cancers and normal tissues. Remarkably, all of the cells that re-replicated their DNA had been derived from malignant cancers; none were from normal tissues. Moreover, cells derived from normal tissues that were later immortalized with SV40 T-antigen (293T and hFOB 1.19) continued to proliferate normally even when their geminin levels were reduced >10-fold. Similarly, three cell lines derived from cancers (HeLa, WM-266-4, and A375) also were insensitive to suppression of their geminin levels. We attribute these effects to suppression of geminin synthesis, because all three of the





**Figure 5.** Normal cells prevent DNA re-replication through multiple convergent pathways that are dependent on both geminin and cyclin A. Normal skin D1 cells (A) and colon carcinoma HCT116 cells (B) were transfected either with siGL2, siGem, siCcnA, or both siGem and siCcnA. Cells were harvested at 2 d posttransfection and subjected to FACS analysis to determine the fraction of cells with greater than 4N DNA content. The same cells were subjected to Western immunoblotting to determine the relative levels of cyclin A, CDT1, geminin, and Orc1. Protein levels were quantified using Multi Gauge software (Fuji Film). Cdt1 and geminin protein levels were normalized relative to glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), and then to either Cdt1 or geminin in siGL2-treated cells.

siGem oligoribonucleotides tested suppressed geminin protein in both cancer and noncancer cells, and either overexpression of a nondegradable form of geminin (28), or cosuppression of both CDT1 and geminin has been shown to prevent induction of DNA re-replication by two of these same molecules (5, 7). These studies, carried out under the same conditions using the same siRNAs, show that whereas some cancer cells are sensitive to geminin depletion, most primary and secondary mammalian cells are insensitive. Therefore, drugs targeted specifically against geminin activity should be excellent candidates to selectively induce DNA re-replication in some forms of cancer without interfering either with the function or proliferation of noncancer cells.

Previous studies reported that inhibition either of the ATM and ATR protein kinases, or the CHK1 protein kinase 2 days after siGem treatment of HCT116 cells rapidly induced apoptosis in cells that had undergone DNA re-replication (7). Therefore, DNA re-replication induced by geminin depletion activates DNA damage response pathways that prevent cells from entering mitosis until the damage is repaired because suppression of this pathway

resulted in apoptosis. When these pathways are inhibited, induction of apoptosis is immediate (6, 7). Here, we show that induction of apoptosis occurs spontaneously within a few days of siGem transfection without addition of protein kinase inhibitors. Because siGem did not induce DNA re-replication, DNA damage, or DNA damage response genes in either normal or immortalized cells, it did not induce apoptosis. Normal cells continued to proliferate. Therefore, inhibition of geminin activity should, in principle, selectively kill cancer cells *in vivo*.

Induction of DNA re-replication by geminin depletion implies that the level of Cdt1 activity is rate limiting for origin licensing, and that a reduction in geminin results in an increase in Cdt1 activity. However, suppression of geminin in either cancer or noncancer cells resulted in a concomitant decrease of Cdt1 protein, the result of ubiquitin-mediated degradation of Cdt1 when it is not bound to geminin (43). Suppression of both geminin and cyclin A restored Cdt1 protein levels to normal in noncancer cells and induced DNA re-replication. Similar results have been reported with *Drosophila* cells (27), and confirms the existence multiple, convergent pathways that restrict genome duplication to once per cell division. For example, CDT1 activity in humans is down-regulated during S phase in three ways (3, 44, 45). It is phosphorylated by cyclin A:CDK2 and then ubiquitinated by SCF<sup>skp2</sup>. It is also ubiquitinated by CDR<sup>Ddb1</sup> in the presence of proliferating cell nuclear antigen, a protein that stimulates DNA polymerase activity at replication forks and that also binds to CDT1. Both reactions result in degradation of CDT1 by the 26S proteasome, although only SCF requires prior phosphorylation of its substrate. Finally, geminin binds specifically to CDT1, thereby inhibiting its activity while simultaneously protecting CDT1 from ubiquitination and degradation. In addition to inactivation of CDT1, cyclin A-dependent phosphorylation of Orc1 prevents it binding to chromatin during mitosis, and cyclin A-dependent phosphorylation of Cdc6 affects its nuclear localization (3, 40). All of these mechanisms could contribute to restricting genome duplication to once per cell division in normal cells, but the fact that suppression of geminin alone can induce DNA re-replication in some cancer cells means that one or more of these pathways is inactive in those cells.

Because geminin protects CDT1 from ubiquitin-dependent degradation (43), suppression of geminin also reduced CDT1 protein levels in both normal and cancer cells. The fact that cancer cells could still re-replicate their DNA under these conditions can be attributed to the ~10-fold excess of CDT1 (Supplementary Fig. S8) and other preRC proteins (ref. 46)<sup>1</sup> in cancer cells compared with normal cells. The fact that suppression of both geminin and cyclin A restored CDT1 levels in normal cells but not in cancer cells (Fig. 5) suggests that normal cells use the SCF pathway to degrade CDT1, whereas some cancer cells use the CDR pathway. The fact that both cyclin A and geminin prevent DNA re-replication in normal cells but only geminin prevents DNA re-replication in some cancer cells could be explained if both SCF and geminin maintained CDT1 activity rate limiting in normal cells, but only geminin maintained CDT1 activity rate limiting in some cancer cells. However, this does not seem to be the case, because overexpression of CDT1 fails to induce DNA re-replication in mammalian cells (19, 31). Therefore, either cyclin A-dependent phosphorylation of ORC1 or CDC6 plays a role, or

<sup>1</sup> S. Ghosh & M.L. DePamphilis, unpublished results.

there is as yet an undiscovered regulatory pathway in mammalian cells.

## Disclosure of Potential Conflicts of Interest

W. Zhu and M.L. DePamphilis have submitted a patent application.

## References

- Noguchi K, Vassilev A, Ghosh S, Yates JL, DePamphilis ML. The BAH domain facilitates the ability of human Orc1 protein to activate replication origins *in vivo*. *EMBO J* 2006;25:5372–82.
- Kelly TJ, Stillman B. Duplication of DNA in Eukaryotic Cells. In: DePamphilis ML, ed. *DNA Replication and Human Disease*. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 2006. p. 1–30.
- DePamphilis ML, Blow JJ, Ghosh S, Saha T, Noguchi K, Vassilev A. Regulating the licensing of DNA replication origins in metazoa. *Curr Opin Cell Biol* 2006;18:231–9.
- Blow JJ, Gillespie PJ. Replication licensing and cancer—a fatal entanglement? *Nat Rev Cancer* 2008;8:799–806.
- Melixetian M, Ballabeni A, Masiero L, et al. Loss of Geminin induces rereplication in the presence of functional p53. *J Cell Biol* 2004;165:473–82.
- Zhu W, Dutta A. An ATR- and BRCA1-mediated Fanconi anemia pathway is required for activating the G2/M checkpoint and DNA damage repair upon rereplication. *Mol Cell Biol* 2006;26:4601–11.
- Zhu W, Chen Y, Dutta A. Rereplication by depletion of geminin is seen regardless of p53 status and activates a G2/M checkpoint. *Mol Cell Biol* 2004;24:7140–50.
- Montanari M, Boninsegna A, Faraglia B, et al. Increased expression of geminin stimulates the growth of mammary epithelial cells and is a frequent event in human tumors. *J Cell Physiol* 2005;202:215–22.
- Wohlschlegel JA, Kutok JL, Weng AP, Dutta A. Expression of geminin as a marker of cell proliferation in normal tissues and malignancies. *Am J Pathol* 2002;161:267–73.
- Shreeram S, Sparks A, Lane DP, Blow JJ. Cell type-specific responses of human cells to inhibition of replication licensing. *Oncogene* 2002;21:6624–32.
- Feng D, Tu Z, Wu W, Liang C. Inhibiting the expression of DNA replication-initiation proteins induces apoptosis in human cancer cells. *Cancer Res* 2003;63:7356–64.
- Machida YJ, Teer JK, Dutta A. Acute reduction of an origin recognition complex (ORC) subunit in human cells reveals a requirement of ORC for Cdk2 activation. *J Biol Chem* 2005;280:27624–30.
- Schwob E, Labib K. Regulating Initiation Events in Yeasts. In: DePamphilis ML, ed. *DNA Replication and Human Disease*. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 2006. p. 295–312.
- Baldiringer T, Gossen M. Binding of *Drosophila* ORC proteins to anaphase chromosomes requires cessation of mitotic cyclin-dependent kinase activity. *Mol Cell Biol* 2009;29:140–9.
- Wu JR, Gilbert DM. The replication origin decision point is a mitogen-independent, 2-aminopurine-sensitive, G<sub>1</sub>-phase event that precedes restriction point control. *Mol Cell Biol* 1997;17:4312–21.
- Wu JR, Gilbert DM. Lovastatin arrests CHO cells between the origin decision point and the restriction point. *FEBS letters* 2000;484:108–12.
- Wu JR, Keezer SM, Gilbert DM. Transformation abrogates an early G<sub>1</sub>-phase arrest point required for specification of the Chinese hamster DHFR replication origin. *EMBO J* 1998;17:1810–8.
- Sivaprasad U, Dutta A, Bell SP. Assembly of Pre-replication Complexes. In: DePamphilis ML, ed. *DNA Replication and Human Disease*. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 2006. p. 63–88.
- Vaziri C, Saxena S, Jeon Y, et al. A p53-dependent checkpoint pathway prevents rereplication. *Mol Cell* 2003;11:997–1008.
- Nishitani H, Lygerou Z, Nishimoto T. Proteolysis of DNA replication licensing factor Cdt1 in S-phase is performed independently of geminin through its N-terminal region. *J Biol Chem* 2004;279:30807–16.
- Thomer M, May NR, Aggarwal BD, Kvoek G, Calvi BR. *Drosophila* double-parked is sufficient to induce rereplication during development and is regulated by cyclin E/CDK2. *Development* 2004;131:4807–18.
- Arias EE, Walter JC. Replication-dependent destruction of Cdt1 limits DNA replication to a single round per cell cycle in *Xenopus* egg extracts. *Genes Dev* 2005;19:114–26.
- Maiorano D, Krasinska L, Lutzmann M, Mechali M. Recombinant Cdt1 induces rereplication of G2 nuclei in *Xenopus* egg extracts. *Curr Biol* 2005;15:146–53.
- Yoshida K, Takisawa H, Kubota Y. Intrinsic nuclear import activity of geminin is essential to prevent reinitiation of DNA replication in *Xenopus* eggs. *Genes Cells* 2005;10:63–73.
- Li A, Blow JJ. Cdt1 downregulation by proteolysis and geminin inhibition prevents DNA re-replication in *Xenopus*. *EMBO J* 2005;24:395–404.
- Castellano M, Boniotti MB, Caro E, Schnittger A, Gutierrez C. DNA replication licensing affects cell proliferation or endoreplication in a cell type-specific manner. *Plant Cell* 2004;16:2380–93.
- Mihaylov IS, Kondo T, Jones L, et al. Control of DNA replication and chromosome ploidy by geminin and cyclin A. *Mol Cell Biol* 2002;22:1868–80.
- Tachibana KE, Gonzalez MA, Guarguaglini G, Nigg EA, Laskey RA. Depletion of licensing inhibitor geminin causes centrosome overduplication and mitotic defects. *EMBO Rep* 2005;6:1052–7.
- Gonzalez MA, Tachibana KE, Adams DJ, et al. Geminin is essential to prevent endoreduplication and to form pluripotent cells during mammalian development. *Genes Dev* 2006;20:1880–4.
- Hara K, Nakayama KI, Nakayama K. Geminin is essential for the development of preimplantation mouse embryos. *Genes Cells* 2006;11:1281–93.
- Liu E, Lee AY, Chiba T, Olson E, Sun P, Wu X. The ATR-mediated S phase checkpoint prevents rereplication in mammalian cells when licensing control is disrupted. *J Cell Biol* 2007;179:643–57.
- Yan W, Shao R. Transduction of a mesenchyme-specific gene perostin into 293T cells induces cell invasive activity through epithelial-mesenchymal transformation. *J Biol Chem* 2006;281:19700–8.
- Otsuki Y, Li Z, Shibata MA. Apoptotic detection methods—from morphology to gene. *Prog Histochem Cytochem* 2003;38:275–339.
- Kerns SL, Torke SJ, Benjamin JM, McGarry TJ. Geminin prevents rereplication during *xenopus* development. *J Biol Chem* 2007;282:5514–21.
- Blow JJ, Dutta A. Preventing re-replication of chromosomal DNA. *Nature Reviews* 2005;6:476–86.
- Tada S. Cdt1 and geminin: role during cell cycle progression and DNA damage in higher eukaryotes. *Front Biosci* 2007;12:1629–41.
- DePamphilis ML, Blow JJ. Regulating Initiation Events in Metazoa. In: DePamphilis ML, ed. *DNA Replication and Human Disease*. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 2006. p. 313–34.
- Saxena S, Dutta A. Geminin-Cdt1 balance is critical for genetic stability. *Mutation research* 2005;569:111–21.
- Kulartz M, Knippers R. The replicative regulator protein geminin on chromatin in the HeLa cell cycle. *J Biol Chem* 2004;279:41686–94.
- Machida YJ, Dutta A. The APC/C inhibitor, Emi1, is essential for prevention of rereplication. *Genes Dev* 2007;21:184–94.
- Teer JK, Dutta A. Human Cdt1 lacking the evolutionarily conserved region that interacts with MCM2–7 is capable of inducing re-replication. *J Biol Chem* 2008;283:6817–25.
- Tatsumi Y, Sugimoto N, Yugawa T, Narisawa-Saito M, Kiyono T, Fujita M. Deregulation of Cdt1 induces chromosomal damage without rereplication and leads to chromosomal instability. *J Cell Sci* 2006;119:3128–40.
- Ballabeni A, Melixetian M, Zamponi R, Masiero L, Marinoni F, Helin K. Human Geminin promotes pre-RC formation and DNA replication by stabilizing CDT1 in mitosis. *EMBO J* 2004;23:3122–32.
- Fujita M. Cdt1 revisited: complex and tight regulation during the cell cycle and consequences of deregulation in mammalian cells. *Cell Division* 2006;1:22.
- Hook SS, Lin JJ, Dutta A. Mechanisms to control rereplication and implications for cancer. *Curr Opin Cell Biol* 2007;19:663–71.
- McNairn AJ, Gilbert DM. Overexpression of ORC subunits and increased ORC-chromatin association in transformed mammalian cells. *J Cellular Biochem* 2005;96:879–87.