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An ATR- and BRCA1-Mediated Fanconi Anemia Pathway Is Required for Activating the G_2/M Checkpoint and DNA Damage Repair upon Rereplication†

Wenge Zhu and Anindya Dutta*

Department of Biochemistry and Molecular Genetics, University of Virginia School of Medicine, Charlottesville, Virginia 22908

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The timely assembly of prereplicative complexes at replication origins is tightly controlled to ensure that genomic DNA is replicated once per cell cycle. The loss of geminin, a DNA replication inhibitor, causes rereplication that activates a G_2/M checkpoint in human cancer cells. Fanconi anemia (FA) is an autosomal recessive and X-linked disorder associated with cancer susceptibility. Here we show that rereplication activates the FA pathway both for the activation of a G_2/M checkpoint and for repair processes, like recruitment of RAD51. Both ATR and BRCA1 are required to activate the FA pathway. The G_2/M checkpoint-mediated arrest of the cell cycle is critical for the prevention of both apoptosis and the accumulation of cells with rereplicated DNA, because the loss of ATR, BRCA1, or FANCA promotes apoptosis and suppresses the accumulation. The accumulation of cells with rereplicated DNA is restored by the artificial induction of a G_2-phase arrest even when ATR, BRCA1, or FANCA is absent. Therefore, the ATR- and BRCA1-mediated FA pathway is required for the activation of a G_2/M checkpoint and for DNA damage repair in response to the endogenous signal of rereplication. In its absence, the cells rapidly lose viability when faced with rereplication.

Licensing of origins through the assembly of prereplicative complexes is tightly controlled to ensure that genomic DNA is replicated once per cell cycle. Multiple mechanisms have evolved in cells to prevent rereplication (3, 19). The balance between Cdt1 and geminin is one such mechanism (23, 31). Cdt1 is a replication initiation factor that promotes the assembly of the prereplicative complex (20, 28). Overexpression of Cdt1 in human cells results in rereplication (43), and increased expression of *Drosophila melanogaster* Cdt1 (Dup) in diploid cells is sufficient to induce polyploidization (40). In *Xenopus laevis* egg extract, addition of Cdt1 after one round of DNA replication causes rereplication (2). Failure to degrade Cdt1 in S phase causes rereplication in *Caenorhabditis elegans*, *Xenopus*, and human cells (16, 35, 49). Geminin plays a critical role in preventing rereplication by limiting Cdt1 activity (34, 46). Rereplication has been reported for both human and *Drosophila* cells upon depletion of geminin by small interfering RNA (siRNA) (22, 24, 50). Rereplication by loss of geminin activates a G_2/M checkpoint that prevents cells from entering mitosis and thus restricts the proliferation of cells with overreplicated DNA. Abrogation of this checkpoint in geminin-depleted cells leads to apoptosis (22, 50). Not much is known about the proteins that sense the rereplication products and activate the checkpoint pathways or about the repair pathways activated by rereplication.

Several genes implicated in chromosome instability syndromes are critical for sensing different types of DNA damage. Fanconi anemia (FA) is an autosomal recessive and X-linked disorder associated with cancer susceptibility (5). FA cells are hypersensitive to DNA cross-linking agents. Eleven FA genes have been cloned out of 12 FA complementation groups, and among these, eight FA proteins (A, B, C, E, F, G, L, and M) form a nuclear complex (FA core complex) that is required for monoubiquitination of FANCD2 after DNA cross-links. Monoubiquitinated FANCD2 interacts with BRCA2 and promotes the assembly of BRCA2 foci. BRCA2 is itself mutated in familial breast cancers and in FA patients from the FA-D1 complementation group and is involved in homology-directed repair (HDR) through its interaction with RAD51 (6, 25, 44, 47). Consistent with the involvement of HDR in this pathway, FANCD2 colocalizes with RAD51. In addition, the FA pathway is required for the formation of RAD51 nuclear foci in response to DNA damage (7, 44, 48). FANCA, FANCG, and FANCD2 also promote HDR (27). Thus, the FA pathway is important for activating HDR DNA repair pathways after DNA damage induced by cross-linking agents.

In addition to their role in DNA repair, FA proteins are involved in checkpoint activation after DNA damage (11, 26, 29, 37). FANCD2 was phosphorylated by ATM and shown to be required for intra-S-phase checkpoint activation but not for G_2/M checkpoint activation (37). In contrast, FANCC from the FA core complex was not required for the intra-S-phase checkpoint activation (37) but was required for the activation of the G_2/M checkpoint in response to ionizing radiation treatment (10).

Accumulating evidence also indicates that there are genetic and physical interactions between FA genes and the breast cancer susceptibility gene BRCA1. For instance, in response to mitomycin C, the BRCA1$^{-/-}$ cell line HCC1937 shows phenotypes similar to those of FA cells: chromosome instability and increased triradial and tetraradial chromosome formation

* Corresponding author. Mailing address: Department of Biochemistry and Molecular Genetics, University of Virginia School of Medicine, Box 800733, Jordan Hall 1240, 1300 Jefferson Park Avenue, Charlottesville, VA 22908. Phone: (434) 924-1277. Fax: (434) 924-5069. E-mail: ad8q@virginia.edu.

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(32, 41). BRCA1 is required for monoubiquitination and nuclear focus formation of FANCD2 (11). Furthermore, BRCA1 physically interacts with both monoubiquitinated FANCD2 and FANCA (9, 11).

Despite the implication of the FA/BRCA pathway in the cellular response to exogenous DNA-damaging agents, not much is known about whether this pathway responds to endogenous errors of DNA replication. Here, we examine whether the FA/BRCA pathway is involved in the cell's response to rereplication. We find that DNA rereplication generates single-stranded DNA (ssDNA), which is colocalized with chromatin-associated monoubiquitinated FANCD2 and RAD51 nuclear foci, suggesting activation of the HDR pathway. The formation of RAD51 foci is dependent on the activation of FANCD2. The chromatin association of monoubiquitinated FANCD2 is suppressed by silencing of ATR, BRCA1, or FANCA. ATR, BRCA1, or FANCA is required both for the accumulation of rereplicated cells and for G_2/M checkpoint activation in geminin-depleted cells. The suppression of ATR, BRCA1, FANCA, or FANCD2 in rereplicating cells leads to apoptosis, suggesting that induction of rereplication may be selectively toxic for cancers with complete loss of BRCA1 or FA pathways. Therefore, the FA/BRCA pathway is involved in responding to rereplication in order to activate checkpoints that prevent cell cycle progression and promote homology-directed repair.

MATERIALS AND METHODS

Cell culture. Human colorectal cancer HCT116 cell lines were grown in 10% fetal bovine serum and 1% penicillin-streptomycin in McCoy's 5A modified medium (Cellgro). HCC1937 cells were grown in 10% fetal bovine serum and 1% penicillin-streptomycin in RPMI 1640 medium (Cellgro).

siRNA. siRNA oligonucleotides (Invitrogen) were made to the following target sequences (sense): geminin (GEM) and control GL2, as described in reference 50; ATR, AAGACGGTGTGCTCATGCGGC (12); BRCA1, CCUGUCUCCA CAAAGUGUG; FANCA, GGGUCAAGAGGGAAAAAUA (1); FANCD2, GGAGAUUGAUGGUCUACUA; and cyclin A, AGCCAGTGAGTGTTAA TGA. Transfections were performed with 100 nM siRNA oligonucleotide duplexes by using Oligofectamine (Invitrogen) according to the instructions of the manufacturer.

Antibodies, immunoblotting, and immunofluorescence. Rabbit antigeminin was raised as described earlier (46). Rabbit anti-phospho-H2AX (serine 139), rabbit anti-PARPp24, rabbit anti-PARPp85 (Upstate), mouse anti-β-actin (Sigma), rabbit anti-phospho-Cdc2 (Cell Signaling Technology), rabbit anti-cyclin A (H432), mouse anti-Cdc2, mouse anti-FANCD2, goat anti-ATR (Santa Cruz), rabbit anti-Rad51, mouse anti-BRCA1 (Oncogene), rabbit anti-ORC2 (BD), rabbit anti-FANCA (gift from Alan D'Andrea, Dana-Farber Cancer Institute), and mouse anti-RPA70 (gift from Bruce Stillman, Cold Spring Harbor Laboratory) were used for immunoblotting and immunofluorescence. Western blotting was performed as described earlier (50). Immunofluorescence was performed as follows. Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min and permeabilized with 0.2% Triton X-100 for 10 min at room temperature. Coverslips were blocked with 3% bovine serum albumin in PBS with 0.02% Tween 20 and incubated with primary antibody for 1 to 3 h at room temperature. Cells were then washed and incubated with tetramethyl rhodamine isothiocyanate- or fluorescein isothiocyanate-conjugated secondary antibody (Dako Corporation). Cells were mounted with a solution containing DAPI (4′,6′-diamidino-2-phenylindole) (Vector Lab) before being examined under a microscope. To detect ssDNA, cells were labeled with 10 μM BrdU after the first transfection until harvest 72 h later.

Fluorescence-activated cell sorter (FACS) analysis. Cells were collected by trypsinization and fixed with 70% ethanol overnight at 4°C. After fixation, cells were centrifuged and stained in 1 ml of propidium iodide solution (0.05% NP-40, 50 μg/ml propidium iodide, and 10 μg/ml RNase A). The labeled cells were analyzed with a Becton Dickinson flow cytometer by using Cellquest software.

Single-cell gel electrophoresis. Single-cell gel electrophoresis was performed using a Comet assay kit (Trevigen, Inc., Gaithersburg, MD) as described in the manual. Briefly, harvested cells were washed two times with cold PBS at a concentration of 1.5×10^6 cells/ml. Cells were then suspended in molten agarose and transferred to glass slides, which were immersed in lysis buffer for 1 h at 4°C. For neutral unwinding conditions, slides were transferred to a horizontal electrophoresis apparatus in Tris-borate-EDTA and subjected to 10 V for 20 min. Slides were then fixed in 70% ethanol, air dried, and stained with SYBR green. For alkaline unwinding conditions, cells were immersed in 300 mM NaOH for 1 h prior to electrophoresis. To score cells, nuclei with a tail larger than 2 nuclear diameters were counted as positive for DNA damage. The percentage of nuclei with comet tails was the number of nuclei positive for DNA damage divided by the total number of nuclei counted.

RESULTS

Rereplication by depletion of geminin generates both ssDNA and double-stranded DNA breaks. Although single-stranded DNA is generated during rereplication (22), the activation of Chk2 (50) suggests that double-stranded DNA breaks must also be generated. The appearance of ssDNA was measured by detecting previously incorporated BrdU by immunofluorescence with anti-BrdU antibody without prior DNA denaturation (22). Consistent with previous results, BrdU foci were detected in rereplicating cells and the foci colocalized with foci of the single-stranded DNA binding protein RPA70 (data not shown). Accompanying this, there was an increase in association with the chromatin of RPA70, the largest subunit of RPA, upon rereplication by geminin depletion (see Fig. 2A).

Phosphorylated H2AX was detected in geminin-depleted cells (22, 50), suggesting but not proving the presence of double-stranded breaks. To directly look for double-stranded breaks, we utilized single-cell gel electrophoresis (Comet assay). Under neutral conditions, the Comet assay detects primarily double-stranded breaks, while under alkaline conditions, the assay detects both double-stranded breaks and single-stranded breaks. Under both neutral and alkaline unwinding conditions, the Comet assay detected significantly more comet tails in geminin-depleted cells than in control-treated cells (Fig. 1A and B). Thus, double-stranded DNA breaks are generated in geminin-depleted cells, explaining why Chk2 is activated (50).

In addition, the sites of generation of double-stranded breaks could be different from the sites of ssDNA generation. Phosphorylated H2AX, however, colocalized with BrdU (Fig. 1C), suggesting that phosphorylated histone H2AX is physically at the same sites where ssDNA is formed upon rereplication. These results suggest that the single-stranded interruptions in rereplicating chromosomes are close to sites of double-stranded breaks (15).

FA pathway is activated in cells undergoing rereplication. While screening for damage-responsive pathways that might sense rereplication, we discovered that the FA pathway is activated in geminin-depleted cells. In control GL2-treated cells, a small fraction of FANCD2 was associated with chromatin, and the chromatin-associated FANCD2 had lower mobility on electrophoresis, consistent with its monoubiquitinated status (Fig. 2A) (44). In geminin-depleted rereplicating cells, however, a large fraction of FANCD2 was associated with chromatin and was highly monoubiquitinated compared to the GL2-treated cells (Fig. 2A), indicating that the FA pathway is activated in cells undergoing rereplication.

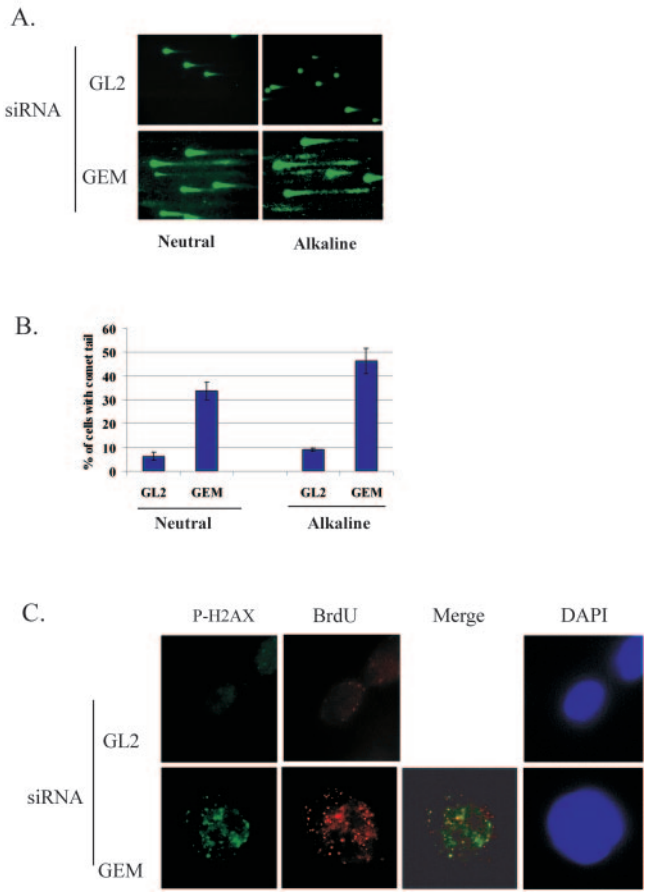


FIG. 1. Depletion of geminin leads to generation of both ssDNA and double-stranded breaks in human cancer cells. (A) Double-stranded breaks are generated in geminin-depleted cells. HCT116 cells were transfected with control siRNA oligonucleotide GL2 or geminin siRNA oligonucleotide GEM twice and harvested 72 h after the first transfection. Shown are results from the Comet assay done under neutral conditions, which detects primarily double-stranded DNA breaks, and alkaline unwinding conditions, which detects both double-stranded and single-stranded DNA breaks. (B) Percentages of cells with DNA damage under both neutral and alkaline unwinding conditions as shown in panel A. At least 80 cells were counted for each experiment. Means ± standard deviations from two experiments are shown. (C) ssDNA is generated in geminin-depleted cells. HCT116 cells were transfected with control siRNA oligonucleotide GL2 or geminin siRNA oligonucleotide GEM with the addition of BrdU after the first transfection and harvested 72 h after the first transfection. Coimmunostaining with anti-BrdU antibody without denaturation and phospho-H2AX antibody (P-H2AX) (Ser139) is shown.

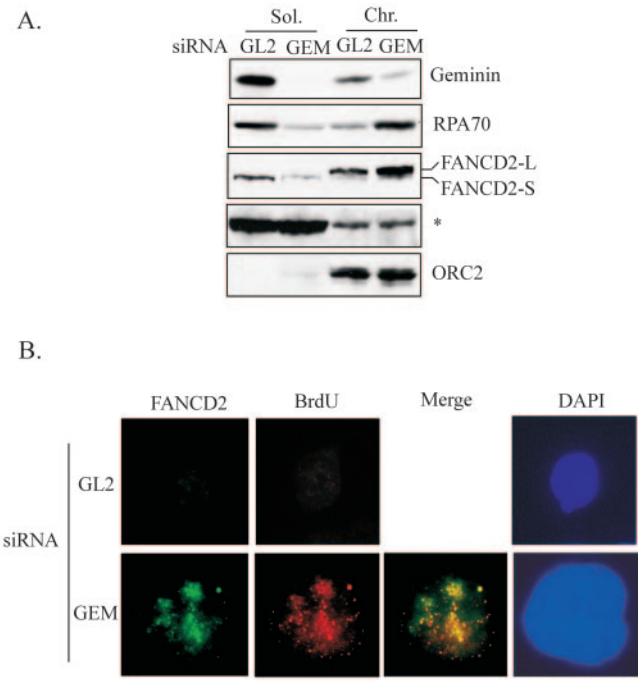


FIG. 2. FA pathway is activated in geminin-depleted cells. (A) Association of RPA70 and FANCD2 with chromatin. HCT116 cells were transfected with control siRNA oligonucleotide GL2 or geminin siRNA oligonucleotide GEM as described in the legend for Fig. 1A. Chromatin fractionation was performed as described by Zou et al. (51). The resultant fractions were resolved on sodium dodecyl sulfate-polyacrylamide gels and immunoblotted with the indicated antibodies (FANCD2-L is monoubiquitinated, and FANCD2-S is unubiquitinated). Orc2 served as a loading control for the chromatin fractions (Chr.) *, protein cross-reacting with anti-ORC2 antibody served as a loading control for the soluble fractions (Sol.). (B) FANCD2 colocalizes with single-stranded BrdU in geminin-depleted cells. HCT116 cells treated as described in the legend for Fig. 1A were immunostained with anti-FANCD2 and anti-BrdU antibodies without denaturation.

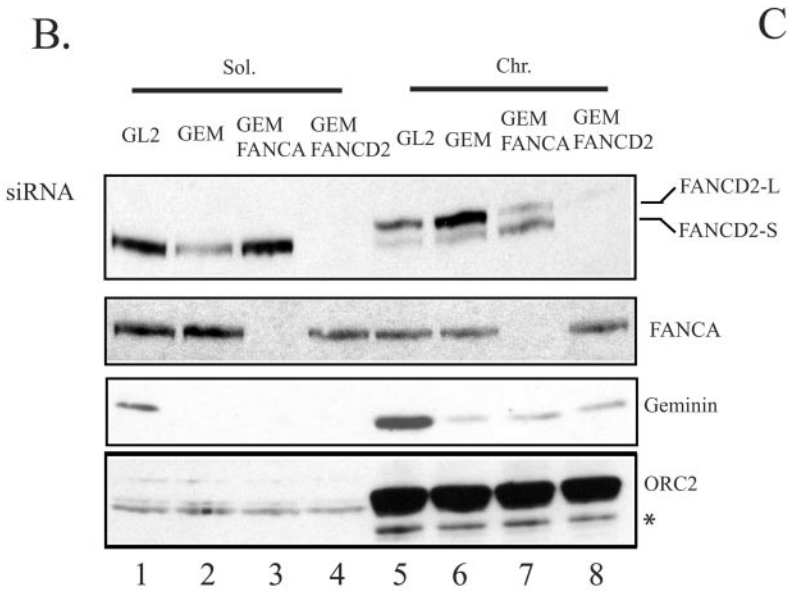
To determine the relationship of FANCD2 loading relative to the sites of rereplication, we took advantage of the fact that the sites of rereplication could be detected cytologically as sites enriched in ssDNA. FANCD2 foci were detected in geminin-depleted cells and colocalized with sites where BrdU was detected without denaturation of DNA (Fig. 2B), indicating that activated FANCD2 was recruited to sites near where ssDNA was produced by rereplication. Therefore, FANCD2 is activated and loaded on chromatin at or near sites of rereplication.

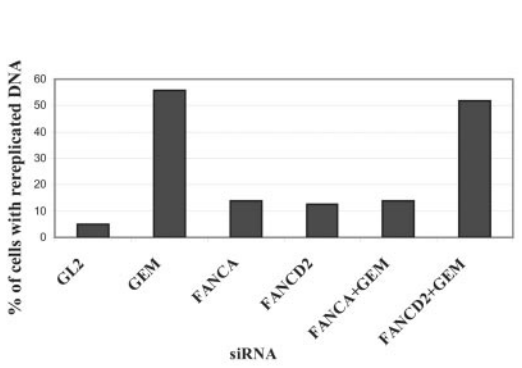
FA proteins involved in FANCD2 activation and checkpoint activation in cells undergoing rereplication. The activation of FANCD2 in geminin-depleted cells led us to test whether FA core complex proteins are involved in this activation. We first examined whether FANCA is required for the loading of FANCD2 to chromatin in geminin-depleted cells, as has been shown after other types of DNA damage (21). In cells with both FANCA and geminin silenced, less FANCD2 was monoubiquitinated and associated with chromatin (Fig. 3A and B, lane 7). In addition, there was an increase in the soluble fraction of FANCD2 (Fig. 3B, lane 3). Therefore, FANCA is upstream from FANCD2 in activation after rereplication.

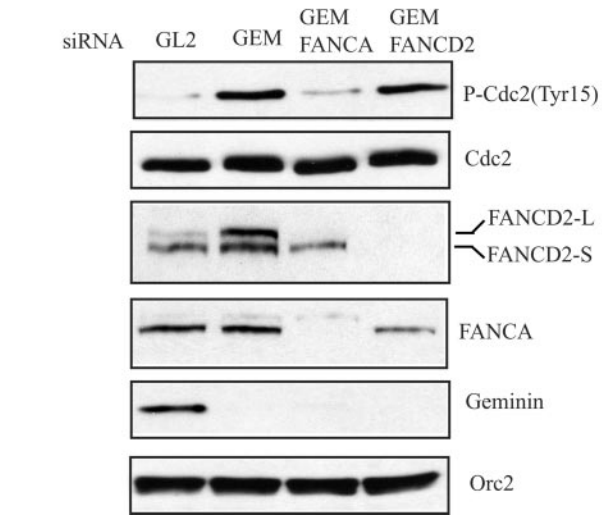
We next examined the role of the FA pathway in activating the G_2/M checkpoint. Abrogation of the G_2/M checkpoint suppresses the accumulation of rereplicated cells (50), and so an easy screen for checkpoint activation is to assay the percentage of cells in the culture with more than 4N DNA content. As shown in Fig. 3C (also see Fig. S1 and S5 in the supplemental material), silencing FANCA significantly suppressed the accumulation of rereplicated cells upon geminin depletion. Additionally, the silencing of FANCL suppressed the accumulation of rereplicated cells (data not shown). Therefore, the Fanconi core complex may be required for checkpoint activation. Surprisingly, the cosilencing of FANCD2 with geminin had no influence on the accumulation of rereplicated cells

A.

siRNA	GL2	GEM	FANCA	FANCD2	FANCA+ GEM	FANCD2+ GEM
DAY1	GL2	GL2	FANCA	FACND2	FANCA	FANCD2
DAY2	GL2+GL2	GEM+GL2	FANCA+GL2	FANCD2+GL2	FANCA+GEM	FANCD2+GEM
DAY3	GL2	GEM	GL2	GL2	GEM	GEM
DAY4	Harvest cells for analysis					







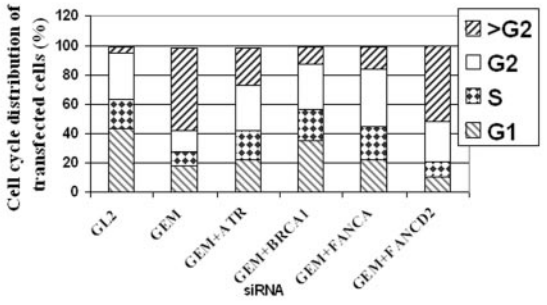


FIG. 3. FA proteins are required for rereplication and activation of the G_2/M checkpoint in geminin-depleted cells. (A) Schematic of transfection protocol. (B) HCT116 cells treated as shown in panel A were fractionated as described in the legend for Fig. 2A. The resultant fractions were resolved on sodium dodecyl sulfate-polyacrylamide gels and immunoblotted with the indicated antibodies. See the legend for Fig. 2A for additional explanations. (C) HCT116 cells treated as shown in panel A were harvested for flow cytometry analysis. Cells with more than G_2/M DNA content are regarded as cells with rereplicated DNA. Primary data are shown in Fig. S1 in the supplemental material. (D) HCT116 cells treated as shown in panel A were immunoblotted for the indicated proteins. (E) Cell cycle distribution of transfected cells. HCT116 cells treated as shown in panel A or Fig. 5A were stained with propidium iodide and subjected to FACS analysis. >G2, cells with more than G_2/M DNA content (Cellquest).

(Fig. 3C) (also see Fig. S1 and S5 in the supplemental material). Therefore, FANCD2, unlike FANCA, was not required for the survival of rereplicated cells after the depletion of geminin.

To confirm the observations made above, we measured the activation of the G_2/M checkpoint pathway biochemically. Consistent with our previous results (50), deletion of geminin elevated inhibitory phosphorylation of Cdc2 at Tyr15 (Fig. 3D) (also see Figure S2 in the supplemental material). The silencing of FANCA in geminin-depleted cells suppressed the inhibitory phosphorylation of Cdc2, while the silencing of FANCD2 had no effect on this phosphorylation. Thus, FANCA, but not FANCD2, is required for G_2/M checkpoint activation after rereplication, in agreement with the accumulation of rereplicated cells after FANCA, but not FANCD2, depletion.

When the G_2/M checkpoint is activated, fewer cells are expected to be in active mitosis. We therefore verified the observations made above by two-color FACS analysis for phospho-histone H3 staining and DNA content (see Fig. S3 in the supplemental material). Consistent with the conclusions described above, geminin depletion decreased the phospho-histone H3-positive 4N cells from 2.73% to 0.88%. Entry into mitosis was restored by codepletion of FANCA (2.03%) but not of FANCD2 (0.38%). A minor (background) percentage of cells had more than 4N DNA content and phosphoH3 staining in all preparations.

Since FANCD2 is monoubiquitinated during S phase in the cell cycle (36), a trivial explanation of the observations discussed above would be that the increase or decrease of FANCD2 ubiquitination merely reflected the percentage of cells in active DNA replication. We therefore measured the distribution of cells in the cell cycle after the various manipulations described above. The percentage of cells in S phase was decreased in geminin-depleted cells compared to the percentage in GL2-treated cells (Fig. 3E), and so an increase in replicating cells cannot explain the ubiquitination of FANCD2. In addition, codepletion of FANCA with geminin increased the S-phase population but decreased FANCD2 ubiquitination. Therefore, the changes in FANCD2 ubiquitination cannot be explained by changes in the population of cells in active DNA replication.

Together, these results suggest that rereplication in human cancer cells leads to monoubiquitination of FANCD2 through FANCA activation and also that G_2/M checkpoint activation is dependent on some other target of FANCA.

FANCD2 is required for the accumulation of RAD51 foci in cells undergoing rereplication. Although FANCD2 is activated, FANCD2 is not required for G_2/M checkpoint activation in geminin-depleted cells. We wondered, therefore, whether FANCD2 activation is required for activating DNA repair pathways in rereplicating cells. Rereplication by loss of geminin led to formation of Rad51 foci (Fig. 4A). The cosilencing of FANCD2 did not suppress the formation of giant rereplicated nuclei in geminin-depleted cells (Fig. 4A), consistent with the fact that FANCD2 is not required for the accumulation of rereplicated cells (Fig. 3C). Loss of FANCD2, however, dramatically suppressed the accumulation of Rad51 foci in the cells with the giant nuclei (Fig. 4A and B), suggesting that active FANCD2 is involved in recruiting Rad51 to sites of rereplication, presumably for DNA damage repair.

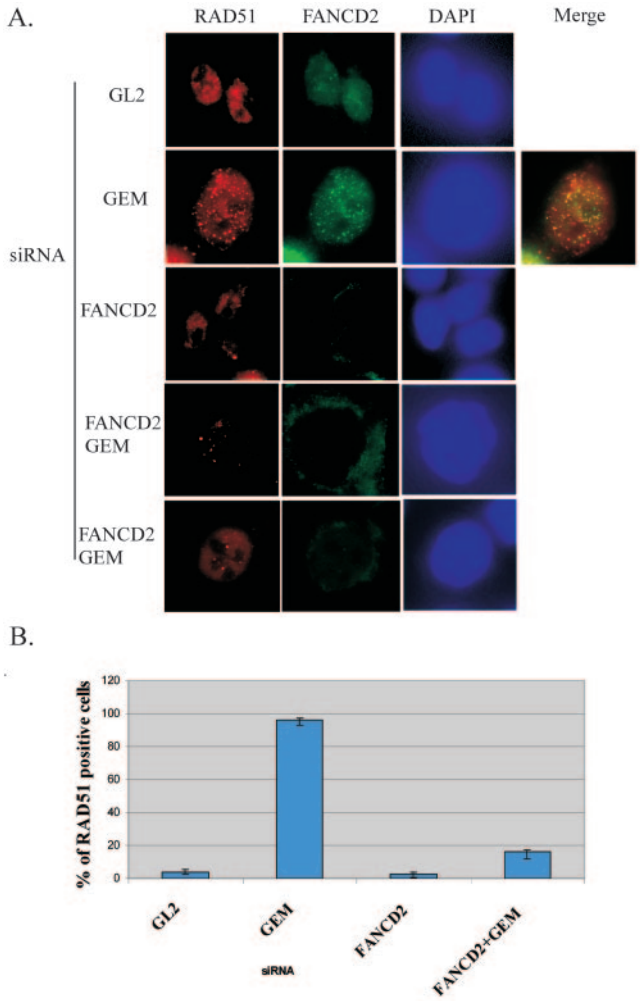


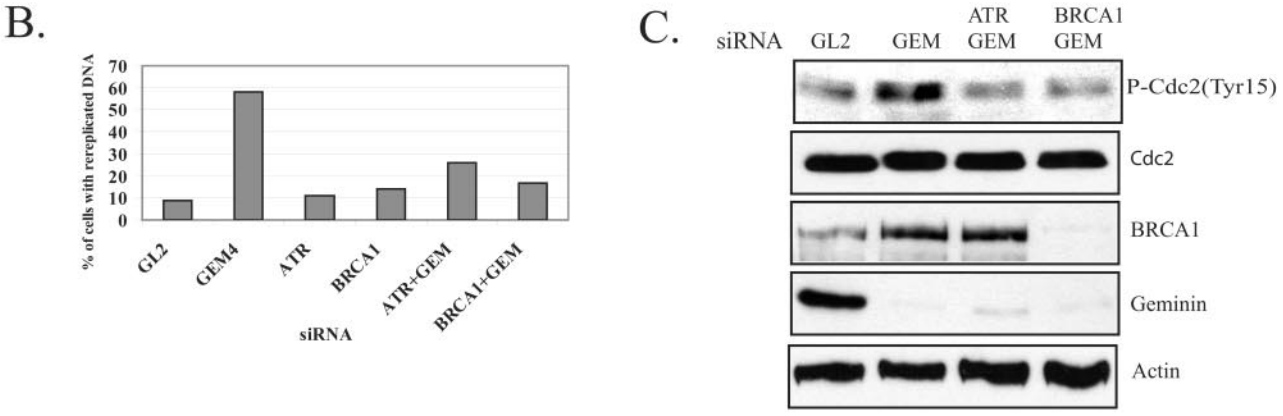
FIG. 4. FANCD2 is required for the accumulation of Rad51 foci in cells undergoing rereplication. (A) HCT116 cells transfected as shown in Fig. 3A were coimmunostained with anti-Rad51 and anti-FANCD2 antibodies. (B) Rad51 focus-positive cells after different siRNA transfections. Cells containing more than five Rad51 foci in the nucleus were scored as focus-positive cells. For GEM or GEM plus FANCD2 siRNA-transfected cells, only cells with giant nuclei were counted. At least 100 cells were examined and the percentages of positive cells calculated from two independent experiments.

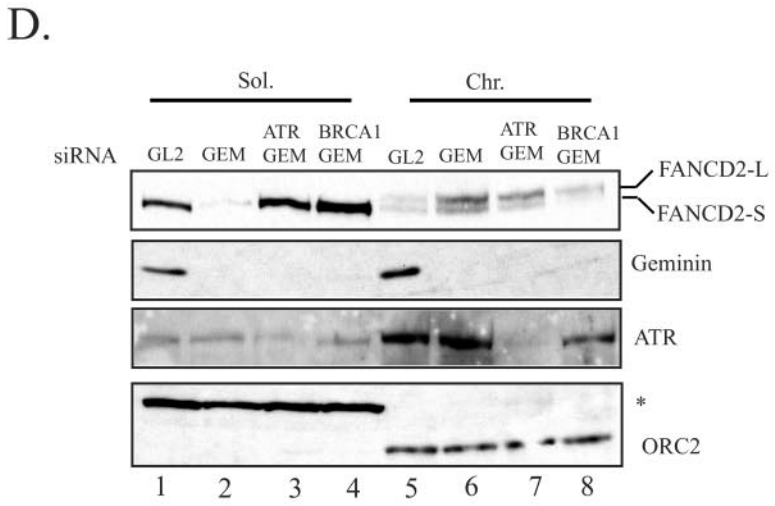
ATR and BRCA1 are required for FA pathway activation in cells undergoing rereplication. The involvement of the FA proteins raised the possibility that proteins known to be upstream from FA are also involved in the cell's response to rereplication. ATR and RPA are required for efficient activation of the FANCA pathway in response to DNA damage (1). As shown in Fig. 5B (also see Fig. S1 in the supplemental material), the silencing of ATR suppressed the appearance of nuclei with rereplicated DNA. The silencing of ATR in geminin-depleted cells also suppressed the monoubiquitination of FANCD2 (Fig. 5D, lane 7) and increased the amount of FANCD2 in the soluble fraction (Fig. 5D, lane 3). Therefore, ATR is required upstream of the FA complex to sense rereplication.

BRCA1 is also involved in the activation of the FA pathway after DNA damage. Consistent with this, we found that the

A.

siRNA	GL2	GEM	ATR	BRCA1	ATR+ GEM	BRCA1+ GEM
DAY1	GL2	GL2	ATR	BRCA1	ATR	BRCA1
DAY2	GL2+GL2	GEM+GL2	ATR+GL2	BRCA1+GL2	ATR+GEM	BRCA1+GEM
DAY3	GL2	GEM	GL2	GL2	GEM	GEM
DAY4	Harvest cells for analysis					





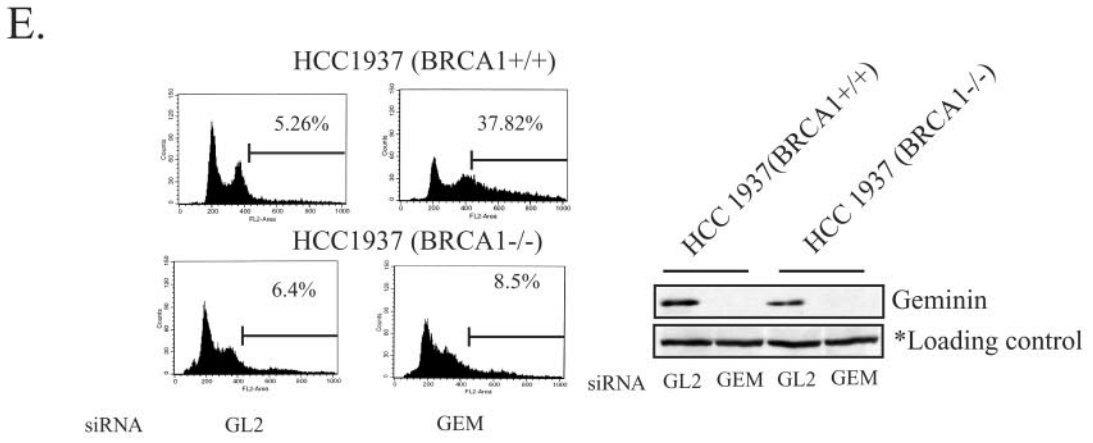


FIG. 5. ATR and BRCA1 are required for FA protein-mediated G_2/M checkpoint activation. (A) Schematic of transfection protocol. (B) HCT116 cells treated as shown in panel A were harvested for flow cytometry analysis. Cells with more than G_2/M DNA content are regarded

accumulation of cells with more than 4N DNA content was suppressed in BRCA1$^-$ cells (HCC1937) but not in BRCA1$^+$ cells (Fig. 5E). To confirm this, we used siRNA to silence BRCA1 together with geminin in HCT116 cells. Silencing BRCA1 also decreased the appearance of rereplicated nuclei (Fig. 5B) (also see Fig. S1 in the supplemental material), suppressed monoubiquitination of FANCD2 (Fig. 5D, lane 8), and released FANCD2 from the chromatin fraction to the soluble fraction in geminin-depleted cells (Fig. 5D, lane 4). Thus, BRCA1 is also required for the activation of the FA pathway in geminin-depleted cells.

Again we measured the percentage of cells in active DNA replication after ATR or BRCA1 depletion (along with geminin) to assess whether the decrease in FANCD2 activation could be accounted for by a decrease in S-phase population (Fig. 3E). The increase in S-phase population under both of these conditions ruled out this explanation.

If ATR or BRCA1 is upstream from the FA proteins, it is expected to be required for G_2/M checkpoint activation in geminin-silenced cells. In agreement with this, the cosilencing of either ATR or BRCA1 with geminin decreased the inhibitory phosphorylation of Cdc2 at Tyr15 (Fig. 5C). Thus, ATR and BRCA1 are required to activate the G_2/M checkpoint in cells with rereplicated DNA, most likely through the Fanconi core complex but not through FANCD2.

Premitotic arrest is required for rereplication caused by loss of geminin. Until now, there has been a perfect concordance between failure to activate the G_2/M checkpoint and failure to accumulate cells with rereplicated DNA, leading to the hypothesis that the G_2 arrest by the ATR-, BRCA1-, and FANCA-mediated G_2 checkpoint is required for cells to accumulate with rereplicated DNA. An alternate hypothesis, however, is that ATR, BRCA1, and FANCA are required for the stabilization of replication forks and for the actual rereplication process. We therefore tested whether a premitotic arrest created by silencing cyclin A would substitute for G_2/M checkpoint activation, as far as the accumulation of cells with rereplicated DNA was concerned. As shown in Fig. 6B, the silencing of cyclin A rescues the appearance of cells with overreplicated DNA even in the absence of ATR or BRCA1, indicating that cell cycle arrest before mitosis is essential for detecting this phenotype. In cyclin A, geminin, and ATR triple-silenced cells, Cdc2 phosphorylated at Tyr15 is decreased (Fig. 6C), indicating that the checkpoint has indeed been inactivated. Thus, introducing a G_2-phase arrest in cells by reduction of cyclin A obviates the need for the G_2/M checkpoint, supporting our notion that the accumulation of cells with rereplicated DNA is dependent on a premitotic arrest. Reduction of cyclin A also restores the appearance of rereplicated cells when FANCA is codepleted with geminin (Fig. 6E).

Restoration of the rereplicated phenotype by cyclin A depletion indicates that ATR, BRCA1, and FANCA are not required for the actual rereplication process but are required specifically for allowing the accumulation of cells with the rereplicated DNA.

Depletion of geminin induces apoptosis in cells when ATR, BRCA1, FANCA, or FANCD2 is downregulated. PARP-1 is a zinc finger nuclear protein that is cleaved by active caspase-3 and caspase-7 to 24- and 85-kDa fragments during apoptosis (8, 39). PARPp24 and PARPp85 cleavage fragments were not detected in geminin-depleted cells when the checkpoint pathway was intact (Fig. 7A). However, depletion of geminin in cells with downregulated ATR, BRCA1, or FANCA induced the generation of 24- and 85-kDa PARP-1 cleavage products (Fig. 7A). Thus, the loss of ATR, BRCA1, or FANCA makes cells with rereplicated DNA vulnerable to apoptosis. We therefore examined cell viability by an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay with cells where geminin and ATR, geminin and BRCA1, or geminin and FANCA are codepleted. As shown in Fig. 7B, the silencing of geminin in ATR-, BRCA1-, or FANCA-downregulated cells decreased the cell viability. The Fanconi core complex is required both for activating the G_2/M checkpoint and for activating DNA repair pathways through monoubiquitination of FANCD2, so either of these activities may be important for preventing apoptosis upon rereplication.

Since the cosilencing of cyclin A facilitated the persistence of cells with rereplicated DNA, we initially thought that failure to activate the G_2/M checkpoint is the primary reason why rereplicated cells with the loss of ATR, BRCA1, or FANCA undergo apoptosis. The cosilencing of FANCD2 and geminin, however, revealed that although FANCD2 was not required for the activation of the G_2/M checkpoint or the accumulation of rereplicated nuclei, it too was required to protect cells from apoptosis after rereplication (Fig. 7A). This suggests that activation of the DNA repair pathways by monoubiquitinated FANCD2 is the critical function required for prevention of apoptosis in rereplicated cells and is independent of any role of the Fanconi core complex in the activation of the G_2/M checkpoint. Given the evidence that FANCD2 is required for assembly of RAD51 foci in geminin-depleted cells (Fig. 4) and that RAD51 focus formation specifically protects cells from undergoing apoptosis after DNA damage (30), it is likely that the apoptosis induced by loss of FANCD2 in rereplicated cells is due to the loss of functional nuclear RAD51 structure.

DISCUSSION

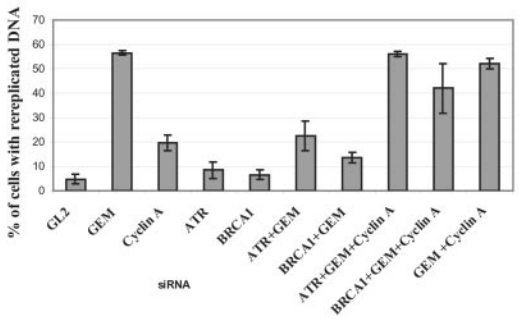
The loss of geminin in human cells causes rereplication, which activates a G_2/M checkpoint (22, 50). In this paper, we demonstrate that ATR, BRCA1, and the Fanconi core complex are required for activating the G_2/M checkpoint in response to this error of DNA replication. Abrogation of the FA pathway or of ATR or BRCA1 leads to apoptosis in geminin-

as cells with rereplicated DNA. (C) HCT116 cells treated as shown in panel A were immunoblotted for the indicated proteins. (D) HCT116 cells were treated as shown in panel A. The resultant fractions were resolved on sodium dodecyl sulfate-polyacrylamide gels and immunoblotted with the indicated antibodies. See the legend for Fig. 2A for additional explanations. (E) HCC1937 (BRCA1$^{+/+}$) and HCC1937 (BRCA1$^{-/-}$) cells were transfected with control oligonucleotide GL2 or GEM twice and harvested 72 h after the first transfection for flow cytometry analysis (left panel) or immunoblotting with the indicated antibodies (right panel). *, protein cross-reacting with antigeminin antibody served as a loading control. FL2, filter that detects fluorescence with emission range at 595 ± 20 nm.

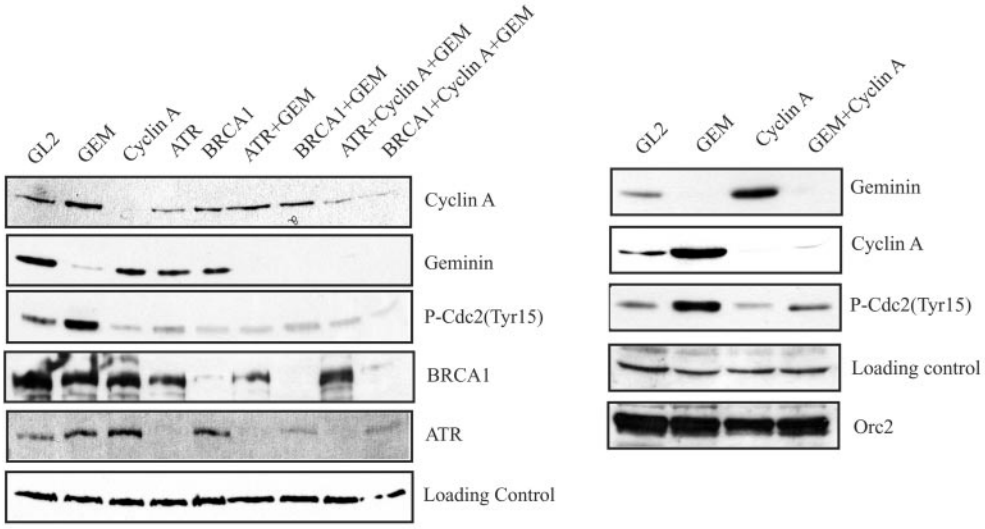
A.

siRNA	GL2	GEM	Cyclin A	ATR	BRCA1	ATR+ GEM	BRCA1 +GEM	ATR+ Cyclin A +GEM	BRCA1 +Cyclin A +GEM	Cyclin A +GEM
Day1	Gl2+ GL2	GL2+ GL2	CyclinA+ GL2	ATR+ GL2	BRCA1+ GL2	ATR+ GL2	BRCA1+ GL2	CyclinA+ ATR	CyclinA+ BRCA1	Cyclin A+ GL2
Day2	GL2+ GL2+ GL2	GEM+ GL2+ GL2	CyclinA+ GL2+ GL2	ATR+ GL2+ GL2	BRCA1+ GL2+ GL2	ATR+ GL2+ GEM	BRCA1+ GL2+ GEM	CyclinA+ ATR+ GEM	CyclinA+ BRCA1+ GEM	Cyclin A+ GL2+ GEM
Day3	GL2	GEM	GL2	GL2	GL2	GEM	GEM	GEM	GEM	GEM
Day4	Harvest cells for analysis									

B.



C.



D.

siRNA	GL2	GEM	Cyclin A	FANCA	FANCD2	FANCA +GEM	FANCD2 +GEM	FANCA+ Cyclin A +GEM	FANCD2 +Cyclin A +GEM
Day1	Gl2+ GL2	GL2+ GL2	CyclinA+ GL2	FANCA+ GL2	FANCD2+ GL2	FANCA+ GL2	FANCD2+ GL2	CyclinA+ FANCA	CyclinA+ FANCD2
Day2	GL2+ GL2+ GL2	GEM+ GL2+ GL2	CyclinA+ GL2+ GL2	FANCA+ GL2+ GL2	FANCD2+ GL2+ GL2	FANCA+ GL2+ GEM	FANCD2+ GL2+ GEM	CyclinA+ FANCA+ GEM	CyclinA+ FANCD2+ GEM
Day3	GL2	GEM	GL2	GL2	GL2	GEM	GEM	GEM	GEM
Day4	Harvest cells for analysis								

E.

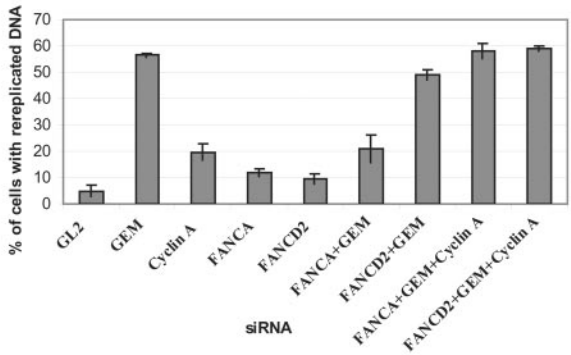


FIG. 6. Premitotic arrest bypasses the requirement of a checkpoint pathway for cells to accumulate rereplicated DNA. (A) Schematic of transfection protocol. (B) Silencing cyclin A rescues the accumulation of cells with overreplicated DNA in geminin and ATR or geminin and BRCA1 cosilenced cells. HCT116 cells treated as shown in panel A were harvested for flow cytometry analysis. (C) HCT116 cells treated as shown in panel A were immunoblotted with the indicated antibodies. (D) Schematic of transfection protocol. (E) Silencing cyclin A rescues the accumulation of cells with overreplicated DNA in geminin and FANCA cosilenced cells. HCT116 cells treated as shown in panel D were harvested for flow cytometry analysis.

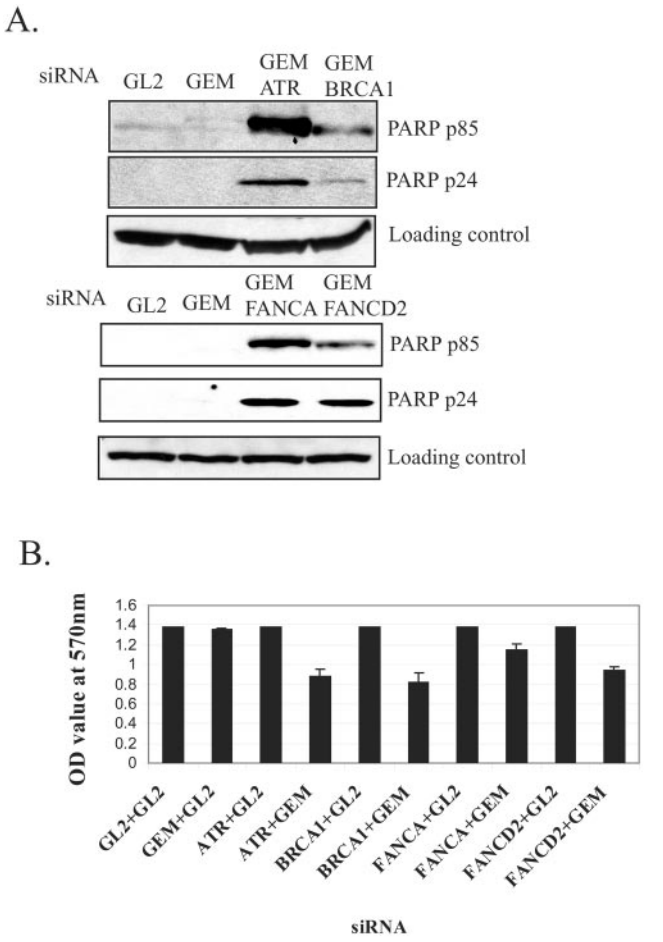


FIG. 7. Downregulation of geminin in ATR-, BRCA1-, FANCA-, or FANCD2-depleted cells causes apoptosis. (A) HCT116 cells were codepleted of geminin and ATR (Fig. 5A), BRCA1 (Fig. 5A), FANCA (Fig. 3A), or FANCD2 (Fig. 3A) and harvested 72 h after the first transfection for immunoblotting using the indicated antibodies. (B) HCT116 cells were treated as shown in Fig. 3A or 5A, and cell viability was measured by an MTT cell growth assay kit (Promega) according to the manufacturer's instructions. OD, optical density.

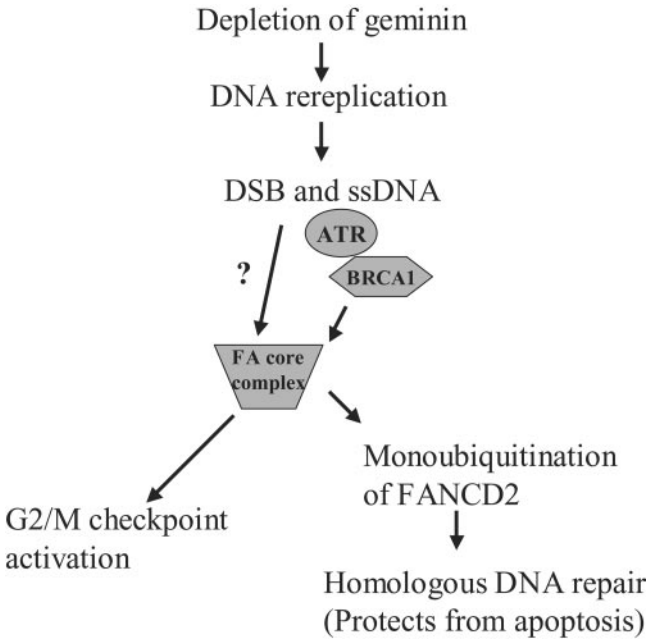


FIG. 8. Rereplication-induced activation of the FA pathway is required for activation of the G_2/M checkpoint and DNA damage repair. Rereplication by loss of geminin generates double-stranded breaks (DSB) and ssDNA. The ssDNA is sensed through ATR and BRCA1, resulting in activation of the FA pathway. The pathway bifurcates at the Fanconi core complex to activate the G_2/M checkpoint and to monoubiquitinate FANCD2. The DNA damage repair initiated by active FANCD2 appears essential to prevent apoptosis and short-term loss of viability.

depleted cells. We also found that an artificial premitotic arrest allows cells to accumulate with rereplicated DNA even with an inactive G_2/M checkpoint, suggesting that the checkpoint is not required for rereplication per se but for the accumulation of cells with sufficient rereplication to produce a detectable phenotype.

The increase in the number of DNA replication forks in rereplicating nuclei might account for the excess of single-stranded DNA in rereplicating cells, as seen after increased initiation of DNA replication due to increased Cdk activity (33). Additionally, the uncoupling of MCM helicase and DNA polymerase activities by an inhibitor of DNA polymerase alpha, aphidicolin, induces ssDNA in *Xenopus* egg extract (4). If the abnormal loading of the replicative helicase in the absence of geminin leads to helicase activity in the absence of polymerase, then such uncoupling could also generate excess ssDNA. Alternatively, rereplication might lead to fork collapse. If collapsed forks are processed as double-stranded breaks, then processing of the double-stranded breaks by the Mre11 nuclease might lead to the formation of extensive single-stranded DNA (14). It is also possible that single-strand stretches in replicating chromosomes might lead to double-stranded breaks (15). Thus, there are multiple possible pathways by which double-stranded breaks and single-stranded DNA might be created in rereplicating cells.

FANCC, a component of the FA core complex, is required for maintaining the G_2/M checkpoint in mouse embryo fibroblasts in response to ionizing radiation (10). Here we find the role of FANCA, another component of the FA core complex, in activating the G_2/M checkpoint in response to rereplication. The FA core complex associates with chromatin during S phase, indicating a possible role of the complex in monitoring the normal progression of replication forks (45). The FA core complex interacts with the BLM helicases, which are involved in the direct resolution of stalled replication forks (13). Therefore, it is not surprising that the FA core complex is activated in response to rereplication and helps in transducing the signal for G_2/M checkpoint activation (Fig. 8).

Unlike FANCA, FANCD2 appears dispensable for activating the G_2/M checkpoint. This result is consistent with the observation by Taniguchi et al. that FANCD2 mutant cells show a defect in the intra-S-phase checkpoint but not in the G_2/M checkpoint (37). However, another study recently showed that the G_2/M checkpoint is induced but cannot be maintained in FANCD2-deficient FA-D2 fibroblasts treated with ionizing radiation (10). Of course, the mode of DNA

damage (ionizing radiation versus rereplication) might influence the exact pathway of checkpoint activation and partially explain the difference. Either way, FANCD2 is downstream from the FA core complex and is less essential for G_2/M checkpoint activation by rereplication. Since FANCA is essential for G_2/M checkpoint activation but FANCD2 is not, we suggest that there must be other targets of the FA core complex that link FA to G_2/M checkpoint activation (Fig. 8).

Instead, FANCD2 may be involved in the damage repair process, which is in turn important for preventing apoptosis in the rereplicated cells. We show that active FANCD2 is targeted to the sites of ssDNA and is required for the accumulation of DNA-repair foci containing RAD51 in cells with rereplicated DNA (Fig. 8). Given the evidence that monoubiquitinated FANCD2 is required for the loading of BRCA2 to chromatin and that it colocalizes with BRCA2 (44), as well as the evidence that BRCA2 stabilizes stalled DNA replication forks (17), FANCD2 may be involved in recruiting BRCA2 to chromatin to stabilize forks on rereplicated DNA.

We attempted to demonstrate the requirement of the Fanconi core complex in G_2/M checkpoint activation by using fibroblast lines carrying mutations in FANCA or FANCD2. However, even in the FANCA-corrected control cell lines, decrease of geminin failed to produce any rereplication (see Fig. S4 in the supplemental material). Several cell lines (e.g., HeLa and A549) respond to geminin depletion with a G_1 block and do not exhibit any rereplication. The reason for this is unclear, but since the fibroblasts we tested appear to belong to the same category, we will have to await methods to induce rereplication in these cell lines before we can use FA mutant cell lines to confirm the findings reported here.

Both ATR and BRCA1 are involved in the FA pathway following exposure to ionizing radiation or cross-linking agents. ATR is required for the monoubiquitination of FANCD2 (1, 11). BRCA1 colocalizes with activated monoubiquitinated FANCD2 and is required for FANCD2 monoubiquitination and focus formation (1, 11). Although another study indicates that BRCA1 is not essential for the monoubiquitination of FANCD2 in response to mitomycin C or ionizing radiation (42), our data show that the activation of the FA core complex and of FANCD2 by errors of DNA replication uses both ATR and BRCA1.

Given the evidence that ATR is present at the replication fork and stabilizes replication forks during S phase (18, 38), one possibility is that ATR is required to stabilize replication forks during rereplication. An alternative possibility, supported by the rescue of rereplication by an artificial premitotic arrest, is that the ATR-mediated G_2/M checkpoint causes a G_2-phase arrest that is required for cells to accumulate rereplication. The intra-S-phase checkpoint is not activated in response to rereplication (50), and consistent with this, the deletion of FANCD2, which is required for the intra-S-phase checkpoint, has no effect on rereplication.

Silencing geminin in ATR-, BRCA1-, FANCA-, or FANCD2-depleted cells causes apoptosis. Since many cancer cells have mutated checkpoint proteins, including BRCA1 and FANCA, these results are significant because they suggest that the induction of rereplication (perhaps by targeting geminin) would be particularly toxic to cancers that have these mutations.

BRCA1 and FA are usually activated by DNA damage induced by exogenous signals, like radiation, hydroxyurea, or mitomycin C. This report shows that disorders of endogenous processes, like DNA replication, can also activate BRCA1 or FA pathways. An interesting possibility is that the low-level activation of FANCD2 seen with all cultures synchronized in S phase is actually in response to low levels of rereplication that are not detectable by FACS analysis. Such subdetectable amounts of rereplication may be sufficient to activate the FA and G_2/M checkpoint pathways in the few affected cells. Resolution of the rereplicated segments by HDR may relieve the checkpoint and allow the cell cycle to proceed. Alternatively, a small percentage of cells might be irreversibly arrested in G_2/M and eventually lose viability and be lost from the culture.

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