DNA replication is tightly controlled to ensure accurate chromosome duplication and segregation in each cell cycle. Inactivation of Geminin, an inhibitor of origin licensing, leads to re-replication in human tumor cells within the same cell cycle and triggers a G2/M checkpoint. We find that the primary pathway to signal that re-replication has been detected is the ATR kinase and the Rad9-Rad1-Hus1 (9-1-1) clamp complex together with Rad17-RFC clamp loader. ATM kinase and the Mre11-Rad50-Nbs1 complex do not appear to play significant roles in the checkpoint. Chk1 activation occurs at early stages, whereas Chk2 activation occurs much later. Overall we conclude that ATR/Chk1 pathway is activated at an early time point after the loss of Geminin and contributes to checkpoint arrest essential for the accumulation of re-replicated cells, whereas activation of the ATM/Chk2 pathway is a by-product of DNA re-replication at a later period.

Duplication of chromosomal DNA is a key event in the cell cycle. Cells have developed multiple mechanisms to ensure accurate duplication of genetic materials. In eukaryotes, DNA replication initiates at areas known as replication origins, which are recognized by a six-subunit complex called origin recognition complex (1–3). Cdc6 and Cdt1 are then independently recruited at recognition complex-associated origins either in the late mitosis or early G1 phase (1, 3, 4). The MCM2–7 complex is subsequently recruited by Cdc6 and Cdt1 to the replication origins to initiate DNA replication (5, 6).

Geminin, an inhibitor of DNA replication initiation (7–9), can physically interact with and inhibit the activity of Cdt1, ensuring firing of origins once per cell cycle. Geminin depletion by short interfering RNA (siRNA)2 can induce DNA re-replication and activate G2/M checkpoint in both human and Droso phila cells (10–12). It was shown previously that Geminin depletion causes the activation of DNA damage protein kinases ATR (ataxia telangiectasia and Rad-3-related), Chk1, and Chk2 and induces G2/M cell cycle arrest in human colon cancer cells (12–14). Abrogation of this checkpoint by ATR depletion leads to apoptosis (12, 13). Thus the accumulation of re-replicated cells after Geminin depletion was critically dependent on G2/M checkpoint activation.

Because re-replication is expected to lead to gene amplification, we consider it important to understand the re-replication-induced checkpoint pathway. Although not much is known about the sensor proteins involved in the re-replication induced checkpoint pathway, a lot is known about the DNA damage pathways. Studies in yeast and mammals have implied that there are several important proteins working as DNA damage sensors in checkpoint pathways.

Rad9, Rad1, Hus1, and Rad17 proteins are required for the checkpoint activation (15–17). Rad9, Rad1, and Hus1 form a heterotrimeric protein complex 9-1-1 and structurally resemble a proliferating cell nuclear antigen-like sliding clamp in both yeast and human cells (18–20). However, Rad17 is a checkpoint protein, which shares homology with replication factor C 1 (RFC1) in structure and associates with four small RFC subunits (RFC2–5) to form a complex related to the proliferating cell nuclear antigen clamp loader (16, 19, 21). Rad17 binds to chromatin before DNA damage and recruits Rad9-Rad1-Hus1 complex in response to DNA damage, probably acting as a clamp loader to load the 9-1-1 complex onto the damage sites. Once the 9-1-1 complex is bound to chromatin, it facilitates the phosphorylation of the substrates (like Chk1 kinase) by ATR (22). In cells lacking ATR, the Chk1 phosphorylation is blocked (23). Furthermore, in cells with reduced Rad17 or lacking Hus1, this phosphorylation is also inhibited (22, 24).

The Rad17-RFC and 9-1-1 complexes are involved in ATR/Chk1 pathway, whereas Mre11-Rad50-Nbs1 (MRN) complex is implicated to play an important role in ATM/Chk2 pathway (16, 25). MRN complex was suggested to work in both DNA damage checkpoint and repair pathways (26, 27). Upon the induction of double-stranded breaks, MRN complex gets recruited to the proximity of DNA damage sites independent of ATM (28) and is involved in the initial process of double-stranded breaks because of Mre11 nuclease activity (29). Furthermore, MRN complex is suggested to enhance ATM accumulation at damage sites and facilitate ATM activation (16, 30–32), which is in turn necessary for the activation Chk2 kinase (33–35). Additionally, in vitro studies using either Xenopus extracts or purified human proteins have shown that ATM activation requires MRN complex for its DNA-tethering and ATM-binding ability (36, 37). Collectively, MRN complex is suggested to function as a DNA damage sensor and amplifier for the ATM/Chk2 signaling pathway.

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2 The abbreviations used are: siRNA, short interfering RNA; ATR, ataxia telangiectasia and Rad-3-related; ATM, ataxia telangiectasia mutated; RFC, replication factor C; MRN, Mre11-Rad50-Nbs1; ssDNA, single-stranded DNA; FACS, fluorescence-activated cell sorter.
ATR Pathway and Re-replication

Re-replication produced both single-stranded DNA and double-stranded DNA breaks (10, 13), and both ATR/Chk1 and ATM/Chk2 pathways are activated. To measure the relative importance of the two pathways, we therefore decided to check whether the cofactors for ATR or ATM activation in the DNA damage pathways are equally important for the checkpoint activation because of re-replication (Fig. 1).

EXPERIMENTAL PROCEDURES

Cell Lines and Drugs—Human colorectal cancer cell line HCT116 (p53+/−) was grown in 10% fetal bovine serum and 1% penicillin-streptomycin in McCoy’s 5A modified medium (Cellgro). The concentration of daunorubicin (Sigma) used was 0.05 μM.

siRNA—Short interfering (siRNA) oligonucleotides (Invitrogen) were made to follow target sequences (sense): Geminin, UGCCAACUCUGGAAUCAAA (12); Rad9, GUCUUUCCUGUCUGUCUUC; Rad17, CAGACUGGGUGACCAACUC; Mre11, ACAGGAGAAGAGUAACACU; ATM, GCGCCU-GAUUCGAGAUC; and control oligonucleotide (GL2), AACGUACCGGAAACUUCGGA. Transfections were performed with 100 nM siRNA oligonucleotide duplexes with Lipofectamine RNAiMAX (Invitrogen) to 1 × 10^6 HCT116 cells per 6-cm dish according to the instructions of the manufacturer.

Antibodies and Immunoblotting—Rabbit anti-Geminin was raised as described earlier (9). Rabbit anti-Rad9, rabbit anti-Rad17, rabbit anti-ATM, mouse anti-CDC2, mouse anti-FANCD2 (Santa Cruz Biotechnology); rabbit anti-Mre11 (Novus Biologicals); rabbit anti-phospho-Chk1 (Ser-317), rabbit anti-phospho-Chk2 (Thr-68), rabbit anti-phospho-CDC2 (Tyr-15) (Cell Signaling Technology); mouse anti-actin, mouse anti-Chk1, and mouse anti-Chk2 (Sigma) were used for Western blotting. Cells were lysed in lysis buffer containing 0.1% Nonidet P-40, 50 mM Tris-HCl 7.4, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4, and protease inhibitor mixture (Sigma). Equal amounts of cell lysates were resolved on SDS-PAGE, and then proteins were transferred and blotted with indicated antibodies.

FACS Analysis—Cells were collected by trypsinization and fixed with 70% ethanol overnight in −80°C. Cells were centrifuged and stained with 500 μl of propidium iodide solution (0.05% Nonidet P-40, 50 ng of propidium iodide/ml, and 10 μg of RNaseA/ml) after fixation. The labeled cells were analyzed on a Becton Dickinson flow cytometer with CellQuest Pro software.

RESULTS

Rad9 and Rad17 Proteins Are Required for G2/M Checkpoint Activation after Geminin Depletion—ATR was previously shown by our lab to be crucial in arresting cells in response to re-replication (13). Because 9-1-1 (Rad9-Rad1-Hus1) and Rad17-RFC complexes are known to be important for regulating ATR activity, we chose to address whether one representative member of each complex is involved in the G2/M checkpoint induced by Geminin depletion.

RNA interference was performed to silence the expression of either Rad9 or Rad17 protein alone or together with Geminin in HCT116 cells (Fig. 2A). As shown in Fig. 2C, Rad9 or Rad17 protein levels were significantly decreased in cells transfected with Rad9 or Rad17 siRNA but not with the control siRNA (GL2) or Geminin siRNA. In addition, Geminin protein levels were extensively reduced only in cells transfected with Geminin siRNA.

Previous results have shown that co-depletion of checkpoint protein (like ATR) and Geminin abrogates checkpoint activation and in turn decreases the accumulation of re-replicated cells because the cells enter mitosis with re-replicated chromosomes and undergo apoptosis (12, 13). Therefore, an easy way to verify the importance of a protein in the checkpoint pathway is to assay the percentage of cells accumulating with >4N DNA content by FACS analysis. Fig. 2B and Table 1 show that knockdown of Rad9 or Rad17 suppressed the accumulation of re-replicated cells after depletion of Geminin. Depletion of Rad9 or Rad17 alone does not have any effect on re-replication. Therefore, both the proteins are likely important in the G2/M checkpoint pathway. Consistent with this, biochemical analysis showed that the activation of checkpoint proteins was decreased in cells where Rad9 or Rad17 proteins were knocked down together with Geminin. Geminin depletion causes Chk1, Chk2 phosphorylation and Cdc2 phosphorylation on Tyr-15 (Fig. 2C, lanes 2 and 6). In addition, the Fanconi anemia core complex gets activated after Geminin depletion and monoubiquitinates FANCD2, activating DNA repair pathways (12–14, 38). In the absence of Rad9 or Rad17 proteins, phosphorylation of Chk1, inhibitory phosphorylation on Cdc2, and monoubiquitination of FANCD2 were decreased after Geminin depletion (Fig. 2C, lanes 4 and 8). Therefore both Rad9 and Rad17 proteins are independently required for the checkpoint activation after re-replication.

Mre11 and ATM Are Not Required for Checkpoint Pathway and Over-replication in Geminin-depleted Cells—It was shown previously that both single-stranded DNA and double-stranded DNA breaks are generated during DNA re-replication (13). Because double-stranded DNA breaks activate ATM (26), we wanted to test whether ATM is required for the checkpoint activation and accumulation of re-replicated cells. Furthermore, MRN complex is implicated to be the DNA damage sen-
sor for detecting double-stranded DNA breaks. We decided to choose Mre11 protein as a representative member of the complex to test the requirement of the complex for the checkpoint activation upon Geminin depletion.

In Fig. 3B, either Mre11 or ATM protein levels were notably reduced in cells treated with the corresponding siRNA. Surprisingly, the accumulation of re-replicated cells was not affected by depletion of either Mre11 or ATM together with Geminin protein (Fig. 3A and Table 1). Consistent with the FACS results, Chk1 activation and Cdc2 phosphorylation on Tyr-15 were not influenced by the co-depletion of Mre11 or ATM (Fig. 3B, lanes 4 and 8). Additionally, FANCD2 monoubiquitination was not changed.

Although the percentage of re-replicated cells did not change after co-depletion, Chk2 phosphorylation on Thr-68 was significantly decreased (Fig. 3B, lanes 4 and 8). This is consistent with the previous reports that ATM and MRN are required for the

TABLE 1

Percentage of re-replication after indicated siRNA

HCT116 cells were transfected with indicated siRNA duplex following the protocol described in Fig. 2A. The cells were harvested for fluorescence-activated cell sorter analysis. Cells with greater than 4N DNA content are regarded as cells undergoing re-replication. Mean ± S.E. from three experiments are shown.

| Checkpoint protein knocked down in columns 4 and 5 | GL2 RNAi | GEM RNAi | Checkpoint protein RNAi | Checkpoint protein + GEM RNAi |
|-----------------------------------------------|----------|----------|-------------------------|------------------------------|
| Rad9 knockdown                                | 5.57 ± 1.09 | 52.93 ± 2.51 | 6.65 ± 0.54             | 25.46 ± 1.97                 |
| Rad17 knockdown                               | 4.73 ± 1.68 | 49.85 ± 0.88 | 7.43 ± 0.73             | 24.14 ± 4.00                 |
| Mre11 knockdown                               | 3.94 ± 0.31 | 56.60 ± 1.25 | 3.44 ± 0.16             | 55.06 ± 1.39                 |
| ATM knockdown                                 | 3.43 ± 0.46 | 52.38 ± 2.33 | 6.03 ± 1.15             | 52.69 ± 3.44                 |

FIGURE 2. Rad9 and Rad17 proteins are required for the accumulation of re-replicated cells and G2/M checkpoint activation after Geminin depletion in HCT116 cells. A, schematic of transfection protocol. B, histogram of cells transfected with indicated siRNA duplex following the protocol described in A. The cells were harvested and stained with propidium iodide for DNA content before flow cytometry analysis. Y-axis, cell count; X-axis, propidium iodide fluorescence. The percentage of cells containing greater than 4N DNA is shown. C, G2/M checkpoint activation is suppressed without Rad9 or Rad17 in Geminin-depleted cells. HCT116 cells treated as in A were immunoblotted for the indicated proteins.

FIGURE 3. Mre11 and ATM proteins are not required for the accumulation of re-replicated cells and G2/M checkpoint activation in HCT116 cells. A, histogram of cells with indicated siRNA transfection following protocol in Fig. 2A. The percentage of cells with >4N DNA is shown. B, checkpoint activation after indicated siRNA transfection. HCT116 cells treated as in A were immunoblotted for the indicated proteins.
activation of Chk2 (33, 35, 37, 39). It also suggests that ATM and Mre11 knockdowns have sufficiently decreased the corresp
spective protein levels to deregulate their functions. Together, these data suggest that the ATM/MRN pathway is not involved to induce G2/M arrest and re-replication after Geminin depletion.

Chk1 Is Required for Chk2 Activation after Geminin Depletion

To our surprise, Chk2 phosphorylation on Thr-68, which is normally a consequence of ATM activation (16, 33–35), was similarly reduced after co-depletion of Geminin and Rad9 or Rad17 (Fig. 2C, lanes 4 and 8). This could be either a direct or an indirect result because of the decrease of ATR activity. To distinguish the two possibilities, we performed siRNA against Chk1, the downstream substrate of ATR, together with Geminin and examined Chk2 activation. If ATM/Chk2 and ATR/Chk1 pathways work in parallel, and the reduction in Chk2 activation is simply because of the cross-talk between the two pathways, Chk1 depletion will not affect Chk2 activation. Otherwise, ATM/Chk2 pathway can be probably located downstream of ATR/Chk1 pathway.

In Fig. 4, Chk1 protein level was significantly decreased in cells treated with the siRNA. After Geminin depletion, Chk2 was activated and phosphorylated on Thr-68 (Fig. 4, lane 2). However, the phosphorylation was reduced without the presence of Chk1. The results suggested Chk1 activation is probably upstream of Chk2 activation. Additionally, instead of functioning in parallel and overlapping with each other, ATM/Chk2 and ATR/Chk1 pathways might work together after Geminin depletion.

Chk1 Is Activated Earlier in Re-replication whereas Chk2 Activation Occurs at Later Stages—As shown above, the Rad9/ATR/Chk1 pathway is required for the accumulation of re-replicated cells but MRN/ATM/Chk2 is not. In addition, Chk1 activation is possibly upstream of Chk2 activation. We wondered whether Chk2 activation was truly a later event in cells undergoing re-replication.

Around 18 h after Geminin siRNA transfection, a G2/M cell cycle arrest was observed, and there was a little more re-replication in Geminin-depleted cells compared with control siRNA (GL2)-treated cells (Fig. 5A). 24 h after the knockdown, a more obvious G2/M arrest was observed in Geminin knockdown cells, and the percentage of re-replicated cells increased further. The phosphorylation of Chk1 protein at these two time points was similar (Fig. 5B). However, Chk2 protein phosphorylation was not notably seen until 30 h after the siRNA transfection, when 32.5% of re-replication was detectable in the cells in the population. In summary, Chk1 was activated around the time of G2/M cell cycle arrest and before the immense accumulation of re-replicated cells, whereas convincing Chk2 activation was
observed relatively late, much after the significant accumulation of re-replicated cells.

**DISCUSSION**

The loss of Geminin in human cancer cells causes a G$_2$/M checkpoint activation and DNA re-replication. In this paper, we demonstrate that Rad9 and Rad17 proteins are both required for the checkpoint activation and accumulation of re-replicated cells. The downstream substrate Chk1 in the same pathway is phosphorylated probably before the huge accumulation of re-replicated cells. Nevertheless neither Mre11 nor ATM is necessary in this process. The Chk2 protein, normally activated in an ATM-dependent manner (16, 33–35), is activated comparatively late in the process.

Geminin is an inhibitor of Cdt1, a pre-replicative complex component (7–9, 40). After Geminin depletion in mammalian cells, an extra round of DNA replication is initiated, and cells with greater than G$_2$ DNA content appear. Generally, the activation of ATR and Chk1 is considered as a consequence of the formation of single-stranded DNA, whereas the ATM and Chk2 activation is observed more in cells with double-stranded DNA breaks (41). The generation of single-stranded DNA in the re-replicated cells might therefore account for the induction of the G$_2$/M checkpoint mediated by ATR, Rad9-Rad1-Hus1, and Rad17-RFC. DNA re-replication continues as the cells are arrested in G$_2$/M phase. Upon further re-replication, more forks collapse, which possibly produces double-stranded DNA breaks together with single-stranded DNA. During the process, the MRN complex probably works as a sensor for the double-stranded DNA breaks and as an amplifier to activate ATM and in turn Chk2 kinase. Additionally, the Mre11 nuclease might help to process the double-stranded breaks to single-stranded DNA and contribute to the ATR-regulated pathway. The ATM/MRN/Chk2 pathway activation is therefore a by-product of the accumulated re-replication that amplifies the downstream signals (inhibition of Cdc25c and Cdc2) for checkpoint activation, but is not the major determinant for the accumulation of re-replicated cells (Fig. 6).

A primary role of ATR/Chk1 in the G$_2$/M checkpoint activation following re-replication is consistent with previous studies in *Drosophila*, *Xenopus*, and human colon cancer cells. In *Drosophila*, Chk2 inactivation does not create an immense change on Geminin deficiency-induced re-replication (11). In *Xenopus* egg extracts, depletion of Geminin causes a Chk1-dependent G$_2$ arrest (14). In human cancer cells, co-depletion of Chk2 does not produce a significant effect on the accumulation of re-replicated cells after Geminin silencing as compared with Chk1 co-depletion (12).

Despite the distinctive functions of ATR/Chk1 and ATM/Chk2 in DNA damage checkpoint, there is plenty of evidence showing cross-talk between the two pathways (27), which means that either Chk1 or Chk2 can be the substrate of both ATM and ATR proteins. We speculate that at the early time point after Geminin depletion, the ATR activation signal induced by single-stranded DNA has not reached the threshold to result in noteworthy phosphorylation of Chk2. As cells were arrested by G$_2$/M checkpoint, more DNA re-replication occurred, and the ATR activation signal was amplified and joined by the ATM activation signal to eventually activate Chk2. Thus although the ATR and ATM kinases respond to DNA damage and activate both Chk1 and Chk2, our results suggest that the lesions caused initially by re-replication are primarily the generation of small-stranded DNA leading to the preferential activation of ATR and Chk1.

Given the many reports of activation of p53 after the over-activity of Cdt1 and Cdc6 (42–44), our results also suggest a hierarchical process of checkpoint activity leading to the activation of p53. The ATR/Chk1 pathway initially causes the G$_2$/M arrest that allows re-replication to continue. At later stages of re-replication, fork collapse and replication across single-stranded nicks (on a template with unligated Okazaki fragments) create double-stranded DNA breaks that activate ATM/Chk2 and p53. Such a graduated response to re-replication would give the cell a chance to repair minor degrees of transient re-replication during the G$_2$ phase although preserving the use of p53 to induce apoptosis only when there is extensive and prolonged re-replication.

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