Human TopBP1 Participates in Cyclin E/CDK2 Activation and Preinitiation Complex Assembly during G₁/S Transition*‡§

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Human TopBP1 with eight BRCA1 C terminus domains has been mainly reported to be involved in DNA damage response pathways. Here we show that TopBP1 is also required for G₁ to S progression in a normal cell cycle. TopBP1 deficiency inhibited cells from entering S phase by up-regulating p21 and p27, resulting in down-regulation of cyclin E/CDK2. Although co-depletion of p21 and p27 with TopBP1 restored the cyclin E/CDK2 kinase activity, however, cells remained arrested at the G₁/S boundary, showing defective chromatin-loading of replication components. Based on these results, we suggest a dual role of TopBP1 necessary for the G₁/S transition: one for activating cyclin E/CDK2 kinase and the other for loading replication components onto chromatin to initiate DNA synthesis.

During successive eukaryotic cell cycles, accurate duplication of the genome is important for the transmission of intact genetic information to daughter cells. The beginning of S phase marks the initiation of chromosomal replication and is therefore strictly regulated by various and complex control systems, necessary for cells to duplicate their genome at the proper time and only once during each cell cycle.

Beginning from late M phase to early G₁ phase, the origin recognition complex binds to replication origins and recruits Cdc6 and Cdt1 (1). These two licensing factors collaborate with ORC to load MCM2–7 onto chromatin, which completes assembly of the pre-replicative complex (pre-RC). The onset of S phase in mammalian cells requires the activity of two important kinase complexes, cyclin E/CDK2 and Cdc7/Dbf4 (2–4). These kinases activate the pre-RC, facilitating Cdc45 loading at replication origins and recruitment of DNA polymerases and assembly of the preinitiation complex (pre-IC). Cyclin E/CDK2 kinase complex plays pivotal roles in G₁/S transition. It inactivates retinoblastoma protein (pRb) by phosphorylation preventing formation of pRb-chromatin remodeling complexes. As a result, E2F transcription is de-repressed and genes required for DNA replication and S phase progression are up-regulated (5, 6). Cyclin E/CDK2 is also believed to be directly implicated in initiation of chromosomal replication by activating replication components via phosphorylation, although its substrates and the activation mechanisms remain to be elucidated (7). CDK2 activity is regulated in a cell cycle-dependent manner by its regulatory partner cyclin E, whose protein level is the highest at the G₁/S boundary (8). It is also negatively regulated by CKD specific inhibitors such as p21 and p27 which bind directly to the cyclinE/CDK2 complex (9, 10).

Human TopBP1 was first identified as a protein interacting with topoisomerase IIα (11). Since TopBP1 contains eight BRCA1 C terminus domains, which are commonly found in proteins involved in DNA repair or cell cycle checkpoints, studies of TopBP1 have mainly focused on its function in the DNA damage response. TopBP1 participates in checkpoint control by responding to various DNA damages (12–14), and this function is conserved from yeasts to mammals (15–20). Additional roles of TopBP1 in transcriptional regulation and chromatin remodeling have been reported: TopBP1 physically interacts with transcriptional factors such as Miz-1 and E2F1 on their target gene promoters and negatively regulates gene transcription (21, 22). TopBP1 homologues such as Cut5/Rad4 in Schizosaccharomyces pombe, Dbp11 in Saccharomyces cerevisiae, Mus101 in Drosophila, and Xcut5/Xmus101 in Xenopus are all known to be required for DNA replication and S phase progression in a normal cell cycle as well as DNA damage response. In both fission yeast and Drosophila, their cut5/mus101 mutants show a block in DNA synthesis (19, 23). Dbp11 in budding yeast physically interacts with DNA polymerase ε (DNA pol-ε) and loaded onto replication origins together in a complex, which is required for recruitment of DNA polymerase α (24). Xenopus Xcut5, also referred to as Xmus101, is required for recruiting Cdc45 to chromatin (25, 26), which is also true of Cut5 in fission yeast (27).

In human cells, however, the role of TopBP1 in DNA replication or S phase progression has yet to be determined; only the physical interaction between TopBP1 and DNA pol-ε implies its involvement in DNA replication (28). In this report, we showed that TopBP1 depletion using small interfering RNAs (siRNAs) induced G₁ arrest via down-regulation of cyclin E/CDK2 activity, by increasing levels of p21 and p27. However,
although cyclin E/CDK2 kinase activity was restored by co-depletion of p21 and p27 with TopBP1, cells still could not progress into S phase due to a defect in pre-IC formation. Therefore our results suggest a sequential mode of TopBP1 function during G₁/S transition: one for activating cyclin E/CDK2 kinase and the other for loading replication components onto chromatin to initiate DNA synthesis.

EXPERIMENTAL PROCEDURES

Cell Culture and Fluorescence-activated Cell Sorter Analysis—U2OS and Saos-2 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Highly confluent cultures promote p27 accumulation, especially in Saos2 cells; therefore, final confluency of all cultured samples was maintained at less than 80%. Cell cycle profiles were analyzed by flow cytometry with standard propidium iodide staining methods.

siRNA Transfection—siRNA oligonucleotides (Samchully Pharm Co.) were synthesized to the following target sequences: TopBP1 #1, ACCGUCGUUACACCUCUUAG; TopBP1 #2, ACCGAGUACGCCACUCUCA; p21, CCAGC- ATGACGAGTTTCTA; p27, CGACGATCTTCTACTCAA; control siRNA, CUUACGUGAUACUUCAUGT (GL3). Cells were transfected with siRNAs at a final concentration of 240 μM using oligofectamine (Invitrogen) according to the instructions of the manufacturer.

Transfections were performed up to three times at 24-h intervals for TopBP1 depletion, while one transfection was enough for both p21 and p27 depletion, and this depleted state lasted for 72 h. Thus, for co-depletion with TopBP1, p21 and/or p27 RNAi was performed once at 24 h with the second transfection of TopBP1 siRNA, at which point both p21 and p27 levels have yet to increase in response to TopBP1 depletion.

Antibodies and Western Blot Analysis—Anti-TopBP1 polyclonal antibody was raised against the TopBP1 fragment corresponding to amino acids 1019–1167. Anti-Rb and anti-DNA pol-ε antibodies (TD Transduction Laboratories), anti-β-actin antibody (Sigma), anti-cyclin D1 antibody (NeoMarkers), anti-MCM2 (Abcam Inc.), and antibodies to phospho-Chk1 (Ser317) and phospho-Chk2 (Thr68) (Cell Signaling) were purchased from the indicated companies. All remaining antibodies were purchased from Santa Cruz Biotechnology.

Immunoprecipitation and in Vitro Kinase Assay—Cells were treated with lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 50 mM NaF, 1 mM Na₃VO₄, and protease inhibitors) for 1 h on a rotator at 4 °C. 100–400 μg of lysates was immunoprecipitated for 1.5 h with anti-cyclin E and -Cdc7 antibodies immobilized on protein G-Sepharose beads (Amersham Biosciences). Kinase assays with the immunoprecipitates were described as performed previously (29, 30). Anti-Cdc7 antibody and GST-MCM2 (carrying N-terminal 60 amino acid residues) used as a substrate for the Cdc7/Dbf4 kinase assay were kind gifts from Dr. Joon-Kyu Lee (Seoul National University).

Immunostaining—Cells grown on coverslips were extracted with 0.5% Triton X-100 in CSK buffer (supplemented with 50 mM NaF, 1 mM Na₃VO₄, and protease inhibitors) for 5 min at 4 °C, followed by 15 min of methanol fixation at −20 °C. For bromodeoxyuridine (BrdUrd) staining, cells on coverslips were incubated with 15 μM BrdUrd (Sigma) for 24 h. After fixing with 4% paraformaldehyde for 15 min at room temperature, cells were permeabilized with 0.5% Triton X-100 in phosphate-buffered saline for another 15 min. Anti-BrdUrd antibody (Amersham Biosciences) was supplemented with 1 unit/μl micrococcal nuclease (Worthington) and 20 mM CaCl₂ before use. For all immunostaining experiments, primary antibodies were detected with Cy3-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories), and coverslips were mounted in Vectashield Mounting Medium (Vector Laboratories) containing 1 μg/ml DAPI.

RESULTS

TopBP1-depleted Cells Are Arrested in G₁ Phase—To address the role of human TopBP1 during cell cycle progression, we investigated the consequences of loss of TopBP1 function using RNAi method. Six different siRNA oligonucleotides, each targeting a unique sequence of TopBP1 mRNA, were tested for TopBP1 knockdown using GL3 siRNA as a control. U2OS cells were transfected up to three times with the siRNAs at 24-h intervals and harvested at the indicated times (Fig. 1A). TopBP1 protein levels began to decrease after 48 h, and most of TopBP1 was eliminated by 72 h after the first transfection. This depletion lasted for at least 96 h (Fig. 1B). All of the TopBP1 siRNAs efficiently depleted the target protein, and #1 and #2 were used for the remaining experiments (Fig. 1B and supplemental Fig. 1A).

Cell cycle profiles of siRNA-treated cells were determined by flow cytometry (Fig. 1C). Compared with the control, TopBP1-depleted cells exhibited an increase in G₁ phase cells, while G₂/M and S phase populations were relatively decreased. We also observed a considerable increase in the number of floating cells with TopBP1 RNAi. This is consistent with the larger sub-G₁ populations present in TopBP1 siRNA-treated cells when we used both floating and attached cells for analyses, suggesting a fraction of TopBP1-deficient cells underwent apoptosis as reported previously (13, 22). Since the sub-G₁ populations in both #1 and #2 TopBP1 siRNA-treated cells were not more than 24% until 96 h, we excluded floating cells and focused on non-apoptotic cells in the following experiments. To confirm the cell cycle effects of TopBP1 depletion, cells were synchro-
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FIGURE 1. Depletion of TopBP1 using siRNAs. A, U2OS cells were transfected up to three times with two different TopBP1 siRNAs (#1 and #2) and control siRNA (GL3) and harvested at the indicated time. B, TopBP1 level in each sample was examined by immunoblotting. C, the siRNA-treated samples were also subjected to flow cytometry analysis. The percentages of sub-G1 populations are shown with cell cycle profiles.

FIGURE 2. TopBP1-deficient cells are arrested in G1 phase. 72 h after the indicated siRNA transfection, U2OS cells were treated with thymidine (Thy; 2 mM, 12 h) or nocodazole (Noc; 100 ng/ml, 16 h). S phase-enriched cells were obtained by releasing thymidine-treated cells into fresh medium for another 8 h (Rel). The untreated cells (Un) were harvested together with these S phase samples. Each sample was analyzed for TopBP1 level and cell cycle profile by immunoblotting (A) and flow cytometry (B). C, cyclin levels in untreated or nocodazole-treated samples were examined by immunoblotting. D, 72 h after RNAi treatment, cells were incubated with BrdUrd for 24 h. BrdUrd-incorporated nuclei were detected with anti-BrdUrd antibody while total nuclei are shown by DAPI staining. Three independent experiments were performed and greater than 300 nuclei were counted to obtain the percentage of BrdUrd-incorporated cells.

The levels of cyclins after TopBP1 RNAi were examined by immunoblotting (Fig. 2C). Cyclins E, A, and B are all expressed in a cell-cycle dependent manner, but their expression patterns are distinct from one another; cyclin E protein is maximal at the G1/S transition, while cyclin A and B are expressed during S and M phase, respectively. Cyclin D level is relatively constant throughout the cell cycle, with the exception that it is not expressed in quiescent (G0) cells (31). With TopBP1 depletion, no significant changes were observed in the cyclin D levels indicating cells were still in a proliferating state. TopBP1-depleted cells showed an increase in cyclin E accompanied with a decrease in cyclin A and B, even following nocodazole treatment. Considering that cyclin E is degraded in S phase (8), these results suggest that TopBP1-deficient cells failed to progress into S phase resulting in the accumulation of cyclin E. The observed decrease in both cyclin A and B with TopBP1 deple-
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**FIGURE 3.** TopBP1 RNAi causes G₁ arrest in an Rb- and p53-independent manner. The experiments described in Fig. 1 were repeated with Saos-2 cells. Each sample was analyzed by immunoblotting (A) and flow cytometry (B).

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**FIGURE 4.** Cyclin E/CDK2 kinase activity decreases in TopBP1-deficient cells. A, siRNA-treated U2OS cells were harvested at 72 h, and the phosphorylation level of pRb (p-pRb) was examined by immunoblotting. B, cyclin E/CDK2 complexes were isolated from each sample by immunoprecipitation using anti-cyclin E antibody and subjected to an *in vitro* kinase assay using histone H1 as a substrate. C, cyclin E/CDK2 activities represented by the phosphorylation of histone H1 (H1-P) were shown with the value of GL3-siRNA-treated sample normalized to 1. Values represent the average of at least three independent experiments.

E/CDK2 activity with TopBP1 depletion (Fig. 4A). To measure cyclin E/CDK2 activity directly, *in vitro* kinase assays were also performed (Fig. 4B). Since cyclin E protein is increased following TopBP1 depletion (Fig. 2C), immunoprecipitation using anti-cyclin E antibody resulted in more immunoprecipitated cyclin E/CDK2 complexes from the extract of TopBP1-deficient cells compared with the control. However, despite the increased cyclin E/CDK2 protein, the kinetics in TopBP1-depleted U2OS and Saos-2 cells were three to four times lower than the control sample (Fig. 4C). Therefore, we hypothesized that TopBP1 depletion down-regulates cyclin E/CDK2 activity thereby preventing cells from progressing into S phase. The decreased activity also explains the accumulation of cyclin E, since autophosphorylation is required for its degradation via ubiquitin-mediated proteolysis (2).
To elucidate the mechanism of decreased cyclin E/CDK2 activity in TopBP1-depleted cells, we examined several candidate regulators of CDKs. A number of checkpoint pathways activated by various genomic stresses are reported to target cyclin E/CDK2 and arrest cells in G1 phase, most of which are mediated by Chk1 and Chk2 (36–38). When TopBP1 was depleted, however, neither phospho-Chk1 nor phospho-Chk2 was detected in both U2OS and Saos-2 cells, whereas hydroxyurea and \( /H9253\)-irradiation treatments demonstrated robust phosphorylation of Chk1 and Chk2. Instead, we observed a dramatic induction of two CDK inhibitors, p21 and p27, in TopBP1-depleted U2OS and Saos-2 cells (Fig. 5A). Time course analyses revealed that the protein levels of p21 and p27 became elevated by 48 h, and this induced state lasted until the 96 h time point (Fig. 5B). Consistent with these results, cyclin E/CDK2 kinase activity began to decrease at 48 h and was further reduced by 96 h. To validate the involvement of p21 and p27 in down-regulation of cyclin E/CDK2 after TopBP1 depletion, the interaction between these CDK inhibitors and cyclin E/CDK2 was examined by immunoprecipitation using anti-cyclin E antibody (Fig. 5C). The amount of co-immunoprecipitated p21 and p27 was significantly increased with TopBP1 depletion compared with that of co-immunoprecipitated CDK2. These results indicate that the up-regulated p21 and p27 indeed interact with cyclin E/CDK2 and inhibit its action in the absence of TopBP1.

**p21 and p27 Are Induced by Different Mechanisms in the Absence of TopBP1**—To investigate the mechanism of p21 and p27 up-regulation following TopBP1 RNAi, we first determined whether p21 and p27 are transcriptionally up-regulated. The mRNA levels of p21 and p27 in siRNA-treated cells were determined by Northern blotting (Fig. 6A). Compared with the control cells, p21 mRNA was greatly induced after TopBP1 depletion in both U2OS and Saos-2 cell lines and correlated well with the temporal induction pattern of the protein. On the other hand, there was no significant change in the p27 mRNA level.

Transcriptional activation of p21 and p27 promoters in response to TopBP1 depletion was also examined by performing promoter-luciferase assay (Fig. 6B). Consistent with the Northern blotting results, the luciferase activity of p27 promoter in TopBP1-depleted cells was similar to that in control cells, suggesting non-transcriptional regulation of p27. In contrast, p21 promoter showed a 3-fold increase in luciferase activity with TopBP1 RNAi, confirming that p21 is up-regulated by transcriptional activation in response to TopBP1 depletion.

**TopBP1 Function Is Required Not Only for Cyclin E/CDK2 Action but Also for Pre-IC Assembly Necessary for S Phase Progression**—Last, we determined whether depletion of p21 and/or p27 could suppress the G1 arrest caused by the absence of TopBP1. The p21 and p27 siRNAs efficiently depleted corresponding proteins, but the elimination of either p21 or p27 alone only partially restored cyclin E/CDK2 activity in TopBP1-deficient cells. However, co-depletion of both p21 and p27 recovered about 80% of cyclin E/CDK2 activity (Fig. 7, A and B). The elevated protein levels of cyclin E, shown in TopBP1-depleted cells, also returned to the levels seen in control cells by the co-depletion, demonstrating that kinase activity is required for its autophosphorylation and degradation.

Flow cytometry analyses were performed to examine the cell cycle progression of these siRNA-treated cells in the presence or absence of nocodazole (Fig. 7C). When TopBP1 is present, elimination of p21 and/or p27 did not affect cell cycle profiles...
significantly from control cells. In contrast, TopBP1-deficient cells remained primarily arrested in G1 phase. Depletion of p21 or p27 alone could not rescue this G1 arrest, which is consistent with the in vitro kinase assay results showing only partial restoration of cyclin E/CDK2 activity by depletion of one inhibitor. Thus, it was surprising to find that a large population of cells remained arrested in G1 when both p21 and p27 were depleted with TopBP1 despite restoring cyclin E/CDK2 by 80%. Therefore CDK2 kinase activity is not sufficient for G1/S transition in the absence of TopBP1.

These results prompted us to consider Cdc7/Dbf4 kinase, another critical factor for G1/S transition. While cyclin E/CDK2 functions globally for transitioning from G1 to S phase, Cdc7/Dbf4 has been reported to act directly in initiation of DNA replication (39, 40). Substantial evidence from yeasts to mammals indicate that MCM proteins are the main substrates of Cdc7/Dbf4 during G1/S transition and that MCM phosphorylation is required for recruiting additional replication components, such as Cdc45 and DNA polymerases, onto replication origins (3). Therefore, if Cdc7/Dbf4 activity is also down-regulated by TopBP1 depletion, it could explain how TopBP1/p21/p27 triple-depleted cells remain arrested in G1 phase despite the normal cyclin E/CDK2 activity. To estimate Cdc7/Dbf4 activity, we first examined the phosphorylation of MCM2 protein from siRNA-treated samples (Fig. 8A). Cdc7/Dbf4 phosphorylates a specific residue of MCM2 which increases its mobility on SDS-PAGE (41). The phospho-MCM2 band (marked with an asterisk) on the SDS-PAGE disappeared in TopBP1-deficient cells, indicating low Cdc7/Dbf4 activity. However, co-depletion of p21 and p27 from TopBP1-depleted cells restored the Cdc7/Dbf4 activity as shown by the normal extent of phospho-MCM2. Furthermore, in vitro kinase assay for Cdc7/Dbf4 activity showed similar results, in which the reduced Cdc7/Dbf4 activity upon TopBP1 depletion was recovered to 60–80% of the control by co-depletion of p21 and p27 (Fig. 8, A and B). Since p21 and p27 are specific inhibitors of CDKs, not of Cdc7, these results suggest that Cdc7/Dbf4 acts after the cyclin E/CDK2 action, which results in the activation of pre-RC by phosphorylating MCM2.

To precisely define the point in replication arrested by TopBP1 depletion, next we investigated the loading of two replication components onto the chromatin, DNA pol-ε and PCNA, by immunostaining. Chromatin bound proteins were selectively detected by extracting soluble proteins prior to fixation as described previously (42). Without pre-extraction, DNA pol-ε and PCNA were equally detected in all cells of both control and TopBP1-depleted samples (Fig. 8C, non-extracted), consistent with the immunoblotting results (supplemental Fig. 2). However, the chromatin loading of those proteins in TopBP1-depleted cells greatly decreased compared with control cells (Fig. 8C, extracted, and 8D). To enrich for the number of cells staining positive for replication factors, we synchronized cells in S phase by...
incubating siRNA-treated cells with hydroxyurea before immunostaining (Fig. 8D). When TopBP1 was eliminated, however, the chromatin loading of both DNA pol-ε and PCNA was significantly reduced, and the co-depletion of p21 and p27 could not reverse this defect. Consistent with these results, BrdUrd incorporation assays also showed that DNA synthesis in TopBP1/p21/p27 triple-depleted cells was still as diminished as cells depleted of TopBP1 alone.

Theses triple RNAi analyses led us to the following conclusions. The restoration of cyclin E/CDK2 activity validated that p21 and p27 are indeed responsible for the down-regulation of cyclin E/CDK2, either directly or indirectly, by modulating the levels of p21 and p27. Further elucidation of p21 and p27 induction mechanisms following TopBP1 depletion will help to clarify this function of TopBP1.

It is important to note that while TopBP1 function is required for cyclin E/CDK2 activity in human cells, other TopBP1 homologues are generally considered to be involved in the loading of pre-IC components such as Cdc45 or DNA polymerases, which is downstream of CDK action (24, 26, 27). Previous studies using either depletion or mutants of TopBP1 homologues focused primarily on defects in the loading of pre-IC components without examining CDK activity; however, it is possible that the actual arresting point in other organisms is earlier than reported. Sequential action of Xcut5 and S-CDK in Xenopus egg extract system has been reported (25), supporting the possibility that the TopBP1 activation of CDK is common to other TopBP1 homologues.

Finally, depleting all three proteins TopBP1, p21, and p27 provided interesting results. Although cyclin E/CDK2 activity was restored, resulting in the reactivation of Cdc7/Dbf4 as well, these triple-RNAi-treated cells still showed a defect in S phase progression with inefficient chromatin-loading of DNA pol-ε and PCNA (Figs. 7 and 8). These results suggest that there exists another function of TopBP1, discrete from the one required for cyclin E/CDK2 activation, of which execution point is after the Cdc7/Dbf4 action but before the loading of DNA polymerases. This function of TopBP1 is consistent with the role of TopBP1 homologues in the assembly of the pre-IC complex for DNA synthesis.

Our data differ from two recent publications in which they also performed TopBP1 depletion using either siRNA or shRNA. Kim et al. (43) reported that depletion of human TopBP1 using siRNA in HCT116, U2OS, and HeLa cells resulted in cells accumulating in G2/M phase. Even in the absence of external genotoxic stresses, TopBP1-depleted cells
generated spontaneous DNA damage and consequently activated ATM and Chk2. Based on these results, the authors suggest that TopBP1 function is not required for DNA replication but necessary for normal S phase progression by maintaining genomic stability. On the other hand, Liu et al. (44) reported no difference in U2OS cell cycle profile after TopBP1 depletion. One possible explanation for this difference is that their TopBP1 depletion was not as efficient as our results. For example, most samples that Kim et al. (43) used in their report were transfected twice with TopBP1 siRNA before harvested at 72 h, whereas we performed RNAi three times before harvest at the same time point. In Xenopus, Xcut5 shows two different modes of chromatin binding: S-CDK-independent and -dependent. Although the amount of CDK-independent loading is quite small compared with that of CDK-dependent binding, it is enough for initiating DNA replication (25). Recently, the same group also reported that most of Xcut5 whose binding is CDK-dependent is required for checkpoint response (45). These Xcut5 studies could explain the G$_2$/M arrest after TopBP1 RNAi, which Kim et al. (43) observed: in their experiments, the residual TopBP1 protein after its knockdown might be enough for cells to progress into S phase but not enough to maintain genome stability resulting in the G$_2$/M arrest. Even though we cannot completely explain the discrepancies between these previous reports and our data, we observed the G$_1$ arrest with all six TopBP1-siRNAs that we tested (including the siRNA described in Kim et al. (43)) and also utilized several different methods to validate the result. Furthermore, the requirement of TopBP1 for G$_1$/S transition in human cells is in agreement with the results observed in other organisms including yeasts and Xenopus.

Therefore, we conclude that human TopBP1 is crucial for normal cell cycle progression: TopBP1 plays a dual role in G$_1$/S transition by promoting both cyclin E/CDK action and pre-IC formation (Fig. 9).

**p21 and p27 Up-regulation in TopBP1-deficient Cells**—TopBP1 depletion leads to up-regulation of both p21 and p27 by different mechanisms. p21 is transcriptionally activated in response to TopBP1 deficiency. The increase of p21 transcripts in p53-null Saos2 cells indicates that this activation pathway is independent of p53 (Fig. 6, A and B). Besides p53, p21 has been reported to be regulated by numerous transcription factors responding to various signals (46). Miz-1 and E2F1 are potential transcription factors for the p21 induction in the absence of TopBP1, based on previous reports in which TopBP1 negatively regulates transcription of these two proteins in proliferating cells (21, 22). Although Miz-1 involvement is less likely as Miz-1-mediated p21 induction requires p53 (21), E2F1 appears to be a good candidate as suggested by several reports showing that increased E2F1 levels lead to p21-dependent cell cycle arrest (47, 48). In addition, the fact that the TopBP1-E2F1 interaction occurs during G$_1$/S transition suggests the link between TopBP1 and p21. Finally, Liu et al. (49) showed in their recent report that the expression of the TopBP1 mutant that fails to interact with and repress E2F1 could not restore the up-regulated level of p21 caused by TopBP1 depletion, which is strongly supporting the possibility.

In contrast, TopBP1 depletion did not affect the p27 mRNA level, which indicates that p27 is stabilized in protein level, rather than transcriptionally regulated. The different induction mechanisms of p21 and p27 must be helpful in reinforcing the G$_1$ arrest in response to TopBP1 depletion, as each can be a backup system for the other in down-regulating cyclin E/CDK2 activity, which is consistent with that the depletion of either p21 or p27 alone was not enough to restore cyclin E/CDK2 activity (Fig. 7, A and B).

**Cellular Responses upon TopBP1 Depletion**—CDK is often compared with an engine that drives cells into the cell cycle. Therefore, it is no surprising that CDK is a main target for various regulatory pathways during cell cycle progression. Considered recent publications reporting the role of TopBP1 as a chromatin remodeling factor (22, 50), it is possible that TopBP1 deficient cells fail to build proper chromatin structures resulting in genome instability, which leads to the down-regulation of cyclin E/CDK2 activity to prevent improper and disordered DNA replication.

However, the down-regulation of cyclin E/CDK2 by p21 and p27 shown here represents a distinctive pathway from other well known regulation mechanisms which are predominantly mediated by Chk1 or Chk2. In addition, although p53 is the best known upstream regulator of p21, it was dispensable for the p21 induction by TopBP1 deficiency. It is interesting that Orc2 depletion in human cells leads to apparently similar phenomena to those of TopBP1 depletion (51), although one depletion does not affect the level of the other protein (data not shown). Orc2 depleted cells also underwent the p53-independent G$_1$ arrest with reduced cyclin E/CDK2 activity and an increase in p21 and p27. The mechanism is also similar with p21 being transcriptionally activated, whereas p27 is stabilized in the protein level. Another study on Xic1, a p27 homologue in Xenopus, reported that Xic1 was stabilized when pre-RC components or downstream initiation factors such as CDK2, Cdc7, and Cdc45 were depleted (52). This stabilization was independent of Xic1 phosphorylation by CDK2, which is also consistent with our p27 stabilization results. Therefore, it is also possible that TopBP1 is involved in a common surveillance system as other replication components, which drives a novel pathway in response to malfunction of the initiation process of DNA replication.

Additionally, we discovered that cyclin E/CDK2 acts prior to Cdc7/Dbf4 during G$_1$ to S phase progression in human cells. The order of these kinase actions at G$_1$/S transition has been
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controversial. In budding yeast, Cdc7/Dbf4 is required for DNA replication only after S-CDK has been activated (53), whereas Cdc7/Dbf4 function precedes the execution point of cyclin E/CDK2 in Xenopus egg extract system (54). Although in vitro experiments suggested the possibility that CDK activity facilitates the Cdc7/Dbf4 action either directly or indirectly (55), the relationship between these kinases for DNA replication initiation had yet to be determined in human cells. Here we showed that Cdc7/Dbf4 was down-regulated by TopBP1 depletion but restored by depletion of p21 and p27, which is the same pattern observed with cyclin E/CDK2 (Fig. 8, A and B). Since both p21 and p27 do not inhibit Cdc7/Dbf4 kinase action (55, 56), these results suggest that Cdc7/Dbf4 activity is not directly affected by TopBP1 depletion but rather is regulated by cyclin E/CDK2 in human cells.

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