

# Stalled Replication Induces p53 Accumulation through Distinct Mechanisms from DNA Damage Checkpoint Pathways

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## Abstract

**Stalled replication forks induce p53, which is required to maintain the replication checkpoint. In contrast to the well-established mechanisms of DNA damage-activated p53, the downstream effectors and upstream regulators of p53 during replication blockade remain to be deciphered. Hydroxyurea triggered accumulation of p53 through an increase in protein stability. The requirement of p53 accumulation for the replication checkpoint was not due to p21<sup>CIP1/WAF1</sup> as its down-regulation with short-hairpin RNA did not affect the checkpoint. Similar to DNA damage, stalled replication triggered the activation of the MRN-ataxia telangiectasia mutated (ATM)/ATM and Rad3-related-CHK1/CHK2 axis. Down-regulation of CHK1 or CHK2, however, reduced p53 basal expression but not the hydroxyurea-dependent induction. Moreover, p53 was still stabilized in ataxia telangiectasia cells or in cells treated with caffeine, suggesting that ATM was not a critical determinant. These data also suggest that the functions of ATM, CHK1, and CHK2 in the replication checkpoint were not through the p53-p21<sup>CIP1/WAF1</sup> pathway. In contrast, induction of p53 by hydroxyurea was defective in cells lacking NBS1 and BLM. In this connection, the impaired replication checkpoint in several other genetic disorders has little correlation with the ability to stabilize p53. These data highlighted the different mechanisms involved in the stabilization of p53 after DNA damage and stalled replication forks. (Cancer Res 2006; 66(4): 2233-41)**

## Introduction

Checkpoints operate throughout the cell cycle to maintain genome stability. DNA damage checkpoints ensure that damaged DNA is neither replicated nor segregated to daughter cells until repaired. Similarly, the replication checkpoint ensures that cells do not enter mitosis until DNA replication is completed. Cells that harbor defects in these pathways are prone to genome instability and neoplastic transformation.

The tumor suppressor p53 is one of the key mediators of the DNA damage checkpoints (1). Genotoxic stress, including double-strand breaks, stabilizes and activates p53. Several downstream targets of p53 are known to modulate cell proliferation. The expression of the cyclin-dependent kinase inhibitor p21<sup>CIP1/WAF1</sup> is enhanced by p53, facilitating cell cycle arrest. Another target of p53 is the proapoptotic protein BAX, which promotes the release of cytochrome *c* from the mitochondria and activates the apoptotic

program. In unstressed cells, p53 is restrained by binding to murine double minute-2 (MDM2), itself a transcriptional target of p53. MDM2 binds to the transactivation domain of p53 and inhibits its transcriptional activity, shuttles p53 out of the nucleus, and targets p53 for ubiquitin-mediated proteolysis (2).

ATM is mutated in the cancer-prone disorder ataxia telangiectasia (OMIM 208900). Cells derived from patients with ataxia telangiectasia exhibit enhanced radiosensitivity, radioresistant DNA synthesis, and chromosomal instability (3). Following exposure to ionizing radiation and other genotoxic insults that elicit double-strand breaks, ataxia telangiectasia mutated (ATM) is autophosphorylated, which causes dimer dissociation and initiates the activation of the kinase (4). Activated ATM phosphorylates a wide range of proteins implicated in the genome integrity checkpoints (5). Several residues at the NH<sub>2</sub>-terminal region of p53 are phosphorylated by ATM; this prevents the binding to MDM2 and thus stabilizes p53. ATM also activates p53 indirectly by phosphorylating CHK2, which in turn phosphorylates and activates p53. Furthermore, MDM2 is phosphorylated by ATM, which attenuates the p53-inhibitory potential of MDM2. Phosphorylation of p53 further facilitates other posttranslational modifications, including association with coactivators p300 and PCAF and acetylation of the COOH-terminal region (6, 7).

Apart from ataxia telangiectasia, several cancer-prone disorders are functionally linked to ATM and are defective in DNA damage responses. Nijmegen breakage syndrome (NBS; OMIM 251260) is an autosomal recessive chromosomal instability syndrome with clinical resemblance to ataxia telangiectasia and is caused by mutations in *NBS1* (or *nibrin*). *NBS1* binds *MRE11* and *RAD50* to form a MRN complex (8). *MRE11* itself is mutated in another neurologic disorder, ataxia telangiectasia-like disorder (OMIM 604391). The similarity of phenotype between NBS, ataxia telangiectasia-like disorder, and ataxia telangiectasia indicates an integral link between ATM and the MRN complex. Indeed, the MRN complex can stimulate the kinase activity of ATM (9) and phosphorylation of *NBS1* by ATM is necessary for the activation of the DNA damage checkpoint (10–13). ATM and *NBS1* are also required for the ionizing radiation-induced phosphorylation of the Fanconi anemia (OMIM 227650) gene product *FANCD2* (14, 15), providing a link between the DNA cross-link response and the DNA damage checkpoint. Seckel syndrome (OMIM 210600) is a rare autosomal recessive disorder characterized by growth retardation and impaired DNA-damage responses. Interestingly, one locus of Seckel syndrome is identified to be a splicing mutation of the ATM-related gene *ATR* (16). Bloom syndrome (OMIM 210900) is an autosomal recessive disorder characterized by predisposition to malignancy and chromosomal instability and is caused by mutations in the gene encoding a RECQ-like DNA helicase (BLM). BLM is phosphorylated through ATM- and ATM and Rad3-related (*ATR*)-dependent pathways in response to ionizing radiation and replication blockade, respectively, and phosphorylation of BLM is required for proper execution of the checkpoints (17). Finally, mutations of *BRCA1* and *BRCA2* cause

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

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predisposition to a subset of familial breast cancer. BRCA1 is phosphorylated by ATM- and ATR-dependent mechanisms after DNA damage. BRCA1 associates with BLM and the MRN complex to form part of a large complex, termed BRCA1-associated genome surveillance complex (BASC), and colocalizes to large nuclear foci after replication blockade or DNA damage (18).

ATM-CBK2 and ATR-CBK1 have been regarded as largely linear, nonoverlapping pathways. Double-strand breaks caused by ionizing radiation activate ATM-CBK2 whereas stalled replication forks activate ATR-CBK1. More recent data suggest that these pathways are not mutually exclusive as once thought. ATM and ATR each contribute to early delay in M-phase entry after ionizing radiation but ATR regulates a majority of the late responses (19). For the downstream kinases, *CHK2*<sup>-/-</sup> cells have a relatively normal G<sub>2</sub> DNA damage checkpoint except that the checkpoint is not as well maintained over time (20, 21). On the other hand, conditional *CHK1*<sup>-/-</sup> ES cells have a faulty G<sub>2</sub> DNA damage checkpoint (22–24). Similarly, disruption of the ionizing radiation-induced G<sub>2</sub> DNA damage checkpoint was obtained by elimination of *CHK1* by small interfering RNA (25, 26). Moreover, apart from being a substrate of ATR, *CHK1* is also phosphorylated at Ser317 in an ATM-dependent manner following ionizing radiation (25). The relative contribution of ATM-CBK2 and ATR-CBK1 to the replication checkpoint remains to be deciphered.

In comparison with its well-established functions in the DNA damage checkpoints, the precise role of p53 in the replication checkpoint is controversial. It is recognized that replication blockade induces p53 (27, 28). Taylor et al. (29) found that unlike wild-type fibroblasts, p53-null fibroblasts enter mitosis even when DNA synthesis is blocked with hydroxyurea. Premature entry into mitosis results in high levels of phosphorylated histone, condensed chromosomes, and fragmented chromatin, indicating that p53 is essential for the replication checkpoint. On the other hand, Gottifredi et al. (30) found that although hydroxyurea stimulates p53<sup>Ser15</sup> phosphorylation, several p53 transcriptional targets (e.g., *CIP1/WAF1* and *MDM2*) that are rapidly induced by ionizing radiation are weakly or not induced at all by hydroxyurea. These results suggest that the hydroxyurea-induced p53 is transcriptionally impaired. In disagreement with these results, Nayak and Das (31) found that p53 transactivates its target genes, including *CIP1/WAF1*, *GADD45*, and *BAX*, in response to replication blockade.

In this article, we sought to elucidate the relationship between the replication checkpoint and p53. In particular, we examined whether downstream effectors and upstream regulators of p53 during the replication checkpoint are the same as the DNA damage checkpoint. We found that the levels of p53 and p21<sup>CIP1/WAF1</sup> attained after hydroxyurea treatment were significantly lower than those after DNA damage. Knockdown of p21<sup>CIP1/WAF1</sup> did not affect the responses to replication blockade. We also found that disruption of *CHK1* and *CHK2* reduced the basal levels of p53 but did not abolish the induction of p53 by hydroxyurea. Furthermore, p53 induction did not require ATM and was caffeine insensitive, but it was defective in cells lacking NBS1 or BLM. Together, these data reveal that the downstream effectors and upstream regulators of p53 during the replication checkpoint are very different from those after DNA damage.

## Materials and Methods

**Materials.** All reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless stated otherwise.

**DNA constructs.** Short hairpin RNAs (shRNA) were expressed from mU6pro (a generous gift from Dr. David Turner, University of Michigan, MI; ref. 32). p21<sup>CIP1/WAF1</sup> shRNA was created by annealing the primers 5'-TTTGGACCATGTGGACCTGTCACTTCAAGAGAGTGACAGGTCCACATGGTCTTTT-3' and 5'-CTAGAAAAAGACCATGTGGACCTGTCACTTCTTGAAGTGACAGGTCCACATGGTTC-3' into *BbsI-XbaI*-cut mU6pro. *CHK1* shRNA in mU6pro was as described (33). A puromycin-resistant gene was inserted into the *PvuII* site of the shRNA constructs.

**Cell culture.** HepG2 cells (hepatoblastoma), MCF7 cells (mammary gland adenocarcinoma), and U2OS (osteosarcoma) were obtained from the American Type Culture Collection (Manassas, VA). Wild-type (GM03798), ataxia telangiectasia (GM03189), Bloom syndrome (GM03403H), *BRCA1* mutation (GM13705A), *BRCA2* mutation (GM14788), Seckel Syndrome (GM09703), and NBS (GM07078D) lymphoblastoid cells were obtained from Coriell Cell Repositories (Camden, NJ). *CHK2*<sup>-/-</sup> mouse embryonic fibroblasts (MEF; ref. 34) were generous gifts from Dr. Tak W. Mak (University of Toronto, Toronto, Canada). Unless stated otherwise, cells were treated with the following reagents at the indicated final concentration: Adriamycin (200 ng/mL), aphidicolin (5 µg/mL), caffeine (5 mmol/L), camptothecin (0.67 µmol/L), hydroxyurea (1.5 mmol/L), nocodazole (0.1 µg/mL), puromycin (1 µg/mL), and thymidine (2 mmol/L). Ionizing radiation was delivered from a <sup>137</sup>Cs source from a MDS Nordion Gammacell 1000 Elite Irradiator (Ottawa, Ontario, Canada). Cell-free extracts were prepared as described (35). To create stable cell lines expressing shRNA, cells were transfected with the shRNA constructs with a calcium phosphate precipitation method (36). Two days after transfection, the cells were selected in medium supplemented with puromycin. After ~3 weeks of selection, the mixed population of cells was expanded and propagated in normal medium.

**Flow cytometry.** BrdUrd incorporation followed by flow cytometry analysis was done as previously described (37).

**Single-cell gel electrophoresis assays.** Detection of DNA breaks in individual cells was done as described by Singh et al. (38) with the pH >13 electrophoresis buffer. Comet tail movement was quantified with the software CometScore (Tritek Corporation, Sumerduck, VA). Fifty cells were scored for each sample.

**Pulse-chase analysis.** Cells were incubated in Cys/Met-free DMEM (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% (v/v) PBS-dialyzed fetal bovine serum. After 15 minutes, [<sup>35</sup>S]methionine (0.5 mCi/mL; Amersham, Piscataway, NJ) was added to label the cells for 2 hours. The radioactive medium was replaced with normal growth medium and the cells were harvested at different time points. Cell extracts were prepared, immunoprecipitated with p53 antiserum, and applied onto SDS-PAGE. [<sup>35</sup>S]-labeled proteins were detected and quantified with a PhosphorImager (Amersham Biosciences).

**Antibodies and immunologic methods.** Polyclonal antibodies against Ser139-phosphorylated histone H2AX were either gifts from Junjie Chen (Mayo Clinic, Rochester, MN) or obtained from Bethyl Laboratories (Montgomery, TX). Monoclonal antibodies A17 against CDC2 and YL1/2 against tubulin were gifts from Dr. Tim Hunt (Cancer Research UK, South Mimms, United Kingdom). Monoclonal antibodies against *CHK1*, p53, ATM, polyclonal antibodies against 14-3-3σ, Ser10-phosphorylated histone H3, p21<sup>CIP1/WAF1</sup>, p53 (for detection of mouse p53), and Thr68-phosphorylated *CHK2* were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody 2A10 against *MDM2* was obtained from Calbiochem (San Diego, CA). Antibodies against Ser20-phosphorylated p53, Tyr15-phosphorylated CDC2, Ser1981-phosphorylated ATM, and Lys382-acetylated p53 were obtained from Cell Signaling Technology (Beverly, MA). Immunoblotting was done as previously described (35). The intensities of bands were quantified using ImageJ software (NIH, Bethesda, MD).

## Results

**Characterization of p53 induction during the replication checkpoint.** To verify that p53 is induced during the replication checkpoint, cells were treated with different inhibitors of S phase and the expression of p53 was examined by immunoblotting

(Fig. 1A). As expected, phosphorylation of histone H3<sup>Ser10</sup> was abolished after treatment with hydroxyurea, aphidicolin, or thymidine, indicating that entry into mitosis was inhibited. As controls, histone H3<sup>Ser10</sup> phosphorylation was strongly stimulated by nocodazole and inhibited after DNA damage by the topoisomerase poison Adriamycin. As we have previously shown (39), CHK1 was phosphorylated when replication or mitosis was stalled. We found that similar to DNA damage, hydroxyurea and thymidine stimulated p53 accumulation. Interestingly, p53 was not strongly induced by aphidicolin, indicating that not all types of replication block were effective in stabilizing p53. Importantly, whereas p21<sup>CIP1/WAF1</sup> was robustly activated by Adriamycin, it was only weakly induced by hydroxyurea. As a comparison, DNA damage triggered by ionizing radiation inhibited DNA replication and activated both p53 and p21<sup>CIP1/WAF1</sup> (Supplementary Fig. S1). Interestingly, 14-3-3 $\sigma$  (another target of p53 that is implicated in G<sub>2</sub>-M control) was only weakly induced by replication blockade or DNA damage (Fig. 1A).

Although p53 was readily induced by both DNA damage and stalled replication forks, the levels of p53 attained after hydroxyurea treatment were categorically lower than that after DNA damage (Fig. 1B and Supplementary Figs. S2 and S3). This may partly explain why the expression of p21<sup>CIP1/WAF1</sup> after hydroxyurea treatment was significantly lower than after DNA damage. Similar results were obtained for cell lines including HepG2 (Fig. 1A), MCF7 (Fig. 1B), and lymphoblastoid cells (data not shown), indicating that the effect was not cell line specific. We found that

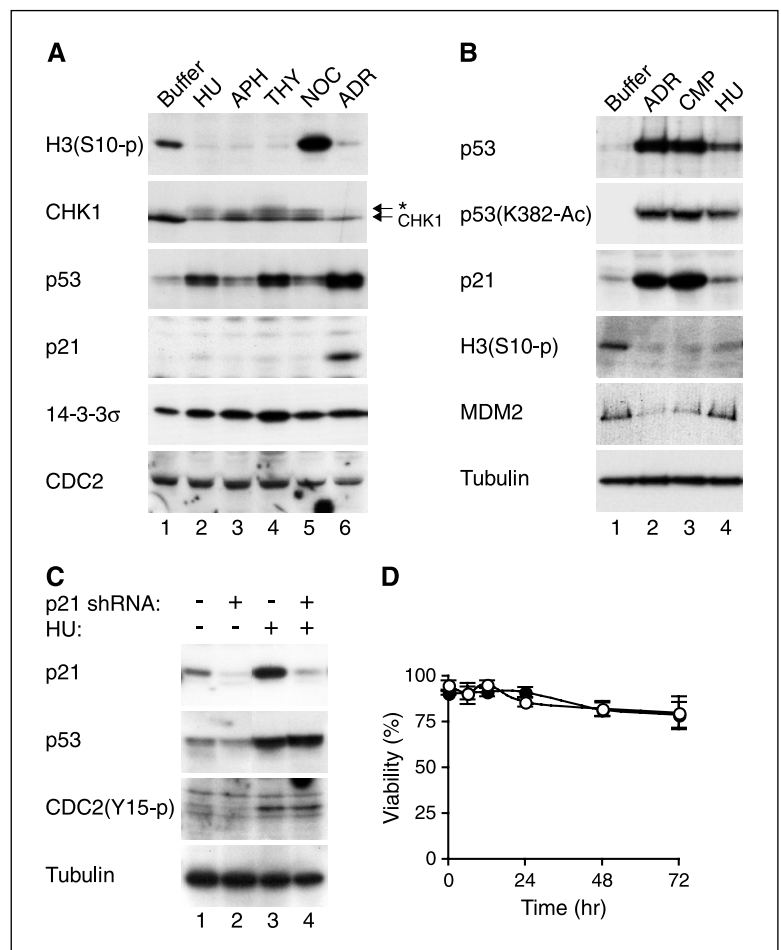
hydroxyurea strongly promoted the acetylation of p53<sup>Lys382</sup> in MCF7 cells (Fig. 1B), suggesting that the accumulated p53 was active as a transcription factor (6). Similarly, hydroxyurea triggered p53 acetylation robustly in HepG2 cells. Although hydroxyurea induced less total p53 than the DNA damaging agents, stronger Lys382 acetylation was promoted by hydroxyurea (Supplementary Fig. S2). Consistent with the relatively low expression of p53 and p21<sup>CIP1/WAF1</sup> after hydroxyurea exposure, hydroxyurea and Adriamycin together did not induce more p53 or p21<sup>CIP1/WAF1</sup> than Adriamycin alone (Supplementary Fig. S3). Interestingly, the expression of MDM2 after DNA damage or replication block displayed an inverse relation with p53 (Fig. 1B), suggesting the accumulation of p53 may in part be determined by MDM2.

Consistent with the increase in p53 during the replication checkpoint, we found that the half-life of p53 was increased after hydroxyurea treatment (Supplementary Fig. S4). Nevertheless, the stability of p53 after hydroxyurea treatment was lower than that after DNA damage.

Collectively, these observations show that although both p53 and p21<sup>CIP1/WAF1</sup> were readily induced during replication blockade, their level of expression was lower than that after DNA damage.

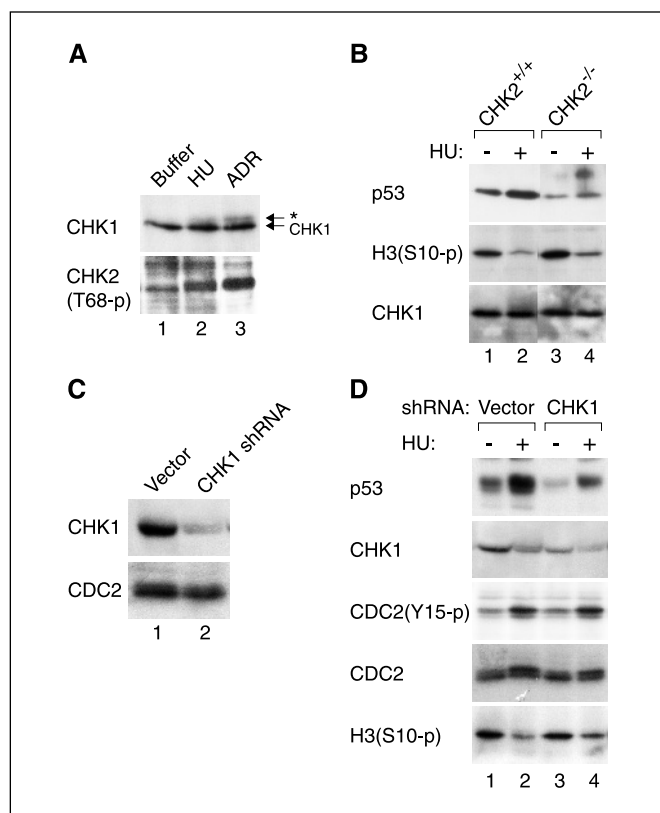
**p21<sup>CIP1/WAF1</sup> is not required for the replication checkpoint.** To see if the activation of p21<sup>CIP1/WAF1</sup> is indeed required for the replication checkpoint, its expression was down-regulated with a shRNA construct. Figure 1C shows that cells with stable integration of the shRNA had significantly lower expression of p21<sup>CIP1/WAF1</sup> in comparison the parental cells (before or after hydroxyurea

**Figure 1.** p53 is induced by both the DNA damage checkpoint and the replication checkpoint. **A**, replication blockade induces p53 but activates very little p21<sup>CIP1/WAF1</sup>. HepG2 cells were treated with control buffer, hydroxyurea (HU), aphidicolin (APH), thymidine (THY), nocodazole (NOC), or Adriamycin (ADR) as indicated for 16 hours. Cell extracts were prepared and were subjected to immunoblotting for phospho-histone H3<sup>Ser10</sup>, CHK1, p53, p21<sup>CIP1/WAF1</sup>, and 14-3-3 $\sigma$  as indicated. Equal loading of lysates was confirmed by immunoblotting for CDC2. \*, position of phosphorylated CHK1. **B**, hydroxyurea induces less p53 and p21<sup>CIP1/WAF1</sup> than topoisomerase poisons. MCF7 cells were treated with buffer, Adriamycin, camptothecin (CMP), or hydroxyurea for 24 hours. Cell extracts were prepared and phospho-histone H3<sup>Ser10</sup>, p53, Lys382-acetylated p53, p21<sup>CIP1/WAF1</sup>, and MDM2 were detected by immunoblotting. Tubulin served to indicate the uniform loading of lysates. **C**, down-regulation of p21<sup>CIP1/WAF1</sup> by shRNA. HepG2 cells or cells with stable integration of p21<sup>CIP1/WAF1</sup> shRNA were treated with either buffer or hydroxyurea for 24 hours. Cell extracts were prepared and the expression of p21<sup>CIP1/WAF1</sup>, p53, and phospho-CDC2<sup>Tyr15</sup> was determined by immunoblotting. Uniform loading of lysates was confirmed by immunoblotting for tubulin. **D**, down-regulation of p21<sup>CIP1/WAF1</sup> does not affect viability after hydroxyurea treatment. HepG2 (○) and HepG2 expressing the p21<sup>CIP1/WAF1</sup> shRNA (●) were treated with hydroxyurea for 24 hours. The cells were then washed and growth was continued in normal growth medium. Viability was measured by trypan blue exclusion analysis. Points, mean of three independent experiments; bars, SD.



treatment). Densitometry analysis indicates that the knockdown was >80%. As expected, the induction of p53 was not affected by the shRNA. The hydroxyurea-activated pathways that lead to the increase in inhibitory phosphorylation of CDC2 were also unaffected by the p21<sup>CIP1/WAF1</sup> knockdown. Despite the knockdown of p21<sup>CIP1/WAF1</sup>, the sensitivity to hydroxyurea was not enhanced in these cells (compare with checkpoint-defective cells, see later; Fig. 1D). These data indicate that the requirement of p53 for the replication checkpoint may not be due to the activation of p21<sup>CIP1/WAF1</sup>.

**CHK1 and CHK2 are involved in maintaining the basal expression of p53 but are not required for p53 accumulation during the replication checkpoint.** We next analyzed the upstream regulators of p53 during the replication checkpoint. We found that CHK2 was activated after DNA damage and replication block as indicated by the CHK2<sup>Thr68</sup> phosphorylation (Fig. 2A).



**Figure 2.** The involvement of CHK1 and CHK2 in p53 regulation during replication blockade. **A**, both CHK1 and CHK2 are activated by replication block. HepG2 cells were treated with buffer, hydroxyurea, or Adriamycin for 24 hours. Cell extracts were prepared and were subjected to immunoblotting for CHK1 and phospho-CHK2<sup>Thr68</sup>. \*, position of phosphorylated CHK1. **B**, CHK2 is not required for the induction of p53 by hydroxyurea. Wild-type MEFs and CHK2<sup>-/-</sup> MEFs were treated with hydroxyurea for 6 hours and cell extracts were prepared. The expression of p53 and phospho-histone H3<sup>Ser10</sup> was detected by immunoblotting. Uniform loading of lysates was confirmed by immunoblotting for CHK1. Each panel was from the same blot and the same exposure (lanes in between were removed). **C**, down-regulation of CHK1 with shRNA. U2OS cells transfected with blank vector or with stable integration of CHK1 shRNA were created as described in Materials and Methods. Cell extracts were prepared and the expression of CHK1 was determined by immunoblotting. Uniform loading of lysates was confirmed by immunoblotting for CDC2. **D**, down-regulation of CHK1 does not affect p53 accumulation during the replication checkpoint. U2OS or U2OS with stable integration of CHK1 shRNA was treated with either buffer or hydroxyurea for 24 hours. Cell extracts were prepared and the expression of p53, CHK1, phospho-CDC2<sup>Tyr15</sup>, total CDC2, and phospho-histone H3<sup>Ser10</sup> was detected by immunoblotting.

Similarly, CHK1 was activated under both conditions (as indicated by mobility shifts). Hence, it is possible that CHK1 and CHK2 are involved in the induction of p53 after hydroxyurea treatment.

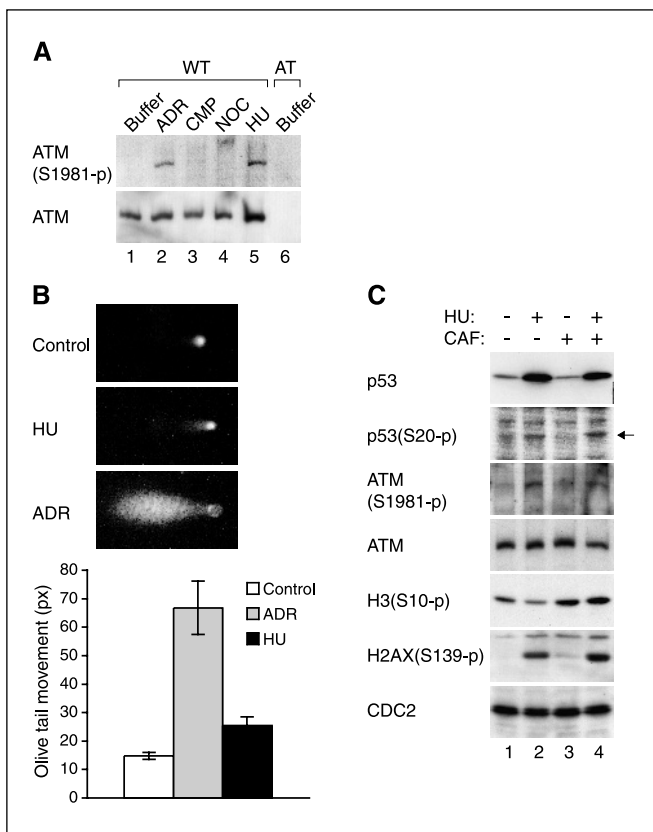
To see if CHK2 is required for the stabilization of p53 by hydroxyurea, we compared MEFs with *CHK2* gene disruption to MEFs in a wild-type background. Figure 2B shows that p53 was stabilized by hydroxyurea in both wild-type and CHK2<sup>-/-</sup> MEFs. It should be noted, however, that the preinduced level of p53 was significantly lower in the CHK2<sup>-/-</sup> cells than in the wild-type cells, suggesting that CHK2 is involved in maintaining the basal expression of p53.

To evaluate the role of CHK1 in the replication checkpoint, CHK1 was down-regulated in U2OS cells by stable integration of a previously characterized shRNA construct (ref. 33; Fig. 2C). Knockdown of CHK1 (densitometry analysis indicates that the knockdown in this cell line was ~75%) significantly reduced the basal expression of p53 but hydroxyurea was still able to induce p53 (Fig. 2D). Due to the lower basal expression, however, the induced level of p53 was lower than that in the control cells.

Checkpoint-proficient cells suppress mitotic events, such as histone H3<sup>Ser10</sup> phosphorylation, in the presence of hydroxyurea. Histone H3<sup>Ser10</sup> phosphorylation was reduced by hydroxyurea in both wild-type and CHK2<sup>-/-</sup> MEFs (Fig. 2B) as well as in the presence or absence of CHK1 shRNA (Fig. 2D), indicating that neither CHK1 nor CHK2 is critical for the replication checkpoint. Similarly, inhibitory phosphorylation of CDC2 (as detected by phospho-Tyr15-specific antibody and gel mobility shift) was triggered by hydroxyurea treatment in the presence of CHK1 shRNA (Fig. 2D). Phosphorylation of histone H3<sup>Ser10</sup>, however, was consistently slightly higher in cells lacking CHK1 or CHK2, suggesting that CHK1 and CHK2 may play a partial role in the replication checkpoint. Taken together, these data show that although both CHK1 and CHK2 were activated by hydroxyurea and contribute to the basal expression of p53, neither CHK1 nor CHK2 completely accounted for the accumulation of p53 after hydroxyurea treatment or the execution of the replication checkpoint.

**Induction of p53 by hydroxyurea correlates with the activation of the MRN-ATM pathway but is insensitive to caffeine.** The major upstream regulators of CHK1 and CHK2 are ATM and ATR. Activation of ATM by double-strand breaks induces autophosphorylation at Ser1981 (4). We found that hydroxyurea also stimulated ATM<sup>Ser1981</sup> phosphorylation (Fig. 3A). ATM was present in mock-treated cells but was not phosphorylated (lane 1) and Adriamycin-mediated ATM<sup>Ser1981</sup> phosphorylation served as a positive control (lane 2); interestingly, the topoisomerase I poison camptothecin did not trigger ATM<sup>Ser1981</sup> phosphorylation. Also similar to the responses to DNA damage, hydroxyurea triggered the phosphorylation of MRE11 (Supplementary Fig. S5) and histone H2AX (see later). In contrast to DNA damage, however, hydroxyurea only induced a low level of DNA breaks as measured by single-cell gel electrophoresis (comet) assays (Fig. 3B). These results indicate that hydroxyurea activates the MRN-ATM-CHK1/CHK2 pathway similar to DNA-damaging agents.

Caffeine can inhibit ATM and ATR, thereby bypassing both the DNA damage checkpoint and the replication checkpoint (40). We next investigated if caffeine affected ATM activation and p53 accumulation during replication blockade. As expected, the decrease of histone H3<sup>Ser10</sup> phosphorylation after hydroxyurea treatment could be restored by caffeine (Fig. 3C). Importantly, the induction of p53 by hydroxyurea was unaffected by caffeine and



**Figure 3.** Induction of p53 by hydroxyurea correlates with the activation of MRN-ATM pathway but is insensitive to caffeine. **A**, ATM is autophosphorylated after DNA damage and replication block. Normal lymphoblastoid cells (WT) were treated with the indicated agents for 24 hours. The levels of phospho-ATM<sup>Ser1981</sup> and total ATM were detected by immunoblotting. Extracts of ataxia telangiectasia lymphoblastoid cells act as negative controls. **B**, hydroxyurea induces significantly fewer DNA breakage than Adriamycin. Wild-type lymphoblastoid cells were mock treated or treated with hydroxyurea or Adriamycin for 16 hours. The cells were then harvested for single-cell gel electrophoresis assay. Representational images are shown and tail movements were quantified. Columns, mean of three independent experiments; bars, SD. **C**, hydroxyurea-induced ATM, p53, and histone H2AX phosphorylation are not suppressed by caffeine. Wild-type lymphoblastoid cells were treated with buffer or hydroxyurea for 6 hours before buffer or caffeine was added for another 6 hours. Cell extracts were prepared and the expression of p53, phospho-p53<sup>Ser20</sup>, phospho-ATM<sup>Ser1981</sup>, ATM, phospho-histone H3<sup>Ser10</sup>, and phospho-histone H2AX<sup>Ser139</sup> was detected by immunoblotting. Uniform loading of lysates was confirmed by immunoblotting for CDC2.

caffeine itself did not activate p53. Moreover, caffeine did not inhibit the ATM<sup>Ser1981</sup> phosphorylation induced by hydroxyurea. In agreement with previously published results (41), we found that hydroxyurea increased the phosphorylation of histone H2AX at Ser139 (also called  $\gamma$ -H2AX). Foci of  $\gamma$ -H2AX colocalize with several proteins, including MRN complexes and BRCA1, and may be important in retention of repair factors to double-strand breaks (42). We found that caffeine had no effect on histone H2AX<sup>Ser139</sup> phosphorylation. As a control, caffeine was able to reduce the phosphorylation of ATM (data not shown) and p53 (Supplementary Fig. S6) caused by topoisomerase poison-mediated DNA damage. Collectively, these data show that the induction of p53 during the replication checkpoint is carried out by a caffeine-insensitive mechanism.

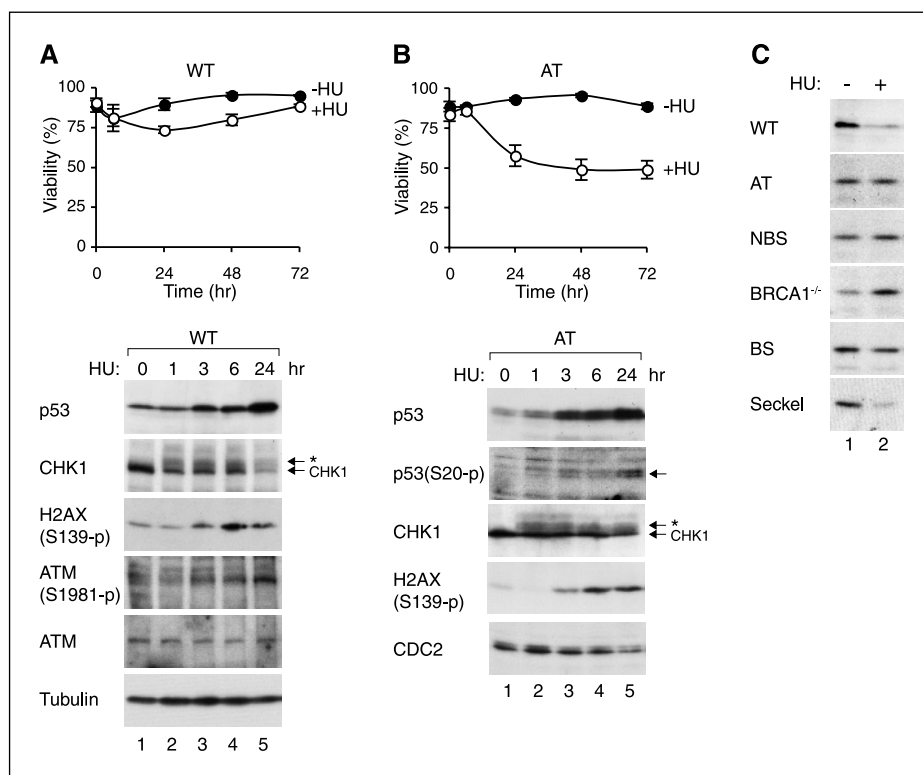
**ATM is not required for the induction of p53 by the replication checkpoint.** Given that ATM was activated by hydroxyurea, we next investigated directly whether ATM is

required for the stabilization of p53 during the replication checkpoint. We compared the hydroxyurea responses in cells derived from normal and ataxia telangiectasia individuals. Figure 3A verified that endogenous ATM was absent in the ataxia telangiectasia lymphoblastoid cells. The cells were treated with hydroxyurea for 24 hours before they were allowed to recover for another 48 hours. The idea is that checkpoint-defective cells would not halt the cell cycle even in the presence of hydroxyurea, resulting in premature chromosome condensation and cell death. Figure 4A shows that the viability of wild-type lymphoblastoid cells remained constant during and after hydroxyurea treatment. Furthermore, cell proliferation was promptly restored after hydroxyurea was removed (data not shown). In marked contrast, the viability of ataxia telangiectasia cells was significantly reduced in the presence of hydroxyurea and remained low after hydroxyurea was removed (Fig. 4B), indicating that the replication checkpoint was defective in ATM<sup>-/-</sup> cells.

In wild-type lymphoblastoid cells, p53 was induced by hydroxyurea in a time-dependent manner (Fig. 4A). Consistent with the activation of the replication checkpoint, CHK1 was rapidly phosphorylated (mobility shift was detected 1 hour after hydroxyurea addition). Interestingly, histone H2AX<sup>Ser139</sup> phosphorylation was only transient and reduced at later time points. Despite their hypersensitivity to hydroxyurea, ataxia telangiectasia lymphoblastoid cells accumulated p53 normally (Fig. 4B). Side-by-side comparison showed that ataxia telangiectasia cells contained comparable levels of p53 to wild-type cells after hydroxyurea treatment (data not shown). Also similar to wild-type cells, both CHK1 and histone H2AX were phosphorylated in ataxia telangiectasia cells after hydroxyurea treatment.

For a comparison with the responses to hydroxyurea, the responses to DNA damage in wild-type and ataxia telangiectasia cells were also examined. We found that p53 was stabilized after irradiation in both cell types (Supplementary Fig. S7). Similarly, ionizing radiation triggered histone H2AX<sup>Ser139</sup> phosphorylation and reduced histone H3<sup>Ser10</sup> phosphorylation in a dose-dependent manner. The increase in histone H3<sup>Ser10</sup> phosphorylation at higher ionizing radiation doses was probably due to apoptosis triggered by severe DNA damage as previously reported (40, 43). In spite of the induction of p53, the ionizing radiation-mediated DNA damage checkpoint was clearly abnormal in ataxia telangiectasia cells. DNA damage in wild-type cells was associated with an inhibition of DNA synthesis (Supplementary Fig. S7). In contrast, the G<sub>1</sub> population decreased and DNA synthesis continued after irradiation in ataxia telangiectasia cells (radioresistant DNA synthesis). These results indicate that the defects in the replication checkpoint and DNA damage checkpoint in ataxia telangiectasia cells are not due to a faulty mechanism to induce p53.

**Induction of p53 does not correlate with defective replication checkpoint in various genetic disorders.** The above results show that the checkpoint defects in ataxia telangiectasia cells do not correlate with any impairment in p53 induction. To see if p53 induction is generally not correlated with the replication checkpoint, we next examined cells from several genetic disorders that display hypersensitive to replication stress. The replication checkpoint was displayed both in terms of inhibition of histone H3<sup>Ser10</sup> phosphorylation and recovery after hydroxyurea treatment. Figure 4C shows that whereas histone H3<sup>Ser10</sup> phosphorylation decreased after hydroxyurea treatment in wild-type lymphoblastoid cells, it remained phosphorylated in lymphoblastoid cells with defective ATM, NBS1, BRCA1, or BLM.



**Figure 4.** Induction of p53 by the replication checkpoint does not require ATM. *A*, wild-type cells remain viable after hydroxyurea treatment. Lymphoblastoid cells were treated with either buffer or hydroxyurea for 24 hours before they were washed and incubated in normal growth medium. Viability was measured by trypan blue exclusion analysis (*top*). Points, mean of three independent experiments; bars, SD. Cells were treated with hydroxyurea for the indicated time and subjected to immunoblotting for the indicated proteins (*bottom*). The expression of tubulin confirms uniform loading of extracts. \*, position of phosphorylated CHK1. *B*, ataxia telangiectasia cells have a normal p53 response but are hypersensitive to hydroxyurea. Lymphoblastoid cells from ataxia telangiectasia patient were treated with either buffer or hydroxyurea for 24 hours before they were washed and incubated in normal growth medium. Viability was measured by trypan blue exclusion analysis (*top*). Points, mean of three independent experiments; bars, SD. Cells were treated with hydroxyurea for the indicated time and subjected to immunoblotting for the indicated proteins (*bottom*). *C*, defective inhibition of histone H3<sup>Ser10</sup> phosphorylation after hydroxyurea treatment in various genetic disorders. Lymphoblastoid cells from normal individual (WT), ataxia telangiectasia, NBS, BRCA1-mutated, Bloom syndrome, or Seckel syndrome were treated with hydroxyurea for 12 hours. Cell extracts were prepared and phospho-histone H3<sup>Ser10</sup> was detected by immunoblotting. Uniform loading of lysates for each cell type was confirmed by immunoblotting for tubulin (data not shown).

The hydroxyurea sensitivity, induction of p53, CHK1 phosphorylation, and histone H2AX<sup>S139</sup> phosphorylation in various disorders are summarized in Table 1. For an illustration, Fig. 5*A* and *B* shows that BRCA1<sup>-/-</sup> cells, but not BRCA2<sup>-/-</sup> cells, were hypersensitive to hydroxyurea. Despite the differences in their sensitivity to hydroxyurea, p53 was induced in both cell types (Fig. 5*A* and *B*). CHK1 and histone H2AX<sup>Ser139</sup> were also similarly phosphorylated after hydroxyurea treatment in both cell types. Indeed, we found that there was no obvious correlation between the ability to induce p53 and the sensitivity to hydroxyurea in these disorders (Table 1).

Several lines of evidence suggest that hydroxyurea does not trigger histone H2AX<sup>Ser139</sup> phosphorylation through the same pathway as double-strand breaks. We found that hydroxyurea only induced a low level of double-strand breaks in comparison with DNA damaging agents (Fig. 3*B*). Furthermore, the hydroxyurea-induced histone H2AX<sup>Ser139</sup> phosphorylation was insensitive to caffeine (Fig. 3*C*), suggesting that ATM or related kinases are not involved. In agreement with this, histone H2AX<sup>Ser139</sup> was still phosphorylated after replication block in ATM<sup>-/-</sup> cells (Fig. 4*B*). Histone H2AX<sup>Ser139</sup> phosphorylation also

**Table 1.** Hydroxyurea responses in lymphoblastoid cells

| Cells                      | p53 accumulation | CHK1 phosphorylation | H2AX phosphorylation | Hydroxyurea sensitivity |
|----------------------------|------------------|----------------------|----------------------|-------------------------|
| Wild-type                  | +                | +                    | +                    | -                       |
| Ataxia telangiectasia      | +                | +                    | +                    | +                       |
| Bloom syndrome             | -                | -                    | -                    | +                       |
| Breast cancer (BRCA1)      | +                | +                    | +                    | +                       |
| Breast cancer (BRCA2)      | +                | +                    | +                    | -                       |
| Fanconi anemia (FANCD2)    | +                | +                    | +                    | -                       |
| Nijmegen breakage syndrome | -                | +                    | +                    | +                       |
| Seckel syndrome            | +                | -                    | +                    | +                       |

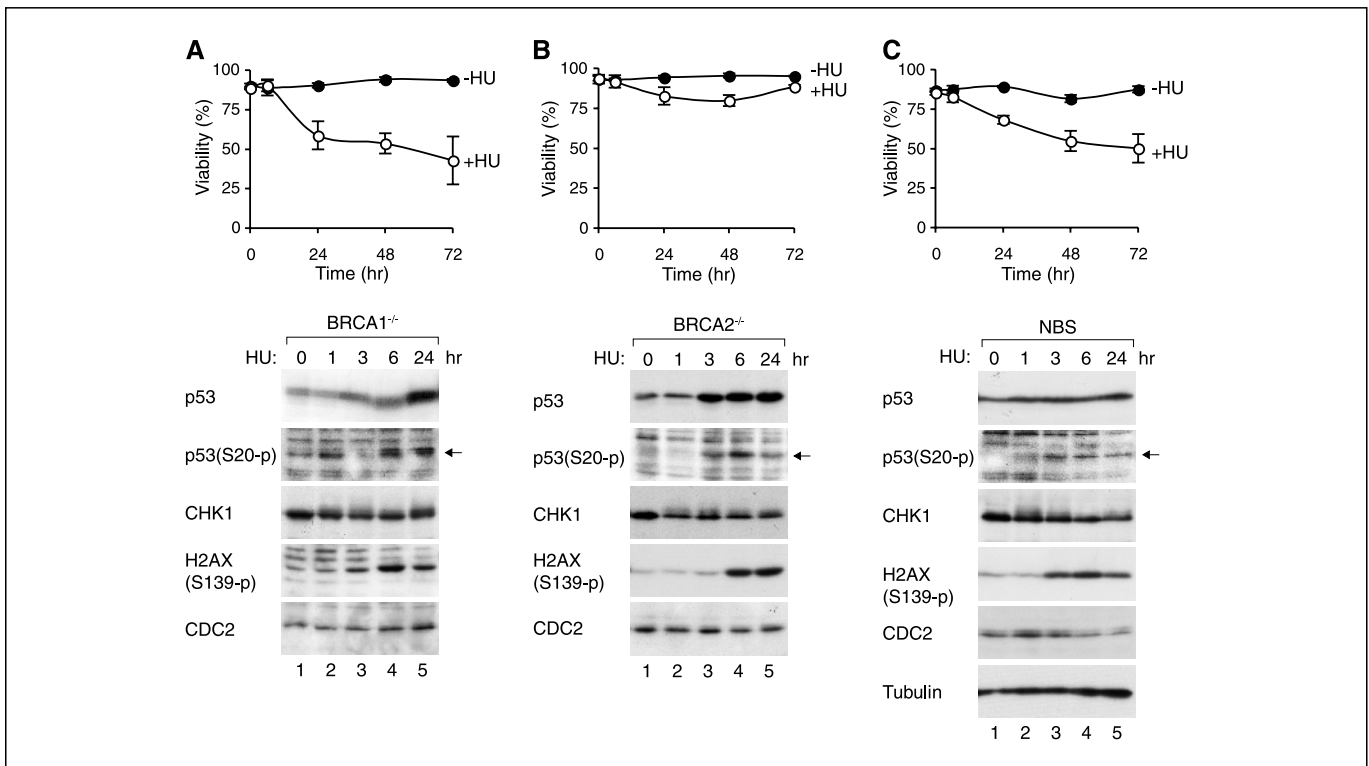
occurred after hydroxyurea treatment in cells lacking BRCA1, BRCA2, or NBS1 (Table 1). In contrast, histone H2AX<sup>Ser139</sup> phosphorylation was only poorly stimulated by hydroxyurea (or ionizing radiation) in cells lacking BLM. This differs from the results obtained by Sengupta et al. (44), which showed that  $\gamma$ -H2AX foci are induced by hydroxyurea even in Bloom syndrome fibroblasts. Interestingly, following an initial increase, histone H2AX<sup>Ser139</sup> phosphorylation invariably decreased at later time points after hydroxyurea treatment. This suggests that histone H2AX<sup>Ser139</sup> phosphorylation may not be required for the maintenance of the checkpoint.

We found that NBS cells (and, to a lesser extent, Bloom syndrome cells) were defective in p53 induction during replication block. NBS cells were hypersensitive to hydroxyurea (Fig. 5C) and p53 was only slightly induced (Fig. 5C). Responses to stalled replication forks were not completely deregulated in NBS cells because phosphorylation of CHK1 and histone H2AX<sup>Ser139</sup> still occurred normally. Similarly, DNA damage-mediated p53 accumulation was also impaired in NBS cells (Supplementary Fig. S8A). Analysis of BrdUrd incorporation indicated that DNA synthesis was inhibited after DNA damage (Supplementary Fig. S8B). The sub-G<sub>1</sub> population (indicative of apoptotic cell death) was significantly increased after ionizing radiation exposure in comparison with wild-type cells (Supplementary Fig. S8C), underscoring the importance of NBS1 for the DNA damage checkpoint. These data indicate that functional NBS1 and BLM are important for the p53 response during the replication checkpoint.

## Discussion

The basis of this study is that stalled replication forks induce p53 and the replication checkpoint itself cannot be maintained in the absence of p53 (see Introduction). Mechanistically, exactly how p53 contributes to the replication checkpoint remains elusive. This is accentuated by the conflicting reports on whether p53 is transcriptionally competent to activate downstream targets during the replication checkpoint (30, 31). Hydroxyurea effectively activated the replication checkpoint as indicated by the loss of histone H3<sup>Ser10</sup> phosphorylation (Fig. 1A), maintenance of cell viability (Fig. 4A), and an early S-phase block (37, 39). Side-by-side comparison with DNA damage indicated that hydroxyurea only induced p21<sup>CIP1/WAF1</sup> weakly in several p53-containing cell lines, including HepG2 (Fig. 1A), MCF7 (Fig. 1B), and lymphoblastoid cells (data not shown). Although p53 was readily induced by hydroxyurea in different cell lines, the levels attained were significantly lower than those after DNA damage. This may partly be explained by the relatively high level of MDM2 (Fig. 1B). The degree of p53 accumulation may explain the discrepancies in the previously published data on whether p53 transcriptional targets like p21<sup>CIP1/WAF1</sup> are activated during the replication checkpoint (30, 31).

Our experiments indicated that p21<sup>CIP1/WAF1</sup> was only weakly induced and was not required for maintaining viability after hydroxyurea treatment (Fig. 1C and D). What then is the function of p53 during the replication checkpoint? One possibility is that other transcriptional targets of p53 (like PIC3) are important for preventing cell cycle progression during replication blockade.



**Figure 5.** Cells lacking BRCA1 and BRCA2 have different hydroxyurea sensitivities but normal p53 responses, and NBS cells are defective in hydroxyurea-induced accumulation of p53. *A*, cells lacking BRCA1 are hypersensitive to hydroxyurea. BRCA1<sup>-/-</sup> lymphoblastoid cells were treated with hydroxyurea and analyzed by trypan blue exclusion as in Fig. 4A (top). Cells were treated with hydroxyurea for the indicated time and subjected to immunoblotting for the indicated proteins (bottom). *B*, cells lacking BRCA2 cells are not sensitive to hydroxyurea. Experiments were done as in (A) except that BRCA2<sup>-/-</sup> cells were used. *C*, NBS cells are hypersensitive to hydroxyurea. NBS lymphoblastoid cells were treated with hydroxyurea and the viability analyzed by trypan blue exclusion as in Fig. 4A (top). Cells were treated with hydroxyurea and harvested at the indicated time. Cell extracts were prepared and were subjected to immunoblotting for the indicated proteins (bottom). Equal loading of lysates was confirmed by immunoblotting for tubulin.

Another possibility is that the replication checkpoint may involve transcriptional repression by p53, as is proposed for the G<sub>2</sub> arrest function of p53 (45). Not surprisingly, induction of p53 alone was not sufficient to maintain the replication checkpoint as several genetic disorders that displayed defective replication checkpoint still accumulated p53 after hydroxyurea treatment (Table 1). This indicates the potential complexity of the replication checkpoint mechanisms.

In reflection of its central role in cell proliferation, p53 is known to be regulated by a myriad of pathways after different types of stress. Yet, redundancies seem to be the norm in these pathways. Similar to after DNA damage, ATM is activated during replication blockade (Figs. 3A and 4A). However, p53 accumulated after hydroxyurea treatment in both wild-type and ataxia telangiectasia cells (Fig. 4A and B), indicating that ATM-independent mechanisms are involved. This is not too surprising as p53 was also stabilized after ionizing radiation-induced (Supplementary Fig. S7) or topoisomerase poison-induced damage (40) in ataxia telangiectasia cells. Interestingly, whereas caffeine could bypass the DNA damage-induced p53 (Supplementary Fig. S6), the hydroxyurea-induced p53 was insensitive to caffeine (Fig. 3C). Moreover, caffeine could abolish ATM activation after DNA damage but not after replication block.

CHK2 lies downstream of ATM and can be activated by stalled replication forks (46, 47). Side-by-side comparison with wild-type MEFs indicated that unstressed CHK2<sup>-/-</sup> MEFs contained low level of p53 (Fig. 2B), suggesting that CHK2 controlled the basal expression of p53. Despite its low expression, p53 was nevertheless stabilized by hydroxyurea in CHK2<sup>-/-</sup> cells, suggesting that the hydroxyurea-induced stabilization of p53 does not require CHK2. A good candidate for regulating p53 during the replication checkpoint is CHK1. CHK1<sup>-/-</sup> MEFs cannot be obtained as disruption of *CHK1* gene results in early lethality. We found that knockdown of CHK1 expression by shRNA diminished the basal expression of p53 (Fig. 2D). We have previously shown that the same CHK1 shRNA construct abolished the replication checkpoint in HeLa cells (which lack p53 due to the presence of human papillomavirus E6; ref. 33). Similar to cells with *CHK2* disruption, p53 was still induced by hydroxyurea in cells expressing CHK1 shRNA, suggesting that the hydroxyurea-induced stabilization of p53 does not require CHK1. A caveat is that we cannot exclude the possibility that p53 was still regulated by the residual CHK1 after the knockdown.

If the ATM/ATR-CHK1/CHK2 axis is indeed not essential for p53 induction by hydroxyurea, what is the major mechanism of p53 accumulation during stalled replication? One possibility is the modification of p53 by acetylation. We found that hydroxyurea strongly induced the acetylation of p53 on Lys382 (Fig. 1B and

Supplementary Fig. S2). It would be interesting to see if p300, CREB binding protein, or other proteins that are known to modify the COOH-terminal lysine residues are activated during the replication checkpoint.

Although p53 accumulated normally after hydroxyurea treatment in ataxia telangiectasia cells, these cells have impaired replication checkpoint (Fig. 4B and C). Similarly, although hydroxyurea was able to induce p53 in cells with BRCA1 mutations and from Seckel syndrome, these cells were hypersensitive to hydroxyurea (Table 1). The Seckel syndrome cells used here were probably not caused by a disruption of *ATR*, as *ATR* protein was detectable in the lysates by immunoblotting.<sup>1</sup> In contrast, both the hydroxyurea-induced p53 and the replication checkpoint seemed to be functional in cells with a wild-type background, BRCA2, or FANCD2 mutations. The lack of correlation between p53 induction and hydroxyurea sensitivity indicates that p53 is not the sole determinant of the replication checkpoint. Interestingly, induction of p53 by hydroxyurea was impaired in NBS cells (Fig. 5C) and Bloom syndrome cells. Indeed, the BLM helicase is known to be involved in the efficient localization of the MRN complex (48) and p53 (49) to sites of stalled DNA replication forks. Another indication that the MRN complex may be involved in the replication checkpoint is that MRE11 was phosphorylated during replication block (Supplementary Fig. S5).

Aside from their usefulness in studying the replication checkpoint, replication blockers are important chemotherapeutic agents. Hydroxyurea is used to control the chronic phase of myeloid leukemia and other hematologic malignancies. It is interesting that cells from many genetic disorders examined here are hypersensitive to hydroxyurea, suggesting that replication blockers may be attractive agents for treatment of cancers associated with these mutations.

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<sup>1</sup> Unpublished data.

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## Stalled Replication Induces p53 Accumulation through Distinct Mechanisms from DNA Damage Checkpoint Pathways

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