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A Role of the Mitotic Spindle Checkpoint in the Cellular Response to DNA Replication Stress

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Abstract Replication stress is a frequent and early event during tumorigenesis. Whereas the cellular responses to a persistent block of replication fork progression have been extensively studied, relatively little is known about how cells respond to low-intensity replication stress. However, transient replication fork perturbations are likely to occur even more frequently in tumor cells than a permanent replication arrest. We report here that transient, low intensity replication stress leads to a rapid activation of the DNA replication checkpoint but to a significantly delayed apoptotic response in a small but significant number of cells. This late apoptotic response was independent of p53 and we found evidence for cell death during mitosis in a proportion of cells. To further explore the role of p53 in the response to replication stress, we analyzed mouse embryonic fibroblasts (MEFs) deficient of p53 in comparison to wild-type or p63- or p73-deficient MEFs. We detected a significant increase of apoptosis and morphological signs of failed mitosis such as multinucleation in p53-deficient MEFs following replication stress, but not in wild-type or p63- or p73-deficient cells. Multinucleated p53-deficient MEFs frequently retained cyclin B1 expression indicating a persistently activated mitotic spindle checkpoint. Collectively, our results suggest that the cellular response to replication stress involves the mitotic spindle checkpoint in a proportion of cells. These findings imply that the mitotic spindle checkpoint may act in concert with DNA damage and cell-cycle checkpoints as an early anti-tumor barrier and provide a possible explanation for its frequent relaxation in human cancer. J. Cell. Biochem. 99: 759–769, 2006.

Key words: replication stress; mitotic spindle checkpoint; p53; cancer

DNA damage and cell-cycle checkpoints are critical to protect genome integrity and to suppress tumorigenesis [Kastan and Bartek, 2004]. DNA replication is a particularly vulnerable phase of the cell division cycle. The major S phase-associated checkpoints are the DNA replication checkpoint, the intra-S-phase checkpoint, and the S–M checkpoint [Osborn et al., 2002; Bartek et al., 2004]. The DNA replication checkpoint is activated in response to stalled or perturbed replication forks. This so-called replication stress can originate from suboptimal deoxyribonucleotide (dNTP) levels, damaged or otherwise difficult to replicate DNA, or inhibition of DNA polymerase activity. The main functions of the DNA replication checkpoint are to downregulate DNA replication from unfired origins through modulation of cyclin/CDK and CDC7–DBF4 kinase activity [Jares et al., 2000] and to protect replication fork integrity in order to facilitate their restart when optimal replication conditions are restored.

A key event during DNA replication checkpoint activation is the phosphorylation of CHK1 by the *Ataxia teleangiectasia* mutated-Rad3-related kinase (ATR) [Osborn et al., 2002]. Activated CHK1 phosphorylates downstream targets including CDC25C, a phosphatase that removes the inhibitory phosphorylation of the mitotic CDK1 at tyrosines 14 and 15 [Enoch et al., 1992; Parker and Piwnica-Worms, 1992]. Phosphorylation of CDC25C by CHK1 inhibits its phosphatase activity and hence suppresses CDK1 activation and mitotic entry. Other

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proteins that can be phosphorylated by ATR are p53 [Tibbetts et al., 1999] and histone variant H2AX [Ward and Chen, 2001].

It is conceivable that DNA replication stress is a frequent event during unscheduled DNA replication and it has recently been shown that persistent oncogene activation leads to altered replication dynamics and prolonged activation of the ATR–CHK1 signaling pathway [Bartkova et al., 2005]. DNA damage checkpoint activation can be detected already in precancerous lesions [Bartkova et al., 2005; Gorgoulis et al., 2005] and it has, therefore, been proposed that the DNA damage response functions as an initial anti-tumor barrier [Bartkova et al., 2005]. Support for this notion stems also from experiments showing that deregulated cyclin expression can cause a high level of replication fork stalling due to impaired assembly of pre-replicative complexes [Tanaka and Diffley, 2002].

Given the primarily protective role of the DNA replication checkpoint on fork integrity and function [Lopes et al., 2001; Tercero et al., 2003], re-start of S phase [Desany et al., 1998] and cell viability [Cho et al., 2005], the question arises how cells that have acquired intolerable DNA damage during replication stress are eliminated. In most studies, cells are treated with replication arrest-inducing agents for a prolonged period of time. It is highly likely, however, that transient replication fork perturbations are even more common in tumor cells than a permanent replication block. Therefore, we focused here on the cellular responses to low-intensity and transient replication stress. We found that transient replication stress elicits a rapid activation of CHK1 that is associated with a temporary downregulation of DNA replication. An apoptotic response, however, occurred with a considerable delay in a small but significant proportion of cells and involved mitotic catastrophe. When mouse embryonic fibroblasts (MEFs) with permanently deleted p53 were challenged with transient replication stress, cells showed signs of aberrant exit from mitosis and a persistently activated mitotic spindle checkpoint. Collectively, our results suggest a role of the mitotic spindle checkpoint in the cellular response to transient replication stress. Given the high frequency of replication stress in tumor cells, these findings help to explain the selection pressure leading to relaxation of the mitotic spindle checkpoint function during transformation [Lengauer et al., 1998]. In addition, our results have implications for the use of chemotherapeutic agents that cause replication stress in tumors that have lost the p53 tumor suppressor protein.

METHODS

Cell Lines and Treatment

U-2 OS cells (ATCC, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium (DMEM; Cambrex, Walkersville, MD) supplemented with 10% fetal bovine serum (FBS; Mediatech, Herndon, VA), 50 U/ml penicillin (Cambrex), and 50 μg/ml streptomycin (Cambrex). MEFs deficient of p53 were kindly provided by Dr. Tyler Jacks (Massachusetts Institute of Technology, Cambridge, MA). MEFs deficient of p63 or p73 were a generous gift of Dr. Elsa Flores (M. D. Anderson Cancer Center, Houston, TX). Early passage MEFs were cultured in DMEM containing heat-inactivated FBS and supplements as described above. Hydroxyurea (HU; Calbiochem, San Diego, CA) was dissolved in distilled water and added to FBS-containing DMEM cell culture media to a final concentration of 1 mM. Cells were treated for 1 h (U-2 OS) or 24 h (MEFs) or left untreated followed by washing with normal growth media and incubation in normal media for the indicated time intervals. Cells exposed to γ-irradiation were used as positive controls for DNA damage checkpoint activation and phosphorylation of histone H2AX. Briefly, U-2 OS cells grown in DMEM supplemented with 10% FBS as well as 50 U/ml penicillin and 50 μg/ml streptomycin were plated into 60 mm tissue culture plates and grown to about 50% confluency. Plates were irradiated with 10 Gy γ-irradiation (Gammacell, Nordion International, Kanata, ON, Canada) or left unirradiated and whole cell lysates were prepared after 3 h.

Immunological Methods

Whole cell lysates were prepared by scraping cells into lysis buffer (1% NP-40, 50 mM Tris-HCl pH 8.0, 100 mM NaF, 30 mM $Na_4P_2O_7$, 2 mM Na_2MoO_4, 5 mM ethylenediamine tetraacetic acid, 2 mM Na_3VO_4) containing protease inhibitors (10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 μM phenylmethylsulfonyl fluoride). Lysates were incubated for 1 h with shaking at 4°C and then cleared by centrifugation for 30 min at 13,000 rpm. Protein concentrations

were determined by Bradford assay (BioRad, Hercules, CA). Proteins (30 μg) were loaded on 4–12% *Bis-Tris* gels (Invitrogen, Carlsbad, CA) and blotted onto nitrocellulose membranes. Primary antibodies used for immunoblotting were directed against phosphorylated CHK1 (serine 345 and serine 317; Cell Signaling, Beverly, MA), CHK1 (Cell Signaling), cleaved caspase-3 (Cell Signaling), γ-H2AX (Upstate, Charlottesville, VA), PCNA (Santa Cruz, Santa Cruz, CA), p53 (Santa Cruz) and actin (Sigma, St. Louis, MO).

For immunofluorescence analyses, cells grown on coverslips were briefly washed in phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde (Sigma) in PBS for 15 min at room temperature. Cells were then washed in PBS and permeabilized with 1% Triton-X 100 (Sigma) in PBS for 15 min at room temperature followed by washing in PBS and blocking with 10% normal donkey serum (Jackson Immunoresearch, West Grove, PA) in distilled water for 15 min at room temperature. Cells were then incubated with anti-γ-H2AX primary antibodies (Trevigen, Gaithersburg, MD) or anti-cyclin B1 antibodies (Lab Vision, Fremont, CA) at a 1:100 dilution in PBS overnight at 4°C in a wet chamber and incubated for another 3 h at 37°C the next morning. After a brief wash in PBS, cells were incubated with Rhodamine Red- or FITC-conjugated secondary antibodies (Jackson Immunoresearch) at a 1:1,000 dilution or 1:200 dilution, respectively, for at least 2 h at 37°C, washed with PBS and counterstained with DAPI (Vector Laboratories, Burlingame, CA). For double-immunofluorescence, cells were sequentially stained for γ-H2AX and cyclin B1. Cells were analyzed using an Olympus AX70 epifluorescence microscope equipped with a SpotRT digital camera.

BrdU Labeling

Immunofluorescence detection of 5-bromo-2′-deoxyuridine (BrdU) incorporation into cellular DNA was performed in order to determine the fraction of cells with ongoing DNA replication according to the manufacturer's protocol (Roche Molecular Biochemicals, Indianapolis, IN). Mean fluorescence intensity of positive cells was measured using NIH Image software.

Small Interfering RNA (siRNA)

Synthetic RNA duplexes were used to reduce p53 protein expression [Elbashir et al., 2001]. Oligonucleotides targeting p53 and control oligos were obtained commercially (siGENOME SMARTpool; Dharmacon). For each experiment, U-2 OS cells were grown on coverslips in 60-mm tissue culture dishes with 2 ml DMEM free of antibiotics. Cells were transfected with 12 μl of 20 μM annealed RNA duplexes using Oligofectamine (Life Technologies) transfection reagent. After 24 h, the growth medium containing the RNA duplexes was removed and cells were treated for 1 h with 1 mM HU. After 1 h, cells was washed and the RNA duplex-containing growth medium were put back into the culture dishes for additional 48 h.

RESULTS

Transient Replication Stress Causes a Delayed Apoptotic Response

To explore the cellular response to transient replication stress, human U-2 OS osteosarcoma cells were treated for 1 h with 1 mM of the ribonucleotide reductase inhibitor HU or left untreated (Fig. 1A). HU induces a reversible stalling of replication forks and cells can re-enter the cell cycle after removal of the drug. U-2 OS cells have been extensively used for DNA damage studies and have *bona fide* intact DNA damage-sensing checkpoints [Lukas et al., 2003]. Cells were first analyzed at sequential time points for BrdU incorporation to measure DNA replication (Fig. 1). Because of the short treatment interval, we did not measure BrdU positivity but determined the BrdU staining intensity per cell. A decrease of DNA replication in HU-treated cells compared to untreated controls was detected immediately after the HU pulse (0 h; Fig. 1B,C). BrdU incorporation gradually recovered after 1 h and 3 h after the HU pulse and reached normal levels at 24 and 48 h (Fig. 1B,C).

To analyze activation of the DNA replication checkpoint, we performed immunoblot analyses of whole cell lysates from HU-treated cells at sequential time points and compared the results to untreated controls (Fig. 2). We wished to compare the level of the DNA damage response following low-intensity, transient replication stress to conditions under which a strong activation of the DNA damage response occurs. Treatment of cells with 10 Gy γ-irradiation has been previously reported to lead to a robust stimulation of DNA damage checkpoint signaling including CHK1 activation and

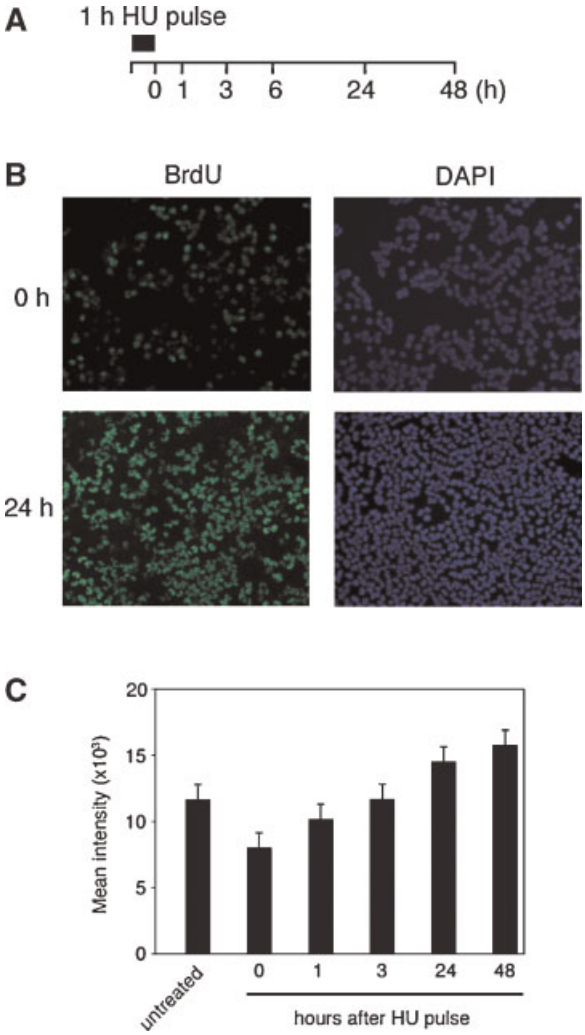

Fig. 1. Rapid downregulation of DNA replication after transient, low-intensity replication stress. **A**: Schematic representation of the experimental setup. **B**: Analysis of S phase progression in U-2 OS cells treated with 1 mM HU for 1 h using a BrdU incorporation assay at the indicated time points. Nuclei are stained with DAPI. **C**: Quantification of the mean fluorescence intensity of BrdU-positive cells either untreated or after 1 h HU pulse at the indicated time points. Mean and standard errors of triplicate measurements of at least 50 cells are shown. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

phosphorylation of H2AX [Paull et al., 2000; Gatei et al., 2003]. We, therefore, used whole cell lysates from U-2 OS populations harvested 3 h after 10 Gy γ-irradiation as positive controls (Fig. 2).

Phosphorylation of CHK1 at serine residues 345 and 317 by ATR is a hallmark of DNA replication stress checkpoint signaling [Zhao and Piwnica-Worms, 2001]. We found that 1 h pulse treatment of cells with HU results in a rapid increase of CHK1 phosphorylation at

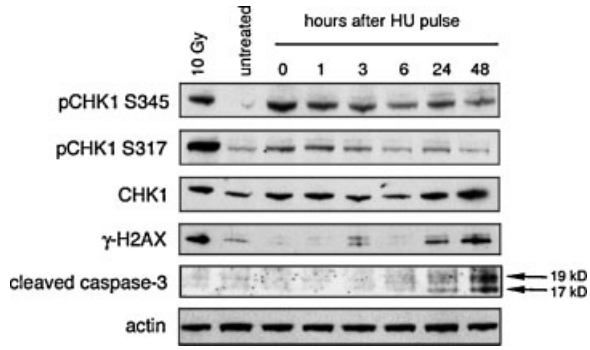

Fig. 2. Apoptosis is a late event after low-intensity replication stress. Immunoblot analysis of U-2 OS cells at sequential time points after 1 h HU treatment in comparison to untreated cells or cells exposed to 10 Gy γ-irradiation. Representative immunoblots for CHK1 phosphorylated at serine 345 (pCHK1 S345) and serine 317 (pCHK1 S317), total CHK1, γ-H2AX, and cleaved caspase-3. Immunoblot for actin is shown to demonstrate equal loading.

serine 345 (pCHK1 S345) when compared to untreated cells (Fig. 2). This increase of CHK1 phosphorylated at S345 immediately after the HU pulse (0 h) was similar to the level of CHK1 phosphorylation detected in cells exposed to 10 Gy γ-irradiation indicating that even transient replication stress elicits a vigorous checkpoint response. pCHK1 S345 levels did not return to the virtually undetectable level we found in untreated cells within 48 h. We also detected an increase of CHK1 phosphorylation at serine 317 (pCHK1 S317). The increase of pCHK1 S317 was less pronounced than that of pCHK1 S345 and pCHK1 S317 levels returned to control levels within 6 h after the HU pulse.

Histone variant H2AX is a critical nexus between DNA damage and activation of DNA damage signaling pathways [Rogakou et al., 1998; Celeste et al., 2002; Bassing et al., 2003] and becomes phosphorylated by ATR in response to replication stress [Ward and Chen, 2001]. Surprisingly, we found that the serine 139 phosphorylated form of H2AX (γ-H2AX) does not increase until 24 h after the HU pulse with highest levels at 48 h (Fig. 2). This late response together with the notion that H2AX becomes phosphorylated following cell death [Rogakou et al., 2000] and the fact that we did not use histone extraction methods in our assay prompted us to investigate whether cells undergo enhanced apoptosis. Caspase-3 is an effector caspase that is activated during cell death by limited proteolysis giving rise to protein fragments of 17 and 19 kDa [Boatright

and Salvesen, 2003]. We found that cleavage of caspase-3 was initiated at 24 h after removal of HU and that maximum levels were reached after 48 h (Fig. 2). The lack of caspase-3 cleavage in cells treated with 10 Gy γ-irradiation is most likely related to the fact that cells were harvested only 3 h post-irradiation.

To further corroborate that the increase of γ-H2AX was due to enhanced apoptosis in contrast to DNA double strand breaks (DSBs), an immunofluorescence and morphological analysis was performed (Fig. 3A). In cells with DNA DSBs, γ-H2AX forms foci in morphologically intact nuclei. When H2AX becomes phosphorylated during apoptosis [Rogakou et al., 2000], cells show nuclear changes typically associated with cell death including shrinkage and fragmentation and a more diffuse γ-H2AX staining pattern [Rogakou et al., 2000]. At 48 h after HU treatment, the proportion of cells with γ-H2AX foci in morphologically normal nuclei remained unchanged when compared to untreated cells (Fig. 3B). By contrast, a modest, but statistically significant 7.1-fold increase of γ-H2AX-positive cells undergoing apoptosis was detected in HU-treated populations in comparison to controls (1.2% vs. 0.17%; $P \leq 0.05$, Student's t-test for independent samples; Fig. 3B). Although these results cannot rule out the possibility that DNA DSBs form before cells undergo apoptosis, they suggest that the elevated γ-H2AX levels detected by immunoblotting are in fact caused by increased apoptosis.

Transient Replication Stress Can Lead to Mitotic Catastrophe

To explore whether re-entry into the cell division cycle (Fig. 1C) and enhanced apoptosis (Fig. 3B) may be functionally related, we tested the possibility that cells are eliminated by mitotic catastrophe. Two major subtypes of mitotic catastrophe have been proposed: cell death during metaphase and cell death after a failed mitosis [Castedo et al., 2004]. Failure to complete mitosis can also be associated with a chronic activation of the mitotic spindle checkpoint and adaptation of cells resulting in abnormally large interphase nuclei that are frequently multinucleated and accompanied by micronuclei [Weaver and Cleveland, 2005]. Double-immunofluorescence staining of HU-treated and untreated cells was performed 48 h after the HU pulse and the number of apoptotic cells co-expressing γ-H2AX and the mitotic cyclin B1 was determined (Fig. 4). In control cells, cyclin B1 expression was limited to pro-metaphase and metaphase cells whereas most apoptotic cells stained negative for cyclin B1 but had a diffuse γ-H2AX expression associated with the apoptotic chromatin (Fig. 4). In

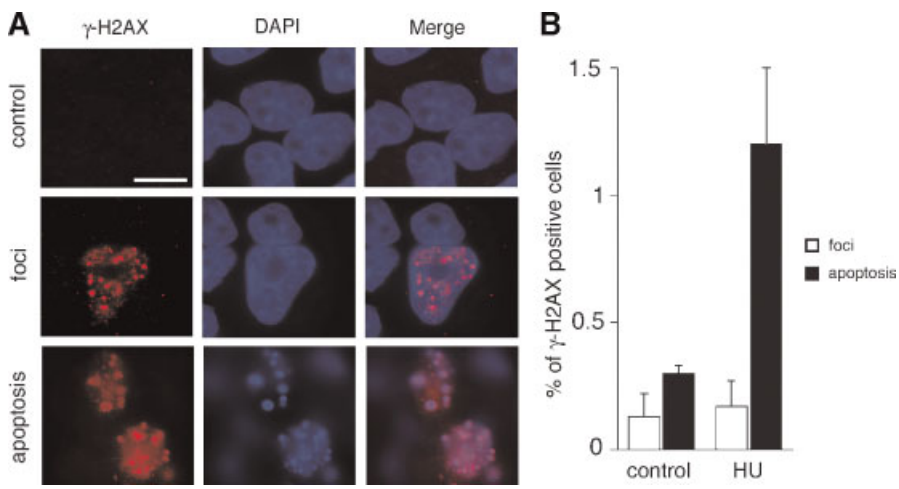

Fig. 3. γ-H2AX upregulation is associated with enhanced apoptosis after low-intensity replication stress. **A**: Immunofluorescence analysis of U-2 OS cells for γ-H2AX at 48 h after 1 h HU pulse treatment. Cells lacking γ-H2AX staining are shown as control (**top panels**). The proportion of cells displaying γ-H2AX foci in morphologically intact nuclei indicating DNA DSB repair (**middle panels**) remained unchanged. In contrast, a significant increase of γ-H2AX-positive cells with an apoptotic nuclear morphology was detected in HU pulse-treated populations (**bottom panels**). Nuclei stained with DAPI. Scale bar indicates 10 μm. **B**: Quantification of cells displaying γ-H2AX foci in intact nuclei or diffuse γ-H2AX staining associated with an apoptotic morphology in either untreated control populations or 48 h after 1 h HU pulse treatment. Mean and standard error of three independent experiments with at least 500 cells counted per experiment are shown. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

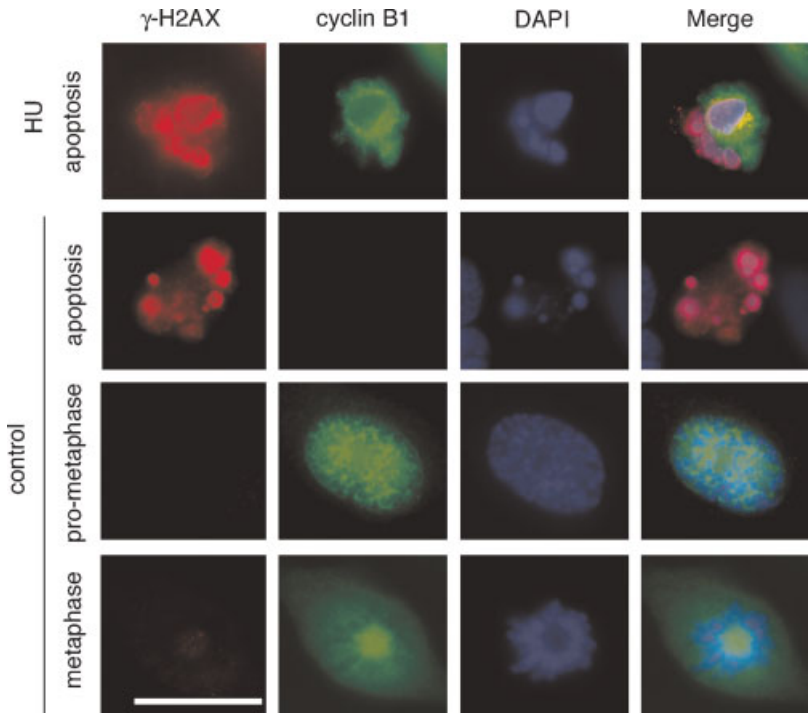

Fig. 4. Cell death during mitosis increases after low-intensity replication stress. Double-immunofluorescence analysis of U-2 OS cells undergoing apoptosis 48 h after a 1 h HU pulse. Staining for γ-H2AX (red) and cyclin B1 (green) shows co-expression of both proteins in a HU-treated apoptotic cell (**top panels**). Apoptosis in an untreated control cell is associated with diffuse positivity for γ-H2AX, but not cyclin B1 (**top control panels**). Normal cyclin B1 staining pattern in a pro-metaphase cell (**middle control panels**) and metaphase cell (**bottom control panels**) is shown in comparison. Nuclei stained with DAPI. Scale bar indicates 10 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

HU-treated cell populations, we found 43 out of 217 apoptotic cells that co-expressed γ-H2AX and cyclin B1 (19.8%) compared to 4 out of 148 apoptotic cells in untreated control populations (2.7%). The co-incidence of apoptotic chromatin condensation, diffuse γ-H2AX staining, and cyclin B1 expression led us to conclude that a proportion of cells dies during or close to metaphase. Our results, therefore, suggest that mitotic catastrophe is involved in the elimination of a fraction of cells that have acquired intolerable damage during replication stress.

Depletion of p53 Does not Prevent Replication Stress-Induced Apoptosis

To determine whether the apoptotic response following transient replication stress depends on p53, we performed small interfering RNA (siRNA) experiments to deplete p53 protein levels in U-2 OS cells before cells were treated with 1 mM HU for 1 h (Fig. 5A,B). Short-term replication stress-induced apoptosis was not reduced in cells in which p53 protein has been knocked down (Fig. 5C) suggesting that this response does not require p53.

Transient Replication Stress in p53-Deficient Cells Causes Prolonged Mitotic Spindle Checkpoint Activation

To further explore the role of p53 and p53 family members in the cellular response to transient replication stress, we used MEFs derived from animals deficient of p53, p63, or p73, respectively [Flores et al., 2002]. Since a 1 h HU pulse did not result in increased apoptosis (data not shown), we extended the HU treatment interval to 24 h followed by a biochemical analysis at 24 h, 48 h, and 72 h after removal of the drug (Fig. 6A). An increased cleavage of caspase-3, which is associated with apoptosis, was detected in HU-treated $p53^{-/-}$ MEFs and these cells failed to downregulate proliferation as measured by PCNA expression when compared to wild-type cells. In contrast, $p63^{-/-}$ and $p73^{-/-}$ MEFs showed a partial downregulation of proliferative activity in response to HU treatment (Fig. 6B).

A morphological analysis of MEFs at 72 h revealed an increase of interphase cells with multinucleation (three or more nuclei per cell)

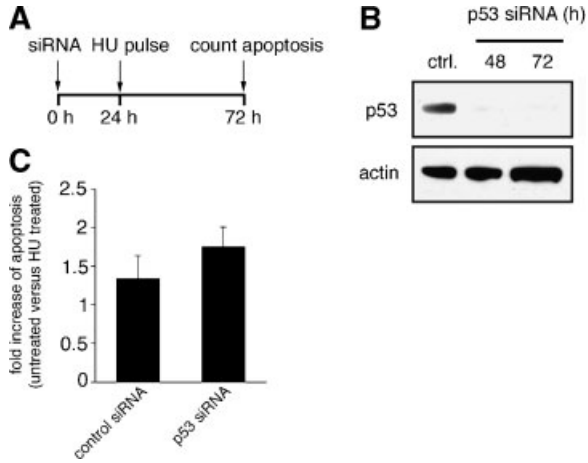

Fig. 5. Depletion of p53 does not prevent replication stress-induced apoptosis. **A**: Schematic representation of the experimental setup. **B**: Immunoblot analysis of p53 protein expression in cells transfected with siRNA duplexes targeting p53 or control RNA duplexes at the indicated time points post transfection. Whole cell lysates were used. Immunoblot for actin is shown to demonstrate equal loading. **C**: Quantification of fold increase of apoptotic cells after 1 h HU pulse treatment of cell populations transfected with either control siRNA or p53 siRNA. All counts were performed at 48 h post HU pulse treatment. Fold-changes represent averages from at least three independent experiments.

in $p53^{-/-}$ populations, which was frequently accompanied by extensive micronucleus formation (Fig. 6C). Micronuclei are typically found in cells that have undergone mitotic catastrophe in the sense of aberrant exit from an attempted but failed mitosis. In addition, $p53^{-/-}$ MEFs with multinucleation after HU treatment frequently showed numerous γ-H2AX foci indicating DNA damage (Fig. 6C). A quantitative analysis revealed a significant increase of multinucleated cells displaying γ-H2AX foci in Hu-treated $p53^{-/-}$ MEFs (34.4%; Fig. 6D) when compared to Hu-treated wild-type cells (4%; $P \leq 0.001$; Student's t-test for independent samples). There was no significant increase of such cells in $p63^{-/-}$ or $p73^{-/-}$ MEF populations or untreated MEF ($P > 0.05$; Student's t-test for independent samples).

Cyclin B1 accumulates in the cytoplasm during S phase and G2 and is imported into the nucleus at the end of G2 [Takizawa and Morgan, 2000; Pines and Rieder, 2001]. During a normal cell division, cyclin B becomes degraded by the anaphase promoting complex (APC) at the metaphase–anaphase transition. Mitotic spindle checkpoint activation inhibits

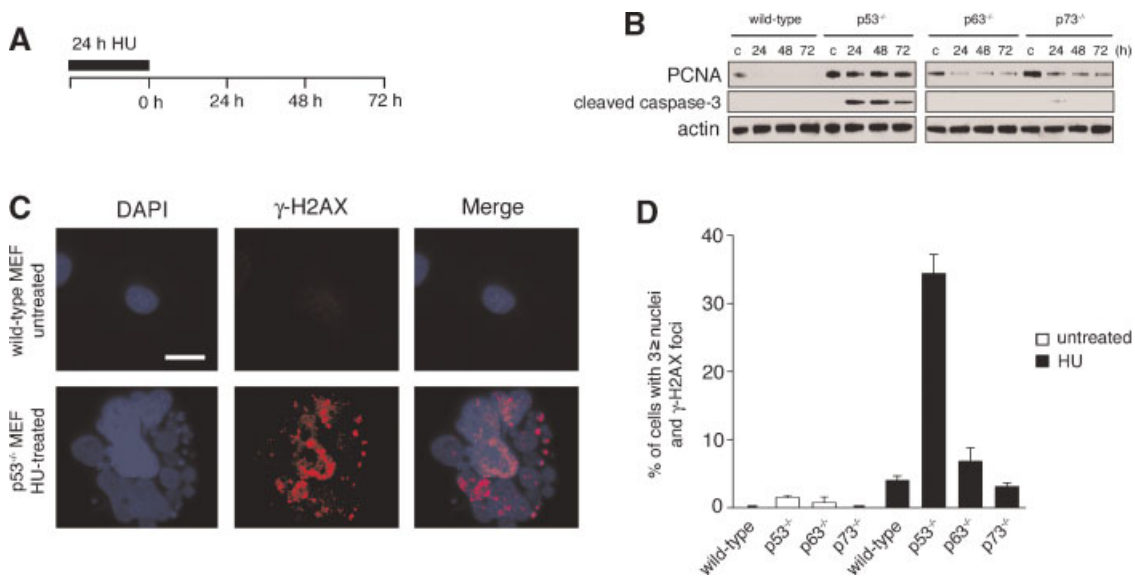

Fig. 6. Transient replication stress in p53-deficient MEFs promotes apoptosis and an accumulation of multinucleated cells. **A**: Schematic representation of the experimental setup. **B**: Immunoblot analysis of wild-type MEFs in comparison to $p53^{-/-}$, $p63^{-/-}$, or $p73^{-/-}$ MEFs for PCNA and cleaved caspase-3 at the indicated time points following 24 h HU treatment or left untreated. Immunoblot for actin is shown to demonstrate equal loading. **C**: Immunofluorescence analysis of wild-type (**top panels**) or $p53^{-/-}$ MEFs (**bottom panels**) for γ-H2AX expression either untreated or 72 h after HU treatment. Note the multinucleation and extensive formation of γ-H2AX-positive micronuclei in a HU-treated $p53^{-/-}$ MEF (**bottom panels**). Nuclei stained with DAPI. Scale bar indicates 10 μm. **D**: Quantification of MEFs with three or more nuclei and γ-H2AX foci either untreated (open bars) or 72 h after HU treatment (black bars). Mean and standard error of three independent experiments with at least 100 cells counted per experiment are shown. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

the APC thereby preventing the drop of cyclin B1 protein expression. Prolonged or aberrant expression of cyclin B1 can, therefore, be used to determine activation of the mitotic spindle checkpoint [Whitfield et al., 1990; Geley et al., 2001]. We analyzed p53-deficient MEFs for cyclin B1 expression at 72 h after removal of HU using immunofluorescence microscopy (Fig. 7A). No increase of cells with apoptotic chromatin condensation and cyclin B1 expression was detected. However, we found that 37.5% of $p53^{-/-}$ multinucleated MEFs with decondensed chromatin expressed predominantly nuclear cyclin B1 compared to 2% in

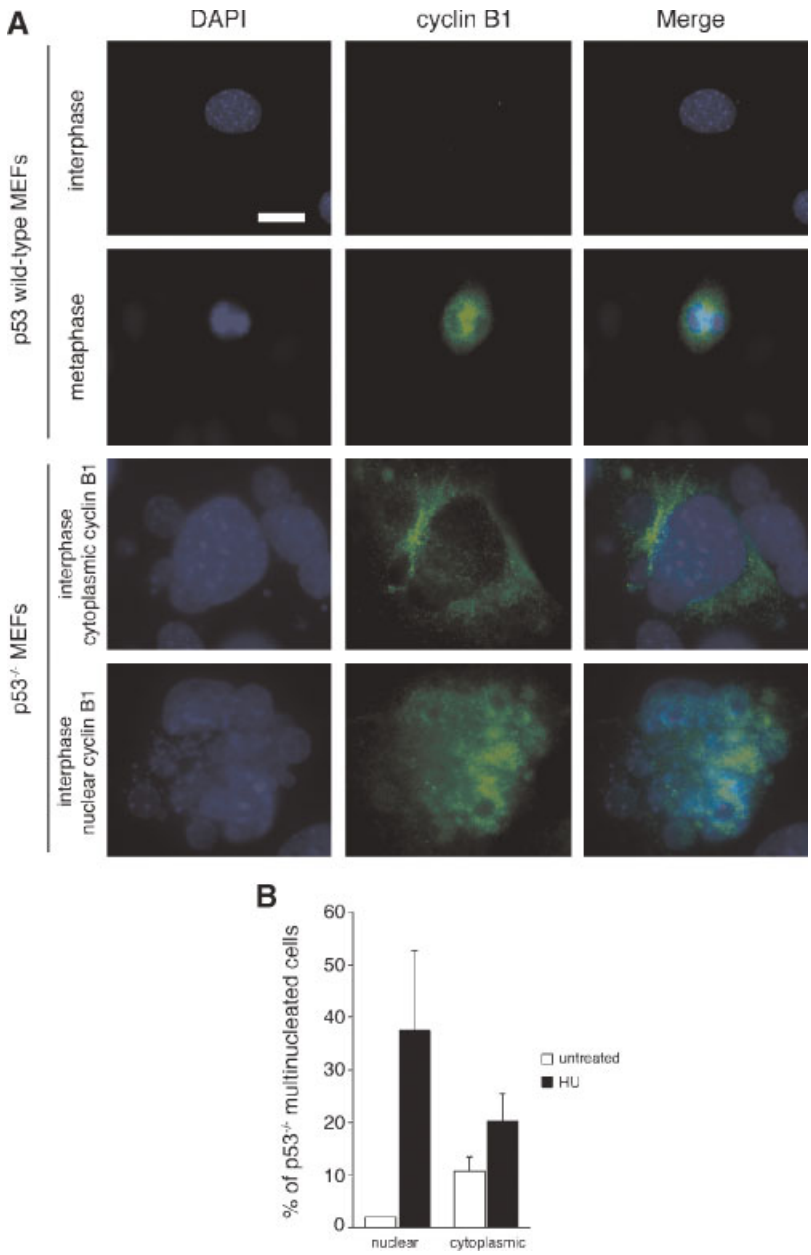

Fig. 7. p53-deficient multinucleated MEFs show persistent cyclin B1 expression. **A**: Immunofluorescence analysis of wild-type and $p53^{-/-}$ MEFs for cyclin B1 expression. Cyclin B1 is non-detectable in non-dividing MEFs (**top panels**) and is expressed at high levels in metaphase cells (**second from the top panels**). Note the accumulation of cytoplasmic (**second from the bottom panels**) or nuclear cyclin B1 (**bottom panels**) in interphase nuclei of $p53^{-/-}$ MEFs. Nuclei stained with DAPI. Scale bar indicates 10 μm. **B**: Quantification of $p53^{-/-}$ MEFs for nuclear or cytoplasmic cyclin B1 expression either untreated or 72 h after HU treatment. Mean and standard errors of a representative experiment with triple quantification of at least 50 cells are shown. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

untreated populations ($P \leq 0.05$; Mann–Whitney *U*-test; Fig. 7B). We also detected an increase of cells with decondensed chromatin and mostly cytoplasmic cyclin B1, albeit less pronounced, in HU-treated $p53^{-/-}$ MEFs (20.2%) in comparison to untreated MEFs (10.7%; $P > 0.05$; Mann–Whitney *U*-test). These results suggest a persistently active mitotic spindle checkpoint in a fraction of $p53^{-/-}$ MEFs following transient replication stress.

DISCUSSION

Prolonged oncogenic signaling can cause a persistent DNA damage checkpoint activation, which presumably involves the induction of DNA replication stress [Bartkova et al., 2005; Gorgoulis et al., 2005]. In contrast, relatively little is known about the cellular responses to transient or low-intensity replication stress. It is conceivable, however, that such events occur frequently during tumorigenesis for example, after unscheduled initiation of DNA replication [Tanaka and Diffley, 2002]. Based on the primarily protective functions of the DNA replication checkpoint on the integrity of replication forks [Bartek et al., 2004], we asked how cells that have acquired intolerable DNA damage during transient replication stress are eliminated.

In this report, we show that the cellular response to transient replication stress involves the mitotic spindle checkpoint in a proportion of cells. In p53-positive U2-OS cells exposed to transient, low-intensity replication stress, a small but significant fraction of cells was found to express cyclin B1 in the presence of apoptotic chromatin condensation and a diffuse γ-H2AX staining suggesting mitotic catastrophe during or close to metaphase. This form of cell death requires an active mitotic spindle checkpoint [Nitta et al., 2004] and it is important to mention that previous results showed an abrogation of HU-induced apoptosis by spindle depolymerizing agents [Kumar et al., 2005]. Although the overall number of apoptotic cells was relatively low in our experiments, it is still remarkable that a measurable increase of cell death can be detected given that cells were treated with HU for only one 1 h and the long interval between replication fork perturbation and cell death (48 h). This finding reflects the situation in many human cancers where apoptosis is usually a relatively rare event. In keeping with the notion that cell death during or close to metaphase may be p53 independent [Castedo et al., 2004], siRNA-mediated depletion of p53 did not impede the apoptotic response (Fig. 5). It is likely, however, that the requirement of p53 for replication stress-induced apoptosis is cell-type dependent [Kumar et al., 2005].

When similar experiments were performed with primary MEFs (Fig. 6), several differences were noticed. Since a 1 h treatment with HU did not result in enhanced apoptosis, we extended the treatment interval to 24 h. When cells deficient of p53 were compared to wild-type or p63- or p73-deficient cells, only $p53^{-/-}$ MEFs showed enhanced apoptosis as measured by caspase-3 cleavage. However, no significant increase of cells with apoptotic chromatin condensation in combination with cyclin B1 expression was detected as seen in U-2 OS cells. In contrast, a significant increase of cells with multinucleation, micronuclei, and γ-H2AX foci in the presence of decondensed chromatin was found suggesting that a fraction of cells failed to complete mitosis and underwent aberrant mitotic exit. Moreover, the fact that the majority of multinucleated p53-negative MEFs still expressed cyclin B1 indicates a persistent activation of the mitotic spindle checkpoint [Weaver and Cleveland, 2005]. We cannot rule out, however, that the well-established ability of p53-deficient cells to reduplicate their DNA after mitotic exit contributes to the observed phenotype [Lanni and Jacks, 1998]. Human cells deficient of p53 have previously been reported to arrest in response to DNA damage before they undergo mitotic catastrophe directly from metaphase [Nitta et al., 2004]. The fact that we did not detect cell death during metaphase in MEFs may be related to cell type-specific differences and our experimental set-up that may select for surviving multinucleated cells at 72 h after removal of HU. It is noteworthy that transient replication stress can trigger a similar phenotype than what has been observed following microtubule disruption. A further analysis of the underlying molecular events may provide important support for a potential use of replication stress-inducing drugs in p53-negative cancers. Strikingly, p53-proficient wild-type and $p63^{-/-}$ or $p73^{-/-}$ MEFs did not show enhanced apoptosis and multinucleation but instead downregulated cell proliferation in response to HU, which further supports the notion that p53-negative cells may be sensitive to replication stress-inducing drugs.

The mitotic spindle checkpoint senses tension of attached microtubules and the DNA replication machinery has also been implicated in establishing cohesion between sister chromatids [Carson and Christman, 2001]. It is, therefore, conceivable that perturbed DNA replication may not only result in stretches of incompletely replicated DNA but also impact on chromosome cohesion. Whether and to what extent these chromosomal alterations can activate the mitotic spindle checkpoint as a consequence of replication stress requires further investigation.

In summary, our results suggest a model in which cells that have acquired intolerable damage during transient replication stress are eliminated in a process that at least in a fraction of cells involves the mitotic spindle checkpoint. Our findings may help to understand the high incidence of spindle checkpoint relaxation in tumor cells [Lengauer et al., 1998]. Given that many chemotherapeutic agents function through an induction of replication stress, the results presented here may help to identify approaches to increase their efficiency, for example by promoting entry into mitosis through attenuation of the G2 checkpoint [Yu et al., 2002; Cho et al., 2005; Gudkov and Komarova, 2005] or by reconstituting a functional mitotic spindle checkpoint. Moreover, our results imply that replication stress-inducing drugs may retain antineoplastic activity in p53-negative tumors.

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