Protein phosphatase 1 γ is responsible for dephosphorylation of histone H3 at Thr 11 after DNA damage

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The DNA-damage-induced transcriptional suppression of cell cycle regulatory genes correlates with a reduction in histone H3-Thr 11 phosphorylation (H3-pThr 11) on their promoters that is partly mediated by the dissociation of Chk1 from chromatin. In this study, we identify protein phosphatase 1 γ (PP1 γ) as a phosphatase responsible for DNA-damage-induced H3-pThr 11 dephosphorylation. PP1 γ is activated after DNA damage, which is mainly mediated by a reduction in Cdk-dependent phosphorylation of PP1 γ at Thr 311. The depletion of PP1 γ sensitizes HCT116 cells to DNA damage. Our results suggest that the ataxia telangiectasia, mutated and Rad3-related–Chk1 axis regulates H3-pThr 11 dephosphorylation on DNA damage, at least in part by the activation of PP1 γ through Chk1-dependent inhibition of Cdk.

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INTRODUCTION

Eukaryotic cells have coordinated systems to respond to DNA damage, such as cell cycle arrest mechanisms, DNA repair pathways and the apoptotic response. Together, these maintain genomic integrity (Sancar *et al*, 2004). These systems are partly regulated by transcriptional activation and repression (Zhan *et al*, 1993; Engelberg *et al*, 1994). We and others have recently identified histone H3-Thr 11 phosphorylation (H3-pThr 11) as a new transcriptional marker (Metzger *et al*, 2008; Shimada & Nakanishi, 2008; Shimada *et al*, 2008) that rapidly decreases after DNA damage. Under normal conditions Chk1 associates with chromatin (Smits *et al*, 2006; Niida *et al*, 2007) and phosphorylates H3-Thr 11. In response to DNA damage, Chk1 is rapidly released from chromatin and H3-pThr 11 is reduced.

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Mammalian serine (Ser)/threonine (Thr)-specific protein phosphatases (PPs) are represented by eight distinct prototypes: PP1, PP2A, PP2B, PP2C, PP4, PP5, PP6 and PP7 (Moorhead *et al*, 2007; Swingle *et al*, 2007). Of these, PP1, PP2A and PP4 have all been identified as histone phosphatases: PP1 dephosphorylates H1, which is phosphorylated in a cell-cycle-dependent manner (Paulson *et al*, 1996). The PP1 homologue dephosphorylates H3-pSer 10 at mitotic exit in yeast and worms (Hsu *et al*, 2000), and PP2A contributes to its dephosphorylation in *Drosophila* (Nowak *et al*, 2003). Phospho-H2AX (γ -H2AX) is immediately dephosphorylated after DNA repair by PP2A and PP4 in mammals and yeasts (Chowdhury *et al*, 2005, 2008; Keogh *et al*, 2006; Nakada *et al*, 2008). However, the phosphatases that catalyse H3-pThr 11 dephosphorylation in response to DNA damage have yet to be identified. In this study, we identify PP1 γ as a phosphatase responsible for DNA-damage-induced dephosphorylation of H3-pThr 11.

RESULTS AND DISCUSSION

Dephosphorylation of pThr 11 is okadaic acid sensitive

To identify phosphatases that might be capable of DNA-damage-induced dephosphorylation of H3-pThr 11, we treated HeLa cells with different concentrations of okadaic acid (OA) and examined the status of H3-pThr 11 after ultraviolet (UV) irradiation. H3-pThr 11 was reduced after UV irradiation as previously reported (Shimada *et al*, 2008). Treatment with ≥ 25 nM OA resulted in an increase in H3-pThr 11 in the absence of UV, making it difficult to evaluate the effect of OA treatment on DNA-damage-induced dephosphorylation of H3-pThr 11 (Fig 1A). We have previously reported that H3-pThr 11 is strongly enhanced at mitosis (Shimada *et al*, 2008). To establish whether the induction of H3-pThr 11 on OA treatment simply reflected an increase in the number of mitotic cells, we stained OA-treated cells with 4',6-diamidino-2-phenylindole (DAPI) and calculated the mitotic index (Fig 1B). The mitotic index value increased more than threefold after OA treatment. However, in interphase cells, H3-pThr 11 is detected throughout the nuclear region with some exclusion of the peri-centromeric heterochromatin foci (represented by DAPI foci in Fig 1C). By contrast, at mitosis, strong

Fig 1 | The phosphatase responsible for H3-pThr11 dephosphorylation is sensitive to okadaic acid. (A) HeLa cells were pre-treated with the indicated concentrations of OA for 4 h. After irradiation with (+) or without (−) 100 J/m² UV, cells were cultured for an additional 2 h in the medium containing OA. WCE and Chr fractions were subjected to immunoblotting using the indicated antibodies. H3-pThr11 was quantified using NIH Image 1.62 Software and normalized relative to the total H3. The results were expressed at the bottom of the second panel as relative levels of H3-pThr11 compared with non-treated samples (0 h). (B) HeLa cells were fixed after the 6 h incubation with the indicated concentrations of OA and were stained with DAPI. The mitotic index (MI) indicates the percentage of cells with condensed chromosomes. Data are shown as means ± s.d. of four independent experiments. White arrowheads indicate mitotic cells. (C) MEFs were double-stained with H3-pThr11 antibodies and DAPI. Typical staining patterns of H3-pThr11 at interphase and mitosis are shown. (D) A scheme of the experimental strategy using HeLa cells and the MIs expressed as in (B) are shown. RO3306, 9 μM; OA, 100 nM. (E) HeLa cells irradiated with UV according to the strategy shown in (D) were collected, and WCE and Chr fractions were analysed by immunoblotting using the indicated antibodies. H3-pThr11 was quantified as in (A) and the results are shown at the bottom of the second panel. (F) HeLa cells incubated with fostriecin for 48 h were treated with (+) or without (−) UV. After 2 h incubation, WCE and chromatin fractions were subjected to immunoblotting. Chr, chromatin; DAPI, 4',6-diamidino-2-phenylindole; MEF, mouse embryonic fibroblast; MI, mitotic index; NIH, National Institutes of Health; OA, okadaic acid; UV, ultraviolet; WCE, whole-cell extracts.

signals for H3-pThr11 are detected in condensed chromosomes, as reported previously (Preuss *et al*, 2003).

To evaluate the precise effect of OA treatment on H3-pThr11 independent of mitotic entry, we concomitantly treated cells with RO3306. Treatment with 9 μM RO3306 preferentially inhibits Cdk1 and thus prevents mitotic entry (Vassilev *et al*, 2006). The OA treatment did not result in an increase in the number of mitotic cells in the presence of 9 μM RO3306 (Fig 1D). Cell cycle arrest at G2 phase was induced as expected (supplementary Fig S1 online). DNA-damage-induced H3-pThr11 dephosphorylation was obvious at <100 nM OA, but almost completely abrogated at ≥100 nM OA in the presence of RO3306 when bands responsible for H3-pThr11 were quantified (Fig 1E). We observed that dephosphorylation of H3-pThr11 was not reduced after DNA damage, even at concentrations of 500 nM

fostriecin—a specific inhibitor of PP2A—and its closely related PP4 and PP6 isotypes (Walsh *et al*, 1997; Fig 1F). At 100 nM, cyclin B1 had accumulated, demonstrating that inhibition was effective (Cheng *et al*, 1998). These results suggest that PP2A and its related phosphatases are not involved in DNA-damage-induced dephosphorylation of H3-pThr11.

PP1 γ is a H3-pThr11 phosphatase

The OA sensitivity and fostriecin insensitivity in DNA-damage-induced dephosphorylation of H3-pThr11 suggested that the corresponding phosphatase might be a PP1 family member. We depleted PP1 by the using small interfering RNA (siRNA) that could suppress the expression of all three isoforms (PP1 α , PP1 β/δ and PP1 γ) equally. Cells were treated with control, PP1 or PP2A siRNAs for 36 h in the presence of 0.5 mM hydroxyurea,

Fig 2 | Protein phosphatase 1 γ is responsible for DNA-damage-induced dephosphorylation of H3-pThr 11. (A) The experimental strategy is shown (upper panel). Whole-cell extracts with (+) or without (–) 100 J/m² UV were subjected to immunoblotting. (B) HCT116 cells were transfected with control, PP1 or PP2A siRNAs. After 70 h, cells were treated with (+) or without (–) 100 J/m² UV. After 2 h incubation, cells were collected and whole-cell extracts were subjected to immunoblotting. (C) MEFs were exposed to UV (100 J/m²) irradiation and collected at the indicated times. Endogenous PP1 γ on the chromatin fraction was immunoprecipitated and the precipitates were subjected to immunoblotting and to an *in vitro* phosphatase assay using chromatin as a substrate. Phosphatase activity was examined by using immunoblotting with H3-pThr 11 antibodies. H3-pThr 11 was quantified and normalized relative to the total H3 as described in Fig 1E. The results are expressed as relative levels of H3-pThr 11 compared with non-treated samples (0 h). (D) The experimental strategy is shown (upper panel). Total RNAs were prepared from HCT116 cells and quantitative real-time RT-PCR was performed using a set of primers within *cdk1*, *cyclin B1* and *cyclin A2* transcripts. * $P < 0.003$. (E) HCT116 cells transfected with control or PP1 γ siRNAs for 72 h in 96-well plates were irradiated with UV (0, 40, 70 and 100 J/m²). The cell viability was determined 72 h after UV by MTT assay as described in the Methods section. The results were expressed as the percentage ratio of non-irradiated cells. MEF, mouse embryonic fibroblast; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PP, protein phosphatase; RT-PCR, reverse transcriptase PCR; siRNA, small interfering RNA; UV, ultraviolet.

which prevents mitotic arrest by PP1 or PP2A depletion. After 36 h, cells were released from hydroxyurea arrest and incubated with RO3306 for 16 h. Finally, cells were irradiated with UV and sampled 2 h later (Fig 2A, upper panel). The depletion of PP1, but not of PP2A, resulted in compromised H3-pThr 11 dephosphorylation after UV (Fig 2A).

We further demonstrated that PP1 γ depletion specifically compromised DNA-damage-induced H3-pThr 11 dephosphorylation in both HCT116 (Fig 2B) and HeLa (supplementary Fig S2A,B online) cells. Depletion of individual PP1 subunits did not result in gross changes in cell cycle distribution, although PP1 γ depletion mitotic index resulted in a slight increase in the mitotic index

value (supplementary Fig S3 online). It is interesting to note that PP1 γ phosphatase activity towards H3-pThr 11 was activated as early as 1 h and maintained for up to 4 h after DNA damage (Fig 2C). Changes in the expression of cell cycle regulatory genes after PP1 γ depletion were examined in cells treated with RO3306 to prevent low-level induction of mitotic cells. Treatment with RO3306 did not affect the expression of these genes (supplementary Table S1 online). The depletion of PP1 γ suppressed DNA-damage-induced transcriptional repression of *cdk1*, *cyclin B1* and *cyclin A2* (Fig 2D). Importantly, PP1 γ depletion sensitized HCT 116 cells to UV when cell survival was evaluated by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide)

Fig 3 | Protein phosphatase 1 γ -pThr311 on chromatin is decreased after DNA damage. (A) HCT116 cells were treated with UV (100 J/m²) and collected at the indicated times. The chromatin and soluble fractions were subjected to immunoblotting using the indicated antibodies. IKK α and lamin B1 were used as markers of soluble proteins. PP1 γ -pThr311 and H3-pThr11 were quantified and normalized relative to the total PP1 γ and H3, respectively. The results are expressed at the bottom of the second and seventh panels as relative levels of PP1 γ -pThr311 and H3-pThr11 compared with non-treated samples (0 h). (B) Chromatin fractions from UV-irradiated HCT116 cells were solubilized and subjected to IP-western analysis using the indicated antibodies. (C) To separate the isoforms of PP1 by SDS-PAGE, chromatin fractions from cells depleted of the indicated PP1 isoforms with (+) or without (–) UV were subjected to immunoblotting using an SDS-PAGE gel containing 10 μ M Phos Tag. Arrows indicate bands corresponding to PP1 α , β and γ isoforms. PP1 γ -pThr311 was quantified and the results were expressed at the bottom of the first panel as relative levels of PP1 γ -pThr311 compared with control (–UV). (D) The wild type or the T311A mutant of His-PP1 γ purified from Sf9 cells were incubated with chromatin from HCT116 cells for 1 h at 30 °C. The status of H3-pThr11 was monitored by immunoblotting using H3-pThr11 antibodies. H3-pThr11 was quantified and normalized relative to the total H3. The results are expressed at the bottom of the second panel as relative levels of H3-pThr11 compared with the control sample. IKK α , I κ B kinase α ; IP, immunoprecipitation; PP, protein phosphatase; SDS-PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; UV, ultraviolet.

assay (Fig 2E), suggesting the physiological importance of PP1 γ -dependent transcriptional repression after DNA damage. Although the molecular mechanism by which PP1 γ regulates H3-pThr11 dephosphorylation after DNA damage has remained elusive, isoform-specific functions for PP1 have been reported in the regulation of SRp38 dephosphorylation (Shi & Manley, 2007) and in the control of chromosomal architecture (Trinkle-Mulcahy *et al*, 2006).

Regulation of PP1 γ activity in response to DNA damage

The activity of PP1 α is reported to be downregulated by Cdk-dependent Thr320 phosphorylation (Dohadwala *et al*, 1994). The equivalent threonine residue is conserved in all three PP1 isoforms (Thr316 in PP1 β / δ and Thr311 in PP1 γ). Indeed, cyclin B1–Cdk1-phosphorylated PP1 γ -Thr311 *in vitro* (supplementary Fig S4A online). Cdk activity is strongly inhibited after DNA damage, in a manner dependent on Chk1 (Harper & Elledge, 2007). We therefore examined changes in the phosphorylation status of PP1 subunits after UV irradiation by using a combination of isoform-specific antibodies and a phospho-specific Thr320 (PP1 α) antibody (Fig 3A). All PP1 isoforms were similarly localized in both chromatin and soluble fractions of HCT116 cells. As a control, I κ B kinase α (IKK α)—a typical cytosolic protein—and lamin B1—a marker of the nuclear soluble fraction—were predominantly detected in the soluble fraction, indicating that

the subcellular fractionation was successful. The PP1-pThr320-specific signal for each isoform was rapidly dephosphorylated in response to UV, particularly in the chromatin fraction, with kinetics that were similar to that of H3-pThr11. As the PP1 α -pThr320-specific antibody can recognize the corresponding phosphorylations of PP1 β / δ and PP1 γ , we further examined whether PP1 γ -pThr311 was dephosphorylated after UV treatment. We observed that PP1 γ -pThr311 in PP1 γ immune complexes was reduced (Fig 3B). We separated the three isoforms of PP1 using sodium dodecyl sulphate–polyacrylamide gel electrophoresis containing Phos Tag (Kinoshita *et al*, 2009). Treatment of HCT116 cells with each isoform-specific siRNA or their combinations revealed that PP1 γ migrated fastest, followed by PP1 α and PP1 β (Fig 3C), although the amounts of each isoform and their phosphorylations at Thr311 were induced after depletion of the other isoforms, presumably due to a compensatory mechanism. The PP1 γ -pThr311-specific signal was consistently decreased in response to UV irradiation when bands responsible for PP1 γ -pThr311 were quantified. Similar kinetics were observed when cells were treated with bleomycin (supplementary Fig S4B online) or ionizing radiation (supplementary Fig S4C online), and the extent of PP1 γ -pThr311 dephosphorylation was found to be dependent on the UV dose (supplementary Fig S4D online). Importantly, the phosphorylation-defective mutant of PP1 γ (T311A) was more active *in vitro* than in the wild-type PP1 γ (Fig 3D).

Fig 4 | Cdk-dependent phosphorylation of protein phosphatase 1 γ at Thr 311 is involved in DNA-damage-induced transcriptional repression. (A) HCT116 cells were pre-incubated in the presence (+) or absence (–) of 5 mM caffeine for 4 h and then treated with (+) or without (–) UV (100 J/m²). Cells were collected 2 h after UV treatment and their chromatin fractions were analysed by immunoblotting using the indicated antibodies. (B) HCT116 cells were transfected with control or ATR siRNAs. After 70 h, cells were treated with (+) or without (–) UV (100 J/m²) and incubated for an additional 4 h. Chromatin fractions were analysed by immunoblotting. (C) HCT116 cells were treated with 10 μ M purvalanol A for 22 h, irradiated with UV (100 J/m²) and incubated for an additional 4 h. The chromatin fractions were analysed by immunoblotting. (D) HCT116 cells were transfected with control, *cdk1* and/or *cdk2* siRNAs. After 70 h, cells were treated with (+) or without (–) UV (100 J/m²) and incubated for an additional 4 h. The chromatin fractions or WCE were analysed by immunoblotting using the indicated antibodies. (E) HCT116 cells were infected with adenoviruses expressing LacZ as a control or the constitutively active Cdk1 mutant (Cdk1AF), and treated with (+) or without (–) UV (100 J/m²) 24 h after infection. Cells were collected 4 h after UV treatment and their chromatin fractions were analysed by immunoblotting using the indicated antibodies. ATR, ataxia telangiectasia, mutated and Rad3-related; Chr, chromatin; PP, protein phosphatase; siRNA, small interfering RNA; UV, ultraviolet; WCE, whole-cell extracts.

ATR–Chk1 axis regulates PP1 γ activity

Treatment with caffeine—an inhibitor of the ataxia telangiectasia, mutated and Rad3-related (ATR) and ataxia telangiectasia, mutated kinases—partly suppressed DNA-damage-dependent dephosphorylation of PP1 γ -pThr311 (Fig 4A). The knockdown of ATR by siRNA compromised UV-dependent PP1 γ -pThr311 dephosphorylation (Fig 4B). Treatment with purvalanol A, a specific Cdk inhibitor, resulted in almost complete reduction in PP1 γ -pThr311 and H3-pThr11 in the absence of DNA damage (Fig 4C). Both PP1 γ -pThr311 and H3-pThr11 were significantly reduced when Cdk1 was depleted, and further reduced when both Cdk1 and Cdk2 were depleted (Fig 4D). By contrast, ectopic expression of a constitutively active Cdk1 mutant (Cdk1AF) inhibited UV-induced dephosphorylation of PP1 γ -pThr311 and H3-pThr11 (Fig 4E). These results suggest that ATR/Chk1-dependent inhibition of Cdk activity results in dephosphorylation of PP1 γ -pThr311 and activation of PP1 γ , leading to dephosphorylation of H3-pThr11 (Fig 5).

In this study, we demonstrate that the dephosphorylation of H3-pThr11 and the subsequent transcriptional repression of cell cycle regulatory genes after DNA damage are regulated at least in part by the ATR–Chk1–Cdk–PP1 γ axis. This pathway seems to

have an important role in cell survival after DNA damage. Although the precise mechanism by which PP1 γ regulates cell viability remains unknown, levels of cyclins and Cdks might be a crucial determinant of cell viability after DNA damage.

METHODS

Cell culture and adenovirus infection. HCT116 cells were cultured in McCoy's 5a medium containing 10% fetal bovine serum. HeLa cells and mouse embryonic fibroblasts were cultured in Dulbecco's modified Eagle medium containing 10% fetal bovine serum. All cells were cultured at 37 °C in 5% CO₂. Adenovirus-Cdk1AF (Niida *et al*, 2005) or LacZ (control) was used to infect HCT116 cells at multiplicity of infection 40.

Immunoblotting. Subcellular fractionation was performed as previously described (Niida *et al*, 2007). To solubilize the chromatin fraction, pellets were suspended in immunoprecipitation kinase buffer (50 mM HEPES (pH 8.0), 150 mM NaCl, 2.5 mM EGTA, 1 mM dithiothreitol, 0.1% Tween-20, 10% glycerol and protease inhibitors). For preparation of whole-cell extracts, cells were lysed in immunoprecipitation kinase buffer.

Phosphatase assay. Chromatin-bound PP1 γ was solubilized and immunoprecipitated with PP1 γ antibody. The precipitates were

Fig 5 | Schematic model of DNA-damage-induced dephosphorylation of H3-pThr11 on the promoters of cell cycle regulatory genes. Under normal conditions (–UV), Chk1 associates with chromatin and phosphorylates H3-Thr11 to activate transcription of cell cycle regulatory genes. PP1 γ is inactivated through Cdk-dependent phosphorylation at Thr311. In response to DNA damage (+UV), activated ATR phosphorylates Chk1 and the phosphorylated Chk1 then dissociates from chromatin. ATR-dependent release of Chk1 from the promoters of cell cycle regulatory genes indirectly suppresses Cdk activity, which results in activation of PP1 γ through a reduction in Thr311 phosphorylation. PP1 γ -mediated dephosphorylation of H3-pThr11 represses transcription of genes involved in cell cycle regulation. ATR, ataxia telangiectasia, mutated and Rad3-related; PP, protein phosphatase; UV, ultraviolet.

incubated with chromatin at 30 °C for 1 h in phosphatase buffer (10 mM HEPES, 10 mM MgCl₂, 1 mM MnCl₂ and 1 mM DTT). His-tagged wild-type PP1 γ or PP1 γ -Thr 311A was purified from Sf9 cells and phosphatase assays were performed using a chromatin fraction as a substrate.

Knockdown experiments by siRNA transfection. HCT116 or HeLa cells were transfected with either a control siRNA (Silencer Negative Control, Ambion) or an siRNA for ATR, PP1, PP1 α , PP1 β , PP1 γ , PP2A or Cdk1 and Cdk2 using Lipofectamine 2000 (Invitrogen). The sequences of these siRNAs were described in the supplementary Table S2 online.

Immunofluorescence. Cells were grown on glass coverslips or chamber slides, washed with phosphate buffered saline (PBS) and fixed with 2% paraformaldehyde in PBS for 15 min, and stained with the indicated antibodies diluted in 5% blocking buffer (Convince, normal serum block) and DAPI.

Quantitative real-time RT–PCR for measurement of *Cdk1*, *cyclin B1* and *cyclin A2* mRNA expressions. The RNA was extracted using ISOGEN (Wako) according to the manufacturer's protocol. Total RNAs were subjected to reverse transcription. *Cdk1*, *cyclin B1* and *cyclin A2* mRNA levels were measured by Taqman quantitative PCR, normalized to *GAPDH* gene expression and expressed relative to the un-irradiated sample.

MTT assay. HCT116 cells were incubated with medium containing MTT solution (0.5 mg/ml) for 4 h. After removing the medium, cells were incubated with DMSO and the optical density of the resultant supernatant was measured at 535 nm.

Antibodies. The antibodies used for immunoblotting were ATR (sc-1887; Santa Cruz Biotechnology), β -actin (ab6276; Abcam), Chk1 (sc-8408 and sc-56291; Santa Cruz Biotechnology), Myc (sc-789 and sc-40; Santa Cruz Biotechnology), Cdk1 (sc-54; Santa Cruz Biotechnology), Cdk2 (sc-163; Santa Cruz Biotechnology), FLAG (M2; Sigma), H3 (ab1791; Abcam), IKK α (sc-7182; Santa Cruz Biotechnology), H3 (9715; Cell Signaling Technology), pT11 (ab5168; Abcam), PP1 (sc-7482; Santa Cruz Biotechnology), pThr320-PP1 (2581; Cell Signaling Technology), PP2A (05-421; Upstate), PP1 α (06-221; Upstate), PP1 β (ab53315; Abcam) and PP1 γ (sc-6108; Santa Cruz Biotechnology, 07-1298; Millipore).

Supplementary information is available at EMBO reports online (<http://www.emboreports.org>).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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