A PP4-Phosphatase Complex Dephosphorylates γ-H2AX Generated during DNA Replication

Dipanjan Chowdhury,2,3,4,* Xingzhi Xu,1,* Xueyan Zhong,6 Fariyal Ahmed,2,3 Jianing Zhong,1 Ji Liao,1 Derek M. Dykxhoorn,2,3 David M. Weinstock,5 Gerd P. Pfeifer,6 and Judy Lieberman2,3,*

1Laboratory of Cancer Biology, College of Life Science, Capital Normal University, Beijing 100037, China
2Immune Disease Institute
3Department of Pediatrics
Harvard Medical School, Boston, MA 02115, USA
4Department of Radiation Oncology
5Department of Medical Oncology
6Department of Biology, City of Hope National Medical Center, Duarte, CA 91010, USA
*Correspondence: xingzhi.xu@mail.cnu.edu.cn (X.X.), dipanjan.chowdhury@dfci.harvard.edu (D.C.), lieberman@idi.harvard.edu (J.L.)

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SUMMARY

The histone H2A variant H2AX is rapidly phosphorylated in response to DNA double-stranded breaks to produce γ-H2AX. γ-H2AX stabilizes cell-cycle checkpoint proteins and DNA repair factors at the break site. We previously found that the protein phosphatase PP2A is required to resolve γ-H2AX foci and complete DNA repair after exogenous DNA damage. Here we describe a three-protein PP4 phosphatase complex in mammalian cells, containing PP4C, PP4R2, and PP4R3β, that specifically dephosphorylates ATR-mediated γ-H2AX generated during DNA replication. PP4 efficiently dephosphorylates γ-H2AX within mononucleosomes in vitro and does not directly alter ATR kinase activity, suggesting that PP4 acts directly on γ-H2AX in cells. When the PP4 complex is silenced, repair of DNA replication-mediated breaks is inefficient, and cells are hypersensitive to DNA replication inhibitors, but not radiomimetic drugs. Therefore, γ-H2AX elimination at DNA damage foci is required for DNA damage repair, but accomplishing this task involves distinct phosphatases with potentially overlapping roles.

INTRODUCTION

DNA breaks occur constantly from endogenous (e.g., reactive oxygen species, metabolic byproducts, DNA replication, and DNA recombination) and exogenous (e.g., genotoxic chemicals, ionizing radiation [IR], and UV irradiation) sources. Each type of DNA damage elicits a specific cellular repair response (Harrison and Haber, 2006). One of the earliest events in the double-stranded DNA break (DSB) response is the phosphorylation of the histone H2A variant, H2AX, at Ser139 by members of the PI(3)K (phosphatidylinositol-3-OH kinase)-like kinases, ATM (ataxia telangiectasia mutated), ATR (ATM- and Rad3-related), and DNA-PK (DNA-dependent protein kinase) (Fernandez-Capetillo et al., 2004). The three kinases have significant functional redundancy, but they are activated in a stress-specific manner. ATM and DNA-PK redundantly phosphorylate H2AX induced by ionizing radiation and radiomimetic drugs, whereas ATR seems to respond to endogenous or exogenous agents that interfere with DNA replication (Shiloh, 2003).

Phosphorylated H2AX (γ-H2AX) has a role in repair, replication, and recombination of DNA and cell cycle regulation (Fernandez-Capetillo et al., 2004). The large γ-H2AX domains generated at each DSB, visualized as nuclear foci, stabilize cell-cycle and DNA repair factors (cohesins, MDC1, Mre11, BRCA1, 53BP1, etc.) at the break site (Petrini and Stracker, 2003; Stucki and Jackson, 2006). Recent studies in mouse B cells suggest that γ-H2AX stabilizes the broken DNA ends during class switching, giving the repair machinery sufficient time to make appropriate joins (Franco et al., 2006; Ramiro et al., 2006). Importantly, loss of a single H2AX allele compromises genomic integrity and enhances cancer susceptibility in mice. The H2AX gene maps to a cytogenetic region frequently altered in human cancers, implicating similar functions in man (Bassing et al., 2003; Celeste et al., 2003a). Therefore, the formation of γ-H2AX is of utmost importance for DNA repair. Although the kinases and stimuli involved in γ-H2AX formation have been intensely investigated, how γ-H2AX is eliminated in mammalian cells and the functional consequences of having constitutively phosphorylated H2AX remain unclear.

Two recent studies, one in mammals and the other in S. cerevisiae, identified roles for PP2A family phosphatases in γ-H2AX dephosphorylation (Chowdhury et al., 2005; Keogh et al., 2006). The PP2A family of serine/threonine phosphatases includes four distinct catalytic components in mammals—two closely related PP2A enzymes (PP2Acζ, PP2Acβ), PP4C and PP6C (Honkanen and Golden, 2002). The most closely homologous yeast enzymes are Pph21 and Pph22, and Pph3 and Sit4, respectively (Zabrocki et al., 2002). The catalytic components of these enzymes form dimeric or trimeric complexes with regulatory subunits that confer substrate specificity, confer tissue/cell type-specific targeting, and control the extremely vigorous
Figure 1. PP4C Dephosphorylates γ-H2AX In Vitro, and Silencing PP4C Increases Cellular γ-H2AX in the Presence and Absence of Exogenous DNA Damage

(A) Schematic depiction of the PP4C catalytic domain and sequence comparison of the respective catalytic domains of human PP4C and PP2AC with S. cerevisiae Pph3. Alignment of Pph3 with either PP4C or PP2AC shows relatively stronger homology between Pph3 and PP4C. The bars and highlighted residues represent the amino acids which were identical between PP4C and Pph3 but different for PP2AC. The percentage of identity and similarity between yeast Pph3 and PP4C or PP2AC is represented in a table in the lower right.

(B) Western blots showing γ-H2AX and H2AX expression in the presence and absence of CPT. The expression levels of γ-H2AX and H2AX were monitored using specific antibodies. The blots were divided into three groups: Control, PP4C silencing, and PP2AC silencing.

(C) Western blots showing γ-H2AX and H2AX expression in the presence and absence of PP4C and PP2AC. The blots were divided into three groups: Control, PP2A silencing, and PP4C silencing.
activity of the catalytic subunits. PP2A plays an important role in counteracting oncogenic kinases in cell-cycle control and is the target of the SV40 small T antigen (Janssens et al., 2005; Janssens and Goris, 2001). Little is known about the function of mammalian PP4 and PP6, although their yeast and fly homologs have been implicated in centrosome maturation and microtubule organization, resistance to apoptosis induced by UV irradiation and cisplatin, and recovery from the DNA damage checkpoint (PP4) (Cohen et al., 2005; Gingras et al., 2005; Hastie et al., 2006) and G1-S cell-cycle progression (PP6) (Stefansson and Brautigan, 2007). We previously identified PP2A as a phosphatase that removes γ-H2AX foci formed in mammalian cells in response to DNA damage by the topoisomerase I inhibitor camptothecin (CPT) (Chowdhury et al., 2005). PP2AC colocalizes at γ-H2AX foci, suggesting that PP2A dephosphorylates γ-H2AX near a DSB. Importantly, when PP2AC is inhibited or silenced by RNA interference, γ-H2AX levels following DNA damage increase, γ-H2AX foci persist, and DSB repair is impaired (Chowdhury et al., 2005). In a parallel study in yeast, a deletion screen revealed a trimeric complex (histone H2A phosphatase complex) containing PPH3, PSY2, and YB046W that regulates basal γ-H2AX levels (Keogh et al., 2006).

Although both PP2A and Pph3 efficiently dephosphorylate γ-H2AX in vitro, their roles in cells appear to be distinct. Pph3 does not accumulate at the site of an engineered DSB and, therefore, was hypothesized to dephosphorylate γ-H2AX only after it has been removed from chromatin, whereas PP2A likely works on chromatin-bound γ-H2AX. Pph3 deletion has no effect on repair kinetics and the rate of γ-H2AX loss at a single exogenously induced DSB. Pph3 mutants show a constitutive increase in γ-H2AX levels even in the absence of exogenous DNA damage, while silencing PP2A in mammalian cells does not affect basal γ-H2AX in the absence of an exogenous insult (Chowdhury et al., 2005; Keogh et al., 2006). Although the differences between PP2A and Pph3-mediated removal of γ-H2AX might be due to different regulatory mechanisms in yeast and mammals, we speculated that more than one phosphatase might be involved in γ-H2AX removal in mammalian cells and that another mammalian phosphatase might be functionally analogous to Pph3. PP4C is the closest human homolog of yeast Pph3 (Figure 1A). Although several putative PP4C-containing complexes have been identified, their biological function is not well defined (Gingras et al., 2005). Genetic deletion of the PP4 catalytic subunit PP4C in mice results in early embryonic lethality. Conditional deletion of PP4C in thymocytes leads to an early block in thymic development at the DN3 stage and enhanced apoptosis (Shui et al., 2007). Few physiological substrates of mammalian PP4C have been identified. PP4C has been implicated in TNF-α signaling (Mihindukulasuriya et al., 2004; Zhou et al., 2002) and NF-κB regulation (Hu et al., 1998; Yeh et al., 2004). Recently, histone deacetylase 3 (HDAC3) and a mitotic regulatory protein NDEL1 were shown to be regulated by a specific PP4 complex (Toyooka et al., 2008; Zhang et al., 2005).

Here we show that PP4C, like yeast PPH3, controls basal levels of γ-H2AX. PP4C dephosphorylates γ-H2AX incorporated in mononucleosomes in vitro with comparable efficiency as PP2AC. Silencing any component of a PP4C complex, containing mammalian homologs of Psy2 and Yb046W, increases basal γ-H2AX foci that associate with MRE11. The increase in γ-H2AX when PP4 is knocked down is ATR-mediated and requires DNA replication. Unresolved γ-H2AX foci occur only in dividing cells and bind PCNA, suggesting they form at stalled replication forks. However, PP4-deficiency does not alter ATR kinase activity or checkpoint protein phosphorylation, suggesting that γ-H2AX is a direct substrate of PP4.

RESULTS

Silencing PP4C Increases γ-H2AX Basal Levels

To identify a Pph3 functional analog in mammalian cells, we first looked at whether PP4C might fulfill that role. Although all the PP2A family catalytic subunits are highly homologous, PP4C is the closest human homolog of the yeast phosphatase Pph3, especially in the catalytic metal-binding domain and active site (residues 74–125) (Figure 1A). To investigate a possible role of PP4C in regulating γ-H2AX, small interfering RNAs (siRNA) were designed to downregulate PP4C expression specifically without altering expression of the related PP2A family members (Pandey et al., 2003). HeLa cells transfected with two PP4C siRNAs duplexes were assayed for phosphatase proteins by immunoblot 3 days later. Only PP4C siRNA duplex no. 1 efficiently silenced PP4C, and this duplex was used in subsequent experiments. PP4C siRNA had no effect on PP2AC and vice versa (Figure 1B, upper panel). We next investigated how PP4C deficiency affected γ-H2AX levels (Figure 1B, middle panel). HeLa cells transfected with control or PP4C siRNAs (Figure 1B, middle panel) or PP2AC-specific siRNAs (Figure 1B, lower panel) were treated with CPT for 1 hr, and γ-H2AX levels were analyzed by immunoblot (Figure 1B, middle panel). In the absence of PP4C or PP2AC siRNAs, γ-H2AX was not detected in cells not treated with CPT, began to be detected immediately after adding CPT, peaked 2.5 hours after CPT removal, and returned to background by 10 hr. However, in the presence of either PP4C or
PP2AC siRNA, γ-H2AX levels were significantly higher and were only diminished 10 hr after removing CPT. However, the pattern of γ-H2AX, but not total H2AX, protein varied in PP2AC- and PP4C-silenced cells. The most striking result was a significant increase in basal γ-H2AX in PP4C-deficient cells before adding CPT, which was not observed when PP2AC was silenced. However, γ-H2AX levels increased in response to CPT most dramatically in PP2A-deficient cells compared to control or PP4C-silenced cells, and this increase lasted for at least 5 hr after DNA damage. Ten hours after CPT treatment, γ-H2AX was detected only in PP4C-silenced cells and was comparable to basal γ-H2AX levels in PP4C-deficient cells that were not subjected to exogenous DNA damage. To confirm the specificity of the PP4C siRNA effect, we cotransfected HeLa cells with the PP4C siRNA that effectively silenced PP4C and a Flag-tagged PP4C expression plasmid containing silent mutations in the siRNA recognition site. Expressing the siRNA-resistant PP4C eliminated the PP4C siRNA-mediated increase in basal γ-H2AX (Figure S1). These results suggest that, although both PP2A and PP4 regulate the cellular pool of γ-H2AX, there are key kinetic and contextual differences in the regulatory pathways.

**PP4C Dephosphorylates γ-H2AX as Efficiently as PP2AC In Vitro**

To determine whether PP4C can dephosphorylate γ-H2AX directly and compare the efficiency of dephosphorylation with PP2AC, we compared the in vitro activity of the two enzymes against Ribosomal S6 kinase 1 (Rsk1)-phosphorylated human γ-H2AX reconstituted in mononucleosomes (Figure 1C). The enzymes were obtained by immunoprecipitating HA-tagged PP4C and PP2AC from in vitro transcribed andtranslated rabbit reticulocyte lysates. Both PP2AC and PP4C dephosphorylated nucleosomal γ-H2AX in a dose-dependent manner, and importantly, their in vitro activities on γ-H2AX were comparable.

**Multiple PP4C-Containing Complexes**

PP4C forms several multimeric complexes with proteins that have homology to the subunits of the γ-H2AX regulating yeast phosphatase complex, HTP-C (Cohen et al., 2005). However, the subunit composition and molecular architecture, cellular functions, and subcellular localization of the different PP4-complexes are not well characterized. A recent study using mass spectrometry analysis of TAP-tagged proteins systematically analyzed yeast PPH3- and mammalian PP4C-interacting proteins and identified several distinct PP4C-containing protein complexes (Gingras et al., 2005). To confirm these results and identify which of the PP4 regulatory subunits are involved in the regulation of γ-H2AX in mammalian cells, we produced antibodies against the predicted PP4 subunits, R1, R2, R3α, R3β, and PP4C, and for three other proteins, TIP41, α4, and GEMIN 4, which have also been reported to associate with PP4C (Carnegie et al., 2003; Gingras et al., 2005). We analyzed the association of these proteins by reciprocal immunoprecipitation/immunoblot assays using 293T cell lysates. Immunoprecipitation with nonspecific IgG controlled for specificity. Antibodies against three other phosphatases, PP2AC, PPM1D (also known as PP2Cβ or WIP1) (Figure 2A), and PP6C (data not shown), served as additional controls (Figure 2A). As in the earlier study (Gingras et al., 2005), we identified three distinct PP4C-containing complexes (Figure 2B): (1) a complex containing PP4R2 and PP4R3β, (2) a complex containing PP4R2 and PP4R3α, and (3) a binary complex with PP4R1. None of these PP4C complex subunits interacted with PP2AC, PP6C, or PPM1D. Although PP4R3α and PP4R3β are close homologs, we failed to detect any physical interaction between them. Contrary to earlier results, we did not detect any interaction between PP4C and TIP41 or α4 (Figure 2A) or GEMIN 4 (data not shown). This discrepancy may be due to the difference in cell or the method (i.e., immunoprecipitating overexpressed tagged proteins versus immunoprecipitating endogenous protein).

**A PP4C-R2-R3β Complex Regulates Basal γ-H2AX**

To determine which PP4-complex controls cellular γ-H2AX, we designed siRNA duplexes against all the subunits. Three days after HeLa cells were transfected with siRNA duplexes targeting each subunit, PP4 complex subunit levels were assessed by immunoblot (Figure 2C). siRNAs against each subunit specifically reduced expression of the respective subunit by >90%. More importantly, it did not affect the expression of any other PP4 subunit or tubulin. We next investigated how knockdown of the different PP4 subunits affected γ-H2AX. HeLa cells transfected with control or PP4-subunit siRNAs were treated with CPT for 1 hr, and γ-H2AX levels were analyzed by immunoblot (Figure 2D). After knockdown of PP4C, PP4R2, or PP4R3β, γ-H2AX levels were significantly higher at all times examined. Consistent with Figure 1B, even in the absence of exogenous DNA damage, basal γ-H2AX levels were increased specifically in PP4C−, PP4R2−, or PP4R3β−deficient cells. Knocking down the other two subunits, PP4R1 or PP4R3α, had no consistent effect on γ-H2AX. Together these results suggest that a PP4 complex, comprised of the catalytic subunit and the two regulatory subunits PP4R2 and PP4R3β, controls cellular levels of γ-H2AX, independent of external DNA damage.

**γ-H2AX Foci Increase Independently of Exogenous DNA Damage in Cells Deficient in PP4C, PP4R2, or PP4R3β**

γ-H2AX accumulation at discrete nuclear foci is an early marker of DSB sites undergoing DNA repair. These foci, visible by immunofluorescence microscopy, can be seen at a low frequency in cycling cells, reflecting the occasional DSB that occurs as DNA is replicated. To investigate whether the PP4 complex regulates γ-H2AX focus formation, HeLa cells transfected with control or PP4C siRNAs were either untreated or treated with CPT for 1 hr and observed for γ-H2AX foci 2 hr later (Figure 3A). In untreated cells, γ-H2AX foci increased greatly in the absence of PP4C. Focus formation in response to CPT was not that different in control or PP4C-deficient cells. Of note, PP4C in control cells also did not appear to colocalize with CPT-induced γ-H2AX foci (see below). Therefore, the basal increase in cellular γ-H2AX in PP4C knocked-down cells (as observed in Figures 1B and 2D) also resulted in formation of γ-H2AX foci. The DNA repair factor, MRE11, which colocalizes with DSBs, and PCNA, which specifically colocalizes with DSB generated during DNA replication (Ward and Chen, 2001), also were recruited to γ-H2AX foci generated in PP4C-deficient cells, suggesting that
γ-H2AX is induced in response to DSB generated during DNA replication (Figure S2). We confirmed the role of the PP4C-R2-R3β complex by doing the same set of experiments in PP4R3β-silenced (Figure 3B) and PP4R2-silenced cells (data not shown). The increase in γ-H2AX foci in PP4R2- and PP4R3β-deficient cells that were not subjected to exogenous DNA damage suggested that the PP4 complex as a whole, and not just the catalytic subunit, was responsible for regulating basal γ-H2AX foci.

The PP4C-R2-R3β Complex Is Associated with Chromatin and Does Not Relocalize in Response to Exogenous DNA Damage

The subcellular location of PP4C and its associated subunits is not known. To study PP4 localization in the context of DNA damage, we costained untreated and CPT-treated HeLa cells with antibodies to γ-H2AX and the PP4 complex proteins, PP4C (Figure 4A), PP4R3β (Figure 4B), and PP4R2 (Figure 4C). Total cellular PP4C, PP4R2, or PP4R3β by immunoblot did not change following treatment with...
CPT or the dNTP synthesis-inhibitor hydroxyurea (HU) (data not shown). In untreated cells, PP4C was primarily perinuclear, with some nuclear staining (Figure 4A). This cellular pattern did not change within the first few hours after CPT treatment. Moreover, as noted in Figure 3A, PP4C did not colocalize with CPT-induced γ-H2AX foci. The majority of PP4R3β staining was nuclear prior to CPT treatment and this pattern remained unchanged after DNA damage (Figure 4B). PP4R2 was visible both in the cytoplasm and nucleus before CPT treatment and in the first few hours after treatment (Figure 4C). Although PP4C appears to be mostly extranuclear, these data suggest that the PP4C-R2-R3β complex is primarily nuclear, independently of exogenous DNA damage, and does not relocalize after exogenous DNA damage. Most cellular PP4C, which is primarily cytosolic, is likely associated with other complexes. By biochemical fractionation, the PP4C-R2-R3β nuclear complex was enriched in chromatin and not detected in the nucleoplasm (Figure 4D). Knocking down PP4R2 (and possibly PP4R3β) reduced chromatin-associated PP4C, suggesting that these subunits may direct PP4C to chromatin (Figure S3).

The Basal Increase in γ-H2AX in PP4C-Silenced Cells Is Mediated by ATR

One of the earliest events in the DSB response is H2AX phosphorylation at Ser139 by one of the three PIKK, ATM, ATR, or DNA-PK. However, it is not clear if γ-H2AX forms in the course of a normal cell division cycle and, if so, which kinase is responsible. Recent reports show ATM-dependent H2AX phosphorylation occurs in mitosis (Ichijima et al., 2005; McManus and Hendzel, 2005). ATR phosphorylates H2AX in response to any DNA replication-mediated DNA damage (Furuta et al., 2003). To investigate which kinase was responsible for the basal increase in γ-H2AX in PP4-deficient cells, we assessed γ-H2AX levels in HeLa cells knocked down for PP4C or PP4R3β and treated with the ATM inhibitor KU55933. Lack of ATM autophosphorylation following γ-irradiation verified that ATM was completely inhibited (Figure 5A, lower

Figure 3. Knocking Down PP4C or -R3β Induces γ-H2AX Foci in the Absence of Exogenous DNA Damage

PP4C (A) or PP4R3β (B) or control siRNA-transfected HeLa cells, treated or not with CPT (2 μM, 1 hr), were fixed and stained with anti-γ-H2AX and DAPI and visualized using laser-scanning confocal microscopy. γ-H2AX foci form in untreated cells deficient in PP4C or PP4R3β.
Figure 4. Subcellular Localization of PP4C, PP4R3β, and PP4R2 Does Not Substantially Change in Response to Exogenous DNA Damage

Untreated or CPT-treated (5 μM, 1 hr, 37°C) HeLa cells were washed to remove CPT and incubated for 2 hr before fixing and staining for γ-H2AX and PP4C (A), PP4R3β (B), or PP4R2 (C). Images were visualized using laser-scanning confocal microscopy. PP4C is primarily perinuclear with some nuclear staining, but PP4R3β and PP4R2 are predominantly nuclear, suggesting that the PP4C-R2-R3β complex mostly resides in the nucleus. None of these components substantially changed their localization following CPT treatment. (D) The PP4C-R2-R3β complex is associated with chromatin in fractionated nuclei from HeLa cells, treated or not with 2 mM HU for 24 hr or with 10 μM CPT for 2 or 4 hr. Although PP4R2 was also found in the nuclear soluble fraction, PP4C and PP4R3β were exclusively detected in the chromatin fraction. MDC1 served as a control for the chromatin fraction.
Figure 5. ATR Is Responsible for γ-H2AX Formation in PP4-Deficient Cells and ATR Activity Is Not Regulated by PP4

(A) ATM inhibition does not affect basal γ-H2AX levels in PP4-silenced cells. PP4C, R3β or control siRNA-transfected HeLa cells were incubated with DMSO or 5 µM KU55933 (ATM inhibitor) and whole cell extracts were analyzed by immunoblot probed for γ- H2AX or H2AX (upper panel). To verify ATM inhibition, cells were irradiated (10 Gy) and immunoblotted for phospho1981-ATM or ATM (lower panel).

(B) Inhibition of ATR, but not DNA-PK or ATM, decreases γ-H2AX in PP4-silenced cells. PP4C, -R3β, or control siRNA-transfected HeLa cells were incubated with 20 µM or 100 µM Wortmannin or with 4 mM caffeine, and whole-cell extracts were analyzed by immunoblot for γ-H2AX or H2AX.

(C) Knocking down PP4 in ATR-deficient cells has no effect on basal γ-H2AX levels. PP4C and PP4R3β were knocked down in Seckel fibroblasts (right), and γ-H2AX levels were analyzed by immunoblot in untreated cells or after irradiation (2 Gy). There was no effect of PP4 knockdown on γ-H2AX in these cells (left).

(D) PP4 knockdown does not affect ATR activity. ATR was immunopurified from PP4 subunit-specific or control siRNA-transfected HeLa cells. The kinase activity of the ATR immunocomplex from PP4-deficient cells was assayed relative to the activity in control cells. The lower panel shows comparable amounts of immunoprecipitated ATR and ATRIP. siRNAs used are as follows: black, control; light dots, PP4C; dark dots, PP4R2; white, PP4R3β. Mean ± SD is shown.

(E) PP4 deficiency does not activate the checkpoint proteins or p53. PP4C, PP4R3β, or control siRNA-transfected HeLa cells were incubated with DMSO or 5 mM HU 48 hr after transfection, and immunoblots were performed on whole cell extracts with the indicated antibodies (upper panel). HeLa cells transfected with PP4C, PP4R3β, or control siRNAs were harvested at indicated times after transfection, and immunoblots of whole cell extracts were probed with the indicated antibodies (lower panel).
PP4 Dephosphorylates γ-H2AX during DNA Replication

PP4C Regulation of γ-H2AX Only Occurs Post-DNA Replication

Because PP4C depletion affected basal DNA damage-initiated γ-H2AX, we wanted to determine what kind of endogenous DNA damage might be linked to elevated basal γ-H2AX in PP4-deficient cells. Endogenous DNA damage in a cell may occur during DNA replication or in response to metabolic byproducts and reactive oxygen species (ROS) (Tanaka et al., 2007). In order to determine whether cell division is necessary for basal γ-H2AX formation in PP4-deficient cells, we isolated human peripheral blood monocytes and differentiated them into macrophages. These are terminally differentiated cells that are unable to divide. We partially silenced PP4C and PP2AC (Figure 6A, top panel) and then irradiated the cells to induce DNA damage. PP4C knockdown did not alter γ-H2AX in macrophages either basally or after irradiation (Figure 6A, middle panel; Figure S4). However, PP2AC silencing led to γ-H2AX persistence in irradiated macrophages (Figure 6A, lower panel). This result suggested that γ-H2AX formation is driven by cell division and not by spontaneous DNA lesions in PP4-silenced cells. To confirm this and directly establish a link between DNA replication and γ-H2AX in PP4-silenced cells, we silenced PP4C complex proteins in serum-starved 293T cells. Serum starvation synchronizes cells in the G0/G1 phase. We then released the G0/G1 block by adding serum and added BrdU to label cells undergoing DNA replication. We allowed the cells to proceed through the S phase and then analyzed them for BrdU incorporation and γ-H2AX foci. In control siRNA-treated cells, no γ-H2AX foci (other than a few isolated foci occasionally seen in normally dividing cells) formed in the replicating BrdU+ cells. However, upon partially depleting any of the PP4 complex proteins, only cells that had incorporated BrdU had increased γ-H2AX foci (Figure 6B). These results in nondividing and synchronized cells strongly suggest that γ-H2AX induction in PP4-deficient cells is due to DNA replication-mediated DNA damage.

PP4 D Dephosphorylation of γ-H2AX Is Required for Efficient DNA Damage Repair during Replication

To determine whether PP4 expression and basal γ-H2AX formation affects DNA repair, we measured DSB persistence in HeLa cells transfected with PP4C, PP4R3β, or control siRNAs, using single-cell gel electrophoresis (comet assay). The comet moment quantifies the extent of unrepaired DNA damage. In asynchronously dividing cells not subjected to exogenous DNA damage, only cells that had incorporated BrdU had increased γ-H2AX foci (Figure 6B). These results in nondividing and synchronized cells strongly suggest that γ-H2AX induction in PP4-deficient cells is due to DNA replication-mediated DNA damage.

We also assessed the activation status of other bona fide ATM and ATR substrates that might indirectly regulate γ-H2AX. In PP4-silenced cells, phosphorylation of the checkpoint proteins CHK1 (ATR substrate), CHK2 (ATM substrate), and the ATM/ATR-substrate, P53, was also unaltered (Figure 5D, upper panel). Phospho-CHK1 or phospho-CHK2 were not detected at any time after transfecting HeLa cells with PP4C or PP4R3β siRNAs (Figure 5D, lower panel). This suggests that PP4C does not directly regulate the activation of these factors and that PP4-deficiency does not constitutively activate ATM or ATR. These results, taken together with the efficient γ-H2AX dephosphorylation by PP4C on mononucleosomes and the subcellular localization of the PP4C-R2-R3β complex on chromatin, suggest that PP4C likely dephosphorylates basal γ-H2AX as a direct substrate on chromatin. However, we cannot exclude the possibility that dephosphorylation of other unknown substrates by PP4C might also indirectly alter the status of γ-H2AX.
post-DNA replication suggests that unresolved γ-H2AX foci impede repair of DNA replication-mediated DSB.

DSB induced during DNA replication are typically repaired by homologous recombination (HR) (Ben-Yehoyada et al., 2007). To test whether PP4 complex-deficient cells displayed differences in HR repair, we assayed for HR-mediated repair of an I-SceI-induced DSB using a recombination substrate DR-GFP (Weinstein et al., 2006). With this reporter assay, if the DSB is repaired by HR, GFP is expressed (typically in < 5% of cells), which is quantifiable by flow cytometry. HR was significantly reduced in cells treated with PP4C and PP4R3β siRNAs (Figure 7B). Since DNA replication-mediated DSB are repaired by HR, the effect of the PP4-complex on HR is consistent with its role in resolving DNA lesions during replication. Interestingly, knocking down PP4C affects HR more than PP4R3β, suggesting that other PP4C complexes (R3β-independent) may also participate in regulating HR.

To investigate the biological significance of the increased DNA damage in PP4-deficient cells, we examined their sensitivity to different DNA-damaging agents (Figure 7C). PP4 complex-deficient cells were hypersensitive to both CPT and HU but were not particularly sensitive to bleomycin. HU and CPT induce DNA breaks by inhibiting DNA replication, whereas bleomycin induces DNA damage independently of replication by direct Fe-mediated oxidation of DNA sugars. These results suggest that the PP4 complex may be specifically involved in the removal of γ-H2AX caused during DNA replication, and impeding this step sensitizes cells to further replication-mediated DNA damage. It should be noted that PP4 depletion reduces cell viability even in undamaged cells (data not shown).

**DISCUSSION**

The cellular response to DNA damage inflicted by external sources has been well studied for many decades, but it is
Figure 7. PP4 Is Required to Repair DNA Replication-Mediated DNA Damage

(A) Knocking down the PP4 complex impairs DNA repair in dividing cells. PP4C, PP4R3β, or control siRNA-transfected 293T cells were serum starved for 2 days. Cells were either maintained in low serum or released from G0/G1 block by adding serum and analyzed by single-cell gel electrophoresis (comet assay) 12 hr later. Representative images are shown above. The comet tail moment of 75 cells for each condition was quantified using NIH Image software. The comet moment was normalized to that of control cells (black bar) and expressed as a percentage. DNA damage in the PP4C (white bar, p < 0.02)- and -R3β (dotted bar, p < 0.02)-deficient cells was significantly increased only after DNA replication resumed.

(B) Measurement of HR-mediated repair of an I-SceI-induced DSB. U2OS cells carrying the recombination substrate (DR-GFP) were transfected with PP4C, PP4R3β, or control siRNAs. I-SceI expression plasmid was transfected after 24 hr, and GFP+ cells were measured 48 hr later. HR repair was significantly impaired in cells transfected with PP4C (p < 0.01) or R3β (p < 0.01) siRNA.

(C) PP4-silenced cells are hypersensitive to DNA replication inhibitors. Cell viability was analyzed by MTT assay relative to untreated cells. Curves were generated from three independent experiments. PP4C- or PP4R3β-deficient cells were hypersensitive to CPT (p < 0.001, left) and HU (p < 0.002, right), but not to bleomycin (lower), relative to control cells. In panels (A)–(C), the mean ± SD of representative experiments is shown.
unclear whether all DSB induced during DNA replication elicit a cellular response comparable to exogenous damaging agents. γ-H2AX clearly is important for repairing replication-induced DNA damage (Furuta et al., 2003; Pommier et al., 2003), but its precise role is not certain. Recent studies suggest that it may not be involved in recruiting repair factors (Bartek and Lukas, 2007), but rather play an important role in stabilizing the repair complex and the broken ends (Celeste et al., 2003b).

In this study, we identify a trimeric phosphatase complex (PP4-PP4R2-R3β) that specifically eliminates γ-H2AX formed without exogenous DNA damage. We show that PP4 dephosphorylates γ-H2AX by demonstrating in vitro phosphatase activity on nucleosomal γ-H2AX and increased γ-H2AX and persistence of γ-H2AX foci when any of the PP4 complex components is silenced. γ-H2AX foci detected in the absence of PP4 is induced by ATR specifically in the course of DNA replication and associates with DNA repair factors like MRE11 and PCNA. In PP4-deficient cells, the activation status of ATR and the ATM/ATR target checkpoint proteins, CHK1, CHK2, and P53, were not altered. Although we cannot formally exclude the possibility that PP4 regulates γ-H2AX foci indirectly via some other factor, our data suggest that PP4 directly dephosphorylates γ-H2AX generated during DNA replication. Moreover, unresolved γ-H2AX impedes the repair of DNA damage that occurs during DNA replication as we previously also found for repair of exogenous DNA damage.

In yeast, mutation of the genes encoding for a phosphatase complex composed of PP4C-R2-R3β homologs (Pph3-Yb1046w-Psy2) also led to a basal increase in γ-H2AX, but the cause and consequence of this increase were not clear. Here we address those key issues in the context of PP4 deficiency in mammalian cells. We demonstrate that the cause of the basal increase in γ-H2AX in PP4-deficient cells is DNA replication-mediated damage. PP4-deficient cells are specifically hypersensitive to DNA replication inhibitors. This further supports the idea that PP4 is playing a role in the repair of DNA lesions induced by replication. The only phenotypic effect of Pph3 deficiency in yeast was the phospho-RAD53 mediated prolongation of the DNA damage-induced cell-cycle checkpoint (Keogh et al., 2006). In mammalian cells, there was no effect of PP4 deficiency on the Rad53 homolog, Chk2. The two mammalian homologs of Psy2 are R3β and R3α (Gingras et al., 2005). We failed to detect any interaction between CHK2 and R3β (data not shown). It is possible that some other PP4 complex, perhaps containing R3α, might interact with CHK2. However, a PP2A complex has been shown to dephosphorylate CHK2 in mammalian cells (Dozier et al., 2004). The yeast study with Pph3 deletion mutants and our work with PP4-silenced mammalian cells share many important features; in both systems, a highly homologous complex is required to resolve an increase in basal γ-H2AX levels. Whether or not the major role of yeast HTP-C is to resolve DNA replication-mediated damage will require further study.

In PP4-deficient cells, we found a basal increase in DNA damage even in the absence of an exogenous damaging agent. However, despite unresolved replication-mediated DNA damage, PP4-silenced cells did not arrest during S phase or at the G2/M checkpoint (data not shown), and checkpoint proteins were not activated. Why then does this DNA damage not activate cell-cycle checkpoint proteins such as CHK1 and CHK2? One possibility is that checkpoint activation might require a higher degree of DNA damage than what occurs during ordinary replication. Another explanation could be via “checkpoint adaptation,” originally defined in S. cerevisiae as the ability to divide despite the presence of damaged DNA (Toczyski et al., 1997). The molecular mechanism or consequence of checkpoint adaptation in human cells remains unknown. The effect of PP4-deficiency on checkpoint adaptation needs to be further investigated.

How does persistence of γ-H2AX foci in PP4-deficient cells lead to an increase in basal DNA damage? There are many possible explanations. γ-H2AX recruits and/or stabilizes a DNA repair complex at the site of DSB. The orchestration of DNA repair might involve a series of regulated steps, one of which is blocked by γ-H2AX. Some critical repair factor(s) might either not be recruited or activated until γ-H2AX is resolved. Another possibility is that some repair factors present in limiting amounts and recruited by γ-H2AX might be unnecessarily sequestered by ectopic γ-H2AX in PP4-deficient cells, consequently preventing it from functioning at other DSBs. The association of MRE11 with γ-H2AX foci in PP4C-silenced cells supports this idea. MRE11 is necessary to recognize a DSB, and sequestering MRE11 and keeping it from other DSB sites may affect the efficacy of DSB repair (Assenmacher and Hopfner, 2004). Alternatively, some of the factors recruited include nucleases such as RAD50, which have a limited and transient role at a DSB site (Assenmacher and Hopfner, 2004); sequestering such factors at the DSB could increase DNA damage.

We previously showed that another phosphatase, PP2A, directly dephosphorylates γ-H2AX formed by exogenous DNA-damaging agents (Chowdhury et al., 2005). Why does the cell need multiple phosphatases to eliminate γ-H2AX? Our data suggest that distinct phosphatases differentially regulate γ-H2AX that originates from different stressors and/or from different degrees of DNA damage. For relatively low levels of DNA damage that occur during DNA replication, the PP4 complex is both necessary and sufficient to remove γ-H2AX, but higher levels of damage during DNA replication induced by CPT or hydroxyurea (HU), might require both PP2A and PP4 to eliminate γ-H2AX. The effect of PP4-deficiency on the HR-mediated repair of the I-SceI break (Figure 7B) suggests that PP4 may have a role in the repair of any DSB, induced exogenously or endogenously, that is repaired by HR. Although the kinases, ATM and ATR have distinct roles in γ-H2AX formation, there is also considerable functional overlap in their activity (Abraham, 2004). We speculate that the phosphatases PP2A and PP4 might function in an analogous manner. Future studies with PP4 and PP2A will further elucidate their potentially overlapping roles in eliminating γ-H2AX and enable us to understand the need for multiple phosphatases in regulating γ-H2AX and DNA repair.

**EXPERIMENTAL PROCEDURES**

**Immunofluorescence, Chromatin Fractionation, and Coimmunoprecipitation**

Immunofluorescence, chromatin fractionation, and coimmunoprecipitation were performed essentially as described (Chowdhury et al., 2005; Xu and Stern, 2003) (see the Supplemental Data for details).
In Vitro Enzymatic Analyses
Mononucleosomes containing human γ-H2AX were prepared as described previously (Chowdhury et al., 2005). PP4 and PP2A enzymes were expressed in vitro using PROTEINscript II T7 Kit (Ambion) and immunopurified using anti-HA beads (Sigma). Phosphatase reactions with γ-H2AX mononucleosomes were performed as described (Zabrocki et al., 2002) in 20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 0.2 mM EDTA, 0.2% β-mercaptoethanol for 30 min at 30°C. Reaction mixtures were resolved by 12% SDS-PAGE, and relative phosphatase activity was determined by loss of γ-H2AX immunoreactivity.

Silencing of PP4 Subunits and PP2A
PP4 subunits and PP2A were silenced using standard transfection procedures, and the siRNA sequences and details are provided in the Supplemental Data.

ATR Activity Assay
This assay was done as described (Chiang and Abraham, 2004). For details, see the Supplemental Data.

Single-Cell Gel Electrophoresis/Comet Assay
Single-cell comet assays were performed as per manufacturer’s instructions (Trevigen). For details, see the Supplemental Data.

HR Assay
HR assay was as described (Weinstein et al., 2006). Briefly, 2 × 10⁵ U2OS cells with a single, stably integrated copy of the transgenic reporter DR-GFP plated overnight in 24-well plates were first transfected with PP4C, PP4R3, or control siRNAs and, 24 hr later, transfected with 0.8 μg of I-SceI expression plasmid (pCBA Sce) using Lipofectamine 2000. Two days later, GFP-positive cells were assayed by FACScan.

SUPPLEMENTAL DATA
The Supplemental Data include Supplemental Experimental Procedures and five figures and can be found with this article online at http://www.molecule.org/cgi/content/full/31/1/33/DC1/.

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