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γ-H2AX Dephosphorylation by Protein Phosphatase 2A Facilitates DNA Double-Strand Break Repair

Short Article

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Summary

Phosphorylated histone H2AX (γ-H2AX) forms foci over large chromatin domains surrounding double-stranded DNA breaks (DSB). These foci recruit DSB repair proteins and dissolve during or after repair is completed. How γ-H2AX is removed from chromatin remains unknown. Here, we show that protein phosphatase 2A (PP2A) is involved in removing γ-H2AX foci. The PP2A catalytic subunit [PP2A(C)] and γ-H2AX coimmunoprecipitate and colocalize in DNA damage foci and PP2A dephosphorylates γ-H2AX in vitro. The recruitment of PP2A(C) to DNA damage foci is H2AX dependent. When PP2A(C) is inhibited or silenced by RNA interference, γ-H2AX foci persist, DNA repair is inefficient, and cells are hypersensitive to DNA damage. The effect of PP2A on γ-H2AX levels is independent of ATM, ATR, or DNA-PK activity.

Introduction

DNA double-strand break (DSB) damage triggers a signaling cascade that leads to the rapid formation of a repair complex at the break (Petrini and Stracker, 2003). One of the earliest events in the damage response is phosphorylation of histone H2AX at Ser139 by members of the phosphatidylinositol-3 kinase-like family of kinases (PI3KK) to create γ-H2AX (Fernandez-Capetillo et al., 2004; Thiriet and Hayes, 2005). Within minutes of DNA damage, γ-H2AX appears at discrete nuclear foci (Rogakou et al., 1999) that contain DNA repair factors like the MRN repair complex, 53BP1, BRCA1 (Paull et al., 2000), and MDC1 (mediator of DNA damage checkpoint protein 1) (Goldberg et al., 2003; Lou et al., 2003; Stewart et al., 2003). γ-H2AX plays an important role in recruiting some, but probably not all (Celeste et al., 2003), proteins to the repair focus and in stabilizing the repair focus to recruit late factors like cohesins (Unal et al., 2004). Cohesins tether the sister chromatids, allowing the undamaged strand to serve as a template for homologous recombination (Strom et al., 2004; Unal et al., 2004; Xie et al., 2004). How γ-H2AX is attenuated and sister chromatids segregate after repair is unknown. Two mechanisms are possible: (1) γ-H2AX may be removed from chromatin by histone exchange or (2) γ-H2AX could be dephosphorylated by a protein phosphatase.

Here, we identify a role for protein phosphatase 2A (PP2A) in removing γ-H2AX foci. PP2A directly binds to and dephosphorylates γ-H2AX either in its monomeric form or when incorporated into mononucleosomes in vitro and in cell lysates. The catalytic subunit of PP2A (PP2A[C]) is recruited to DNA damage foci in wild-type cells, but not in H2AX-deficient cells. PP2A regulates the kinetics of γ-H2AX focus formation and persistence in response to DSB. The effect of PP2A on γ-H2AX appears to be independent of ATM, ATR, and DNA-PK. In cells lacking active PP2A after treatment with PP2A inhibitors or small interfering RNA (siRNA), γ-H2AX foci persist much longer than in control cells. Furthermore, PP2A-deficient cells have inefficient DNA repair and are hypersensitive to DNA damage.

Results and Discussion

To investigate whether a phosphatase is involved in downregulating γ-H2AX, K562 cells were exposed to DNA damaging agents in the presence of okadaic acid (OA), an inhibitor of the serine and/or threonine phosphatases 1 (PP1) and 2A (PP2A). At concentrations <50 nM, OA only inhibits PP2A (Honkanen and Golden, 2002). The topoisomerase inhibitor camptothecin (CPT) and dNTP synthesis-inhibitor hydroxyurea (HU) induce DSB by stalling DNA replication forks, whereas H_2O_2 induces single-strand breaks (SSB). Cellular γ-H2AX, which increases after CPT or HU, but not H_2O_2, treatment, is further increased in the presence of 25 nM OA (Figure 1A). This low OA dose also accelerates the kinetics of phosphorylation (Figure 1B). To focus on the repair process, we repeated these experiments but removed CPT after 1 hr before adding a phosphatase inhibitor (Figure 1C). To address the concern that OA may be inhibiting other phosphatases, we substituted 100 nM fostriecin for OA. Fostriecin is 10,000-fold more active in inhibiting PP2A than inhibiting PP1 (Walsh et al., 1997). In the absence of inhibitor, γ-H2AX peaks at 2 hr and returns to background by 8 hr. However, in the presence of fostriecin, γ-H2AX is significantly increased at all times and remains elevated even 8 hr after removing CPT. Enhanced and persistent H2AX phosphorylation in the presence of fostriecin was also seen after ionizing radiation (data not shown). Therefore, PP2A regulates the cellular pool of γ-H2AX after DSB.

γ-H2AX foci are an early marker of DSB sites undergoing DNA repair. These foci, visible by immunofluorescence microscopy, occur at low frequency in cycling cells, reflecting ATM-dependent H2AX phosphorylation that occurs in mitosis (Ichijima et al., 2005; McManus and Hendzel, 2005). To examine the effect of PP2A on focus formation, HeLa cells were treated with 10 μM CPT for 1 hr (Furuta et al., 2003), washed extensively, and

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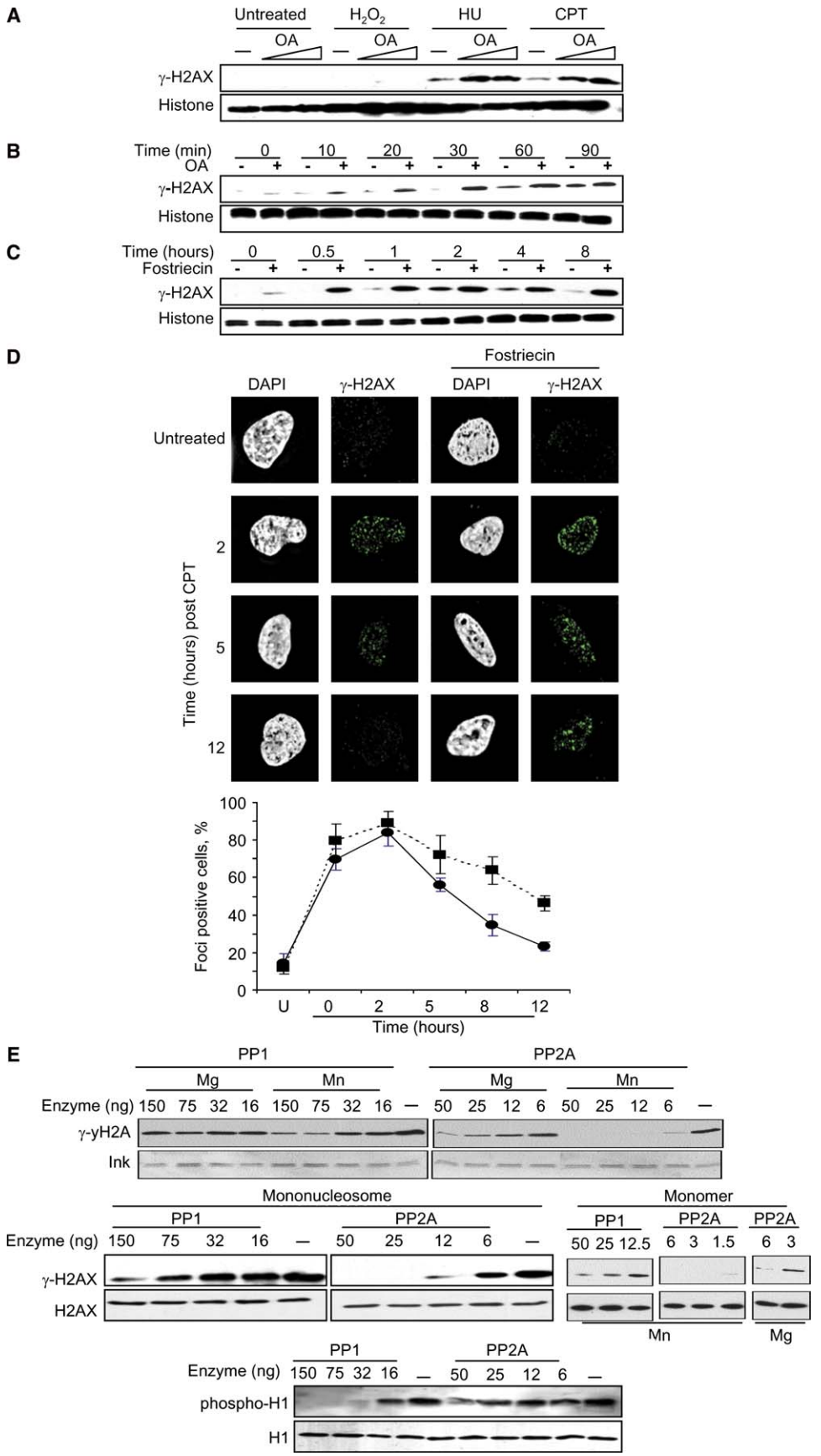

A
Untreated
H2O2
HU
CPT
OA
γ-H2AX
Histone
B
Time (min)
0
10
20
30
60
90
OA
γ-H2AX
Histone
C
Time (hours)
0
0.5
1
2
4
8
Fostriecin
γ-H2AX
Histone
D
Fostriecin
DAPI
γ-H2AX
Untreated
Time (hours) post CPT
2
5
12
Foci positive cells, %
100
80
60
40
20
0
U
0
2
5
8
12
Time (hours)
E
PP1
PP2A
Mg
Mn
Enzyme (ng)
150 75 32 16 150 75 32 16 — 50 25 12 6 50 25 12 6 —
γ-yH2A
Ink
Mononucleosome
Monomer
PP1
PP2A
Enzyme (ng)
150 75 32 16 — 50 25 12 6 —
50 25 12.5
6 3 1.5
6 3
γ-H2AX
H2AX
Mn
Mg
PP1
PP2A
Enzyme (ng)
150 75 32 16 — 50 25 12 6 —
phospho-H1
H1

incubated with or without 100 nM fostriecin (Figure 1D). Fostriecin does not induce γ-H2AX foci in the absence of DNA damage and has no significant effect on focus formation 2 hr after CPT exposure (Figure 1D). However, at 5 hr, the number of foci-positive cells and intensity of γ-H2AX foci was significantly increased by fostriecin treatment. By 12 hr, foci persisted in >50% of fostriecin-treated cells, whereas only ~20% of cells with active PP2A still maintained foci (Figure 1D). Therefore, PP2A regulates chromatin-associated γ-H2AX.

A previous study suggested that PP1 removes γ-H2AX from repaired DSBs based on inhibition by calyculin A and in vitro dephosphorylation of chromatin-associated γ-H2AX (Nazarov et al., 2003). However, calyculin A inhibits both PP1 and PP2A with comparable efficiency (Honkanen and Golden, 2002), and our work suggests that PP2A is the more likely physiological γ-H2AX phosphatase. To resolve this issue, we compared the in vitro activity of the two enzymes against three different substrates: ribosomal S6 kinase 1 (Rsk1)-phosphorylated yeast H2A/H2B dimers and human γ-H2AX, either monomeric or reconstituted in mononucleosomes. Although *S. cerevisiae* does not have an H2AX variant, the yeast H2A (yH2A) C-terminal tail contains a conserved SQE motif that is phosphorylated at DSBs (Shroff et al., 2004) (phosphorylated yeast H2A is referred to here as γ-yH2A). PP2A dephosphorylates γ-yH2A/H2B dimers (Figure 1E, upper) and γ-H2AX in monomeric form or when incorporated into nucleosomes (Figure 1E, middle). PP2A is at least 25-fold more active than PP1 against these substrates. PP2A activity is specific, because even at higher concentrations it does not dephosphorylate histone H1 (Figure 1E, lower). Optimal reaction conditions for each phosphatase were determined empirically and correspond closely to those described previously (Cohen, 1989; Zabrocki et al., 2002). Both enzymes require a divalent cation, with Mn^{2+} preferred over Mg^{2+} or Ca^{2+} (see Figure S1A in the Supplemental Data available with this article online). As expected, PP2A is considerably more sensitive than PP1 to OA inhibition (Figure S1B).

If PP2A dephosphorylates γ-H2AX, it should colocalize at γ-H2AX foci. We therefore costained CPT-treated HeLa cells with antibodies to PP2A(C), γ-H2AX, and Nbs1, a component of the MRN DSB repair complex (Figure 2A, Figure S2A). In undamaged cells, PP2A(C) is primarily nuclear, with detectable amounts in the cytoplasm (Figure 2A and Turowski et al., 1995). Within 1 hr of CPT treatment, PP2A(C) becomes almost exclusively nuclear and punctate foci begin to form. The foci increase in size and intensity by 2.5 hr and overlap extensively (but not completely because PP2A(C) is in excess) with γ-H2AX foci (Figure 2A). Ten hr later, the γ-H2AX foci largely disappear and PP2A(C) returns to its baseline distribution. The DSB repair factor Nbs1 also colocalizes with PP2A(C) and γ-H2AX in these experiments, confirming that foci are active DNA repair sites (Figure S2A).

Some repair and signaling proteins migrate to DSBs independently of H2AX (Celeste et al., 2003). However, foci in *H2AX*$^{-/-}$ mouse-embryo fibroblasts (MEF) are unstable and disintegrate within 60 min. To determine whether PP2A(C) recruitment to DSB is H2AX-dependent, we costained irradiated H2AX-deficient and control MEF with antibodies to 53BP1 and PP2A(C). In control MEF, PP2A(C) and 53BP1 form discrete overlapping foci within 30 min of irradiation. By 60 min, foci are larger and more distinct. Most H2AX-deficient cells display discrete 53BP1 foci within 15 to 30 min, but the foci are not as distinct as in control MEF (data not shown, Figure 2B). As expected, by 60 min there are no visible foci and cells diffusely stain for 53BP1. Nuclear PP2A(C) staining is radically different in irradiated *H2AX*$^{-/-}$ MEF than in control MEF. It remains diffuse and does not concentrate in foci (Figure 2B). Given the abundance of nuclear PP2A, we cannot exclude inefficient PP2A recruitment to DSB in H2AX-deficient cells. Nonetheless, efficient PP2A(C) recruitment to DSB requires H2AX.

To demonstrate a direct interaction of PP2A(C) with γ-H2AX, we did coimmunoprecipitation experiments. Although PP2A(C) does not associate with γ-H2AX or H2AX in untreated cells, PP2A(C), but not PP1, coimmunoprecipitates with γ-H2AX in cell lysates extracted 2.5 hr after CPT-induced DNA damage (Figure 2C). The interaction increases with the extent of DNA damage, with 5 μM CPT inducing greater association of PP2A(C) with γ-H2AX or H2AX than 1 μM CPT (Figure 2B). The interaction of γ-H2AX is direct, as recombinant H2AX, phosphorylated in vitro, coprecipitates with purified PP2A (AC heterodimer), but not with PP1. Unphosphorylated H2AX also binds purified PP2A somewhat

Figure 1. PP2A Dephosphorylates γ-H2AX In Vitro, and Inhibiting PP2A Increases Cellular γ-H2AX and the Persistence of γ-H2AX Foci in Response to DSB

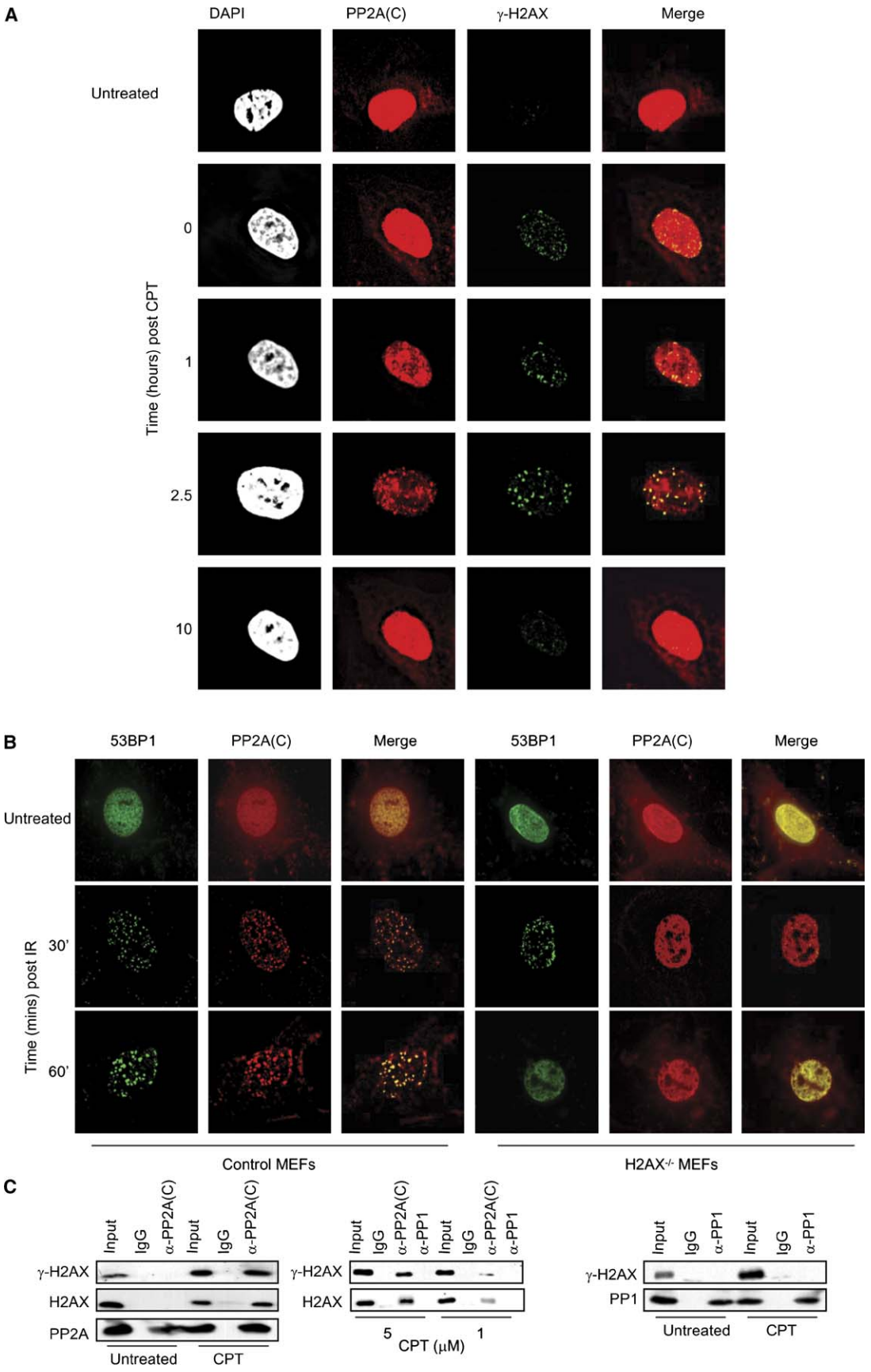
(A) OA increases cellular γ-H2AX in K562 cells treated with genotoxic agents that induce DSB (CPT, HU), but not SSB (H_2O_2). Cells, untreated or pretreated with CPT, HU, or H_2O_2, were then incubated with 25 or 150 nM OA for 2 hr at 37°C before protein extraction. OA treatment alone does not induce γ-H2AX.

(B) OA modulates the kinetics of γ-H2AX induction in CPT-treated K562 cells. Cells were treated with CPT ± OA for up to 90 min before protein extraction.

(C) The PP2A-specific inhibitor fostriecin increases γ-H2AX in CPT-damaged K562 cells. CPT pretreated cells were washed to remove CPT and incubated ± fostriecin for up to 8 hr. In (A)–(C), immunoblots were performed on whole-cell extracts extracted at the indicated times and probed for γ-H2AX (top) or total histones (bottom).

(D) Inhibiting PP2A leads to persistence of γ-H2AX foci in CPT-treated HeLa cells. Untreated or CPT-pretreated cells (10 μM) were washed to remove CPT and incubated ± fostriecin for up to 12 hr. Cells were stained with anti-γ-H2AX and DAPI. The graph indicates the percentage of cells displaying γ-H2AX foci. Error bars represent ±SD. Curves were generated from three independent experiments. Significantly higher numbers of fostriecin-treated cells (■) are foci positive relative to control cells (●) at 5 hr ($p < 0.005$), 8 hr ($p < 0.001$), and 12 hr ($p < 0.002$).

(E) PP2A dephosphorylates γ-yH2A and γ-H2AX in vitro more efficiently than PP1. PP2A dephosphorylates >90% of γ-yH2A under optimal conditions (5 mM $MnCl_2$). In contrast, 25-fold more PP1 dephosphorylates <50% of γ-yH2A under optimal conditions (upper). PP2A dephosphorylates human γ-H2AX assembled in mononucleosomes or as monomers more efficiently than PP1 (middle). PP2A does not efficiently dephosphorylate phospho-H1 (lower). Staining with India ink (upper), immunoblot for total H2AX (middle), or H1 (lower) serve as loading controls.


A
DAPI
PP2A(C)
γ-H2AX
Merge
Untreated
0
1
2.5
10
Time (hours) post CPT
B
53BP1
PP2A(C)
Merge
53BP1
PP2A(C)
Merge
Untreated
30'
60'
Time (mins) post IR
Control MEFs
H2AX-/- MEFs
C
Input
IgG
α-PP2A(C)
Input
IgG
α-PP2A(C)
γ-H2AX
H2AX
PP2A
Untreated
CPT
Input
IgG
α-PP2A(C)
α-PP1
Input
IgG
α-PP2A(C)
α-PP1
γ-H2AX
H2AX
5
1
CPT (μM)
Input
IgG
α-PP1
Input
IgG
α-PP1
γ-H2AX
PP1
Untreated
CPT

(Figure S2B). Coimmunoprecipitation and colocalization of PP2A(C) with γ-H2AX in response to DSB, together with the inhibitor experiments, strongly suggest that PP2A acts directly on γ-H2AX.

PP2A regulates a variety of important cellular processes (Janssens et al., 2005; Virshup, 2000). Its substrates include the DNA repair proteins Chk2, DNA-PK, and ATM (Douglas et al., 2001; Dozier et al., 2004; Goodarzi et al., 2004), the latter two being known H2AX kinases (Fernandez-Capetillo et al., 2004). PP2A enhances the kinase activity of DNA-PK (Douglas et al., 2001) and maintains ATM in an inactive form (Dozier et al., 2004; Goodarzi et al., 2004). In undamaged cells, PP2A associates with ATM (Goodarzi et al., 2004). Upon genotoxic stress, PP2A dissociates, at which point ATM is activated by S1981 autophosphorylation (Bakkenist and Kastan, 2003; Goodarzi et al., 2004). Although ATM phosphorylates H2AX in response to ionizing radiation (IR), γ-H2AX induction in response to CPT is ATM-independent and instead involves ataxia telangiectasia-mutated and Rad3-related (ATR) protein (Furuta et al., 2003). Nonetheless, to exclude the possibility that the increase in γ-H2AX we observed with PP2A inhibition is indirect—mediated by constitutively active ATM—we looked at the effect of fostriecin on γ-H2AX in CPT-treated *ATM*$^{-/-}$ MEF (Elson et al., 1996). In the presence of PP2A inhibitor, γ-H2AX remains elevated in *ATM*$^{-/-}$ MEFs for at least 8 hr (Figure 3A, upper). Therefore, PP2A has an ATM-independent effect on γ-H2AX dephosphorylation. Moreover, PP2A(C) is recruited to γ-H2AX foci 2.5 hr after CPT treatment in *ATM*$^{-/-}$ MEF as in wild-type cells, again suggesting that our results are not an indirect effect of PP2A dephosphorylation of ATM (Figure 3A, lower). We also investigated whether inhibiting PP2A in ATM-deficient cells affects the activity of other PI3K-like kinases that phosphorylate H2AX. Treatment with CPT, fostriecin, or both does not alter ATR kinase activity, suggesting that the difference in γ-H2AX is not due to an effect of PP2A on ATR (Figure 3B). CPT treatment is reported to enhance DNA-PK activity (Shao et al., 1999), whereas fostriecin inhibits DNA-PK (Douglas et al., 2001). Fostriecin also blocks DNA-PK activation in CPT-treated ATM-deficient cells (Figure 3C). Hence, the increase in γ-H2AX levels in PP2A-inhibited cells is not due to an increase in DNA-PK activity. Together, our observations suggest that PP2A does not indirectly reduce γ-H2AX levels by regulating the known H2AX kinases, ATM, ATR, or DNA-PK.

PP2A belongs to a phosphatase family that includes PP4 (65% protein sequence identity) and PP6 (57% identity) (Honkanen and Golden, 2002). OA and fostriecin, the inhibitors used in our experiments, comparably inhibit PP4 and PP2A. To confirm our findings that PP2A dephosphorylates γ-H2AX, small interfering RNAs (siRNAs) were designed to downregulate PP2A expression without affecting PP1, PP4, or PP6. A combination of three siRNAs specifically reduces PP2A expression by 90% (Figure 4A). We next investigated whether γ-H2AX foci persist in PP2A-silenced cells (Figure 4B). HeLa cells transfected with control GFP or PP2A(C) siRNAs were treated with CPT (Figure 4B). Two hr after DNA damage, focus formation was comparable in control or PP2A-deficient cells. However, γ-H2AX foci persist at 8 hr in PP2A-deficient cells, with >50% having increased foci, whereas control cells show significantly reduced numbers of foci. This further supports the conclusion that PP2A dephosphorylates γ-H2AX in cells.

To determine whether PP2A expression and γ-H2AX persistence affect DNA repair, we measured the persistence of DSB in CPT-treated HeLa cells, transfected with PP2A or GFP control siRNAs, using single-cell gel electrophoresis (comet assay, Figure 4C). CPT treatment induces DSBs, visible by increased DNA mobility or "comet tails." Two hr after CPT treatment, control and PP2A-silenced cells have comparable amounts of DNA damage. However, DNA repair is essentially complete by 8 hr in the control population, whereas tails are still visible in PP2A-deficient cells. Based on the comet moments, which quantify the extent of DNA damage, we estimate 3- to 4-fold more unresolved DNA damage in PP2A-deficient cells than control cells at 8 hr. The effect of PP2A silencing on DNA repair is probably via its activity on several proteins, including ATM, DNA-PK, and γ-H2AX. Delaying DNA repair may be biologically significant, and indeed PP2A(C)-silenced cells have reduced viability at all tested doses of CPT relative to control cells (Figure 4D). It should be noted that PP2A(C) depletion reduces population viability even in undamaged cells (data not shown), suggesting that the phosphatase is required to maintain cell health.

Here, we show that PP2A dephosphorylates γ-H2AX by demonstrating in vitro phosphatase activity on monomeric and nucleosomal γ-H2AX, colocalization at DNA repair foci, coimmunoprecipitation in response to DSB, increased γ-H2AX in cells, and persistence of γ-H2AX foci when PP2A activity is inhibited or its expression is silenced. Moreover, we find that PP2A recruitment to DNA damage foci is H2AX-dependent and is required for efficient DSB repair. The effect of PP2A on γ-H2AX is independent of ATM, ATR, and DNA-PK. We therefore hypothesize that PP2A regulates at least two steps in DSB repair. It controls the activity of two of the major kinases (ATM and DNA-PK) that phosphorylate H2AX as a DSB is recognized (Douglas et al., 2001; Goodarzi et al., 2004). γ-H2AX then stabilizes the DNA repair complex and recruits PP2A to the DSB. At a later point, PP2A dephosphorylates H2AX and potentially other factors at the DSB during or after repair is completed. A parallel study in yeast identifies the closely related phosphatase Pph3 (60% identity) in *S. cerevisiae*

Figure 2. H2AX-Dependent Colocalization of PP2A(C) with DNA Damage/Repair Foci and Coimmunoprecipitation of H2AX and PP2A(C) in CPT-Treated Cells

(A) PP2A(C) colocalizes with γ-H2AX in DNA repair foci. Untreated or CPT-treated (10 μM) HeLa cells were washed to remove CPT and incubated for up to 10 hr before fixing and staining for γ-H2AX and PP2A(C).

(B) PP2A(C) colocalizes with 53BP1 and forms discrete foci only in the presence of H2AX. Control or *H2AX*$^{-/-}$ MEF were γ or mock irradiated before fixing and staining at indicated times for PP2A(C) and 53BP1.

(C) PP2A(C) associates with γ-H2AX or H2AX after DSB induction. Lysates from HeLa cells treated or not with CPT (as indicated, 2.5 hr) were immunoprecipitated with mouse IgG, anti-PP2A(C), or anti-PP1 and probed for γ-H2AX, H2AX, PP2A, or PP1.

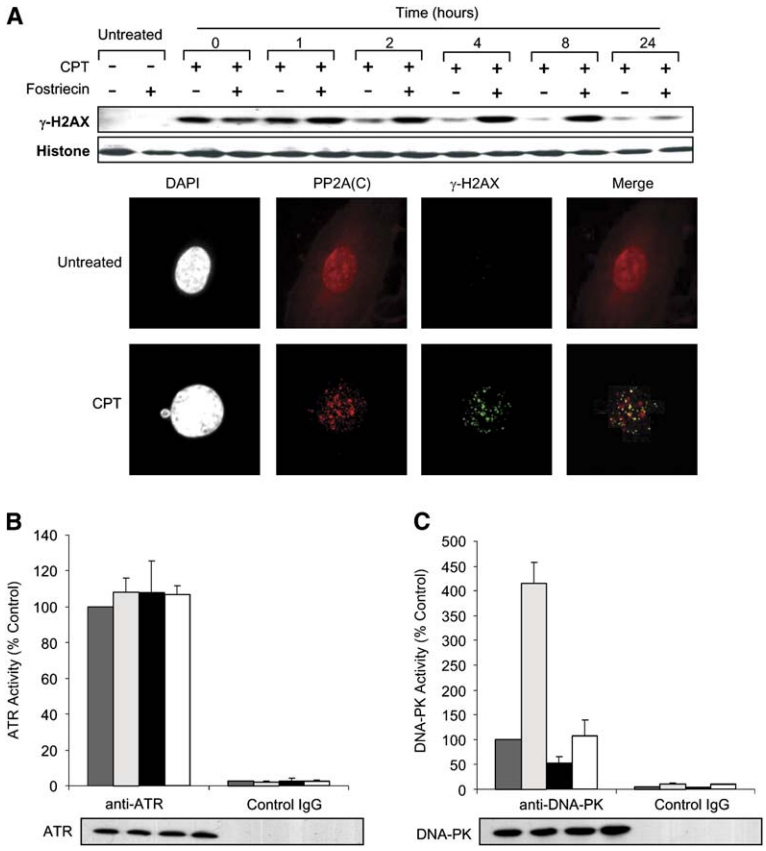


Figure 3. PP2A Reduces γ-H2AX Independently of ATM, ATR, or DNA-PK

(A) In $ATM^{-/-}$ cells, the PP2A inhibitor fostriecin enhances γ-H2AX (upper) and PP2A(C) colocalizes with γ-H2AX (lower). Untreated or CPT-treated $ATM^{-/-}$cells were washed and then incubated ± fostriecin for up to 24 hr. Immunoblots were performed on whole-cell extracts extracted at the indicated times and probed for γ-H2AX (top) or total histone (bottom). Untreated or CPT-treated $ATM^{-/-}$ cells were washed and incubated for 2.5 hr before fixing and staining for γ-H2AX and PP2A(C). Fostriecin does not alter ATR activity (B) but significantly reduces DNA-PK activity (C) ($p < 0.002$) in $ATM^{-/-}$ MEFs. ATR or DNA-PK was isolated by using specific antibodies from lysates prepared from untreated or CPT-treated $ATM^{-/-}$ MEF that were incubated with or without fostriecin. Shown are the mean ±SD values. The kinase activity of the immunocomplex from CPT-treated cells was assayed relative to the activity in untreated cells. The lower panel in (B) and (C) shows comparable amounts of immunoprecipitated ATR or DNA-PK, respectively. Color coding is as indicated: dark gray, untreated; light gray, CPT; black, fostriecin; white, CPT + fostriecin.

as responsible for γ-yH2A dephosphorylation (Keogh et al., 2005).

This study does not address the important question of whether PP2A dephosphorylates γ-H2AX directly on chromatin or whether it dephosphorylates γ-H2AX displaced from the repaired damage site. Our data only show that PP2A regulates the total cellular γ-H2AX pool. The most economical explanation of our findings, given the colocalization of γ-H2AX and PP2A(C) at DSB sites, is that PP2A works on chromatin-associated γ-H2AX. The mobility of GFP-H2AX in live mammalian nuclei studied by fluorescence redistribution after photobleaching was found to be very low (Siino et al., 2002), indicating that turnover by histone exchange may not be the predominant way by which γ-H2AX is removed. However, recent studies have implicated two SNF2-family ATPase complexes in removing γ-yH2A and γ-H2AX by histone exchange: Ino80 in *S. cerevisiae* (Downs et al., 2004; Morrison et al., 2004; van Attikum et al., 2004) and dTip60 in *Drosophila* (Kusch et al., 2004), respectively. Whether the model for dTip60 applies to other species is currently unclear: *Drosophila* contains a histone variant known as H2Av, an amalgam of H2AZ and H2AX not found in human or yeast cells. Indeed, the presumed yeast homolog of dTip60, the SWR-C, catalyzes the exchange of H2AZ rather than H2A (Krogan et al., 2003). In yeast DSB foci, γ-yH2A is found at large stretches ~50 kB surrounding the DSB, but not within 1 to 2 kB of the break, whereas the Ino80 complex appears to be concentrated near the break site (i.e., within 1.5 kb) along with repair factors like the Mre11/Nbs1/Rad50 complex (Downs et al., 2004; Shroff et al., 2004). One possible interpretation is that there may be two mechanisms by which γ-H2AX is eliminated: near the DSB, a chromatin remodeling complex removes γ-H2AX and alters chromatin structure, allowing repair factors access to the DSB; whereas, more distally, γ-H2AX is eliminated by direct PP2A-mediated dephosphorylation. Future studies defining the interaction of PP2A with other DSB repair proteins at γ-H2AX foci may help define the mechanism of γ-H2AX elimination and its role in completing DSB repair.

Experimental Procedures

Cell Lines, Antibodies, and Reagents

K562 and HeLa cells were grown in RPMI 1640 and Dulbecco's modified Eagle's medium (DMEM), respectively, supplemented with 10% fetal calf serum, 2 mM glutamine, 2 mM HEPES, 100 U/ml penicillin, and 100 mg/ml streptomycin. $ATM^{-/-}$ mouse endothelial fibroblasts (kind gift of P. Leder) were maintained in supplemented DMEM as above but containing 1 mM sodium pyruvate and 4 mM glutamine. $H2AX^{-/-}$ mouse endothelial fibroblasts (kind gift of F. Alt) were maintained in supplemented DMEM as above but containing 15% fetal calf serum and 5 mM glutamine. Antibodies were: H2AX and phospho-H1 (rabbit polyclonal, Upstate Biotech); γ-H2AX, H1, and PP2A(C) (monoclonal, Upstate Biotech); PP1(catalytic subunit, monoclonal, Santa Cruz); PP4 (catalytic subunit, rabbit polyclonal, Chemicon International); PP6 (catalytic subunit, rabbit polyclonal, Sigma); Nbs1 (goat polyclonal, Santa Cruz); 53BP1 (rabbit polyclonal, Cell Signaling); ATR (rabbit polyclonal, Santa Cruz); DNA-PK (catalytic subunit, monoclonal, BD Transduction); β-actin (monoclonal, Sigma); and pan-histone (monoclonal, Chemicon International).

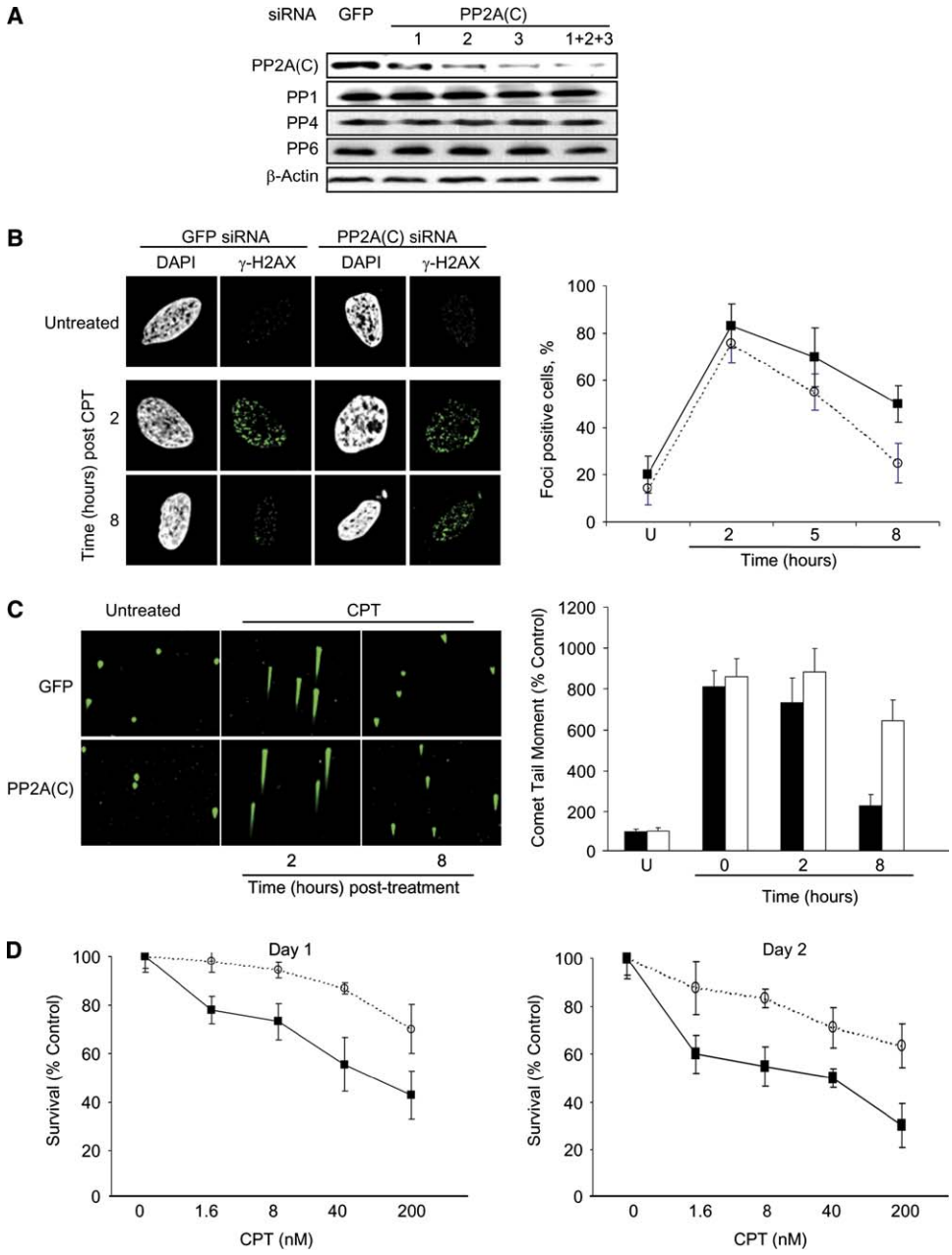


Figure 4. PP2A Is Required for Efficient DNA Damage Repair

(A) Only PP2A(C) protein (and not other phosphatases) is efficiently reduced by PP2A(C)-specific siRNAs. HeLa cells, transfected with control (GFP) or PP2A(C) siRNAs 72 hr earlier were analyzed by immunoblot probed for PP2A(C), PP1, PP4, PP6, or β-actin. Experiments below were performed with a combination of all three PP2A(C) siRNAs.

(B) Silencing PP2A causes a persistence of γ-H2AX foci in CPT-treated cells. GFP or PP2A(C) siRNA-transfected HeLa cells, treated or not with CPT (2 μM), were stained with anti-γH2AX and DAPI. Foci-positive cells were quantified and analyzed as described in Figure 1D. Curves represent mean ± SD from three independent experiments. Significantly more PP2A-silenced cells (■) than control cells (○) are foci positive at 5 hr ($p < 0.005$) and 8 hr ($p < 0.002$).

(C) Silencing PP2A impairs DNA repair in CPT-treated cells. GFP or PP2A(C) siRNA-transfected HeLa cells, treated or not with CPT (2 μM), were analyzed by single-cell gel electrophoresis (comet assay). Representative images are on the left. The comet tail moment of 75 cells (mean ± SD) for each time and condition was quantified with NIH Image software and normalized to that of untreated cells. Color coding is as follows: black bars, GFP siRNA; white, PP2A siRNA.

(D) PP2A-silenced cells are hypersensitive to CPT. Cell viability was analyzed by MTT assay. Curves represent mean ± SD from three independent experiments. PP2A-silenced cells (■) are significantly more sensitive than control cells (○) ($p < 0.001$) to each concentration of CPT when assayed either 1 or 2 days after genotoxic stress.

CPT, HU, and MTT tetrazolium were from Sigma-Aldrich. Phosphatase inhibitors were okadaic acid (OA, 25 nM or indicated dose, CalBiochem) and fostriecin (100 nM, Kamiya Biomedical). PP1 phosphatase was from New England Biolabs. Rsk1, PP2A, and H2AX were from Upstate Biotech. H2AX peptide (134–142) was from AbCam.

DNA Damage

Cells were treated with CPT (1 μM or indicated dose) or 2.5 mM HU for 1 hr at 37ºC or with 1.5 mM H_2O_2 for 30 min at 4ºC.

Enzyme Assays

γ-yH2A or human γ-H2AX was generated with activated rat RSK1 kinase (Upstate) and recombinant yeast H2A/H2B dimers (purified as per Levenstein and Kadonaga, 2002) or human H2AX monomer. After phosphorylation, samples were dialyzed into 20 mM Tris HCl (pH 7.0), 0.1 mM EDTA, 1 mM DTT, and 0.01% Brij-35. γ-yH2A/H2B or γ-H2AX was stored at −70ºC. Mononucleosomes containing human γ-H2AX were made following the method of (Luger et al., 1999) with details in the Supplemental Data. The phospho-H1 substrate was prepared from colcemid-treated HeLa cells (Upstate). Phosphatase reactions were performed in 20 mM Tris HCl (pH 7.4), 50 mM NaCl, 0.2 mM EDTA, and 0.2% β-ME for 30 min at 30ºC as described (Zabrocki et al., 2002). ATR and DNA-PK kinase assays were as described (Chiang and Abraham, 2004), with details in the Supplemental Data.

Immunofluorescence

HeLa cells (2×10^5), *ATM*$^{-/-}$ MEF, and *H2AX*$^{-/-}$ MEF were grown overnight on coverslips. HeLa cells and *ATM*$^{-/-}$ MEF were treated with CPT and fixed with 3.7% paraformaldehyde. *H2AX*$^{-/-}$ MEF were γ irradiated (3 Gy) or mock irradiated and fixed in methanol at indicated times. Cells were stained and analyzed with a Zeiss confocal microscope as described (Keefe et al., 2005). Cells were judged positive for γ-H2AX foci if they displayed five or more discrete bright dots. To quantify foci, at least 200 cells were analyzed for each condition.

Coimmunoprecipitation

Coimmunoprecipitations were as described (Stewart et al., 2003). HeLa cells (10^7) were treated or not with CPT at indicated concentrations for 1 hr at 37ºC and allowed to recover for 2.5 hr before lysis in NETN (50 mM Tris HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, and 1% NP-40). Lysates were precleared and incubated with indicated antibodies and Protein A/G beads. Beads were washed extensively with NETN buffer (containing 0.5% NP-40), resolved by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by immunoblot.

Silencing of PP2A(C)

Synthetic siRNA duplexes were transfected by using Trans IT-TKO (Mirus) as recommended. The PP2A(C) and GFP siRNAs (Dharmacon) were described previously (Fan et al., 2003; Pandey et al., 2003). PP2A(C) downregulation was confirmed 3 days after transfection by immunoblot. For immunofluorescence, HeLa cells (10^5), plated overnight on coverslips, were transfected with siRNAs and treated with CPT 60 hr later.

Cell Viability Assay

siRNA-transfected HeLa cells were seeded (10^3/100 μl) into octuplicate microtiter wells, incubated overnight, and then treated with CPT or medium for 24 or 48 hr. Viability was measured by MTT assay (Furuta et al., 2003). Results were expressed as the OD_{520} relative to that of untreated cells.

Single-Cell Gel Electrophoresis (Neutral Comet) Assay

Single-cell comet assays were performed as per the manufacturer's instructions (Trevigen). For details see the Supplemental Data.

Supplemental Data

Supplemental Data including two figures, Supplemental Experimental Procedures, and Supplemental References are available online with this article at http://www.molecule.org/cgi/content/full/20/5/■■■/DC1/.

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