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Report

Human USP3 Is a Chromatin Modifier Required for S Phase Progression and Genome Stability

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Summary

Protein ubiquitination is critical for numerous cellular functions, including DNA damage response pathways [1, 2]. Histones are the most abundant monoubiquitin conjugates in mammalian cells; however, the regulation and the function of monoubiquitinated H2A (uH2A) and H2B (uH2B) remain poorly understood. In particular, little is known about mammalian deubiquitinating enzymes (DUBs) that catalyze the removal of ubiquitin from uH2A/uH2B. Here we identify the ubiquitin-specific protease 3 USP3 as a deubiquitinating enzyme for uH2A and uH2B. USP3 dynamically associates with chromatin and deubiquitinates H2A/H2B in vivo. The ZnF-UBP domain of USP3 mediates uH2A-USP3 interaction. Functional ablation of USP3 by RNAi leads to delay of S phase progression and to accumulation of DNA breaks, with ensuing activation of DNA damage checkpoint pathways. In addition, we show that in response to ionizing radiation, (1) uH2A redistributes and colocalizes in γ-H2AX DNA repair foci and (2) USP3 is required for full deubiquitination of ubiquitin-conjugates/uH2A and γ-H2AX dephosphorylation. Our studies identify USP3 as a novel regulator of H2A and H2B ubiquitination, highlight its role in preventing replication stress, and suggest its involvement in the response to DNA double-strand breaks. Together, our results implicate USP3 as a novel chromatin modifier in the maintenance of genome integrity.

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Results and Discussion

USP3 Is a Chromatin-Associated DUB

To identify nuclear proteins involved in histone (de)ubiquitination, we employed affinity chromatography on Ub-agarose of chromatin-enriched nuclear fractions. MALDI-TOF mass spectrometric analysis identified the E1 enzyme and the DUBs Ub-specific protease 5 (USP5/isopeptidase T) and Ub-specific protease 3 (USP3) as proteins specifically interacting with Ub-agarose (Figure 1A).

USP3 has been characterized as a functional DUB in vitro, and it is the human DUB most homologous to *S. cerevisiae* Ubp8, which regulates H2B deubiquitination [3–5]. Analysis of USP3 subcellular localization confirmed that USP3 is a nuclear protein and that it is present in the chromatin fraction (Figure S1 in the Supplemental Data available online).

USP3 Regulates the Cellular Levels of Ubiquitinated H2A and H2B

To address a potential function of USP3 in chromatin regulation, we ectopically expressed USP3 in HeLa cells and analyzed the ubiquitination state of histones (Figures 1B–1D). Approximately 5%–15% of histone H2A and less than 1% of H2B are monoubiquitinated, making them the two major monoubiquitinated chromosomal proteins [5]. uH2A was detected by immunoblotting with anti-Ub, anti-H2A antibodies (Ab), or a monoclonal Ab specific for uH2A [6], and direct anti-H2B immunoblot visualized uH2B (Figures 1C and 1D; Figure S2A). Both uH2A and uH2B amounts were significantly reduced upon USP3 overexpression (Figures 1C and 1D). In contrast, USP5 did not affect the levels of uH2A (Figure S2B). USP3 did not alter the total pool of ubiquitinated proteins (not shown).

Next, we knocked down USP3 expression in HeLa cells (USP3-KD) by small interfering RNA (siRNA). Efficient reduction of USP3 protein was accompanied by a significant increase in the levels of uH2A and, to a lesser extent, of uH2B (Figure 1E). Altogether, these data strongly suggest that USP3 is required for H2A and H2B deubiquitination in vivo.

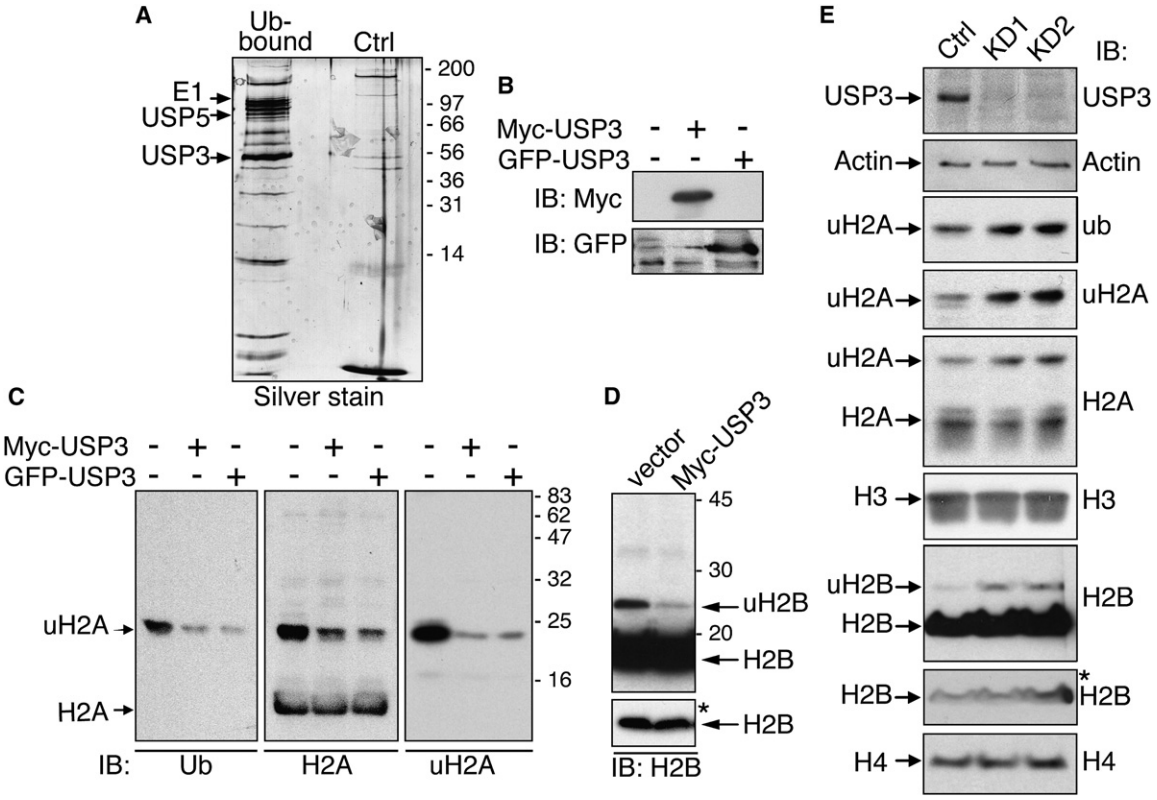


Figure 1. USP3 Is a Regulator of H2A and H2B Ubiquitination In Vivo

(A) Silver stain of nuclear proteins retained on Ub-agarose (Ub-bound) or control (ctrl) beads. Arrows: proteins identified by mass spectrometry; E1 (P22314), USP3 (Q9Y6I4), USP5 (P45974).

(B–D) Overexpression of USP3 decreases uH2A and uH2B. Whole-cell extracts (WCE) (B) and histone fractions (C and D) of HeLa cells transfected with GFP- or Myc-USP3 were IB as indicated. Asterisk indicates IB H2B shorter exposure.

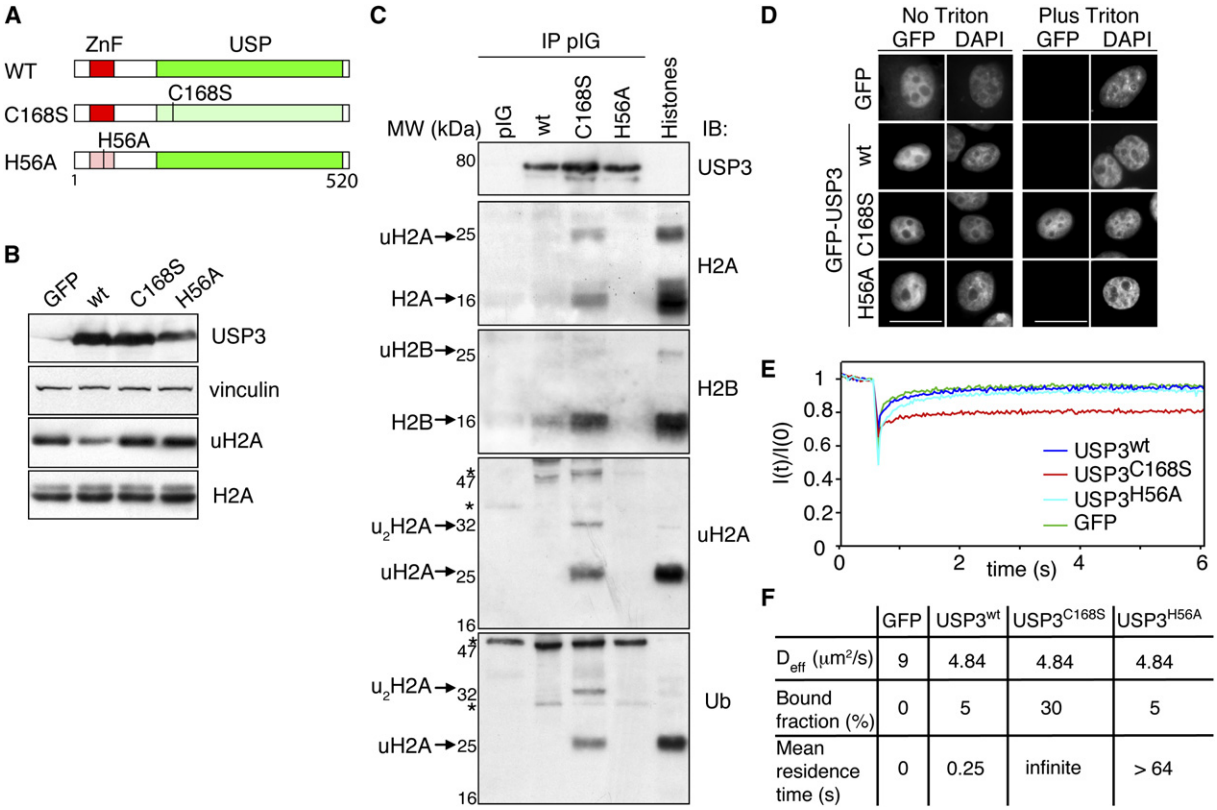
(E) Increased uH2A and uH2B levels in USP3-depleted cells. HeLa cells were transfected with USP3 targeting oligos (USP3 KD1, KD2; 100 nM, two rounds of transfection, cells were harvested at 72 hr) or control siRNA (Ctrl). WCE (anti-USP3 and anti-Actin) and histone fractions were IB as shown. In this and subsequent figures, molecular mass markers are indicated in kDa.

USP3 Binds to uH2A and Dynamically Interacts with Chromatin In Vivo

USP3 harbors two conserved protein domains [3]: a catalytic domain of the Ub-specific protease (USP) class and a zinc finger (ZnF-UBP) Ub-binding domain [1]. USP3 mutants were generated and monitored for their deubiquitination activity and histone binding (Figures 2A–2C). To generate an inactive USP3 mutant, we substituted a serine for the catalytic cysteine of USP3 (Myc-USP3^{C168S}) [7]. To test the potential role of the ZnF-UBP in ubiquitin/ubiquitinated histone interaction, we mutated the Zn-binding, conserved histine to alanine (Myc-USP3^{H56A}) (Figure S3). Only wild-type USP3 significantly reduced the total pool of uH2A, showing that both protease activity and an intact zinc finger are required for H2A deubiquitination (Figure 2B).

To address histone binding, we expressed pIG-USP3^{wt}, pIG-USP3^{C168S}, or pIG-USP3^{H56A} proteins and performed coimmunoprecipitation. uH2A could be efficiently coimmunoprecipitated with IG-USP3^{C168S} but less efficiently with WT USP3 (Figure 2C), possibly because of rapid release of WT USP3 after catalysis. Similarly, strong interaction between uH2A and purified IG-USP3^{C168S} was detected in in vitro pull-down assays, indicating that USP3 can directly contact its substrate (Figure S4). In the IG-USP3^{C168S} immunoprecipitates, we also detected native nonubiquitinated core histones (Figure 2C). However, whereas in bulk chromatin about 5%–15% of total H2A is ubiquitinated [5], in IG-USP3C^{168S} immunoprecipitates, a 1:1 uH2A/H2A ratio was detected, indicating enrichment for uH2A (Figure 2C, IB anti-H2A). Increase of uH2B was less apparent. These results suggest that USP3 is directed to chromatin by a preferential interaction with uH2A. Consistently, the mutation in the ZnF UBP significantly reduced USP3-uH2A interaction both in vivo and in vitro (Figure 2C; Figure S4), indicating that this is the principal domain mediating the interaction with ubiquitin [1]. Immunoblot with anti-Ub Ab revealed that uH2A is the most abundant ubiquitinated protein coimmunoprecipitating with USP3^{C168S}, suggesting that uH2A represents a major USP3 substrate (Figure 2C).

Through an immunofluorescence approach, we could confirm that the interaction of GFP-USP3^{C168S} with its chromatin substrate is more stable than that of GFP-USP3^{wt} (Figure 2D). GFP-USP3^{wt} and GFP-USP3 mutants were all nuclear. However, in Triton X-100-treated HeLa cells, only GFP-USP3^{C168S} maintained its nuclear localization. One reasonable explanation for this is that, whereas the active WT protein transiently interacts with its substrate, the catalytic mutant functions as a "substrate trap."



	GFP	USP3^{wt}	USP3^{C168S}	USP3^{H56A}
D_{eff} ($\mu m^2/s$)	9	4.84	4.84	4.84
Bound fraction (%)	0	5	30	5
Mean residence time (s)	0	0.25	infinite	> 64

Figure 2. USP3 Binds to uH2A and Dynamically Interacts with Chromatin

(A) Schematic representation of wild-type (WT) and mutant USP3 proteins. ZnF, zinc finger ubiquitin binding domain (ZnF UBP); USP, ubiquitin-specific protease domain. All constructs were Myc- (B), GFP- (D–F), or IG- (C) tagged.

(B) 293T cells were transfected with the indicated Myc-USP3 constructs or control GFP and collected after 48 hr. IB was performed on WCE (USP3, vinculin) or histone fractions, with the indicated Ab.

(C) 293T cells were transfected with expression plasmids as indicated. Cell lysates were immunoprecipitated and IB as shown. (u_2H2A), protein band with a molecular weight consistent with form of H2A harboring two ubiquitin moieties. Asterisks: nonspecific bands.

(D) GFP-USP3^{wt}, GFP-USP3^{C168S}, GFP-USP3^{H56A}, and GFP localization was visualized by fluorescence analysis. Before fixation, cells were treated with 0.5% Triton X-100 (plus Triton) or mock treated (no Triton). Scale bar represents 20 μm.

(E) FRAP analysis of GFP-USP3^{wt} (blue, n = 20 nuclei), USP3^{C168S} (red; n = 20 nuclei), USP3^{H56A} (light blue; n = 20 nuclei), and GFP (green; n = 20 nuclei) in HeLa cells. The obtained fluorescence recovery curves were normalized to the prebleach fluorescence set at 1.

(F) Kinetic parameters of GFP-USP3^{wt}, USP3^{C168S}, USP3^{H56A}, and GFP as determined by kinetic modeling.

Next, we determined the nuclear mobility of GFP-USP3^{wt} and mutants by FRAP (fluorescent recovery after photobleaching) [8]. A striking difference in fluorescence recovery between GFP-USP3^{wt} and GFP-USP3^{C168S} was observed (Figures 2E and 2F). Analysis of the fluorescence recovery plots by fitting to curves generated by computer simulation of FRAP [9] revealed that the C168S has a severe impact on both the immobile fraction as on the duration of immobilization. GFP-USP3^{C168S} displayed a significantly larger immobile fraction (30%) compared to the WT protein (5%), and mutant molecules were permanently immobilized (residence time, defined as $1/K_{off}$ = infinite; $K_{off} = 0$), whereas residence time for GFP-USP3^{wt} was in the subsecond range (0.25 s). GFP-USP3^{H56A} immobile fraction was comparable to GFP-USP3^{wt}, whereas its residence time was greatly prolonged (>64 s). This may be explained by a reduced catalytic rate of this mutant, as supported by its inability to deubiquitinate H2A (Figure 2B) and consistent with studies on USP5 [10]. The dynamic properties of the WT protein suggest that a significant fraction of USP3 molecules is recruited with a very high frequency and transiently at target sites, probably through an interaction with its substrate(s) uH2A/uH2B, to execute deubiquitination.

USP3 Depletion Delays S Phase Progression and Mitotic Entry

To examine the role of USP3, we knocked down its expression in U2OS cells by siRNA. USP3-KD resulted in slower growth (Figure S5). To investigate whether USP3 depletion altered cell-cycle progression, we synchronized siRNA-transfected cells at the beginning of S phase by thymidine and followed the cell cycle upon release. USP3-KD cells showed a profound delay in S phase progression with most of the cells in middle S phase at 9 hr after release (Figure 3A). Also, compared to control cells, only a minor fraction of USP3-KD cells incorporated BrdU at all time points (Figure 3B). Therefore, both FACS analysis and 5′-BrdU labeling demonstrated that USP3 depletion causes a replication defect. Finally, we found that very few mitotic cells accumulated over time in USP3-KD, compared to control cells (Figure S6). Accumulation of DNA damage during defective DNA

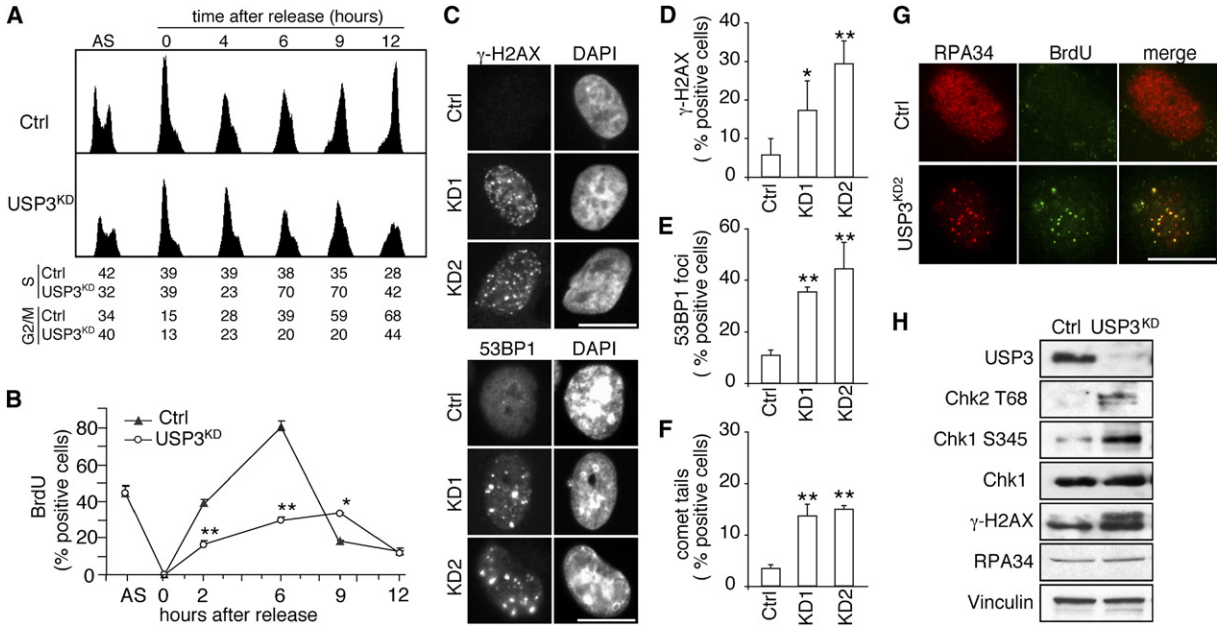


Figure 3. USP3 Knockdown Delays S Phase Progression, Induces DNA Damage, and Activates DNA Damage Checkpoints

(A and B) U2OS cells, transfected with control (Ctrl) or USP3-silencing oligos, were blocked with thymidine for 24 hr and released in the presence of nocodazole. In (A), at the indicated time intervals, cell-cycle profiles were determined by FACS. The approximate distributions of cell-cycle phases (S, G2/M) are shown as percentage. AS, asynchronous cell population. In (B), at the indicated time intervals, cells were pulse labeled for 20 min with 33 μM BrdU, fixed, and stained for BrdU. Results are the mean of two independent experiments. At least 1000 cells were counted for each time point. Shown are the mean ± SD values. *$p < 0.05$; **$p < 0.001$.

(C–F) Induction of DNA damage in USP3-silenced cells. U2OS cells were transfected twice with control (Ctrl) or USP3 siRNA oligonucleotides (KD1 and KD2) and analyzed at 72 hr after transfection. Transfected cells were stained for γ-H2AX or 53BP1 (C), and quantification for γ-H2AX positivity (D) or presence of 53BP1 foci (E) was performed. At least 300 (for γ-H2AX) or 150 (for 53BP1) cells were counted. Cells containing more than three nuclear foci were considered positive for 53BP1. Scale bars represent 10 μm.

(F) DNA breaks in USP3-depleted U2OS cells were assayed by comet assay. At least 200 cells/sample were analyzed. In (D)–(F), the results are the mean of three independent experiments. Shown are the mean ± SD values. *$p < 0.05$; **$p < 0.001$.

(G) USP3-depleted cells contain RPA34-coated ssDNA. U2OS cells were prelabeled with 10 μM BrdU, incubated with USP3 ($USP3^{KD2}$) or control (Ctrl) siRNA for 48 hr, and immunostained for BrdU without denaturation of the DNA. Scale bar represents 10 μm.

(H) DNA damage checkpoint activation in USP3-depleted cells. IB analysis of Ctrl or $USP3^{KD}$ U2OS cell lysates prepared at 48 hr after transfection. Chk1 pS315 and Chk2 pT68 indicate antibodies specific for the phosphorylated amino acid of the protein.

replication might cause this delay (see below). We conclude that USP3 is required for proper progression through S phase and subsequent mitotic entry.

Induction of DNA Damage and Checkpoint Activation in USP3-Silenced Cells

One cause of S phase delay and mitotic arrest is DNA damage together with DNA damage checkpoint activation. Chromatin folding and histone modifications play an important role in DNA damage response (DDR) [11]. Therefore, we tested whether USP3-KD could affect genome integrity in nonchallenging conditions. An early response to DNA damage is the phosphorylation of H2AX (γ-H2AX) at DNA breaks by the ataxia telangiectasia mutated (ATM) and ATM- and Rad3-related (ATR) checkpoint kinases [12]. As shown in Figures 3C–3E, γ-H2AX staining, as well as accumulation of the checkpoint protein 53BP1 [13] in nuclear foci, was enriched in USP3-KD U2OS cells. Complementation experiments showed that USP3 catalytic activity was required to rescue USP3-KD cells from accumulating γ-H2AX (Figure S7).

To investigate whether DNA damage was present in USP3-KD cells, we performed a comet assay under alkaline conditions [14]. DNA breaks were readily detected in USP3-KD cells (Figure 3F). Positive detection of comet tails was not due to a general apoptotic program, because apoptotic-typical nuclear morphology was not evident (Figure 3C) and caspase activity was not significantly induced (not shown).

DNA damage or stalled replication activates the ATR-Chk1 pathway in a manner dependent on the association of the replication protein A (RPA) to single-stranded DNA (ssDNA; [15]). To assess the presence of ssDNA, we measured BrdU incorporation without prior denaturation of the DNA [16]. Only USP3-depleted cells showed numerous BrdU foci colocalizing with the ssDNA binding protein RPA34, indicating the formation of ssDNA (Figure 3G). Consistent with the presence of DNA breaks, there were significantly higher levels of γ-H2AX and of activated ATM and ATR targets, Chk2 (phosphorylated at Thr68) and Chk1 (phosphorylated at Ser345) in USP3-KD cells than in control cells (Figure 3H) [12]. Of note, Ser345 is the preferential site for phosphorylation by ATR in response to stalled replication forks [17], suggesting that the ATR/Chk1 pathway is also activated and that USP3-KD cells undergo replicative stress.

Altogether, these data suggest that USP3 inhibition leads to accumulation of DNA breaks and activation of an ATM/ATR-regulated DNA damage response pathway.

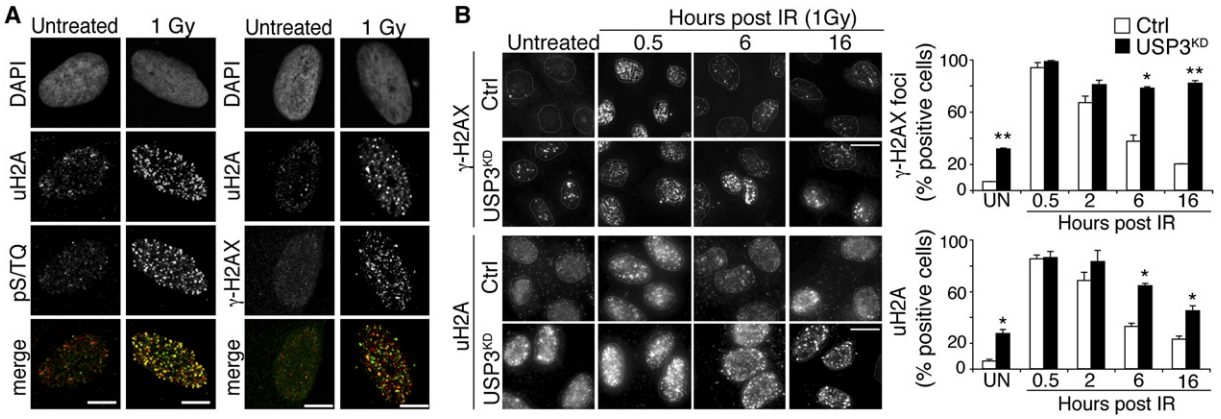


Figure 4. Ionizing Radiation Induces uH2A Nuclear Foci Formation, and Depletion of USP3 Increases the Persistence of uH2A and γ-H2AX Foci in Response to DSB

(A) U2OS cells were exposed to IR, stained for uH2A and γ-H2AX or uH2A and pS/TQ Ab at 30 min after IR, and analyzed by confocal microscope. Scale bars represent 5 μm.

(B) Left: Ctrl or USP3^{KD} U2OS cells were treated with IR (1Gy) at 48 hr after transfection and stained for γ-H2AX (top) or uH2A (bottom) as indicated. Ab staining was merged with DAPI and images were processed to show only the periphery of the nucleus (thin white lines). Scale bars represent 10 μm. Right: γ-H2AX foci-positive cells were quantified as described in Figure 3. Cells containing more than five foci were considered positive for uH2A. The results are the mean of two independent experiments. Shown are the mean ± SD values. *$p < 0.05$; **$p < 0.001$.

Evidence for an Involvement of USP3 in the Response to DNA Damage

The above results led us to hypothesize that USP3, possibly through its ability to deubiquitinate H2A/H2B, might affect the response to and/or the repair of exogenous DNA damage. Previous observations suggest that histones are ubiquitinated upon UV damage [18, 19]. Ubiquitination of H2B in *S. cerevisiae* is required for checkpoint activation upon genotoxic stress [20]. In addition, nuclear foci of conjugated ubiquitin are detected at IR-induced damage sites with an antibody recognizing conjugated Ub (FK2) (Figures S10 and S11; [21, 22]). We analyzed uH2A upon IR. uH2A redistribution to nuclear foci and significant colocalization with γ-H2AX foci and with phosphorylated ATM/ATR substrates (pS/TQ Ab) was observed early upon IR (Figure 4A; Figures S8 and S9). Accumulation of Ub conjugates was present in a patient cell line with reduced ATR expression, but was less evident in cells deficient for ATM, which is primarily involved in DSB-induced signaling (Figure S10).

γ-H2AX is phosphorylated within minutes upon IR, and its dephosphorylation is required for full recovery from the DDR [23, 24]. We therefore examined the kinetic of formation/disappearance of γ-H2AX and uH2A/Ub (FK2) foci upon IR in USP3-silenced cells. At 30 min after IR, there was comparable foci formation in control and USP3-KD cells. However, γ-H2AX and uH2A/Ub foci persisted for a prolonged time, with more than 50% of the silenced cells displaying foci up to 40 hr post IR (Figure 4B; Figure S11). In line with γ-H2AX kinetics, USP3-KD cells entered a IR-induced G2 arrest similarly to control cells but showed a prolonged G2/M checkpoint (Figure S12). These data suggest that USP3 plays a role in deubiquitination events, including H2A deubiquitination, at DNA damage sites, and might thereby contribute to an efficient recovery from the DNA damage checkpoint. Whether loss of USP3 leads to a checkpoint defect or rather a DNA repair defect remains to be established.

Conclusions

Our results identify USP3 as a novel regulator of uH2A and uH2B and suggest that USP3 might have an important role in genome-wide H2A deubiquitination. In addition, USP3 KD was genotoxic, leading to DNA damage checkpoint activation. Thus, while we cannot exclude that the effect on the DNA damage checkpoint is due to other putative targets of USP3, our results suggest that USP3 affects the DDR through deubiquitination of its major substrate, uH2A.

How can H2A ubiquitination influence the DNA damage response? A first possibility is that the ubiquitin moiety on H2A could promote recruitment and/or stabilization of regulatory factors, containing ubiquitin-binding domains [1], that in turn play a role in the DNA damage response/DNA repair. Of note, the ubiquitin-binding protein RAP80 has been shown to bind to ubiquitin conjugates at DSB, thereby promoting BRCA1 recruitment [25, 26]. Second, uH2A could influence post-translational modification of other histones. Such a cross-talk between H2B ubiquitination and H3 methylation has been documented [27].

In USP3-silenced cells, we observe accumulation of DNA damage, S phase delay, and activation of an ATR/ATM-regulated checkpoint response. How do these events relate to each other? One plausible explanation is that USP3 might, directly or indirectly, affect the DDR. Under this scenario, ubiquitin conjugates would most likely function as a signal for the presence of DNA damage. Supporting this hypothesis, a number of ubiquitination events have been described at DSBs, including monoubiquitination of repair proteins and accumulation of ubiquitin polymers [2, 26]. Our results suggest that uH2A may be part of this ubiquitin-signaling network. The role of USP3 might involve removal of ubiquitin marks from chromatin(-bound proteins) to attenuate the signal. Absence of USP3 might, thus, lead to hyperactivation of the checkpoint that, in turn, could result in replication and/or recombination abnormalities and genotoxic

events. Alternatively, the primary function of USP3 might be related to DNA replication. Replicative stress in the absence of USP3 may lead to stalled or damaged replication forks that would generate single-stranded DNA regions and DNA breaks, and, therefore, checkpoint signals.

uH2A and uH2B have been associated with transcriptional regulation [27]. Thus, the possibility that USP3 is involved also in transcription deserves experimental attention.

In summary, our studies implicate USP3 in DNA damage signaling and reveal a crucial function of USP3 in preventing replication stress.

Supplemental Data

Twelve figures and Experimental Procedures are available at http://www.current-biology.com/cgi/content/full/17/22/1972/DC1/.

Acknowledgments

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