Regulation of histone H2A ubiquitination in the maintenance of genome stability

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Human USP3 is a chromatin modifier required for S-phase progression and genome stability
Human USP3 is a chromatin modifier required for S-phase progression and genome stability

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Protein ubiquitination is critical for numerous cellular functions, including DNA damage response pathways [1-2]. Histones are the most abundant mono-ubiquitin conjugates in mammalian cells, however, the regulation and the function of mono-ubiquitinated 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. 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: i) uH2A redistributes and colocalizes in γ-H2AX DNA repair foci, and ii) 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.

Keywords: ubiquitin, histone, histone ubiquitination, deubiquitinating enzymes (DUBs), DNA damage response (DDR)

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 it is present in the chromatin fraction (Figure S1 in the Supplemental Data).
**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 (Figure 1B-D). Approximately 5-15% of histone H2A and less than 1% of H2B are mono-ubiquitinated, making them the two major mono-ubiquitinated chromosomal proteins [6]. uH2A was detected by immunoblotting with anti-Ub, anti-H2A antibodies (Ab) or a monoclonal Ab specific for uH2A [7], while direct anti-H2B immunoblot visualized uH2B (Figure 1C-D and S2A). Both uH2A and uH2B amounts were significantly reduced upon USP3 overexpression (Figure 1C-D). 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.

**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 (UBP) class and a zinc finger (ZnF-UBP) Ub-binding domain [1]. USP3 mutants were generated and monitored for their deubiquitination activity and histone binding (Figure 2A-C). To generate an inactive USP3 mutant, we substituted a serine for the catalytic cysteine of USP3 (Myc-USP3C168S) [8]. To test the potential role of the ZnF-UBP in ubiquitin/ubiquitinated histones-interaction, we

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**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 beads (ctrl). 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,D) of HeLa cells transfected with GFP- or Myc-USP3 were IB as indicated. *: 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; 100nM), two rounds of transfection, cells were harvested at 72h) 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.
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-USP3wt, pIG-USP3 C168S or pIG-USP3 H56A proteins, and performed co-immunoprecipitation. uH2A could be efficiently co-immunoprecipitated 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 non-ubiquitinated core histones (Figure 2C). However, while in bulk chromatin about 5-15% of total H2A is ubiquitinated [6], in IG-USP3 C168S 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 (Figures 2C and 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 co-immunoprecipitating with USP3 C168S, suggesting that uH2A represents a major USP3

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- tagged (C).
B. 293T cells were transfected with the indicated Myc-USP3 constructs or control GFP and collected after 48h. 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. (uH2A), protein band with a molecular weight consistent with form of H2A harboring two ubiquitin moieties Asterisks: non-specific 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). Bar 20 mm.
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 pre-bleach fluorescence set at 1.
F. Kinetic parameters of GFP-USP3 wt, USP3 C168S, USP3 H56A and GFP as determined by kinetic modeling.
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 if 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 hours post 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 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 checkpoints activation. Chromatin folding and histone modifications play an important role in DNA damage response (DDR) [12]. Thus, we tested whether USP3-KD could affect genome integrity in non-challenging 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 [13]. As shown in Figure 3C-E, γ-H2AX staining, as well as accumulation of the checkpoint protein 53BP1 [14] 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 if DNA damage was present in USP3-KD cells, we performed a comet assay under alkaline conditions [15]. DNA breaks were readily detected in USP3-KD cells (Figure 3F). Positive detection of comet tails was not due to a general apoptotic program, since 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
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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 [19-20]. Ubiquitination of H2B in *S. cerevisiae* is required for checkpoint activation upon genotoxic stress [21]. In addition, nuclear foci of conjugated ubiquitin are detected at IR-induced damage sites with an antibody recognizing conjugated Ub (FK2) (Figures S10, S11, and [22-23]). We analyzed uH2A upon IR. uH2A redistribution to nuclear foci and significant co-localization with γ-H2AX foci and with phosphorylated ATM/ATR substrates (pS/TQ Ab) was observed early upon

![Image](3)

**Figure 3.**

**USP3 knock-down delays S-phase progression, induces DNA damage and activates DNA damage checkpoints**

A-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 mM 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 72h post 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 3 nuclear foci were considered positive for 53BP1. Bar 10 mm. 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 3 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 mM BrdU, incubated with USP3 (USP3KD) or control (Ctrl) siRNA for 48 hours and immunostained for BrdU without denaturation of the DNA. Bar 10 mm.

H. DNA damage checkpoint activation in USP3-depleted cells. IB analysis of Ctrl or USP3KD U2OS cell lysates prepared at 48h post transfection. Chk1 pS315 and Chk2 pT68 indicate antibodies specific for the phosphorylated amino acid of the protein.
IR (Figures 4A and S8, 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 [24-25]. We thus examined the kinetic of formation/disappearance of γ-H2AX and uH2A/Ub (FK2) foci upon IR in USP3-silenced cells. At 30 minutes 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 hrs post IR (Figure 4B and 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 [26-27]. Second, uH2A could influence post-translational modification of other histones. Such a cross-talk between H2B ubiquitination and H3 methylation has been documented [28].

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

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 post IR and analyzed by confocal microscope. Bar 5 mm.
B. Left panels. Ctrl or USP3 KD U2OS cells were treated with IR (1 Gy) at 48 h post transfection and stained for γ-H2AX (upper panels) or uH2A (lower panels) as indicated. Ab staining was merged with DAPI and images were processed to show only the periphery of the nuclei (thin white lines). Bar 10 mm. Right panels. γ-H2AX foci positive cells were quantified as described in Figure 3. Cells containing more than 5 foci were considered positive for uH2A. The results are the mean of 2 independent experiments. Shown are the mean ± SD values. *, p < 0.05; **, p < 0.001.
of ubiquitination events have been described at DSBs, including mono-ubiquitination of repair proteins and accumulation of ubiquitin polymers [2, 27]. 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 hyper-activation of the checkpoint that, in turns, 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 which would generate single stranded DNA regions and DNA breaks, and, therefore, checkpoint signals.

uH2A and uH2B have been associated with transcriptional regulation [28]. 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.

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REFERENCES


SUPPLEMENTAL DATA

EXPERIMENTAL PROCEDURES

Cell culture
HeLa, U2OS (human osteosarcoma cells), human embryonic kidney 293T and cells from a modified 293T cell line (Phoenix) were grown in DMEM supplemented with 10% fetal calf serum (FCS). Primary human fibroblast CSRO, WI-38, AT2RO (AT-M), and GM18366 (fibroblast with low levels of ATR protein, obtained from Coriell Institute) were cultured in Ham’s F10 medium supplemented with 15% FCS and antibiotics.

Plasmids
The human USP3 cDNA, generously provided by R. T. Baker [1], was subcloned into pcDNA3.1/myc-His(−) (Invitrogen), and into pEGFP-C (Clontech). The C168S and H56A mutations were inserted by PCR-directed mutagenesis (QuickChangeTM XL, Stratagene). All constructs were sequence verified. pIg3.3-TEV constructs were created to express fusion proteins with the Fc portion of human IgG1. The pIg3.3-TEV vector was obtained by cloning the TEV-cassette into a digested EcoRI/BamHI pIgG3 vector (a pcDNA3 based version of pIg1 [2]). The TEV-cassette was created by annealing the two following primers: pIgTEVF 5’-AGCTTGCTAG CAGTGGTACC AGAAAACCTG TACTTTCAGT CCGCAGGTAA GTG-3’ and pIgTEVR 5’-AATTCACTTA CCTGCGGACT GAAAGTACAG GTTTTCTGGT ACCACTGCTA GCA-3’. The plasmids were verified by DNA sequence analysis.

RNA interference, cell synchronization and FACS analysis.
USP3 siRNAs (Individual siGENOME duplex D-006078-01,-04) and control siRNA (siCONTROL Non-Targeting siRNA, D-001210-01) were from Dharmacon. Transfection was performed with OligofectAMINE (Invitrogen). Directly after transfection, U2OS cells were incubated with thymidine (2.5 mM) for 24 hr to arrest the cells at the G1/S transition. Immediately after removal of thymidine, nocodazole (200 ng/ml) was added to the culture medium. The cells were analyzed on a Becton Dickinson Flow Cytometer.

Protein studies
High salt nuclear extracts were prepared from HeLa cells according to standard procedures. Whole cell extracts preparation, biochemical fractionation, immunoprecipitation and acid extraction of histones were as described [3-4].

Isolation of Ub-binding proteins
High salt HeLa nuclear extracts were incubated with Ub-agarose resin (Invitrogen) in buffer A [20 mM Hepes pH 7.5, 50 mM KCl, 5 mM MgCl2, 2 mM ATP, glycerol 10%, 0.2 mM DTT and protease inhibitors cocktail (SIGMA)], for 2 h at 4 °C, followed by washing with buffer A containing 0.1% NP-40 and 0.3 M KCl, and then with buffer A. Protein identification was by MALDI TOF. Briefly, bands were excised from Silver-stained gels, reduced, alkylated, “in gel” digested with sequencing-grade trypsin (Roche) for 3 h at 37 °C [5]. Samples were analyzed by MALDI time-of-flight and the spectra were recorded on a Voyager-DE STR Biospectrometry workstation (PerSeptive Biosystems, Foster City, CA 94404 USA). Profound Peptide Mapping Software
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(Rockefeller University, version 4.10.5) was used to search a non-redundant protein sequence database (NCBI).

Immunofluorescence studies

Cells, grown on glass coverslips, were either fixed in 4% paraformaldehyde in PBS at room temperature (RT) for 10 min, or permeabilized with 0.5% Triton X-100 in PBS for 10 min on ice before fixation to detect uH2A. IF was performed as described [3, 6], with the appropriate primary/secondary Ab, and analyzed under a Bx61 Olympus Fluorescence Microscope equipped with epifluorescence optics or with a Leica TCS SP2 AOBS confocal microscope. To detect ssDNA, cells were preincubated with 10 µM BrdU (SIGMA), treated with siRNA in the presence of 10 µM BrdU for 48 hours, fixed with methanol for 20 min at -20 °C, and stained with anti-BrdU Ab without prior denaturation of the DNA.

Confocal laser scanning microscopy images of living cells were obtained on a Zeiss LSM 510 META equipped with cell culture microscopy stage. HeLa cells expressing low and comparable levels of wt and mutant proteins were selected. FRAP was performed as described [7] and mobility parameters were calculated by fitting the experimental data to in silico simulated random diffusion models as described [8].

Comet assay

DNA damage was evaluated using the alkaline (pH>13) Single-Cell Gel Electrophoresis assay (CometAssayTM, Trevigen, Inc.). Alkaline conditions allow the detection of both single- and double-stranded DNA breaks [9]. Staining was with SYBR SafeTM DNA gel stain (Molecular Probes). Comet tails were scored (200 cells/slide) using a Bx61 Olympus Fluorescence Microscope.

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**Supplementary Figures**

**A**

USP3

DAPI

**B**

Figure S1. USP3 is a chromatin-associated DUB

A. USP3 is a nuclear protein. U2OS cells were fixed and stained with anti-USP3 antibody. DNA was stained with DAPI. Bar 10 mm. B. USP3 is a chromatin-associated DUB. HeLa cell extracts were fractionated and IB as shown. Fractions: S1, detergent soluble; DNAseI + Ammonium Sulfate (AS); High salt (2M NaCl); Matrix (nuclear). Anti-MeCP2 and anti-LaminB Ab were used to check the purity of the fractions.

**Figure S2.** Overexpression of USP5 does not alter uH2A levels.

A. Detection of uH2A and uH2B. Histone fractions from HeLa cells were IB as shown. B. Phoenix cells were transfected with GFP-USP3, Myc-USP5 (a kind gift of H.L. Ploegh) or pCDNA3-myc, along with pEGFP plasmid. At 48h post tranfection, GFP positive cells were FACS sorted and cell lysates were prepared. WCE and histone fractions were IB as shown. In this and subsequent figures, molecular mass markers are indicated in kDa.
Figure S3. Sequence alignment of ZnF-UBP domains of USP3 orthologs.

A. Schematic representation of USP3 protein domains. ZnF, zinc-finger ubiquitin binding domain (ZnF UBP); USP, Ubiquitin Specific Protease domain.

B. Sequence alignment of ZnF-UBP domains of USP3 orthologs. The alignment was generated with ClustalW based on the reported Zn-UBP consensus alignment (PFAM: PF02148). The accession numbers from top to bottom are: human (NP_006528), mouse (NP_659186), rat (NP_001020595), chicken (XP_413755), frog (xenopus, AA126024), zebrafish (XP_698168), fly (apis mellifera, XP_392160), worm (Caenorhabditis briggsae, CA67996). The residues that chelate zinc are indicated by an asterix. Three asterisks mark the H56A mutation.
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Figure S4. USP3-histones interaction by in vitro pull down.
A. Schematic representation of pIG.3-TEV-USP3 construct. IG, Fc portion of human IgG1; TEV-cleavage, TEV protease recognition site.
B. Purification of USP3-IG proteins. pIG.3-TEV-USP3 constructs were expressed in 293T cells for 96 hours. Cells were lysed in JS buffer (Triton-X100 1%, NaCl 150 mM, Hepes 50mM, Glycerol 2%, MgCl2 1.5 mM, EGTA 5 mM) for 30 min on ice followed by sonication and ultracentrifugation (150,000 x g, 45 min at 4°C). USP3-IG proteins were affinity purified from the supernatant on Protein A sepharose beads (Amersham) and analyzed by Coomassie staining (5 and 10 ml). BSA: 2.5, 5 and 10 mg.
C. Micromolar amounts (0.5-1 mM) of Protein A-purified USP3-IG proteins (as indicated on top), or control protein A beads (pIG), were incubated with equimolar amounts of native histones in PBS for 2 hours at 4°C on a rotating wheel. Beads were washed three times in PBS. Proteins were eluted by boiling in Laemmli Buffer and analyzed by Coomassie staining or IB as shown.
For histone preparation, 293T cells were extracted for 10’ on ice in PBS-Triton X100 0.5%, followed by centrifugation at 10000g for 10 min. Nuclear pellet was washed twice and histones were prepared by overnight incubation in 0.2N HCl at 4°C, followed by TCA precipitation. Protein pellet was resuspended and extensively dialysed in H2O to allow histone octamer refolding.

Figure S5. USP3-KD results in inhibition of cell growth.
U2OS cells were transfected with USP3-specific or control siRNAs. At 48 hours after transfection, cells were counted, seeded in triplicates at 150,000 cells/well in a six well plate and grown for 2 days. Counts were performed automatically with a Multisizer-3 Coulter Counter (Beckman CoulterTM) and p-values were calculated with a TTEST. Bar 60 mm. *, p < 0.05; **, p < 0.001.

Figure S6. USP3 depletion delays mitotic entry
U2OS cells, transfected with control oligos (Ctrl) or USP3-silencing oligos, were blocked with thymidine directly after transfection and released after for 24 hr in the presence of nocodazole. At the indicated time points, cells were fixed and phospho-histone H3-positive cells were counted in IF. 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.
Figure S7. USP3 catalytic activity is required to rescue siRNA-induced DNA damage response.
Expression of a siRNA-resistant USP3wt, but not of catalytically inactive forms, USP3 C168S and USP3 HA-CS, rescues USP3-KD (USP3 KD3) cells from accumulating γ-H2AX, proving that the DDR phenotype is a direct consequence of USP3 depletion and that USP3 DUB activity is required to prevent DDR activation. U2OS cells were transfected with GFP-USP3wt, -USP3C168S, -USP3HA-CS (harboring both H56A and C168S mutations) or GFP by FuGene (Roche). After 20 hours cell were transfected with USP3 siRNA oligonucleotides directed against its 3’_UTR region (USP3KD3, 5’-GCAUGUACUUGUUCGAAUUUU-3’), or with control siRNAs (CTR_UTR, 5’-CUCUCUGUGUGAAGCACCUUU-3’). At 40 hours post siRNA transfection, cells were fixed and stained with γ-H2AX. Upper panel. Quantification for γ-H2AX-positivity was performed in cells displaying low GFP expression. At least 150 GFP-positive cells were counted for each sample on duplicate slides. Results are the mean ± SD values. *, p < 0.01; ***, p > 0.05. Bottom panels. Representative images for each sample are shown. Bar 20 mm. GFP-USP3 HA-CS is impaired in uH2A deubiquitination (not shown).
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Figure S8. Dose response of uH2A to ionizing radiation analyzed by immunofluorescence. Upper panels. U2OS cells were exposed to the indicated doses of IR and stained for uH2A or γ-H2AX and pS/TQ Ab at 30 min post IR. Bar 20mm. Lower panels. Fluorescence analysis was performed with ImageJ 10.2 software. Threshold was set on DAPI channel (blue), and pixel intensity in each nucleus was measured on the second channel, red for uH2A or γ-H2AX, and green for pS/TQ. 50th percentile was used to calculate the mean intensity and standard deviation. The Average Background Intensity (ABI) was measured on untreated cells. Cells with pixel intensity greater than 2X ABI were considered positive for the signal. At least 100 cells per sample were analyzed.
Figure S9. uH2A nuclear foci formation is an early response to ionizing radiation. U2OS cells were exposed to IR (1Gy) and stained for uH2A or γ-H2AX at the indicated time points post IR. Bar 20mm. The percentage of uH2A/γ-H2AX positive cells and the pixel intensity was evaluated as in Figure S8. At least 100 cells per sample were analyzed.
Figure S10. Ubiquitin-conjugates foci formation upon IR is dependent on ATM. Wt human fibroblast CSRO-hTERT and WI-38, and ATR-hTERT or ATM mutant cells were cultured on glass coverslips and either left untreated or irradiated with 3 Gy. Cells were stained for Ub-conjugates (FK2) or γ-H2AX at 30 min post IR and analyzed by confocal microscopy. FK2-foci show significant co-localization with γ-H2AX foci. FK2 foci were efficiently detected in ATR defective cells but not in ATM cells. Bar 10 mm.
Figure S11. Persistence of γ-H2AX and FK2-Ub nuclear foci upon IR in USP3-depleted cells.
Left panels. Ctrl or USP3 KD U2OS cells were treated with IR (2Gy) at 48h post transfection and stained for γ-H2AX (upper panels) or FK2 (lower panels) at the indicated time. Efficient USP3 KD was verified by IF (not shown). Bar 10 mm. Right panels. γ-H2AX foci positive cells were quantified as described in Figure 3. Cells containing more than 5 foci were considered positive for FK2. The results are the mean of 4 (γ-H2AX) or 2 (FK2) independent experiments. Shown are the mean ± SD values. *, p < 0.05; **, p < 0.004.

A. U2OS cells transfected with control (Ctrl) or USP3-silencing oligos were left untreated or irradiated at the indicated doses 48h post transfection. At 1h after IR or 48h after transfection (untreated), cells were fixed and mitotic cells were assessed by histone H3 phosphorylation. A minimum of 1000 cells was counted. Results are the mean of 3 independent experiments. Shown are the mean ± SD values. **, p < 0.001.
B. U2OS cells were transfected with control (Ctrl) or USP3-silencing oligos (two rounds of transfection, 24 hours interval). Cells were treated with IR (6Gy) 5 hours after the second transfection and fixed at 20, 30 or 40 hours post irradiation. Untreated cells were fixed at 72 h post siRNA transfection. Mitotic cells were assessed as in Figure S6. Results are the mean of 3 independent experiments. Shown are the mean ± SD values. *, p < 0.05; **, p ≤ 0.001.