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Deubiquitinases as a Signaling Target of Oxidative Stress

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SUMMARY

Deubiquitinating enzymes (DUBs) constitute a large family of cysteine proteases that have a broad impact on numerous biological and pathological processes, including the regulation of genomic stability. DUBs are often assembled onto multiprotein complexes to assist in their localization and substrate selection, yet it remains unclear how the enzymatic activity of DUBs is modulated by intracellular signals. Herein, we show that bursts of reactive oxygen species (ROS) reversibly inactivate DUBs through the oxidation of the catalytic cysteine residue. Importantly, USP1, a key regulator of genomic stability, is reversibly inactivated upon oxidative stress. This, in part, explains the rapid nature of PCNA monoubiquitination-dependent DNA damage tolerance in response to oxidative DNA damage in replicating cells. We propose that DUBs of the cysteine protease family act as ROS sensors in human cells and that ROS-mediated DUB inactivation is a critical mechanism for fine-tuning stress-activated signaling pathways.

INTRODUCTION

Deubiquitinating enzymes (DUBs) are proteases that cleave ubiquitin or ubiquitin-like proteins from proproteins or conjugates with target proteins (Reyes-Turcu et al., 2009). In doing so, DUBs maintain balance of ubiquitination dynamics, playing an editing role analogous to that of the phosphatases in kinase/phosphatase regulatory pathways. Deubiquitination is a highly regulated process that has been implicated in numerous cellular functions, including cell-cycle regulation (Song and Rape, 2008), proteasome- and lysosome-dependent protein degradation (Guterman and Glickman, 2004), gene expression, kinase activation (Grabbe et al., 2011; Skaug et al., 2009), microbial pathogenesis (Lindner, 2007; Rytkönen and Holden, 2007), and DNA repair (Huang and D'Andrea, 2006; Messick and Greenberg, 2009). Mutations in several DUBs have been linked to diseases ranging from cancer to neurological disorders (Fraile et al., 2012; Sacco et al., 2010; Singhal et al., 2008; Todi and Paulson, 2011). Although a few substrates have been identified for a handful of DUBs, the substrates and physiological roles of most DUBs are poorly defined. In addition, the regulation of DUB activity has only emerged recently. Several studies have shown that DUB activity is dependent on the presence of substrate or a scaffolding protein, which enables conformational changes toward catalytic competency (Cohn et al., 2007; Das et al., 2006; Hu et al., 2002; Yao et al., 2006). DUBs are also posttranslationally modified by phosphorylation, ubiquitination, or sumoylation, which alters activity, localization, or half-life (Reyes-Turcu et al., 2009). Thus, similar to other proteases, DUB activity is carefully controlled to prevent spurious cleavage of substrates; however, the participation of endogenous small molecules in modulating DUB activity has been unexplored.

There are nearly 100 putative ubiquitin-specific DUBs encoded by the human genome, and they belong to five different families (Komander, 2010; Reyes-Turcu et al., 2009). Four families belong to Clan A of cysteine proteases: ubiquitin C-terminal hydrolase (UCH), ubiquitin-specific protease (USP/UBP), ovarian tumor domain (OTU), and Josephin domain (MJD) DUBs (Rawlings et al., 2012). The Cys-dependent deubiquitinases contain a catalytic diad or triad whereby a His residue lowers the pKa of a nucleophilic Cys residue, priming it for attack of the isopeptide linkage of a ubiquitinated substrate. To complete the triad, an Asp or Asn aligns and polarizes the His residue. The fifth family of DUBs belongs to the JAB1/MPN/Mov34 metalloenzyme (JAMM) domain family of zinc-dependent metalloproteases. This family utilizes invariant His, Asp/Glu, and Ser residues to coordinate two zinc ions for activation of a water molecule (Maytal-Kivity et al., 2002; Tran et al., 2003). Thus, two very different catalytic mechanisms exist within the five families of DUBs: one involves nucleophilic attack of the substrate by a Cys thiolate, whereas the other involves direct attack by a water molecule. Little is known about how these differences reveal themselves in terms of susceptibility to cellular signals, including endogenous small molecules.

One such family of endogenous small molecules are reactive oxygen species (ROS), which are generated during mitochondrial oxidative metabolism as well as in cellular response to xenobiotics, cytokines, bacterial invasion, and replication stress (Ray et al., 2012). Oxidative stress is an imbalance between ROS or oxidants and the ability of the cell to mount an effective

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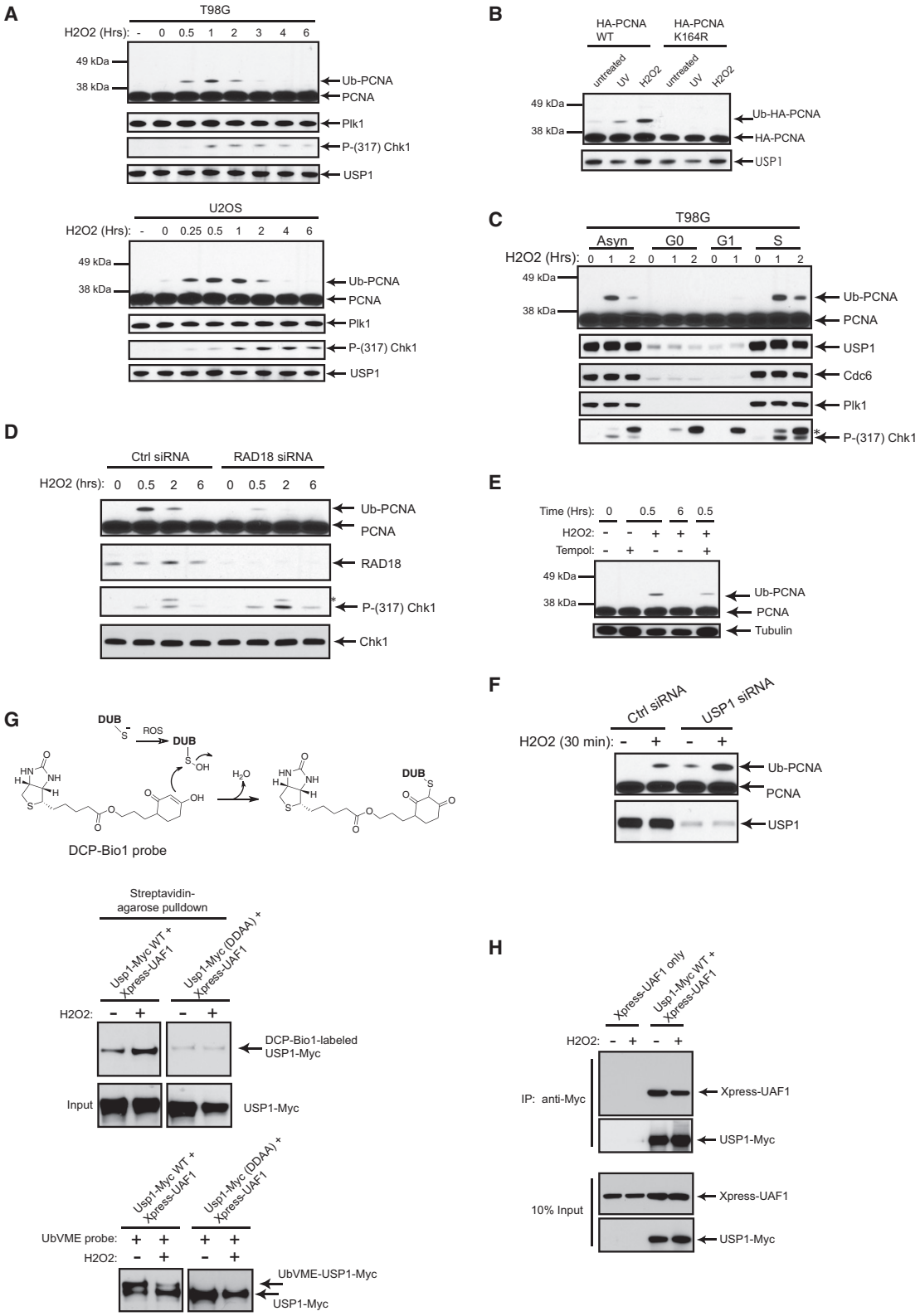

Figure 1. Oxidation of USP1 Is Dependent on Its Catalytic Site Competency

(A) PCNA monoubiquitination is transiently induced by oxidative stress, whereas checkpoint activation is prolonged. T98G and U2OS cells were treated with H_2O_2 (final concentration 1 mM) for the indicated amount of time. Samples were collected, lysed, and analyzed by western blot with the indicated antibodies.

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antioxidant response. Although oxidative stress results in macromolecular damage and is implicated in various disease states, including atherosclerosis, diabetes (Paravicini and Touyz, 2006), neurodegeneration (Andersen, 2004), aging (Haigis and Yankner, 2010), and cancer (Trachootham et al., 2009), accumulating evidence indicates that ROS also serve as critical signaling molecules in cell proliferation and survival. Although there is a wealth of research demonstrating the general effect of oxidative stress on DNA damage and signaling pathways, less is known about the signaling targets of ROS. Protein tyrosine phosphatases (PTPs) are a family of redox-regulated proteins that have been studied in recent years (Tonks, 2006). PTPs can be directly inactivated by ROS through the oxidation of the catalytic Cys residue (den Hertog et al., 2005; Salmeen and Barford, 2005; Tonks, 2005). The inactivation of PTPs by ROS is typically transient and, under physiological conditions, the oxidized Cys residues are often reduced to the catalytically active thiolate. Thus, the reversibility of the reaction fine-tunes the signaling pathways emanating from receptor tyrosine kinases.

In our study, we investigate the susceptibility of DUBs to redox regulation in vivo. Strikingly, we find that the Cys protease family of DUBs can be rapidly targeted by oxidative stress. We show that USP1, a DUB critical for the maintenance of genomic stability (Jones et al., 2011; Kim et al., 2009), is transiently oxidized and inactivated in response to oxidative stress in vivo, suggesting that USP1 is responsible for fine-tuning the proliferating cell nuclear antigen (PCNA) monoubiquitination-dependent DNA damage tolerance response to oxidative DNA damage during S phase. Due to the intractability of USP1 in vitro, we used the DUB USP7 (HAUSP), an important regulator of the p53 tumor suppressor and genotoxic pathways (Khoronenkova and Dianov, 2012; Khoronenkova et al., 2012), to identify the catalytic Cys residue as the point of oxidative modification, explaining how DUBs can be mechanistically inactivated by oxidative stress. Importantly, our in vitro studies show that only Cys-based DUBs, not metalloprotease DUBs, undergo ROS-mediated catalytic inactivation, even though both families are susceptible to direct redox control. Thus, our data suggest that DUBs of the Cys protease family act as ROS sensors in human cells and that ROS-mediated DUB inactivation is a critical mechanism for fine-tuning stress-activated signaling pathways.

RESULTS AND DISCUSSION

Rapid and Transient S Phase-Dependent Monoubiquitination of PCNA Tempers the G2 Checkpoint Activation after Oxidative Stress

Posttranslational modification of PCNA by monoubiquitination on Lys 164 plays an important role in coordinating DNA damage tolerance during DNA replication in mammalian cells (Bienko et al., 2005; Kannouche et al., 2004). The ubiquitin E3 ligase Rad18 monoubiquitinates PCNA in response to blocks in the progression of the replication fork caused by DNA lesions (Hoege et al., 2002; Watanabe et al., 2004). We previously showed that the DUB USP1 is an important negative regulator of the DNA damage tolerance pathway via the deubiquitination of PCNA, which is responsible for preventing the aberrant recruitment of error-prone translesion synthesis (TLS) DNA polymerases to the replication fork (Huang et al., 2006; Jones et al., 2011). Certain DNA-damaging agents, such as UV irradiation, can concomitantly promote the degradation of USP1 to enhance PCNA monoubiquitination (Cotto-Rios et al., 2011; Huang et al., 2006; Niimi et al., 2008). A more recent report showed that cells treated with the oxidizing agent hydrogen peroxide (H_2O_2) results in high levels of PCNA monoubiquitination and, unlike UV irradiation and other DNA-damaging agents, this is a rapid and reversible response (Zlatanou et al., 2011). We found similar kinetics of PCNA monoubiquitination after H_2O_2 treatment in the U2OS osteosarcoma or T98G glioblastoma cell lines (Figure 1A, Figure S1A) and, importantly, the

(B) Oxidative stress-induced PCNA monoubiquitination is dependent on an intact Lys 164 site but independent of USP1 degradation. U2OS cells stably expressing HA-PCNA wild-type (WT) or HA-PCNA K164R mutant were left untreated or treated with 50 J/M^2 UVC for 3 hr or treated with 1 mM H_2O_2 for 30 min. Samples were collected, lysed, and analyzed by western blot with the indicated antibodies.

(C) Oxidative stress-induced PCNA monoubiquitination mainly occurs during S phase. T98G cells were synchronized in G0/G1 by serum deprivation for 72 hr. Cells were then replenished with fresh media and collected at the indicated time points. Cells were either left untreated or treated with a final concentration of 0.5 mM H_2O_2. Synchronized cells were treated at 0 hr after release from serum deprivation for G0, after 4 hr of serum deprivation release for G1 phase, or after 24 hr of release for early S phase. Samples were collected, lysed, and analyzed by western blot with the indicated antibodies. Asterisk indicates a nonspecific, cross-reactive band when probing with anti-phospho Chk1 antibody (p317).

(D) Depletion of Rad18 results in increased Chk1 activation. U2OS cells were transfected for 48 hr with a control (Ctrl) siRNA or Rad18 siRNA. Cells were then treated with H_2O_2 (final concentration 0.5 mM) for the indicated amount of time. Samples were collected, lysed, and analyzed by western blot with the indicated antibodies. Asterisk indicates a nonspecific, cross-reactive band.

(E) The antioxidant activity of Tempol reduces the levels of PCNA monoubiquitination after oxidative stress. U2OS cells in the presence or absence of Tempol (0.5 mM) were treated with H_2O_2 (final concentration 0.3 mM) for the indicated amount of time. Samples were collected, lysed, and analyzed by western blot with the indicated antibodies.

(F) Depletion of USP1 further elevates PCNA monoubiquitination in the presence of oxidative stress. U2OS cells were transfected for 48 hr with a control (Ctrl) siRNA or USP1 siRNA. Cells were then treated with H_2O_2 (final concentration 0.3 mM) for 30 min. Samples were collected, lysed, and analyzed by western blot with the indicated antibodies.

(G) Oxidation of USP1 catalytic Cys to the sulfenic acid intermediate can be captured by DCP-Bio1 probe. U2OS cells were transfected for 48 hr with Usp1-Myc WT or (DDAA)-Myc mutant along with Xpress-UAF1. Cells were then treated with H_2O_2 (final concentration 0.3 mM) for 30 min. Extracts were made and divided for two separate reactions: labeling with DCP-Bio1 probe or with the UbVME DUB activity probe. Input represents 30% of extracts used for the DCP-Bio1 reaction.

(H) Oxidative stress does not disrupt the binding between Usp1 and UAF1 in cells. U2OS cells were transfected with Xpress-UAF1 only or cotransfected with Usp1-Myc WT and Xpress-UAF1 and treated with H_2O_2 (final concentration 1 mM) for 30 min. Extracts were subjected to immunoprecipitation with anti-Myc antibody and probed with indicated antibodies.

See also Figure S1.

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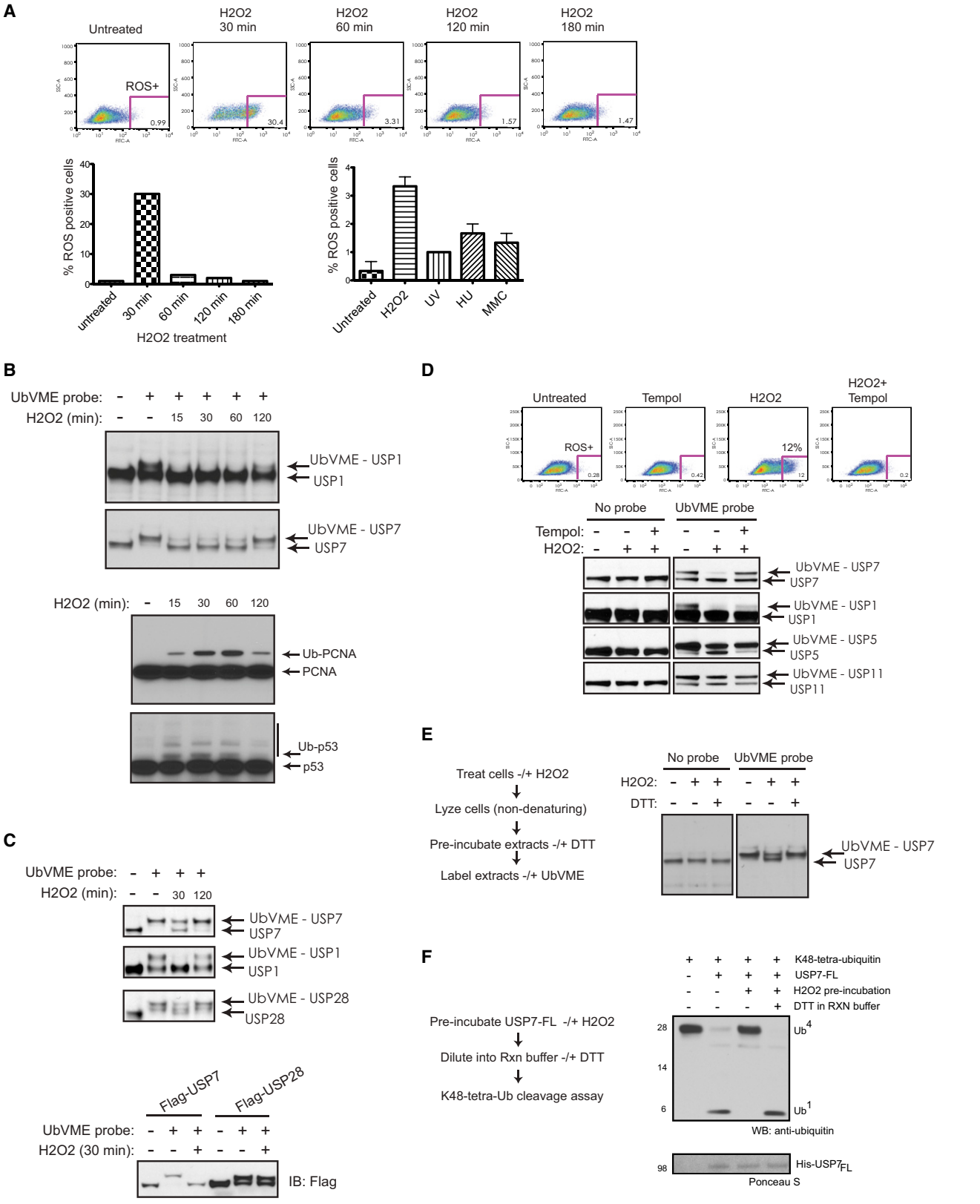

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steady-state levels of USP1 remained unchanged despite the robust activation of PCNA monoubiquitination 30–60 min post-treatment (Figure 1A). This is in contrast to UV-mediated DNA damage whereby USP1 is degraded to enhance PCNA monoubiquitination, suggesting that alternate mechanisms inhibit USP1 activity in a time-dependent manner (Figure 1B). Additionally, the activation of PCNA monoubiquitination by oxidative stress appeared to be most active during S phase and nearly nonexistent in G0 or G1 phase of the cell cycle (Figure 1C). Oxidative stress also activated the DNA damage checkpoint response as shown by the phosphorylation of Ser317 of Chk1 (Figures 1A and 1C). Importantly, depletion of Rad18, which abolished PCNA monoubiquitination, resulted in increased Chk1 phosphorylation after oxidative stress (Figure 1D). This is probably due to the reduced ability of cells to tolerate or bypass oxidative DNA damage, which will cause more stalling of the replication fork, Chk1 signaling, and G2 checkpoint activation. In the absence of checkpoint signaling with caffeine treatment (Kannouche et al., 2001), PCNA K164R-expressing cells are marginally sensitive to H_2O_2 treatment (Figure S1B). Elevated ROS levels are probably the cause of oxidative DNA damage, because treatment of cells with a superoxide scavenger, Tempol, reduced the monoubiquitination of PCNA (Figure 1E).

Oxidation of USP1 Is Dependent on Its Catalytic Competency

To address the role of USP1 in regulating PCNA monoubiquitination after oxidative DNA damage, we used siRNAs to deplete USP1 and found that PCNA monoubiquitination levels were further increased in response to oxidative stress (Figure 1F). This provides evidence that USP1 is indeed a critical regulator of oxidative DNA damage-induced PCNA monoubiquitination. Next, we tested whether USP1 could be a direct target of oxidative stress, and whether this could affect its catalytic activity. Cys oxidation by H_2O_2 results in the formation of sulfenic (−SOH), sulfinic (−SO2H), or sulfonic (−SO3H) acid, which represents the addition of one, two, or three oxygen molecules, respectively. Although sulfenic acid conversion is reversible due to its propensity to react with other thiol groups or thiol-containing proteins to generate a disulfide, the sulfonic and sulfinic acid forms are typically considered irreversible end products (Claiborne et al., 1999). If USP1 is a direct target of oxidative stress, it is important to determine which oxidation state it exists in. Recently, the synthesis of several dimedone-based chemoselective reagents capable of labeling, enriching, and subsequent identification of sulfenic acid-modified proteins was reported (Charles et al., 2007; Poole et al., 2007; Reddie et al., 2008). Herein, we used a biotin-tagged 1,3-cyclohexadione derivative, DCP-Bio1 (Figure 1G), to investigate the oxidation status of USP1 in response to oxidative stress. Because the analysis of the endogenous USP1 oxidation state was difficult to assess due to the low abundance of active USP1 pool in cells (Figure 2B), exogenous USP1 was coexpressed in U2OS cells along with its catalytic cofactor UAF1 to ensure higher levels of active DUB. Upon H_2O_2 treatment, we found that exogenously expressed C-terminally epitope-tagged mouse Usp1 (Usp1-Myc) was labeled by DCP-Bio1 in cell lysates (Figure 1G), suggesting that Usp1 was oxidized to the sulfenic acid intermediate in response to oxidative stress. To determine whether the labeling, and therefore oxidation, of USP1 was dependent on its catalytic competency, we tested labeling of USP1 mutated in the active site His domain. This mutant (DD750/751AA) compromises the nucleophilic ability of the catalytic Cys residue by mutating the Asp in its catalytic triad (Rawlings et al., 2012) and, when expressed, it was not labeled by the DCP-Bio1 probe in an oxidative stress-dependent manner (Figure 1G).

To assess the catalytic activity of the modified USP1, we used ubiquitin vinyl methyl ester (UbVME) suicide substrate probe. In brief, covalent labeling of DUBs by UbVME suggests active protein. Importantly, the DUB activity of exogenously expressed wild-type Usp1 was inhibited after oxidative stress (Figure 1G). As expected, the Asp catalytic triad mutant (DD750/751AA) was not labeled by UbVME. These results demonstrate that the oxidation-induced inhibition of USP1 is dependent on whether or not the active site residues are engaged in the proper conformation for activity, strongly suggesting that DUBs are most susceptible to oxidation when they are catalytically active. Moreover, H_2O_2 treatment did not affect the association of

Figure 2. Reversible Inhibition of DUBs by Oxidative Stress

(A) Temporal analysis of ROS levels inside cells after H_2O_2 treatment. T98G cells were treated with a final concentration of 1 mM H_2O_2 for the indicated amount of time (top and bottom left). Cells were trypsinized, collected, and incubated with CM-H_2DCFDA for 30 min at 37°C prior to FACS analysis. Additionally, T98G cells were left untreated or treated with 0.3 mM H_2O_2 for 30 min, 50 J/M^2 UVC for 30 min, 2 mM HU for 18 hr, or MMC for 18 hr (bottom right) and trypsinized, collected, and incubated with CM-H_2DCFDA for FACS analysis. Error bars represent SD of the experiment done in triplicate.

(B) USP1 and USP7 are reversibly inactivated by oxidative stress and correlate with the ubiquitination status of their respective substrates. U2OS cells were left untreated or treated with a final concentration of 1 mM H_2O_2 for the indicated timecourse. Extracts were made in parallel to assess DUB activity by the UbVME probe and by whole-cell lysis to assess the ubiquitination status of PCNA and p53 proteins.

(C) Ectopically expressed DUBs are similarly affected by oxidative stress as their endogenous counterparts. 293T cells were either transfected or not for 48 hr with wild-type Flag-USP7 or Flag-USP28 expression constructs. Then cells were left untreated or treated with 1 mM H_2O_2 for 30 min or with the indicated timecourse. Samples were then collected and processed for DUB activity assay as in (B) and probed with the indicated antibodies for western blot. Additional bands that appear are probably different isoforms of USP28 (USP28 antibody).

(D) Tempol can reverse the DUB catalytic inhibition by oxidative stress. U2OS cells were left untreated or treated with 0.3 mM H_2O_2 for 30 min in the absence or presence of Tempol. Cells were then trypsinized, collected, and incubated with CM-H_2DCFDA as in (A). In parallel analysis, samples were collected and processed for DUB activity assay as in (B).

(E) ROS-inactivated USP7 in U2OS cells can be reversed after in vitro DTT treatment. U2OS cells were treated with 1 mM H_2O_2 for 30 min. Extracts were then preincubated on ice with 20 mM DTT (or not) prior to the labeling with UbVME as in (B).

(F) Recombinant USP7 can be reversibly inhibited by ROS in vitro. His-tagged recombinant full-length USP7 is subjected to a ubiquitin cleavage assay of K48-linked tetraubiquitin chains in the presence or absence of H_2O_2 (1 mM) preincubation. DUB reaction was done in the presence or absence of 20 mM DTT. See also Figure S1.

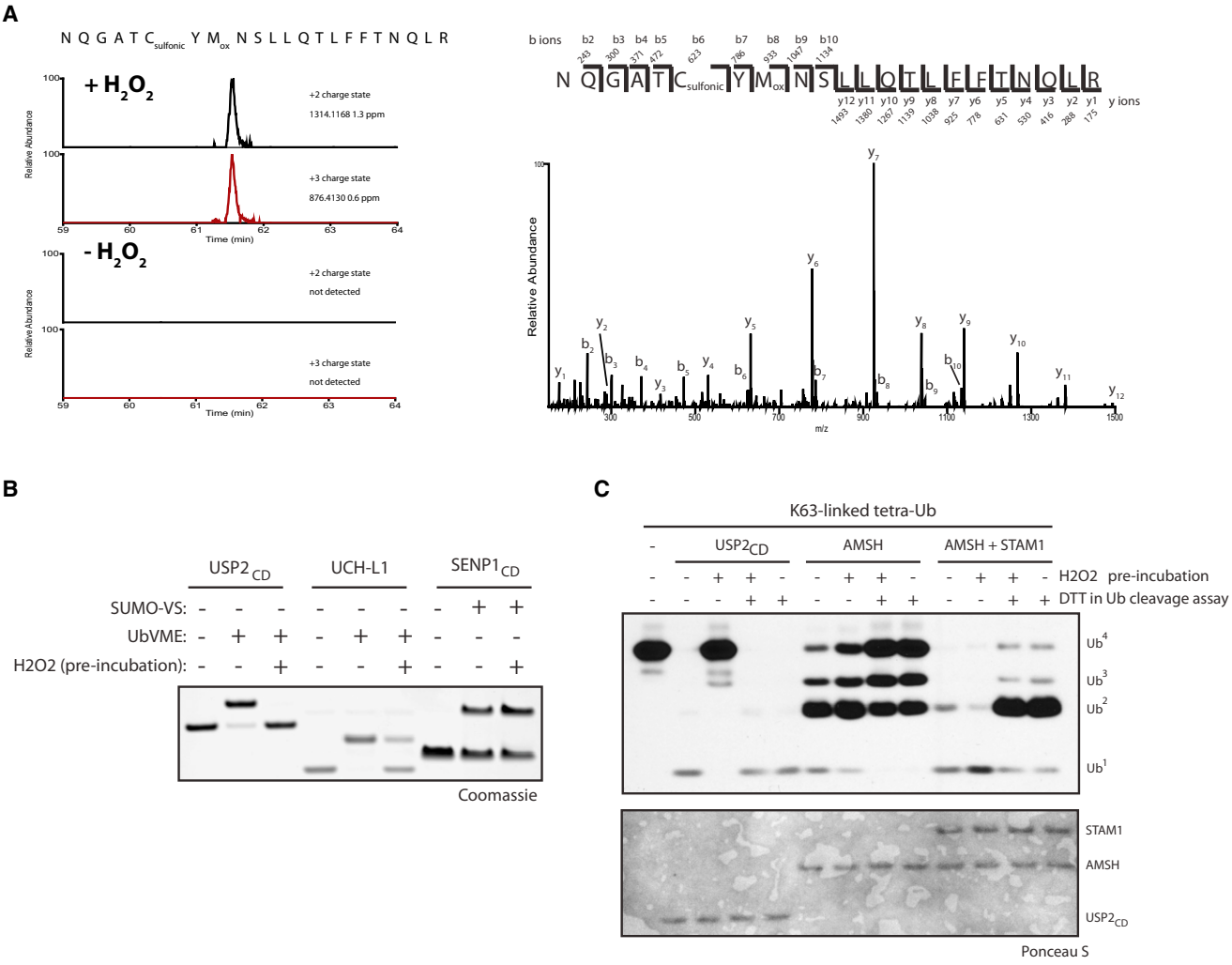

Figure 3. The Metalloprotease AMSH Is Not Susceptible to ROS-Mediated Catalytic Inhibition

(A) Detecting the oxidation of the active site Cys of USP7 by mass spectrometry. Left: extracted ion chromatograms for the peptide harboring the catalytic Cys NQGAT(C)YMNSLLQTLFFTNQLR in the H_2O_2-treated (top) and untreated (left) sample. The m/z values for the doubly (monoisotopic m/z = 1314.1151) and the triply (monoisotopic m/z = 876.4125) charged peptide carrying a sulfonic acid modification on the Cys residue and an oxidation on Met are extracted with a mass window of 0.1 Da. In the H_2O_2-treated sample, the doubly and triply charged precursor of the sulfonic acid-modified peptide with an oxidation on the Met residue could be detected (see also right panel). The mass error is within 2 ppm of the theoretical value. In the untreated sample, there are no ions detected for the doubly or triply charged peptide carrying a sulfonic acid modification and a methionine oxidation. In addition, an m/z value for the peptide carrying just the sulfonic acid

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Usp1 with UAF1 (Figure 1H), suggesting that inhibition of USP1 by oxidative stress is not likely caused by ROS-mediated disruption of the Usp1-UAF1 complex.

Reversibility of Oxidative Stress-Induced Inactivation of DUBs

To directly measure the duration of the ROS burst in cells treated with H_2O_2, we used the cell-permeable 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) probe as a fluorescent indicator for changes in ROS levels in cells. After oxidative stress, ROS levels peaked at 30 min and were reduced to near baseline levels by 60 min (Figure 2A). Importantly, genotoxic agents, including hydroxyurea (HU) or mitomycin C (MMC), have been shown to increase the cellular levels of ROS, which can affect diverse intracellular signaling processes (Ray et al., 2012). To assess whether other DUBs, in addition to USP1, were susceptible to inhibition by oxidative stress, and possibly through other DNA-damaging agents, we treated U2OS cells with H_2O_2 at different time points and then made cell extracts and subjected them to UbVME labeling. Both USP1 and another cysteine protease DUB, USP7, were reversibly inactivated by oxidative stress in the timecourse study (Figure 2B). USP7 plays an important role in regulating genotoxic stress pathways and has been shown to associate with and deubiquitinate both the tumor suppressor protein p53 and its ubiquitin E3 ligase Mdm2 (Li et al., 2004; Meulmeester et al., 2005). Additionally, USP7 may also have a role in modulating histone ubiquitination levels to enable base excision repair of oxidative lesions (Khoronenkova et al., 2011). Despite the importance of this enzyme, the regulation of USP7 activity is still not well understood. Importantly, the DUB activity of both USP1 and USP7 inversely correlated with the ubiquitination state of their respective substrates (Figure 2B), suggesting that oxidative stress can regulate downstream substrates of the DUBs. Interestingly, USP28 was not as susceptible to ROS-induced inhibition as compared to USP7 or USP1, suggesting that other determining factors, such as the local electrostatic environment of the catalytic cysteine or even subcellular localization and/or scaffolding protein partners may modulate the sensitivity of the DUB to oxidative stress. In agreement with their endogenous counterparts, ectopically expressed Flag-USP7 could be inhibited by ROS, whereas Flag-USP28 was more refractory to this inhibition (Figure 2C). Additionally, treatment of cells with Tempol prevents ROS-induced inactivation of DUBs (Figure 2D) and in vivo analysis of only a small subset of DUBs suggests differential regulation of catalytic activity by ROS (Figure 2D). We speculate that this may be due to differences between how each DUB achieves a catalytically competent conformation.

The Catalytic Cysteine Residue of USP7 Is the Target of ROS-Mediated Oxidization

The timecourse study in U2OS cells suggests that USP7 activity is fully recovered 2 hr after oxidative stress (Figure 2B). The ROS-induced inhibition of USP7 activity in cells was shown to be reversible by an in vitro incubation with the reducing agent dithiothreitol (DTT), suggesting that the catalytic Cys of USP7 existed, in part, in a reversibly oxidized state inside the cell (probably in the sulfenic acid form, Cys-SOH) (Figure 2E). To determine whether the catalytic activity of USP7 can be modulated directly by ROS in vitro, we used recombinant full-length USP7 to show that USP7 catalytic activity, as measured by cleavage of K48-linked tetraubiquitin chains or by the UbVME probe, can be inhibited by preincubation with H_2O_2 (Figure 2F and Figure S1C). Importantly, this in vitro ROS-induced inhibition of USP7 is also fully reversible by DTT (Figure 2F). To verify the active site Cys modification, we subjected USP7 to extensive H_2O_2 treatment and demonstrated by tandem mass spectrometry (MS/MS) that the active site Cys is modified to sulfonic acid (Cys-SO_3) (Figure 3A). Because the sulfenic acid intermediate is highly labile, the sulfonic acid is the only form amenable to MS analysis. Our analysis revealed additional oxidized Cys residues on USP7 (data not shown), which may be due to the extensive H_2O_2 treatment. It is possible that under low-level DUB oxidation, the sulfenic acid intermediate is converted to a sulfenamide intermediate or undergoes disulfide or mixed disulfide forms as a possible mechanism to protect the active site Cys residue against further oxidation (Salmeen et al., 2003).

The Metalloprotease Family of DUBs Is Probably Not Susceptible to ROS-Mediated Catalytic Inhibition

Because four of the five families of DUBs are Cys proteases, we predict that the majority of the DUBs may achieve some level of

modification could not be detected (data not shown). Right: MS/MS spectrum of the triply charged ion of peptide NQGAT(C)YMNSLLQTLFFTNQLR carrying a sulfonic acid modification on the Cys residue and an oxidation on Met. Observed peptide bond cleavage is indicated in the sequence. The corresponding theoretical N-terminal (b-type ion) and C-terminal (y-type ions) ion series for the observed fragment ions is shown above and below the sequence, respectively. Note that the mass error on the precursor ion and all fragment ions is better than 2 ppm. Neutral loss of water from y- and b-type ions is not indicated in the spectrum.

(B) UCH-L1, but not SENP1, can be inhibited by ROS in vitro. Recombinant UCH-L1 and $USP2_{CD}$ (catalytic domain) were labeled by UbVME, whereas recombinant $SENP1_{CD}$ was labeled by SUMO1-VS. Samples were preincubated either with or without H_2O_2 (1 mM). Reactions were analyzed by Coomassie-stained SDS-PAGE.

(C) AMSH cleavage of K63-linked tetraubiquitin chains is unaffected by ROS. Recombinant $USP2_{CD}$, AMSH, or AMSH plus STAM1 are subjected to ubiquitin cleavage assay of K63-linked tetraubiquitin chains in the presence or absence of H_2O_2 (1 mM) preincubation. The DUB reaction was done in the presence or absence of 20 mM DTT. Samples were then analyzed by western blot with anti-ubiquitin antibody.

(D) Model depicting how reversible oxidation of USP1 could modulate PCNA monoubiquitination in the presence of oxidative DNA damage. Left: in the absence of DNA damage, USP1 associated with its catalytic cofactor, UAF1, is in the catalytically active state and can readily deubiquitinate or suppress monoubiquitinated PCNA to prevent aberrant recruitment of error-prone TLS polymerases. Right: in response to oxidative stress or oxidative DNA damage, USP1 becomes transiently inactivated by direct oxidation of its catalytic Cys to the sulfenic acid intermediate. This renders USP1 unable to deubiquitinate PCNA, allowing for monoubiquitinated PCNA to engage with TLS polymerases that are responsible for DNA damage tolerance and/or gap repair. In the absence of oxidative DNA damage-induced checkpoint response, the cells become more reliant on the DNA damage tolerance pathway to ensure that the genome is fully replicated in a timely manner during S phase.

susceptibility to ROS-mediated catalytic inhibition. As predicted, using the DUB activity UbVME probe, we showed that UCH-L1, a Cys-dependent DUB belonging to the UCH family, is also inhibited by ROS, like USP2, albeit to a different extent (Figure 3B). Interestingly, a related family of cysteine proteases, SENPs, which process the ubiquitin-like molecule SUMO, have also been reported to undergo reversible inactivation by oxidative stress (Bossis and Melchior, 2006; Xu et al., 2008). Under the conditions that we use to analyze DUBs, however, the degree of ROS sensitivity appears to be different between USP2 and SENP1 (Figure 3B), arguing that the local environment of the catalytic cysteine could be key in determining the threshold for ROS sensitivity of cysteine proteases. To complete the analysis of DUB susceptibility to oxidative stress, we tested the zinc-dependent metalloprotease AMSH (associated molecule with the SH3 domain of STAM) (Clague and Urbé, 2006) for its activity in response to oxidation. AMSH does not appear to be susceptible to ROS-mediated catalytic inhibition; to show this, we assayed the ability of AMSH to cleave K63-linked tetraubiquitin chains in the presence or absence of H_2O_2. Although USP2 was readily inhibited by ROS, the activity of AMSH was unaffected, even in the presence of STAM, the binding partner and catalytic activator of AMSH (Figure 3C). Interestingly, treatment of the proteins with DTT resulted in opposite effects as compared with Cys proteases. Whereas DTT can reverse the ROS-mediated inhibition of Cys proteases, it may slightly inhibit the DUB activity of AMSH (Figure 3C). This is consistent with a structural study on AMSH that showed a potential disulfide bond between two Cys residues adjacent to the active site; the authors reported a marginal loss of DUB activity in the Cys mutant (Davies et al., 2011). Therefore, these studies provide evidence that not all DUBs will react similarly to elevated levels of ROS, and it raises the possibility that metalloproteases may have evolved to respond to an oxidant environment in a different manner than Cys-dependent proteases in order to maintain some baseline level of DUB activity in cells.

In summary, we define a cellular target of oxidative stress-induced signaling that involves ROS-mediated catalytic inhibition of Cys-dependent DUBs. This will probably have broad implications in future studies whereby oxidative stress pathways can regulate specific DUBs and thus their substrates involved in developmental growth control, microbial infection, and human diseases involving inflammation and cancer. Similar to the PTPs, the oxidation of the catalytic Cys of DUBs is transient and reversible, adding a layer of complexity involved in post-translational control of DUB activity. The degree of inhibition by ROS could be highly specific for individual DUBs, because each could reflect differences in basal reactivity of the catalytic site thiol or the geometry of the active site, leading to different ranges of catalytic competency. For example, partially active or inactive DUBs may be less susceptible to the oxidation of its active site cysteine residue when compared to a fully active DUB. There are many different ways DUB activity can be regulated, one of which is substrate-induced active site conformational changes, and another of which involves binding to scaffold or adaptor proteins that can alter the activity of DUBs. Perhaps different mechanisms to control DUB activity exist to counteract adventitious oxidation of constitutively active DUBs, because they would be more susceptible to active site inhibition by physiological fluctuations of ROS inside the cell.

Importantly, we show that USP1 is an attractive cellular target for reversible DUB inhibition by ROS. Transient inhibition of USP1 activity after oxidative DNA damage will ensure a rapid and time-dependent accumulation of monoubiquitinated PCNA, thus allowing for DNA damage tolerance and/or gap repair of oxidized DNA lesions during S phase (Figure 3D). This illustrates an example of how transient inhibition of a DUB by a burst of ROS can modulate cellular signaling pathways, underscoring the broad scope of the role ROS plays in modifying enzyme functions in biology.

EXPERIMENTAL PROCEDURES

DUB Activity Assay for In Vivo Measurements

For measuring DUB activity, we used the chemically modified ubiquitin activity probe UbVME. UbVME DUB activity assay was performed according to Borodovsky et al. (2002) with several modifications. Cells were lysed for 1 hr on ice with gentle tapping (250 mM Tris, 150 mM NaCl, 3 mM EDTA, and 0.5% NP40). Supernatant from cell lysis was collected after 10 min spin at 14,000 rpm at 4°C. Cell extracts and 1–1.5 μg of UbVME probe (Boston Biochem) were incubated at 25°C for 30–90 min in DUB reaction buffer (50 mM Tris, 50 mM NaCl, 10% glycerol, and 1 mM EDTA). Reactions were terminated with 250 mM Tris (pH 6.8), 2% SDS, 20% glycerol, and 200 mM 2-Mercaptoethanol, boiled for 10 min, and processed for western blot analysis.

Measurement of In Vivo ROS Generation

U2OS and T98G cells were treated and harvested between 60% to 80% visible confluency. Cells were treated with hydrogen peroxide (Sigma-Aldrich) with the concentrations and for the amount of time indicated. To measure ROS generation in the cells, we used 5-(and-6)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCF-DA) from Invitrogen. Cells were preincubated with Hoechst 33342 for 30 min (final concentration 5 μg/ml), then cells were washed, trypsinized, and collected. Cells were then incubated with CM-H_2DCF-DA (final concentration 10 μM) and Hoechst 33342 (final concentration 5 μg/ml) for 30 min at 37°C in the dark. Next, cells were immediately placed on ice and analyzed by fluorescence-activated cell sorting (FACS). Flow cytometry analysis was performed using LSRII and analyzed using FACS DIVA software and FlowJ 8.7 V3.1 (Verity Software House). CM-H_2DCF-DA was resuspended in 100% ethanol for a stock concentration of 10 mM (8.6 μl of 100% ethanol into the original vial with 50 mg dye). Dilution of CM-H_2DCF-DA and incubation of cells with CM-H_2DCF-DA was done in warm PBS.

Ubiquitin Cleavage and Activity Probe Assays for Recombinant DUBs

DUBs were incubated for 15 min at room temperature in the presence or absence of 1 mM H_2O_2 (preincubation), added to 300 ng tetraubiquitin chains, and further incubated for 1 hr at 37°C in 50 mM Tris (pH = 7.5), 100 mM NaCl, 1 mM EDTA, and 0.1% Tween-20 in the presence or absence of 20 mM DTT (DUB reaction assay). Reactions were terminated by boiling in SDS-PAGE sample buffer and then analyzed by western blotting. DUBs were used in UbVME activity probes (SENP1-CD used in SUMO-VS activity probe) with similar conditions as described for the ubiquitin cleavage assay. Recombinant tetraubiquitin chains (K48 or K63 linked), UbVME, SUMO1-VS, USP7, AMSH, STAM, SENP1-CD, and UCH-L1 were purchased from Boston Biochem (R&D Systems); DUBs were used at a final concentration of 200 nM.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and one figure and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2012.11.011.

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