Regulation of A20 and other OTU deubiquitinases by reversible oxidation

Yogesh Kulathu, Francisco J. Garcia, Tycho E.T. Mevissen, Martin Busch, Nadia Arnaudo, Kate S. Carroll, David Barford & David Komander

Protein ubiquitination is a highly versatile post-translational modification that regulates as diverse processes as protein degradation and kinase activation. Deubiquitinases hydrolyse ubiquitin modifications from proteins and are hence key regulators of the ubiquitin system. Ovarian tumour deubiquitinases comprise a family of fourteen human enzymes, many of which regulate cellular signalling pathways. Ovarian tumour deubiquitinases are cysteine proteases that cleave polyubiquitin chains in vitro and in cells, but little is currently known about their regulation. Here we show that ovarian tumour deubiquitinases are susceptible to reversible oxidation of the catalytic cysteine residue. High-resolution crystal structures of the catalytic domain of A20 in four different oxidation states reveal that the reversible form of A20 oxidation is a cysteine sulphenic acid intermediate, which is stabilised by the architecture of the catalytic centre. Using chemical tools to detect sulphenic acid intermediates, we show that many ovarian tumour deubiquitinases undergo reversible oxidation upon treatment with H₂O₂, revealing a new mechanism to regulate deubiquitinase activity.
Protein ubiquitination is a key regulatory mechanism in which the small protein ubiquitin (Ub) is covalently attached to, most commonly, Lys residues of proteins. Ubiquitination on substrates can comprise a single molecule (monoubiquitination) or a Ub chain (polyubiquitination). Polyubiquitin (polyUb) signals differ in structure and function depending on the linkage type within the polyUb chain. Indeed, the eight distinct Ub chain linkages coexist in cells, and appear to have distinct functions\(^1\)\(^2\). While Lys48- and Lys11-linked Ub polymers target proteins for proteasomal degradation, Lys63- and Met1-linked polyUb regulate non-degradative functions such as activation of protein kinase cascades during NF-κB (nuclear factor-κB) activation\(^1\)\(^3\).

Ub modifications are reversed by five families of altogether ~80 active deubiquinases (DUBs) in human cells, and many of these DUBs are emerging as important regulators of the Ub system\(^4\)\(^5\). For example, several DUBs have been described to regulate the transcription factor NF-κB (ref. 6), activation of which depends on an intricate network of both degradative and non-degradative Ub signals\(^2\)\(^3\).

The best-studied DUB in this pathway is the ovarian tumour (OTU) enzyme A20 (refs 8,9). A20 is a tumour suppressor, and its deletion in mice results in hyperinflammatory phenotypes consistent with deregulated NF-κB signalling. Mechanistically, A20 establishes a powerful negative feedback loop\(^8\)\(^9\), with A20 thought to perform its functions by ‘editing’ Ub chains, that is, by replacing non-degradative Lys63-linked Ub chains with degradative Lys48-linked Ub chains on key ubiquitination substrates (for example, RIP1) in the NF-κB activation cascade\(^10\)\(^11\). This editing capability is facilitated by an N-terminal OTU DUB domain to hydrolyse Ub chains, and by seven C-terminal ZnF domains that bind Ub and, either directly or indirectly, act as an E3-ligase\(^9\)\(^10\).

Inflammation arising as result of A20 deficiency exemplifies that Ub chain assembly and disassembly have to be carefully regulated to generate an appropriate signal. However, mechanisms of DUB inhibition that could amplify Ub signalling have remained elusive. To elucidate such global mechanisms of enzyme regulation, much can be learned from similar reversible post-translational modification systems, such as protein phosphorylation. Protein phosphatases antagonize the activity of protein kinases, and several mechanisms regulating their activity have been reported; one such mechanism is their regulation by reversible oxidation\(^12\). Many phosphatases are reversibly inactivated by reactive oxygen species (ROS) such as \(\text{H}_2\text{O}_2\), a second messenger that is rapidly produced in cells in response to stimuli such as cytokines, growth factors and DNA damage, leading to increased phosphorylation of cellular proteins\(^13\)–\(^17\).

Mechanistic and structural studies have shown that ROS predominantly target Cys residues in proteins, in particular low \(pK_a\) Cys residues that are commonly found in the active site of enzymes. Such Cys residues can be first oxidized to a sulphenic acid intermediate (SOH, ‘sulphenylated’), which is reversible by reducing agents\(^13\). Sulphenylated Cys residues undergo further oxidation to sulphonic (\(\text{SO}_2\text{H}\)) or sulphonic (\(\text{SO}_3\text{H}\)) acid and these higher oxidation states are irreversible. Other forms of reversible Cys oxidation are the formation of a disulphide bridge between two nearby Cys residues\(^18\), or the formation of a cyclic sulphenamide, as a result of a covalent bond formed between the Cys and the main chain nitrogen of a neighbouring residue\(^19\)\(^20\). These two latter mechanisms prevent overoxidation of susceptible Cys residues to irreversible oxidation states.

As four of the five known DUB classes are Cys proteases that contain low \(pK_a\) Cys residues in their active site, we asked whether DUBs are reversibly regulated by ROS. We found that A20 and other OTU enzymes undergo sulphenylation of their catalytic Cys residue in the presence of low concentrations of \(\text{H}_2\text{O}_2\). High-resolution crystal structures of the A20 OTU domain in different oxidation states reveal how the usually unstable SOH intermediate is stabilised by the architecture of the OTU catalytic centre, in a mechanism that may be shared by many OTU DUBs. Our findings reveal a universal mechanism of DUB inhibition that may impact on the levels of ubiquitination in oxidative stress conditions.

### Results

**Reducing agents activate the A20 DUB.** Previous characterization of A20 DUB activity and linkage-specificity using *in vitro* DUB assays has shown that it cleaves Lys48-linked chains. We performed DUB assays with the catalytic domain of A20 (ref. 21) against three chain types. Purified A20 that had been freeze-thawed showed weak activity towards diUb, in the absence of reducing agents in the reaction (Fig. 1a). Preincubation of A20 with 10 mM dithiothreitol (DTT) before the reaction increased A20 DUB activity against Lys48-linked chains, but not Lys63- or Met1-linked chains consistent with previous studies\(^22\). We further tested the effects of reducing agents on A20 activity by incubating the DUB with increasing amounts of DTT for 15 min, and assaying its activity against Lys48-linked triUb (Fig. 1b). While untreated A20 had weak activity, incubation of A20 with DTT increased the activity of the DUB significantly. DTT treatment had no effect on the proteins oligomeric state, as inactive freeze-thawed A20 was still monomeric, monodisperse and readily crystallized (see below).

The effect of reducing agents on A20 activity suggested that the enzyme is susceptible to regulation by ROS. To test this hypothesis, we first treated 5 \(\mu\)M A20 with increasing amounts of \(\text{H}_2\text{O}_2\) *in vitro* for 15 min. Catalase was added to rapidly deplete \(\text{H}_2\text{O}_2\), and stop oxidation. Samples were then split and to one half, 10 mM DTT was added and incubated for 15 min at room temperature. The proteins were used in DUB assays against Lys48-linked triUb (Fig. 1c). This experiment revealed that \(\text{H}_2\text{O}_2\) concentrations exceeding 10 \(\mu\)M significantly reduced A20 DUB activity. Importantly, this inhibition was completely reversed by DTT up to a \(\text{H}_2\text{O}_2\) concentration of 100 \(\mu\)M, suggesting fully reversible inhibition of A20 DUB activity in the physiologically important range of 10–100 \(\mu\)M \(\text{H}_2\text{O}_2\). Incubation with higher concentrations of \(\text{H}_2\text{O}_2\) (1 mM) led to irreversible inhibition that could not be reversed by subsequent DTT treatment.

Overall, these results indicated that ROS-mediated oxidation of A20 can be reversed by DTT treatment, allowing for reversible regulation of enzyme activity. Our previous A20 structure suggested that the active site of A20 is catalytically competent\(^21\), generating a low \(pK_a\) Cys103 as the most likely target of oxidation. However, the mechanism of reversible oxidation, and the involvement of the remaining six further Cys residues present in the analysed A20 OTU domain construct (amino acids 1–366, (ref. 21)) was unclear (Fig. 2a).

**Identification of an A20 SOH intermediate at Cys103.** Recent advances in chemical biology allow for the direct detection of Cys SOH intermediates in proteins (Fig. 2a). Dimedone derivatives label hydroxylated thiol groups site-specifically, and can be used in conjunction with click-chemistry to append a reporter tag for the detection of sulphenylated proteins for further analysis by western blotting and/or mass-spectrometry\(^23\)\(^24\). To test whether A20 forms SOH intermediates, purified A20 OTU domain was incubated with either dimethylsulphoxide (control) or DAz-2 (ref. 24) during \(\text{H}_2\text{O}_2\) treatment *in vitro*. Subsequently, excess probe was removed and DAz-2 tagged proteins were conjugated to a biotin tag. DAz-2 treated A20 could be readily detected by...
western blotting before H$_2$O$_2$ incubation, and labelling was significantly enhanced in the presence of H$_2$O$_2$ (Fig. 2b), showing that A20 was indeed sulphenylated.

To identify the site(s) of modification, A20 was subjected to mass-spectrometry analysis. The average intact mass of A20 was within 1 Da of the expected mass of unmodified A20 (Fig. 2c). Incubation with DAZ-2 generated a second A20 species with lower abundance in the untreated sample and a highly abundant species with a mass-increase of 194 Da in the H$_2$O$_2$ treated sample. The higher mass corresponds to A20 labelled with one molecule of DAZ-2 (MW 195.2 Da) (Fig. 2c).

Mass-spectrometry (MS)/MS analysis was used to identify the site of A20 modification by DAZ-2. For this, samples were treated with iodoacetamide (IAM) to block unwanted reactions of free Cys residues, and then digested by trypsin. A triply charged precursor ion derived from the tryptic peptide containing the catalytic Cys103 was identified to be IAM labelled in untreated sample, and DAZ-2-labelled in the H$_2$O$_2$ treated reaction (Fig. 2d). This revealed that the catalytic Cys103 of A20 undergoes modification to SOH in the presence of H$_2$O$_2$, likely explaining the observed inhibition of enzyme activity by ROS.

**Molecular basis for A20 inhibition by reversible oxidation.** Having identified sulphenylation of A20 at Cys103, we set out to understand how this Cys modification was stabilised within the A20 catalytic site. We used X-ray crystallography to understand A20 regulation by ROS at the molecular level. High-resolution crystal structures are required to resolve oxidation of Cys residues. Published A20 OTU domain structures are either of too low resolution (3.2 Å, (ref. 21)) or suffer from crystal twinning (2.5 Å, (ref. 25)). We obtained a new high-resolution (<2.0 Å) crystal form for the A20 OTU domain (Fig. 3a), and performed oxidation studies on these crystals (see Methods), to analyse the oxidation states of the catalytic Cys.

Crystals were grown in the presence or absence of DTT in mother liquor and protein to generate a reduced, active structure of A20, as well as a reversibly oxidized structure of A20, respectively. Some crystals grown in the absence of DTT were further treated with 50 µM H$_2$O$_2$ for 60 or 270 min in soaking experiments. Data were collected to high resolution (1.84–2.20 Å, Table 1) at the Diamond Light Source, beamline I-02. Crystals belonged to space group P1 with two A20 molecules in the asymmetric unit (Fig. 3b). Structures were determined by molecular replacement using the previously published A20 structure as a search model, and refined to final statistics shown in Table 1.

The high-resolution structure of A20 OTU is almost identical to previously determined structures, with root-mean-square deviation (RMSD) values of <0.8 Å (Fig. 4a). There are no large-scale conformational changes, and most loops absent in the previous structures are also disordered in the new crystal form (Fig. 3a,c). The catalytic centre of the A20 OTU domain is in an identical conformation in which the catalytic His residue deprotonates the thiol group of the catalytic Cys103, increasing its reactivity (Fig. 4a).

Cys103 is unambiguously defined in crystals grown in the presence of DTT (Fig. 4a). Importantly, in the absence of DTT, Cys103 shows additional positive $|F_o|-|F_c|$ electron density at its thiol function in both molecules of the asymmetric unit (Fig. 4b). This additional electron density cannot be explained by alternate side-chain conformations, is too close for a water molecule, and represents Cys SOH (A20 SOH), the reversibly oxidized form of A20 (Fig. 4b). When these crystals were incubated in 50 µM H$_2$O$_2$ for 60 or 270 min, further oxidation occurred that was modelled as either Cys sulphinic acid (A20 SO$_2$H) or Cys sulphonic acid (A20 SO$_3$H) (Fig. 4c,d). Cys103 was the only Cys affected by oxidation as the remaining five ordered Cys thiolate groups were clearly defined in electron density even after long H$_2$O$_2$ incubations (Fig. 4e). Modelling of the different oxidation states of Cys103 in the final refined structures reveal that Cys SOH forms an additional backbone hydrogen with the loop preceding the catalytic Cys (Cys-loop) (Fig. 4f). Irreversibly oxidized states of A20 additionally form a second hydrogen bond with the catalytic His256 residue (Fig. 4f).

The refined A20 SOH structure revealed the molecular basis for A20’s ability to be regulated by reversible oxidation, as the SOH intermediate of Cys103 is stabilised by the architecture of the OTU catalytic centre via hydrogen bond formation with the Cys-loop. This likely decreases the reactivity of Cys103 and with H$_2$O$_2$.
Figure 2 | Detection of SOH in reversibly oxidized A20. (a) Schematic of the reaction. Structure of A20 (pdb-id 2vfj (ref.21)) showing the six ordered Cys residues (green). Treatment of A20 with dimedone derivatives, such as DAz-2, tests for Cys SOH intermediates, which can be detected by western blotting (as in b) or by mass-spectrometry (as in c) (b) Purified A20 was treated with 50 μM H₂O₂ for 60 min at room temperature and incubated with dimethylsulphoxide or DAz-2. Subsequent biotinylation allows detection of A20 modification by Strepavidin-coupled horseradish peroxidase (Strep-HRP). (c) Left, mass-spectra of intact A20 OTU domain (calculated mass 43452 Da, labelled with black asterisks) without H₂O₂ treatment. Middle, untreated A20 OTU domain labelled with DAz-2. Right, A20 OTU domain treated with 50 μM H₂O₂ for 1 h at room temperature, and labelled with DAz-2. Labelling with DAz-2 (195 Da) results in a second peak (labelled with red asterisks) in untreated and H₂O₂ treated samples (observed mass 43654/43646, respectively). The observed mass corresponds to A20 labelled with one molecule of DAz-2. (d) Tryptic analysis of unlabelled (left) and labelled (right) A20 reveals the Cys103-containing peptide to be modified with DAz-2 (see Methods). Shown is the sequence of the Cys103 containing tryptic peptide including b- and y-ions (top), extracted ion chromatograms (middle), and MS/MS spectra after collision-induced dissociation (bottom). The MS/MS spectra after collision-induced dissociation of precursor ion 1074.36 [M + 3H]⁺³ yielded specific b and y ions pertaining to the peptide with DAz-2 modification at Cys103.
stabilises Cys103 against further oxidation, which occurs only after prolonged H₂O₂ treatment (Fig. 4) or at high H₂O₂ concentration (Fig. 1c).

Cys SOH is common in OTU DUBs. Stabilization of SOH by the catalytic architecture of A20 suggested that other OTU DUBs might be regulated in a similar manner. The 14 human OTU DUBs can be subclassified into three different groups based on the size of the catalytic domain5, but available structures show that the catalytic site and the position of the Cys-loop are highly conserved5. To test whether A20-like and other OTU DUBs are sulphenylated, we used a newly developed chemoselective probe, DYn-2, which labels sulphenylated proteins and harbours an alkyne handle for enhanced bioorthogonal ligation26 (Fig. 5a).

Purified OTU DUBs were incubated with increasing concentrations of H₂O₂ for 15 min, and SOH modifications were detected by western blotting. A20 was modified by DYn-2 to a similar extent as compared with DAz-2 (Fig. 5b). Cezanne/OTUD7B is highly similar to A20, and was found to be sulphenylated at low mM H₂O₂ concentrations. Higher concentrations (100 mM) abrogated the DYn-2 signal, perhaps indicating rapid irreversible oxidation (Fig. 5c). Importantly, sulphenylation of Cezanne is not observed when the catalytic Cys194 is mutated to Ala (Fig. 5d), indicating that also this enzyme is exclusively modified at its active site Cys.

Furthermore, sulphenylation was also detected in OTUB1, OTUD1, OTUD2, OTUD3, OTUD5 and OTUD6A (Fig. 5e–j) but not in OTUD6B (Fig. 5k). DYn-2 modification was observed...
Figure 4 | The A20 catalytic triad in different oxidation states. (a–d) Stereo images of the catalytic Cys and His residues and the Cys-loop in A20. A 2|F_o|−|F_c| map (blue) contoured at 1.5 σ, and a |F_o|−|F_c| map (green, thick lines) contoured at 3.5 σ covers shown residues. Left: Molecule A of the asymmetric unit. Right: Molecule B of the asymmetric unit. (a) Structure of A20 in reduced, active state, grown in the presence of DTT. (b) Structure of A20 with reversible oxidized Cys103 SOH, from untreated crystals. (c) Structure of A20 with irreversibly oxidized Cys103 after incubation with 50 μM H₂O₂ for 60 min. Cys residues were modelled as sulphinic and sulphonic acid in the two molecules, respectively. (d) Structure of A20 after 270 min incubation with 50 μM H₂O₂, modelled as in c. (e) The remaining ordered Cys residues in A20 (Cys54, Cys57, Cys86, Cys200, Cys243) are unaffected even after soaking for 270 min in 50 μM H₂O₂. Individual Cys residues are shown with 2|F_o|−|F_c| map (blue, contoured at 1.5 σ) and |F_o|−|F_c| map (green, contoured at 3.5 σ). There is no unmodelled density at these sites. (f) Final, refined models for reduced (A20 SH), reversibly oxidized (A20 SOH) and irreversibly (A20 SO₂H, A20 SO₃H) oxidized forms of A20. Yellow dotted lines indicate hydrogen bonds formed by oxidized Cys103.
in untreated samples of OTUB1, OTUD1, OTUD5 and OTUD6A suggesting that a significant fraction of the purified enzyme is (reversibly) oxidized. OTUD1, OTUD3 and OTUD5 show a similar profile as compared to A20 (Fig. 5), while OTUD2 is sulphenylated only at the highest H$_2$O$_2$ concentration (Fig. 5g).

Collectively, these results show that OTU family DUBs are readily sulphenylated in response to ROS. Furthermore, the observed differences in modification suggest that ROS may gradually regulate DUB activity.

**Discussion**

Generation of ROS in cells constitutes an important signalling event, however its transient nature, high reactivity and high diffusion rate pose challenges for detecting the roles and targets of ROS. To date, protein tyrosine phosphatases are the best-studied enzyme class regulated by ROS, and the impact of ROS production in phosphorylation cascades is unquestioned. Phosphatases were also instrumental to illuminate the different mechanisms of reversible inhibition of enzyme activity by oxidation.

The arrival of new tools to detect modified forms of proteins, such as dimedone derivatives that label SOH or antibodies against cyclic sulphenamides have confirmed many targets of ROS in cells, and have opened new avenues to study the global effects of oxidative stress. Moreover, the application of these tools to proteomics has led to the identification of many new Cys-modified proteins including kinases, peroxidases and DUBs.

We report that OTU DUBs can be regulated by reversible oxidation of the catalytic Cys, and provide structural and biochemical evidence that this is mediated by a Cys SOH intermediate, which is particularly suitable to be stabilised by the architecture of the OTU active site, in which conserved residues in the loop preceding the catalytic Cys residue (Cys-loop) stabilize the added hydroxyl group. Oxidation of DUBs is perhaps not surprising as most are Cys proteases, but what could be significant is the reversibility and the varying sensitivity of OTU DUBs to H$_2$O$_2$ concentrations, as this suggests that some DUBs (for example, Cezanne) are remarkably sensitive to H$_2$O$_2$, while others (for example, OTUD2) are not. This difference in sensitivity could arise from the architecture of the catalytic centre.

Moreover, it is tempting to speculate that inactive conformations observed for some DUBs in the absence of Ub substrate may represent a protective mechanism, where the DUB only forms a reactive catalytic Cys in the presence of its substrate or an activating interaction partner.

**Figure 5 | Detection of sulphenylation in different OTU DUBs.** (a) Chemical structure of DYn-2, which features an alkyne group. (b-k) Different human OTU family members were treated with increasing concentrations of H$_2$O$_2$ for 15 min at room temperature. H$_2$O$_2$ was quenched by adding 200 U catalase and the OTU was labelled with 1 µM DYn-2, which was subsequently biotinylated. The labelled proteins were detected by streptavidin-horseradish peroxidase (Strep-horseradish peroxidase (HRP)) western blot. Ponceau S staining of the membrane confirmed equal loading of proteins. (b) A20 OTU domain (aa 1–366), (c) Cezanne/OTUD7b (aa 124–438), (d) Cezanne/OTUD7b C194A (aa 124–438), (e) OTUB1 (aa 1–271, full length), (f) OTUD1 (aa 287–481), (g) OTUD2 (aa 1–348, full length), (h) OTUD3 (aa 52–275), (i) OTUD5/DUBA (aa 171–358), (j) OTUD6A (aa 128–288), (k) OTUD6B (aa 133–293).
Reversible oxidation of Cys proteases has been studied in detail, for example, Cys Cathepsins, but was unknown for DUBs. While this manuscript was under review, Coto-Rios et al. reported reversible oxidation via SOH intermediates for several USP family members and for UCH-L1, DUBs which are structurally unrelated to OTU DUBs. The authors could show that USP1 was reversibly inactivated in cells in response to oxidative DNA damage, leading to increased PCNA ubiquitination, suggesting in vivo relevance for this new regulatory mechanism. Reversible oxidation has further been studied for small Ub-like modifier (SUMO) proteases of the Secretin/SUMO-specific protease (SENP) family, and while these enzymes were shown to undergo reversible crosslinking as well as sulphhydration, the required concentrations of H2O2 were significantly higher (4–10 mM) as compared with OTU DUBs. In addition, ROS also regulate the activity of Atg8, a Secretin/SUMO-specific protease, and ROS treatment of Atg8 decreased SUMO conjugates by 4–10 mM DTT as compared with OTU DUBs.

Most enzymes in the Ub system utilize catalytic Cys residues. E1 Ub activating enzymes, E2 Ub conjugating enzymes and subclasses of E3 Ub ligases transfer Ub via thioester intermediates. Little is known about ROS regulation of these enzymatic processes. The membrane protein ubiquitin-like protein 3 (ULP3) is a 182 amino acid cytosolic protein that contains a thioredoxin-like domain and two Ub-like domains at the C-terminal end. The thioredoxin domain has been shown to be oxidized and crosslinked upon exposure to ROS. The enzyme has been shown to be involved in the regulation of JNK activation by ROS. The involvement of ROS in the regulation of JNK activation is supported by the observation that JNK is activated by ROS in a ULP3-dependent manner. The crosslinking of the thioredoxin domain to ULP3 has been shown to be mediated by the formation of a disulfide bond between the thioredoxin domain and a cysteine residue in the C-terminal Ub-like domain. The formation of this disulfide bond is thought to stabilize the enzyme and prevent its degradation by proteasomes.

ARTICLE NATURE COMMUNICATIONS | DOI: 10.1038/ncomms2567

labellling of OTU DUBS with DAz-2. A20 was treated with 20 mM DTT for 10 min at room temperature. Excess DTT was removed with a P-6 micro BioSpin column (BioRad) that had been pre-equilibrated with 20 mM Tris-HCl pH 7.2, 150 mM NaCl. 25 mM A20 was labelled with 10 mM DAz-2 and treated with 50 mM H2O2 for 1 h at room temperature while rocking. Protein was buffer exchanged into 25 mM ammonium bicarbonate pH 8.0, using an Amicon ultrafiltration filter with a 10-kDa Molecular weight cut-off (MWCO) (Millipore). The DAz-2 labelled samples were concentrated to dryness via vacuum centrifugation and then resuspended in 50 mM Tris-HCl pH 7.4 and 1% SDS. Probe modified proteins were detected via bioorthogonal Huisgen (3+2) cycloaddition by incubating with 100 µM iodoBn, 1 mM Tris (2-carboxyethyl) phosphine hydrochloride (TCEP), 100 µM triS-[1-benzyl-1H-1,2,3-triazol-4-yl]methylamine ligand (TBT), and 1 mM CuSO4 for 1 h at room temperature while rocking. The protein was methanol precipitated to remove excess reagents. Protein samples were resuspended in Lane buffer containing 10% (v/v) β-mercaptoethanol. The samples were separated by SDS–polyacrylamide gel electrophoresis using Mini-Protein TGX 4–15% Tris-Glycine gels (BioRad) and transferred to a polyvinylidene difluoride (PVDF) membrane (BioRad). After transfer, the PVDF membrane was blocked with 3% bovine serum albumin for 1 h at room temperature. The membrane was washed with Tris-buffered saline Tween-20 buffer (TBST) (2 × 10 min) and then incubated with 1:80,000 Strep-horseradish peroxidase (GE Healthcare). The PVDF membrane was washed with Tris-buffered saline Tween-20 buffer (TBST) (2 × 10 min) and then developed with chemiluminescence (GE Healthcare ECL Plus Western Blot Detection System) and imaged by film. Equal loading was assessed by staining the PVDF membrane with 50% R 250 Coomassie blue (Boston BioProducts) in 5% MeOH for 10 min.

Deubiquitinating enzymes (DUBs). DUB assays were performed according to ref. 22. DUBs were diluted to 2 × 10−6 M in 25 mM Tris (pH 7.5) and 10 mM DTT and used directly, or activated at 23 °C for 10 min. Subsequently, 10 µl of diluted enzyme were mixed with 1–2 µg diUb and 2 µl of 10 × DUB buffer (500 mM NaCl, 500 mM Tris (pH 7.5) and 50 mM DTT) in a 20 µl reaction. For oxidation studies, A20 was preactivated with 10 mM DTT, and subsequently dialysed against degassed reaction buffer lacking DTT. Protein was incubated with indicated concentrations of H2O2 for 15 min, and either used in a DUB reaction, or again DTT-treated (10 mM, 15 min) and used in a DUB reaction.

Reactions were incubated at 37 °C and 6 µl aliquots were taken at indicated times, and mixed with 4 µl lithium dodecyl sulphate (LDS) sample buffer containing 6–8 M urea/100 mM Tris- pH 7.2. The protein was reduced with 10 mM DTT for 1 h at room temperature. The samples were subsequently alkylated with 55 mM IAM for 1 h at room temperature in the dark. Excess IAM was quenched with the addition of 55 mM DTT and allowed to react for 30 min at room temperature. The urea concentration was diluted to less than 2 M by addition of 25 mM ammonium bicarbonate pH 8.0. Sequencing grade trypsin (Promega) was added at a ratio of 1:25 (w/w) and incubated overnight at 37 °C. The sample was concentrated to dryness via vacuum centrifugation and desalted with a C18 column (Next Gen). Peptide mass fingerprints were submitted for intact mass analysis using an electrospray linear ion trap mass spectrometer (LTQ-XL, Thermo Scientific) after separation on an Agilent Eclipse XDB-C8 2.1 mm × 15 mm trap with mobile phases A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile), which was used to trap, desalt and elute the peptides on to a Varian Eclipse reverse-phase C18 monomeric column (2.1 mm × 150 mm, 300 Å, 5 µm) with a gradient of 5 to 100% B in 14 min at a flow rate of 200 µl min−1. Peptide and protein identification was done through the Mascot search programme (http://www.matrixscience.com). Mass lists, in the form of Mascot generic files, were created automatically and used as the input for Mascot MS/MS ion searches against a database containing the full sequence of A20. The search parameters were as follows. The enzyme specificity was Trypsin with one missed cleavage permitted. The variable modifications included Carbamidomethyl (C-SH), DAz-2 (C-SOH) and Oxidation (M). The mass tolerance for the precursor ions obtained from the ES-I/ES-MS was set to ± 2 Da. The fragment mass tolerance was set to ± 0.8 Da. The maximum expectation value (p) for accepting individual MS/MS spectra was set at 0.05.
Synchrotron data was collected at the Diamond Light Source, beamline I-02, at 100 K and at wavelengths of 0.97950 (A20 SH) and 0.92000 (A20 SOH, A20 SO-H). Crystals displayed the space group P1 with two molecules of A20 in the asymmetric unit. Phases were obtained by molecular replacement in Phaser using the previous A20 structure (2vfj, (ref. 21)) as a search model. Subsequent rounds of model building in Coot 18 and refinement in Phenix 19 (using simulated annealing) resulted in final statistics shown in Table 1. Less than 0.5% of all residues were Ramachandran outliers, and these residues were located in flexible loop regions.

Labelling and detection of OTU sulphenylation using DYn-2. Labelling and detection using DYn-2 probes was performed as described20. The different DUBs were first treated with Tris (2-carboxyethyl) phosphine (TCEP) and DTT to completely reduce Cys residues, and subsequently buffer exchanged using P-6 micro Bisspin columns (BioRad) to remove reducing agents. OTU DUBs were treated with H2O2 in the concentration range of 0–100 mM for 15 min at 23°C. Catalase was added to deplete H2O2 and the proteins labelled with DYn-2. After labelling, excess probe was removed and DYn-2 labelled proteins were conjugated to biotin, which could be detected by western blotting with streptavidin-horseradish peroxidase. Ponceau S (Sigma Aldrich) staining was used to detect total protein levels.

References
1. Hershko, A. & Ciechanover, A. The ubiquitin system. Annu. Rev. Biochem. 67, 425–479 (1998).
2. Komander, D. & Rape, M. The ubiquitin code. Annu. Rev. Biochem. 81, 203–229 (2012).
3. Kulathu, Y. & Komander, D. Atypical ubiquitylation—the unexplored world of polyubiquitin beyond Lys48 and Lys63 linkages. Nat. Rev. Mol. Cell. Biol. 13, 508–523 (2012).
4. Reyes-Turcu, F. E., Ventii, K. H. & Wilkinson, K. D. Regulation and cellular roles of ubiquitin-specific deubiquitinating enzymes. Annu. Rev. Biochem. 78, 363–399 (2009).
5. Komander, D., Clague, M. J. & Ubé, S. Breaking the chains: structure and function of the deubiquitinases. Nat. Rev. Mol. Cell. Biol. 10, 550–563 (2009).
6. Harhaj, E. W. & Dixit, V. M. Regulation of NF-kB by deubiquitinases. Immunol. Rev. 246, 107–124 (2012).
7. Skaug, B., Jiang, X. & Chen, Z. J. The role of ubiquitin in NF-kB regulatory pathways. Annu. Rev. Biochem. 78, 769–796 (2009).
8. Hymowitz, S. G. & Wertz, I. E. A20: from ubiquitin editing to tumour suppression. Nat. Rev. Cancer 10, 332–341 (2010).
9. Shembade, N. & Harhaj, E. W. Regulation of NF-kB signalling by the A20 deubiquitinase. Cell. Mol. Immunol. 9, 123–130 (2012).
10. Wertz, I. E. et al. De-ubiquitination and ubiquitin ligases domains of A20 downregulate NF-kB signalling. Nature 430, 694–699 (2004).
11. Newton, K. et al. Ubiquitin chain editing revealed by polyubiquitin linkage-specific antibodies. Cell 134, 668–678 (2008).
12. Salmeen, A. & Barford, D. Functions and mechanisms of redox regulation of cysteine-based phosphatases. Antioxid. Redox Signal. 7, 560–577 (2005).
13. Denu, J. M. & Tanner, K. G. Specific and reversible inactivation of protein tyrosine phosphatase superfamily. Cell. Mol. Immunol. 9, 103–112 (2012).
14. Lin, S. et al. Targeting the reversibly oxidized cysteine oxidation products of A20 have been deposited in the Protein Data Bank under accession codes 3vfj: Coordinates and structure factors for the reduced and oxidized forms of A20 SH: 5ejj (A20 SOH), 3izj (A20 SO-H) and 3izl (A20 SO-H). Competing financial interests: DK is a consultant for Mission Therapeutics. Additional information Accession codes: Coordinates and structure factors for the reduced and oxidized forms of A20 have been deposited in the Protein Data Bank under accession codes 3vfj (A20 SH), 5ejj (A20 SOH), 3izj (A20 SO-H) and 3izl (A20 SO-H). Competing financial interests: DK is a consultant for Mission Therapeutics. Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/ How to cite this article: Kulathu, Y. et al. Regulation of A20 and other OTU deubiquitinases by reversible oxidation. Nat. Commun. 4:1569 doi: 10.1038/ncomms2567 (2013).