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De-ubiquitinating protease USP2a targets RIP1 and TRAF2 to mediate cell death by TNF

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Components of the TNFR1 complex are subject to dynamic ubiquitination that impacts on their effects as signalling factors. We have found that the ubiquitin-specific protease USP2a has a pivotal role in the decision for cell death or survival by the TNFR1 complex. This enzyme is a novel component of the TNFR1 complex that is recruited upon ligand binding and controls the signalling activity of the TNFR1-interacting protein RIP1 by removing its K63-linked ubiquitin chains. USP2a similarly de-ubiquitinates TRAF2, a ubiquitin-ligase recruited to the TNFR1 complex. During the TNF response the activity of USP2a on RIP1 and TRAF2 is required for the efficient reappearance of IκBα, which is essential to inactivate the anti-apoptotic transcription factor NF-κB. The effects of USP2a culminate in the conversion of the anti-apoptotic TNFR1 complex I into the pro-apoptotic TNFR1 complex II. Consequently, downregulation of USP2a promotes NF-κB activation and protects cells against TNF-induced cell death.
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Tumour necrosis factor (TNF) is a pleiotropic cytokine that can, through the TNF receptor 1 (TNFR1), elicit various cellular responses ranging from inflammation, cell proliferation, cell survival to apoptosis. The interaction of this cytokine with TNFR1 results in the recruitment of the adaptor TRADD to its intracellular death domain. RIP kinase 1 (RIP1), the ubiquitin ligase TNF receptor-associated factor 2 (TRAF2), and the inhibitors of apoptosis cIAP1 and cIAP2 then associate in the so-called complex I.[1] Following its recruitment to the complex I, RIP1 is ubiquitinated mainly by K63 ubiquitin chains.[2–5] The E3 ubiquitin ligase TRAF2 was the first to be described as being responsible for K63 ubiquitination of RIP1[5–7] together with the lipid shingosine-1-phosphate.[8] Other publications have shown that cIAP1/2 can attach K63 ubiquitin chains to RIP1[9–12] or that TRAF2 and cIAPs cooperate for the ubiquitination of RIP1[6,13,14]. These ubiquitin chains bind and associate the TAB/TAK (TAK1/TAB2/TAB3) and NEMO/IKK (IKKα, β, γ) kinases complex.[3,4,12,15] By proximity, TAK1 activates IKKβ by phosphorylation, which in turn phosphorylates IκBα to promote its K48-linked poly-ubiquitination and degradation by the proteasome. The anti-apoptotic transcription factor NF-κB is then free to translocate to the nucleus and activate gene transcription leading to survival of the cells.[7,15–20] Upon internalisation of the complex and in the absence of K63 ubiquitination, RIP1 is able to form the so-called complex II with FADD and pro-caspase-8 to initiate apoptosis.[1,16] Hence, ubiquitin conjugation has a crucial role in allowing cells to decide between cell death and survival in the TNF signalling cascade. Although a number of ubiquitin ligases, that presumably modify components of the TNFR1 complex, have been identified, so far only two de-ubiquitinating proteases have functionally been found in the TNF signalling pathway that impact on cell death, A20 and CYLD (cylindromatosis tumour suppressor).[21–24] A20 is a bi-functional enzyme that acts on RIP1 both as a ubiquitin protease for K63-linked ubiquitin chains and, through a separate protein domain, as a ubiquitin ligase that attaches K48 ubiquitin-linked chains.[5,25] CYLD was identified as a de-ubiquitinating protease that targets TRAF2 and releases its K63-linked ubiquitin chains.[22,26,27] Both enzymes are target genes of NF-κB and their induction is responsible for its efficient downregulation in a negative feed-back loop.[5,28] This suggests that the ubiquitin protease that only regulates the TNFR1 components on initial TNF binding has not yet been discovered.

The gene for the de-ubiquitinating enzyme USP2 produces three different isoforms as a result of alternative splicing.[29,30] These isoforms have different N-termini, but share an identical C-terminal catalytic core, which contains the canonical isopeptidase Cys- and His-boxes comprising the residues critical for catalysis. We have previously shown that the 41-kDa USP2 isoform induces apoptosis in 293T cells.[30] Here we investigated the 69-kDa splice isoform USP2a, which is the most prevalent isoform of the USP2 gene.[29] Given that the short isoform of USP2 impacts on the expression level of cFLIP,[31] we wanted to determine the role of its longest isoform in the TNF signalling pathway. We found that downregulation of USP2a by siRNA promotes NF-κB activation and protects cells against TNF induced cell death. We show that USP2a is recruited to the TNFR1 complex upon ligand binding. It releases the K63 ubiquitin chains from RIP1 and TRAF2, two of its crucial components, and thereby mediates the cell death signal that this receptor can trigger. This is

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Abbreviations: NF-κB, nuclear factor-kB; HA, hemagglutinin; RT-PCR, reverse transcription polymerase chain reaction; SDS, sodium dodecyl sulfate; RING domain, really interesting new gene domain; siRNA, small interfering ribonucleic acid
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accomplished by USP2a through enhancing the conversion of TNFR1 complex I into the pro-apoptotic complex II. Moreover, the downregulation of USP2a led to a complete loss of the stability of the IκBα inhibitor of the transcription factor NF-κB, facilitating the activation of the cell survival pathway. As USP2a is not upregulated by NF-κB, it can only function on first impact of the TNF cytokine. Hence the USP2a protease occupies a crucial and non-redundant position in the activation of the TNF receptor complex and in its life/death decision.

Results

USP2a mediates apoptosis by TNF through Inhibition of NF-κB. When USP2a was downregulated in Hela cells it efficiently protected them against TNF- but not Fas- induced cell death (Figure 1a). We observed the same effect in MCF7 cells (Supplementary Figure S1A). As the attachment of ubiquitin chains to TNFR1 subunits triggers the survival pathway via the activation of NF-κB[16] we asked whether the NF-κB activity is modified when USP2a is absent. As shown in Figure 1b (top and bottom panels), in a standard assay for NF-κB activation,[32] we detected a pronounced increase of its activity and of the cell number positive for NF-κB activation upon TNF treatment in the cells treated with siRNA against USP2a when compared with the scramble control cells. In line with this, the NF-κB activation was significantly reduced when USP2a was co-transfected and TNF applied to the cells (Supplementary Figure S1B). In addition, quantitative

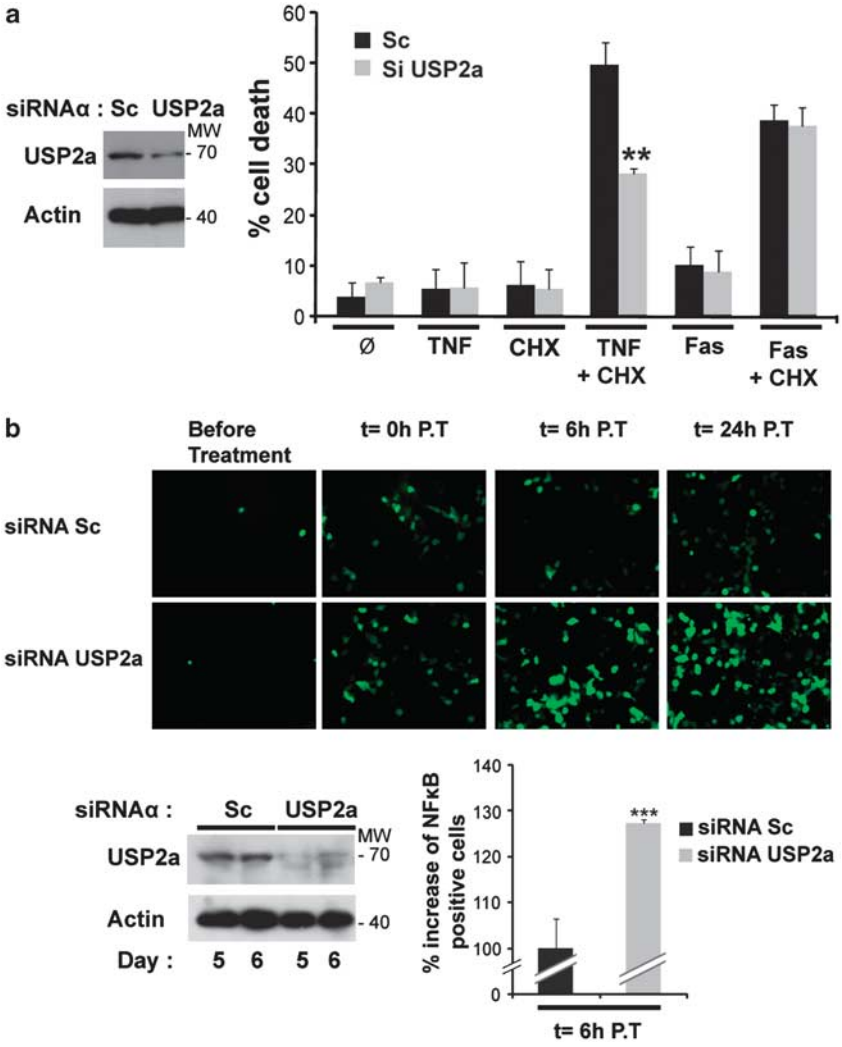

Figure 1 USP2a mediates cell death in the TNF signalling pathway. (**a**) USP2a downregulation protects cells against TNF-induced apoptosis. HeLa cells were transfected with a siRNA construct against USP2a or with a control scrambled siRNA (Sc). The effect on apoptosis induction by TNF upon USP2a downregulation was determined by the sub-G1 cell population using PI staining. At 48 h after transfection, cells were treated with TNF (100 ng/ml) or Fas ligand (100 ng/ml) in the presence or absence of cycloheximide (CHX) overnight. HeLa cells were harvested and stained with PI to quantify cell death. Shown are the means of three independent experiments done in duplicate for each construct. Bars are means ± S.D. ***t*-test, $P<0.01$. (**b**) USP2a downregulation enhances NF-κB activity induced by TNF. 293T cells were transfected with siRNA against USP2a or with a control scrambled siRNA construct (Sc). Forty-eight hours post transfection, cells were transfected with a reporter plasmid for NF-κB, pNF-κB-hrGFP. After 24 h, the cells were treated with TNF (20 ng/ml) for 4 h. The activity of the reporter plasmid was documented by representative confocal microscopy pictures (magnification $\times$ 40) at the indicated time points (top panels). The 6-h time point post treatment (P.T) was quantified by FACS for the GFP fluorescence (right hand bottom panel). Bars denote ± S.D. ****t*-test, $P<0.005$

RT-PCR showed that the NF-κB target gene TNF was enhanced (Supplementary Figure S1C), whereas TWEAK-signalling remained unaffected (Supplementary Figure S1D). We speculated that USP2a is interacting with components of the TNFR1 complex upon TNF treatment and set up immunoprecipitations to follow the fate of endogenous USP2a. We observed that upon TNF stimulation this enzyme becomes associated with the TNFR1 complex members RIP1, TRAF2, TRADD and the TNFR1 itself (Figure 2a, top panels). Interestingly, USP2a also weakly interacted with RIP1 in untreated cells. These results indicated that USP2a could affect the TNFR1 pathway. When we performed a time-course experiment after TNF stimulation we detected the recruitment of endogenous USP2a together with TRAF2 and RIP1 to the TNFR1 complex (Figure 2b). We also tested A20 and CYLD, two other de-ubiquitinases involved in the TNF pathway. In comparison with USP2a, A20 was recruited at later time points and CYLD was not found associated with the TNFR1 complex within the observed time frame (Supplementary Figure S2A).

Evidence for a physical interaction between USP2a and RIP1 was also provided by additional immunoprecipitations. Lysates from USP2a-HA- or USP2aC276A-HA (a catalytically inactive USP2a mutant)-transfected cells were immunoprecipitated with an anti-HA antibody. Western blot analysis revealed the presence of endogenous RIP1, suggesting the existence of a complex containing both proteins (Supplementary Figure S2B). The mutant form of USP2a, in contrast, did not associate with RIP1 (Supplementary Figure S2B). To explore a potential interaction between USP2a and TRAF2 as previously suggested by the two-hybrid system,[33]

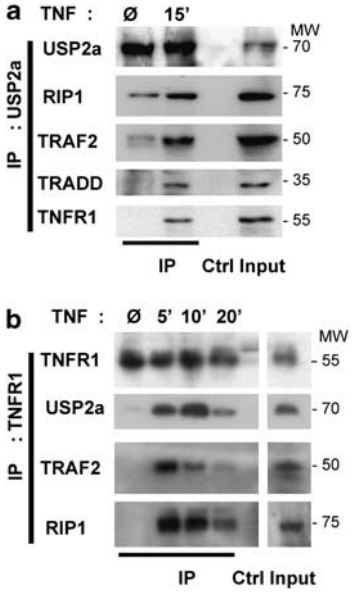

Figure 2 USP2a associates with components of the TNFR1 upon TNF application. (**a**) MCF7 cells were left untreated or treated with TNF for 15 min after which immunoprecipitations against endogenous USP2a were performed and the indicated co-precipitated proteins detected on a western blot. (**b**) 293T cells were left untreated or treated with TNF for 5, 10 or 20 min after which immunoprecipitations against the endogenous TNFR1 were performed and the co-precipitated proteins detected on a western blot as indicated

we conducted a co-immunoprecipitation between USP2a-HA and endogenous TRAF2. As shown in Figure S2B, western blots of the immunoprecipitates pulled down with an anti-HA antibody revealed an association between TRAF2 with USP2a but not with the catalytically inactive USP2a mutant. Co-immunoprecipitations with deletion mutants indicated that the RING domain in TRAF2 is required for this association (Supplementary Figure S2C, lower panels).

RIP1 and TRAF2 are targets of USP2a. Western blotting against all HA-ubiquitin conjugated proteins in whole cell lysates revealed that USP2a is an enzyme that is able to remove WT ubiquitin chains and also K63 polyubiquitin chains from a broad range of proteins. This effect strictly depended on the integrity of the cysteine residue 276 in the catalytic core localised in the C-terminal part of USP2a (Figure 3a). To validate that RIP1 is a direct target for USP2a, we conducted an *in vitro* de-ubiquination assay, which showed that the ubiquitin chains from RIP1 could be efficiently detached by recombinant USP2a, an activity that was inhibited by the irreversible inhibitor N-ethylmaleimide (NEM; Figure 3b, left panels). When we conducted the same experiment for TRAF2 we observed, as with RIP1, the efficient removal of ubiquitin by recombinant USP2 (Figure 3b, right panels). As RIP1 and TRAF2 are conjugated to K63 ubiquitin chains upon TNF application,[4,16] (Supplementary Figure S3), we monitored the effect of USP2a on K63 ubiquitin chains in an *in vivo* de-ubiquitination assay for which we co-transfected USP2a or its C276A mutant with ubiquitin WT-HA, or its mutant K63-HA (K63 only, other lysines mutated to arginines). After the cells were lysed in SDS 1% and then diluted in a dissociation buffer, endogenous RIP1 or TRAF2 were immunoprecipitated and a subsequent western blot revealed the presence of HA-tagged K63 ubiquitin chains. Upon TNF treatment, we detected an efficient removal of K63 ubiquitin variants from RIP1 (Figure 3c, left panels) and TRAF2 (Figure 3b, right panels). RIP1 and TRAF2 can also be conjugated to K48-ubiquitin chains and hence we analysed the activity of USP2 for these ubiquitin chains. As shown in Figure S4, USP2 could remove the K48-ubiquitin chains from RIP1 but not from TRAF2. Moreover, as shown in Supplementary Figure S5, USP2 was not able to release K63 ubiquitin chains from NEMO and hence does not target all the components of the TNFR1 pathway. In these experiments we used SDS 1% in order to remove all proteins not linked covalently to RIP1 or TRAF2 from the complex. We then analysed the effect of USP2a downregulation on the ubiquitination status of RIP1 upon TNF treatment in 293T cells (Figure 4, upper panels). The K63-ubiquitination level of endogenous RIP1 was increased and sustained at later points of the TNF treatment in the cells that received siRNA against USP2a compared with the control cells. Similar results were observed with TRAF2 (Figure 4, lower panels). We then studied the effect of USP2a knockdown on the TNFR1 complex I and complex II. We immunoprecipitated the TNFR1 protein, which is only present in complex I.[1] Figure 5a reveals that in cells lacking USP2a the stability of the complex I increased as evidenced by the fact that the presence of the RIP1 and TRAF2 components could be

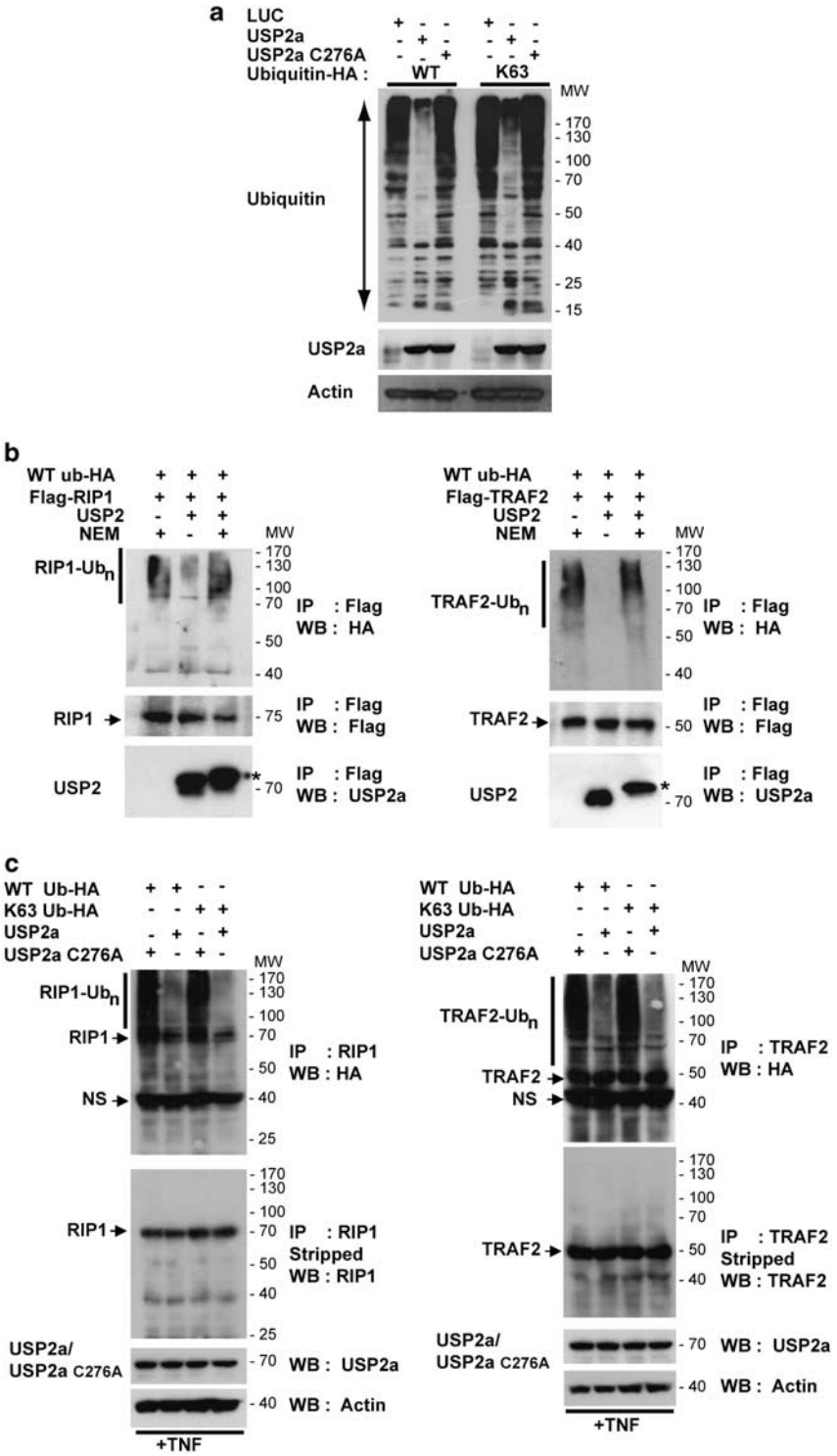

Figure 3 USP2a de-ubiquinates RIP1 and TRAF2 on K63-linked chains. (**a**) USP2a can disassemble the K63-linked ubiquitin chains of a broad range of proteins. 293T cells were transfected with USP2a or its catalytically inactive mutant C276A together with plasmids coding for WT ubiquitin-HA, or K63 ubiquitin-HA. Overall protein ubiquitination was detected in a western blot with an antibody against HA. USP2a and USP2a C276A expression were confirmed in an additional protein blot using an antibody against USP2a. Actin was used as a loading control. (**b**) USP2a de-ubiquitinates RIP1 and TRAF2 *in vitro*. 293T cells were transfected with plasmids coding for Flag-RIP1 (left panels) or Flag-TRAF2 (right panels) and WT ubiquitin-HA. Twenty-four hours after transfection cells were treated with TNF (20 ng/ml) for 15 min. The cell lysates were immunoprecipitated with an anti-Flag antibody and incubated for 1 h in a de-ubiquitination buffer in the presence or absence of the recombinant USP2 protein (with or without NEM). Western blots were probed with the indicated antibodies. NEM binds covalently the cystein protease and as a consequence it increases the apparent molecular weight of USP2a (*). (**c**) USP2a disassembles K63-linked ubiquitin chains from endogenous RIP1 and TRAF2. 293T cells were transfected with the expression plasmids for USP2a or its catalytically inactive mutant C276A together with a plasmid coding for WT ubiquitin-HA or K63 ubiquitin-HA mutants. Twenty-four hours post transfection, cells were treated with TNF (20 ng/ml) for 15 min (for K63, right panels). Cells were lysed in the denaturating dissociation buffer. Endogenous RIP1 (left panels) and TRAF2 (right panels) were pulled down and blots probed with the indicated antibodies. 'NS', non-specific

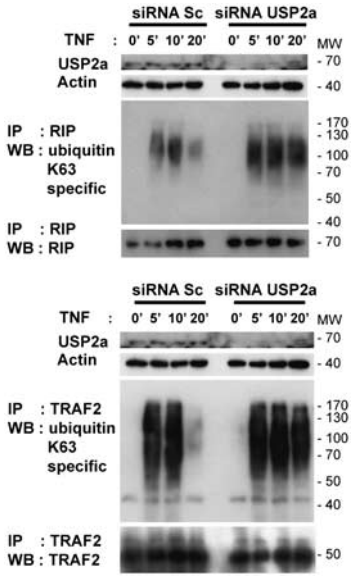

Figure 4 Effect of USP2a KD cells on the ubiquitination of RIP1 and TRAF2. RIP1 (upper panels) and TRAF2 (lower panels) ubiquitination levels in KD USP2a cells. 293T cells were transfected with scramble siRNA (Sc) or siRNA against USP2a. The cells were treated by adding TNF (20 ng/ml) over a period of 20 min. Cells were lysed and endogenous RIP1 or TRAF2 was pulled-down and the presence of ubiquitin was detected by western blot using antibodies specifically designed against K63 ubiquitin chains. USP2 downregulation was confirmed by western blot (top panels)

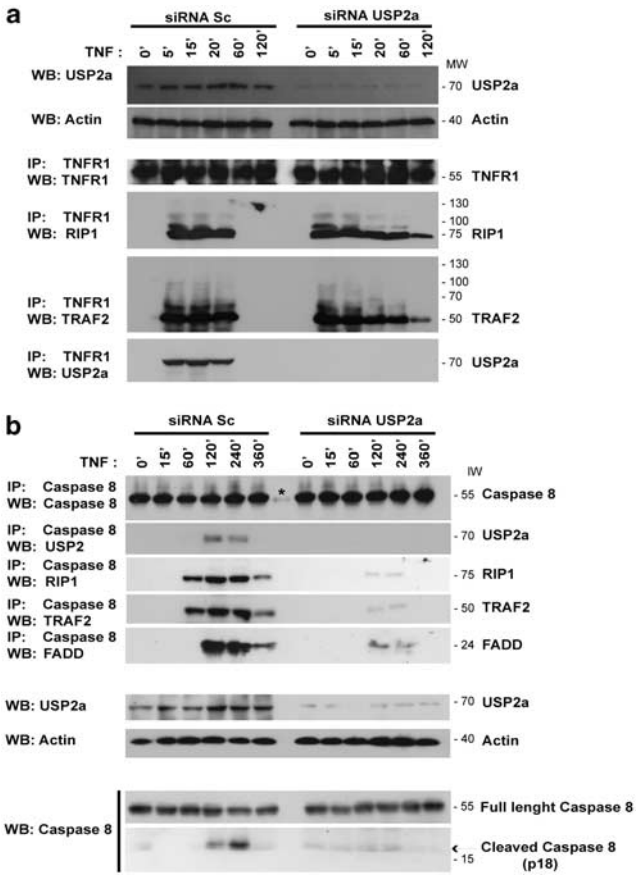

Figure 5 Effect of USP2a knockdown on TNFR1 complex I and II. (**a**) MCF7 transfected with scramble siRNA (Sc) or siRNA against USP2a were left untreated or treated with TNF (20 ng/ml) for 5, 10, 20, 60 or 120 min after which immunoprecipitations against the endogenous TNFR1 were performed and the co-precipitated proteins detected on a western blot as indicated. USP2 downregulation was confirmed by western blot (upper panels). (**b**) MCF7 transfected with scramble siRNA (Sc) or siRNA against USP2a were left untreated or treated with TNF (100 ng/ml) for 0, 15, 60, 120, 240 or 360 min after which immunoprecipitations against the endogenous caspase 8 were performed and the co-precipitated proteins detected on a western blot as indicated. USP2 downregulation was confirmed by western blot (middle panels). Caspase 8 cleavage over the time-course was detected in scramble cells or USP2a siRNA cells (bottom panels). *Cross reaction with protein size marker

observed for longer associated to TNFR1. To investigate the TNFR1 complex II we targeted caspase-8, which is only a subunit of complex II.[1] The assembly of the pro-apoptotic complex II almost completely relied on the presence of USP2a as demonstrated by the disappearance of the interaction between caspase-8 and the other complex II constituents FADD, RIP1 and TRAF2 when USP2a was knocked down (Figure 5b).

One of the genes activated by NF-κB is its inhibitor IκBα, which restores the inactive NF-κB during the response to TNF, thereby creating a negative feedback loop. As USP2 downregulation leads to an increase of NF-κB activation (Figure 1), we monitored the degradation of IκBα in these cells. As Figure 6a indicates, the re-accumulation of this protein was completely repressed at all time points tested when USP2a was knocked down with siRNA. Nevertheless, in those cells, the mRNA level of the NF-κB target gene IκBα was increased compared with the scramble cells (Figure 6b). The sustained signalling for IκBα degradation was evident upon reduction of USP2a expression by an increase in the phosphorylation of IκBα, p38 and JNK (Figure 6c). The ubiquitin-specific proteases A20 and CYLD have been shown to be target genes of NF-κB.[5,28] A quantitative RT-PCR confirmed this and revealed that in contrast to those enzymes USP2a was not significantly induced upon TNF application over a range of 24 h (Figure 7). We also followed the effect of USP2a downregulation on the protein expression levels of CYLD and A20 over time upon TNF application. Supplementary Figure S6 shows that both proteins were unchanged in comparison with the control cells up to 6 h after TNF stimulation.

Discussion

Our study shows that the ubiquitin specific protease USP2a performs two enzymatic reactions that mediate cell death by TNF, both based on the release of K63-linked ubiquitin chains. One activity targets RIP1 (Figure 4, Supplementary Figure S2B), which, when deconjugated from K63 ubiquitin chains, is rendered unable to stimulate NF-κB for cell survival but can activate caspase-8 and causes apoptosis.[11,16,34] The second enzymatic reaction targets TRAF2 (Figure 4, Supplementary Figure S2B), which, without its K63-linked ubiquitin chains, similarly loses its potential to assemble the activation complex for NF-κB[20,22] and possibly also its function as a K63-ubiquitin ligase for RIP1,[35] which in turn reinforces the direct effect of USP2a on RIP1. Consequently, we observed a potent inhibition of apoptosis induced by TNF when USP2a was knocked down (Figure 1a, Supplementary Figure S1A).

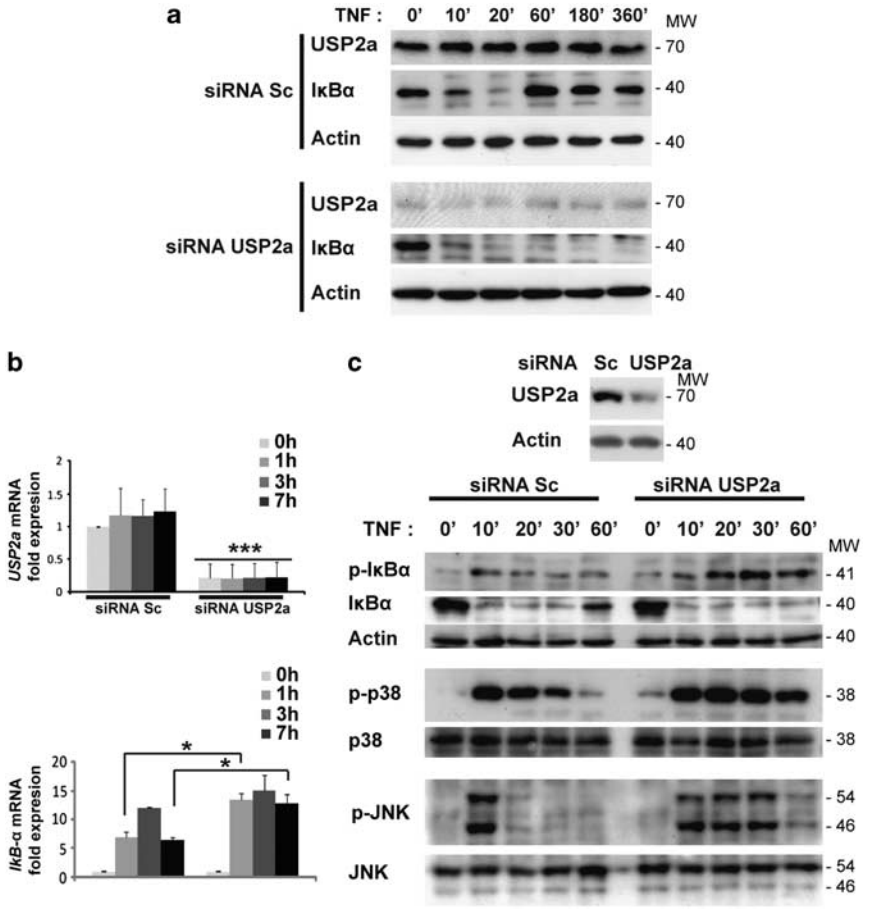

Figure 6 USP2a downregulation leads to prolonged downregulation of IκBα and sustained signalling for IκBα degradation. (**a**) USP2a inhibition attenuates the re-accumulation of IκBα. 293T cells were transfected with siRNA against USP2a or with a control scrambled siRNA construct (Sc). Forty-eight hours post transfection, the degradation of the NF-κB inhibitor IκBα was monitored at different time points in a western blot after TNF addition to both control-transfected cells and cells transfected with siRNA against USP2a. USP2 downregulation was confirmed by western blot and actin was used as a loading control. (**b**) Effect of TNF on the transcriptional upregulation of the NF-κB target gene IκBα. 293T cells were transfected with siRNA against USP2a or with a control scrambled siRNA construct (Sc). A quantitative RT-PCR was performed with primers specific for USP2a and IκBα at the indicated time points after addition of TNF and the results presented as the fold induction in comparison with the respective levels in untreated cells. Means and standard deviation of triplicates are shown from one representative experiment. Bars denote means ± S.D. ***t-test, $P<0.005$, *t-test, $P<0.05$. (**c**) Knockdown of USP2a enhances phosphorylation of IκBα, p38 and JNK upon TNF treatment. 293T cells transfected with siRNA against USP2a or with a control scrambled siRNA construct (Sc). The cells were treated with TNF (20 ng/ml) at the indicated time points and their lysates subsequently immunoblotted with the indicated antibodies. USP2 downregulation was confirmed by western blot and actin was used as a loading control

Our results indicate that USP2a exerts its effect on apoptosis by TNFR1 through the repression of NF-κB, whose activation is increased when USP2a is downregulated (Figure 1b) through blocking the re-appearance of IκBα (Figure 6a). This is a consequence of the longer-lasting – and in the case of RIP1, the stronger – ubiquitination of RIP1 and TRAF2 (Figure 4), leading to a prolonged aggregation of the TNFR1 complex I (Figure 5a). The latter cannot be the result of a possible K48 de-ubiquitinating activity of USP2a as its consequence would be a decrease of the half-life of the TNFR1 components when USP2a is downregulated. Hence, removing K63 ubiquitins by USP2a, rather than only impacting on the signalling for NF-κB activation, also has a destabilising effect on the TNFR1 complex I. In the absence of USP2a the TNFR1 complex II is substantially reduced (Figure 5b). Hence, this enzyme mediates the conversion of TNFR1 complex I into the pro-apoptotic complex II and thereby causes apoptosis by TNFα (Figure 8). A similar activity was shown for CYLD only when cIAP1 and cIAP2 were inhibited at the same time with a Smac mimetic.[34]

The combined effect of USP2a on the stability of the TNFR1 complex I and II and the ubiquitination of its subunits limits the downstream phosporylation pathway that causes the degradation of IκBα (Figure 6c). Hence, we believe that USP2a is responsible for containing the activation of NF-κB and hence the survival pathway upon TNF application. Despite the upregulation of the IκBα mRNA when USP2a is inhibited (Figure 6b), its protein is so efficiently degraded that it does not reappear (Figure 6a) pointing out the central role of USP2a in the regulation of this inducible transcription factor.

With two substrates in the TNF receptor complex, RIP1 and TRAF2, USP2a is a potent pro-apoptotic mediator, but how is this enzyme itself regulated to allow NF-κB activation by TNF? A recent report showed the 41-kDa USP2 isoform is down-regulated by low doses of TNF in murine hepatocytes.[36] This led to a decrease in the cellular level of the ubiquitin ligase

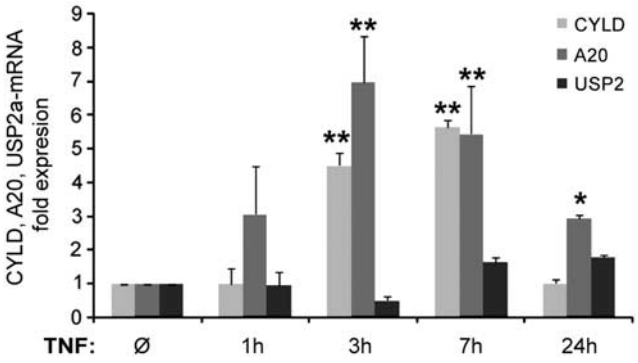

Figure 7 Effect of TNF on the transcriptional upregulation of USP2a and other ubiquitin-specific proteases in 293T cells. A quantitative RT-PCR was performed with primers specific for the USP2a, A20 and CYLD ubiquitin proteases at the indicated time points after addition of TNF and presented as the fold induction in comparison with their levels in untreated cells. Shown are the means of three independent experiments done in triplicate for each condition. Bars denote means ± S.D. **t*-test, $P<0.05$, ***t*-test, $P<0.01$

Itch, a concomitant accumulation of c-FLIP and to apoptosis inhibition. Hence, other isoforms of USP2 could function at additional levels in the activation of the TNFR1 complex.

The enzymatic activity of USP2a for K63 ubiquitin chains transmits cell death by TNF, an effect that is also supported by its associated with the TNFR1 complex and its components (Figure 2). However, its activity on K48 ubiquitin chains could have different and even opposite consequence in other biological scenarios. Previous publications identified cyclin D1, mdm2 and fatty acid synthase as targets, which, upon de-ubiquitination from K48 chains and accumulation, promote cell cycle progression or render cells resistant to cell death.[37–39] Nevertheless, knockdown of USP2a is not causing apoptosis (Figure 1a, Supplementary Figure S1A). Thus, the effect of USP2a on apoptosis could depend on the cell death signal. Alternatively, the protein expression pattern, in particular if changed by mutations in tumour cells, could modulate USP2a's effects and enhance or repress the cellular responses. This would be relevant for efforts to further exploit the ubiquitin–proteasome system for target definition and therapeutic interference.[39]

With CYLD and A20 two other de-ubiquitin proteases that impact on apoptosis have been described in the TNFR1 complex so far. Nevertheless, USP2a seems to occupy a critical and non-redundant position in the TNFR1 signalling pathway as its downregulation led to a complete loss of the re-emergence of the IκBα protein (Figure 6a). Moreover, based on its de-ubiquitinase activities on RIP1 and TRAF2 as described in this study, the USP2a enzyme is unique among de-ubiquitinases that regulate components of the TNF complex. Although A20 has ubiquitin–protease (on K63 linked chains) and –ligase activities (for K48-linked chains) that both target RIP1,[5] CYLD targets solely K63-linked ubiquitin chains in TRAF2.[22,26,27] Both enzymes are NF-κB target genes and seem to function in a negative feedback loop that again downregulates this transcription factor.[5,28] In contrast, our search of databases and publications did not reveal any indication that USP2a is a target gene of NF-κB and a quantitative PCR confirmed this (Figure 7). Hence, in contrast

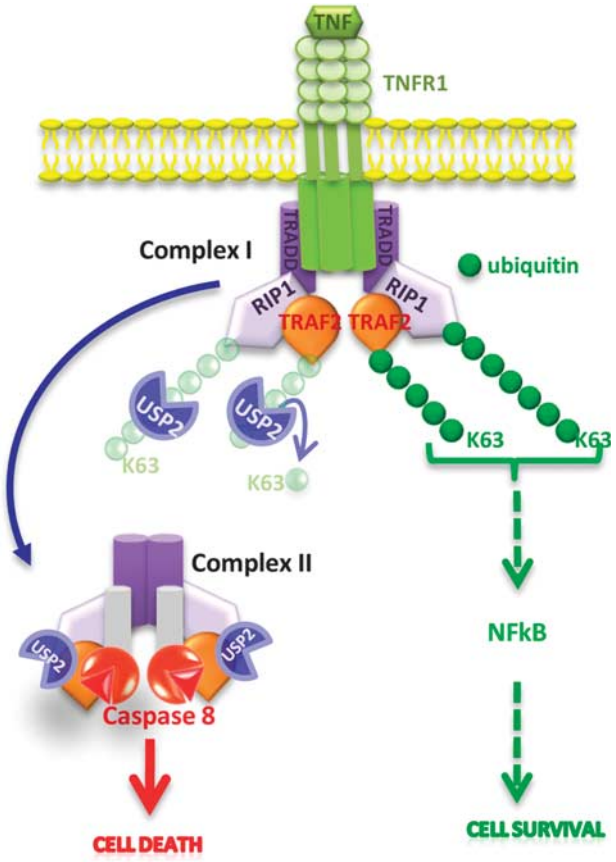

Figure 8 Model of USP2a's activity in the TNFR1 complex for cell death induction. Binding of TNF to the TNFR1 induces its trimerisation and recruitment of TRADD, RIP1 and TRAF2 to its cytosolic domain. The ubiquitin ligase TRAF2 can attach K63-linked ubiquitin chains to itself, which allows it to also conjugate these chains to RIP1. Both ubiquitin chains facilitate NF-κB activation and cell survival (right). USP2a can remove the K63-linked ubiquitin chains both from TRAF2 and from RIP1 (left). This allows RIP1 to activate pro-caspase-8 and induce cell death

to the known DUBs in the TNFR1 complex, USP2a could be the ubiquitin-specific protease that only regulates the components of this complex upon first contact with TNF. On the basis of this and on our findings presented in this report, we believe that USP2a is one of the master regulators of apoptosis by the TNFR1 complex.

In conclusion, the discovery of USP2a as a crucial regulator of the TNFR1 complex through its substrates TRAF2 and RIP1 and its activity to convert complex I into the pro-apoptotic complex II as presented in this study add another level of complexity to the regulation of the TNF response and further emphasises the importance of the K63 ubiquitination of the TNFR1 subunits for its decision about cell death or survival.

Materials and Methods

Reagents and antibodies. Human recombinant TNF and Fas and anti-caspase 8 antibody (C15) were obtained from Alexis Biochemicals (Enzo Life Sciences, Exeter, UK). MG132 was obtained from Calbiochem (Feltham, UK). Polyclonal and monoclonal anti-Haemagglutinin (HA), polyclonal anti-USP2a, polyclonal and monoclonal anti-Flag (M2), monoclonal β-actin, horseradish peroxydase-conjugated (HRP) goat-anti-rabbit were from Sigma-Aldrich (Dorset, UK). Monoclonal antibodies against RIP1, TRAF2 and FADD were purchased from

BD Biosciences (Oxford, UK). The polyclonal antibody against USP2a (cterm L523) was obtained from Abgent (Oxfordshire, UK). Anti-phospho-JNK and anti-phospho-p38 were supplied from Promega (Southampton, UK). Polyclonal anti-PARP, anti-TNFR1, anti-SAPK/JNK, anti-phospho-IκB-α antibodies were supplied from Cell Signalling (Hitchin, UK). Anti-TRAF2 (H-249), -TRAF2 (F4), -c-IAP2 (H85), -TNFR1 (H5), -RIP1 (H207), -p38 (N20) and IκB (C-21) antibodies were from Santa Cruz Biotechnology (Insight Biotechnology, Wembley, Middlesex, UK). Anti-ubiquitin, anti-ubiquitin (lysine K48), anti-ubiquitin (lysine K63) and anti-caspase 8 (N-ter) were purchased from Millipore (Durnham, UK). HRP goat-anti-mouse antibody was obtained from Jackson Laboratories (Invitrogen, Paisley, UK) and goat anti-rabbit Alexa Fluor 555 or goat anti-mouse Alexa Fluor 488 antibodies were from Molecular Probes (Invitrogen, Paisley, UK).

DNA constructs. Mammalian expression vectors coding for human USP2a, human TRAF2 and human RIP1 were from Origene (Rockville, MD, USA). Mammalian expression vectors pRK5-HA coding for ubiquitin wild type (WT) (Addgene plasmid 17608), ubiquitin K48 (Addgene plasmid 17605), ubiquitin K63 (Addgene plasmid 17606) were obtained from Addgene (Cambridge, MA, USA). cDNA coding for USP2a were amplified by PCR and sublconed in the pCDNA3Δ (Invitrogen) in-frame with an HA tag sequence. The catalytically inactive mutant of USP2a (C276A) has been engineered with the QuikChange Site-Directed Mutagenesis Kit from Stratagene (Cheshire, UK). cDNAs coding for RIP1 and TRAF2 were amplified by PCR and subcloned in the pci vector (gift from Remy Sadoul, Inserm U836, Grenoble, France) in-frame with a Flag tag sequence. TRAF2ΔRING was produced with the QuikChange Site-Directed Mutagenesis Kit from Stratagene. The reporter plasmid pNFκB-hrGFP was purchased from Stratagene.

Cell culture and plasmid transfection. 293T, MCF7,[40] HeLa, 3T3, 3T3 RIP$^{-/-}$, cells were maintained in DMEM (Invitrogen) containing 10% FCS (Sigma, Dorset, UK), 2 mM Glutamine (Invitrogen), 100 μg/ml streptamycine, 100 U/ml penicillin (Invitrogen) and sodium pyruvate (Invitrogen). 293T and 3T3s cells were transfected using Superfect (Qiagen, Crawley, UK), MCF7 and HeLa cells were transfected with Effectene (Qiagen) following the manufacturer's instructions.

Quantification of cell death. The percentage of the SubG1 cell population was used to quantify cell death. The supernatant and the adherent cells were harvested and resuspended in lysis buffer (0.1% sodium citrate and 0.1% Triton X-100 in PBS) containing propidium iodide (PI, Sigma, 20 μg/ml). Cells were then analysed using FACS (BD Biosciences) with the CellQuest programme (BD Biosciences).

Detection of active caspase-8. CaspaTagTM fluorescein caspase 8 activity kit (Millipore) allows the detection of active caspase-8 in living cells through the use of a fam-LETD-fmk. This probe passively enters the cells and binds irreversibly to the active caspase-8. Forty-hours after transfection, MCF7 cells were harvested and incubated for 1 h at 37°C with fam-LETD-fmk and with PI in accordance with the supplier's instructions. Fluorescein emission was analysed by FACS.

Detection of active caspase-3 by FACS. HeLa and 293T cells were transfected with GFP, β-Gal, Bax, RIP1 or USP2a using Effectene (Qiagen) or Superfect (Qiagen) and grown for 48 h. Cells were harvested and then fixed in 4% paraformaldehyde in PBS for 20 min at 4°C. After a blocking step with 3% BSA in Tris-HCl 50 mM, NaCl 155 mM, pH 7.6 and 0.02% saponine (TBSS) for 30 min at 37 °C, cells were incubated with the primary antibody for 1 h at RT. Rabbit polyclonal anti-Cleaved caspase-3 antibody (Cell Signalling) was used at a 1/200 dilution in TBSS. After centrifugation, cells were rinsed in TBSS and then incubated with the secondary anti-rabbit Alexa fluor 488 antibody (Molecular Probes, Invitrogen) at a dilution of 1/500 in TBSS. After centrifugation, cells were washed in TBSS. After a last centrifugation, the cell pellet was resuspended in 300 μl of PBS and Alexa 488 emission was analysed by FACS using FL1 channel.

Immunoprecipitation and western blotting. Twenty-four hours after transfection, MCF7 or 293T cells were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris pH 8.0, 1% NP-40, 0.5% deoxycholate, 0.1% SDS and 2 $\times$ proteases inhibitor mixture; Sigma). Cell lysates were cleared by centrifugation at 4°C for 15 min at 13 000 r.p.m. followed by two incubations of 30 min G-Sepharose beads (Amersham Biosciences, Little Chalfont, UK). Cell lysates were incubated overnight at 4°C with 3 μg of anti-Flag monoclonal M2 Ab, 3 μg of anti-HA monoclonal or 15 μg of anti-Nterm USP2a polyclonal Ab or with polyclonal anti-RIP1, -TRAF2 or anti-TNFR1 at 1/100. Immune complexes were precipitated with Proteine G-Sepharose and the beads were washed with RIPA buffer. Immunoprecipitated proteins were separated in a 10% SDS-PAGE and transferred onto a PVDF membrane (Millipore). Membranes were blocked with 3% BSA in PBS containing 0.1% Tween and incubated with the appropriate antibodies. Specific signals were revealed by the ECL detection reagent (Pierce, Cramlington, UK).

***In vitro* de-ubiquitination assay.** Plasmids for Flag-TRAF2, Flag-RIP and plasmids coding for HA-ubiquitin (WT) were transfected into 293T cells. At 24 h after transfection, cells were treated with MG132 (10 μM) for 4 h or with TNF (20 ng/ml) for 15 min. Cells were harvested, lysed and boiled 5 min in 1% SDS (v/v) (denaturating dissociation buffer) to dissociate any non-covalently bound proteins. Lysates were diluted 1:10 in RIPA buffer supplemented by 2 $\times$ protease inhibitor mixture (Sigma). RIP-Flag and TRAF2-Flag were immunoprecipitated as described above and incubated for 1 h at 37°C in a de-ubiquitination buffer (Tris-HCl 50 mM pH 7.5, NaCl 150 mM, EDTA 2 mM pH 8.00, DTT 2 mM) in the presence or absence of USP2 (Enzo Life Sciences) and 10 nM of NEM (Sigma). Samples were subsequently prepared for western blot analysis and probed with the indicated antibodies.

***In vivo* de-ubiquitination assay.** USP2a or the catalytically inactive mutant USP2a C276A and plasmids coding for HA-ubiquitin (WT), HA-ubiquitin (K48) or HA-ubiquitin (K63) were transfected in 293T. At 24 h after transfection, cells were treated with MG132 (10 μM) for 4 h or with TNF (20 ng/ml) for 15 min. Cells were harvested, lysed and boiled 5 min in 1% SDS (v/v) to remove all non-covalently associated proteins. Lysates were diluted 1:10 in RIPA buffer supplemented by 2 $\times$ protease inhibitor mixture (Sigma). TRAF2 or RIP1 were immunoprecipitated using an anti-RIP1 or an anti-TRAF2 antibody as described above. Proteins bound to the beads were then eluted in Laemmli buffer and subjected to immunoblot analysis.

siRNA sc cells or siRNA USP2a cells were harvested, lysed and boiled 5 min in 1% SDS (v/v) (denaturating dissociation buffer) to dissociate any non-covalently bound proteins. Lysates were diluted 1:10 in RIPA buffer supplemented by 2 $\times$ protease inhibitor mixture (Sigma). Endogenous TRAF2 or RIP1 were immunoprecipitated using an anti-RIP1 or an anti-TRAF2 antibody as described above. Proteins bound to the beads were then eluted in Laemmli buffer and subjected to immunoblot analysis.

USP2a downregulation by shRNA in MCF7 cells. The USP2a shRNA was sub-cloned downstream of the human H1 promoter in the pSuper vector (Oligoengine, Seattle, WA, USA). MCF7 cells were transfected by USP2a or scrambled shRNA/pSuper by effectene. The sequences of the synthetic oligonucleotides (Invitrogen) used for USP2a shRNA construct were the following: 5′-GATCCCCGCCGGTCCCCACCAGCAGCTTCAAGAGAGCTGCTGGTGGGGACCGGCTTTTTGGAAA-3′ and 5′-AGCTTTTCCAAAAAGCCGGTCCCCACCAGCAGCTCTCTTGAAGCTGCTGGTGGGGACCGGCGGG-3′.

The sequences of the synthetic oligonucleotides used for scrambled shRNA construct were 5′-GATCCCCGCCCTCGCACACGGCACCGTTCAAGAGACGGTGCCGTGTGCGAGGGCTTTTTGGAAA-3′ and 5′-AGCTTTTCCAAAAAGCCCTCGCACACGGCACCGTCTCTTGAACGGTGCCGTGTGCGAGGGCGGG-3′.

The annealed oligonucleotides were ligated into the *Bgl*II–*Hin*dIII cleavage site of the pSuperGFP vector linearised with the same restriction enzymes. MCF7 cells were transfected by USP2a or scrambled shRNA/pSuper by effectene. At 48 h after transfection, the downregulation of USP2a was checked by western blot for the protein expression level or by semi-quantitative RT-PCR for the mRNA expression level.

USP2a downregulation and NF-κB activity assay in 293T cells. The following siRNA were purchased from HP Guaranteed siRNA service (Qiagen). The siRNAs used to downregulate USP2a were: 5′-ACCCAGAAGCTGGACAGCCAA-3′; 5′-AAGAGACGGCATGAATTCTAA-3′; 5′-CACGCTGTGGGAGACGGGAAA-3′; 5′-CCCTGAATACCTGGTCGACTA-3′ and the control siRNA was: 5′-UGAACACCACCAGGGAUCUTT-3′.

293T cells were transfected with siRNAs using Effectene. At 24 h after transfection, cells were split and seeded in a 24-well plate. The following day, 293T cells were transfected by the reporter plasmid pNFκB-hrGFP. 24 h after the second

transfection, 293T cells were treated for 4 h with TNF (20 ng/ml). Then, cells were washed and collected for FACS analysis at different time points over 24 h.

Detection of phosphorylated proteins. 293T cells downregulated for USP2a or scramble using siRNA (see above) were treated by TNF (20 ng/ml). Cells were lysed in MLB buffer (0.5% NP-40, 100 mM NaCl, 50 mM Tris-Cl pH 7.5, 50 mM NaF, 1 mM Na_3Vo_4, 30 mM napyrophosphate, 0.5 mM PMFS, 10 μM chymostatin, 0.002 mg/ml leupeptin, 0.002 mg/ml antipain and 2 $\times$ proteases inhibitor mixture; Sigma). After 45 min on ice, cell lysates were cleared by centrifugation at 4°C for 45 min at 20 000 r.p.m. Proteins were separated in a 10% SDS-PAGE and transferred onto a PVDF membrane (Millipore). Membranes were blocked with 5% BSA in TBS containing 0.1% Tween for 2 h and incubated with the appropriate antibodies. Specific signals were revealed by the ECL detection reagent (Pierce).

Statistical analysis. Statistical analysis was performed using the unpaired Student's *t*-test. Data were regarded as statistically significant if $P < 0.05$ based on Student's *t*-test.

Conflict of Interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)