SUPPLEMENARY INFORMATION

Deubiquitinating protease USP2a targets RIP1 and TRAF2 to mediate cell death by TNF

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Supplementary Experimental Procedures

Reagents and antibodies.

Human recombinant TWEAK was purchased from R&D systems (1090TW/CF). Mouse monoclonal IKKγ/NEMO (72C627) and mouse monoclonal CYLD (E-10) were purchased from Santa-Cruz (UK). Rabbit polyclonal A20 and CYLD antibodies were from Cell Signalling (UK).

Quantitative real-time RT-PCR. 293T were left untreated or treated with TNF (20 ng/ml) for various time points. Total RNA was isolated using RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. 3 µg of RNA was used to synthesize cDNA by SuperScriptase III (Invitrogen) with oligo (dT)20 primers (Invitrogen). To detect USP2a, CYLD, A20, IκB-α, TNF, mRNAs, we used the SYBR green method (Invitrogen). The following primers were used:

Gene	Forward sequence (5'-3')	Reverse sequence (5'-3')
A20	AATGCGGCACCCTTGGAA	CGCTGGCTCGATCTCAGTTG
CYLD	GATCGGGATGGTGGTCAGA	GCACAGCCTTGGATTCTCCT
IκB-α	CCTGGTGTCACTCCTGTT	GTGAGCTGGTAGGGAGAATA

PPIA	CTGCACTGCCAAGACTGA	GCCATTCCTGGACCCAAA
TNF	AACCTCCTCTCTGCCATCAA	GGAAGACCCCTCCCAGATAG
Tubulin	GCCAAGCGTGCCTTTGTTC	CACACCAACCTCCTCATAATCC
USP2a	TCGGCCCGCTACACAGATG	GGACCGGCTTGAAACCAA

USP2a, CYLD, A20, IκB-α, TNF mRNA levels were normalized to tubulin and PPIA mRNA.

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, UK) used for the USP2a shRNA construct were the following:

5'GATCCCCGCCGGTCCCCACCAGCAGCTTCAAGAGAGCTGCTGGTGGGGACC GGCTTTTTGGAAA-3' and 5'-AGCTTTTCCAAAAAGCCGGTCCCCACCAGCAGC TCTCTTGAAGCTGCTGGTGGGGACCGGCGGG-3'

The sequences of the synthetic oligonucleotides used for scrambled shRNA construct were:

5'-GATCCCCGCCCTCGCACACGGCACCGTTCAAGAGA CGGTGCCGTGTGCGAGGGCTTTTTGGAAA-3' and 5'-AGCTTTTCCAAAAA GCCCTCGCACACGGCACCG TCTCTTGAA CGGTGCCGTGTGCGAGGGC GGG-3'

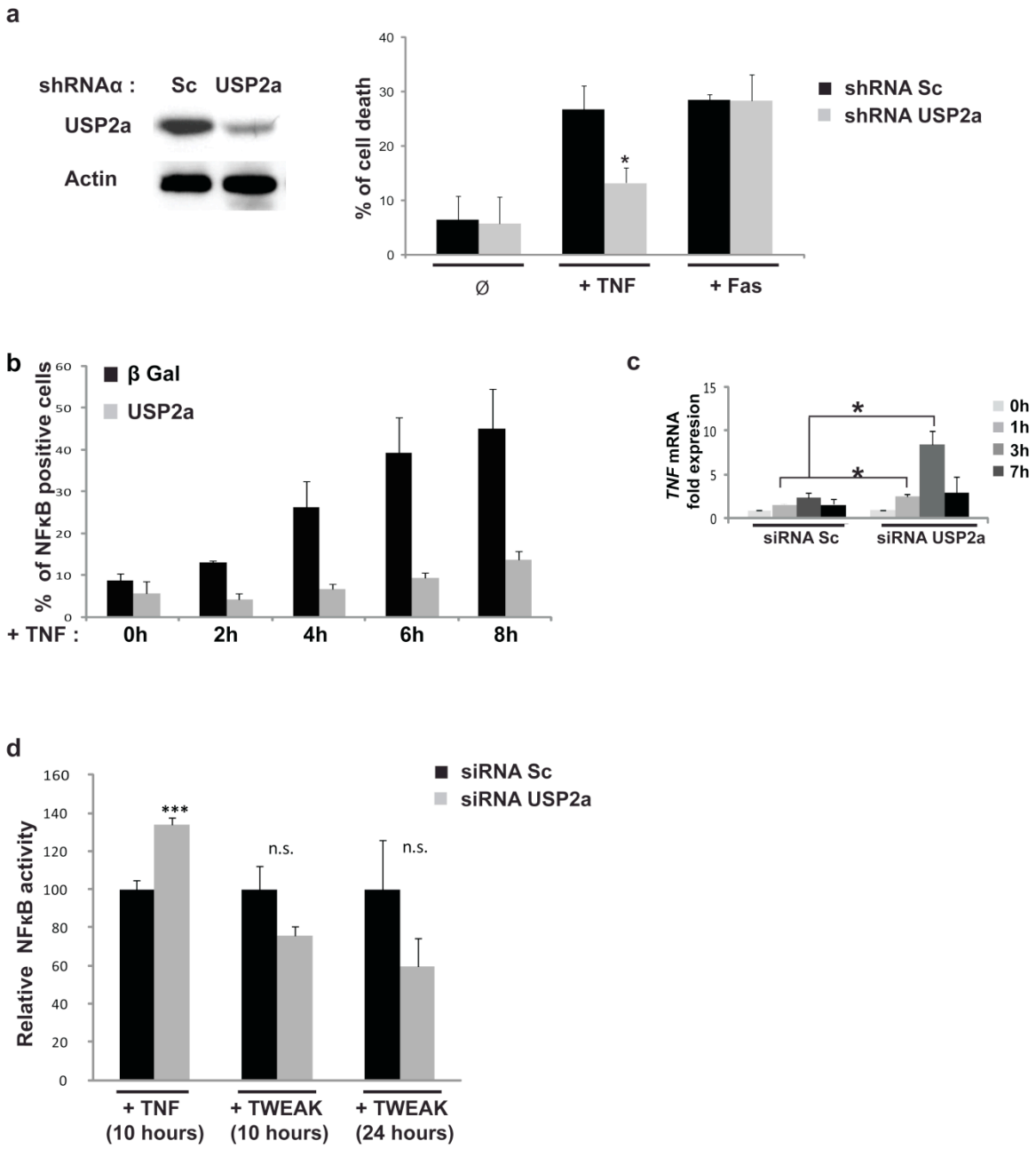
The annealed oligonucleotides were ligated into the BglII-HindIII cleavage site of the pSuperGFP vector, which was linearised with the same restriction enzymes. MCF7 cells were transfected by USP2a or scrambled shRNA/pSuper by Effectene. 48

hours post-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 HeLa cells.

Hela cells cells were transfected with siRNA against USP2a or with a control scrambled siRNA construct (sc). 48 hours post transfection, cells were transfected with a reporter plasmid for NF-κB, pNF-κB-hrGFP. 24 hours later the cells were treated with TNF (40 ng/ml) or with human recombinant Tweak (2μg/ml); for the TNF treatment, cells were washed after 4hrs. The activity of the reporter plasmid was quantified by FACS for the GFP fluorescence at the indicated time points.

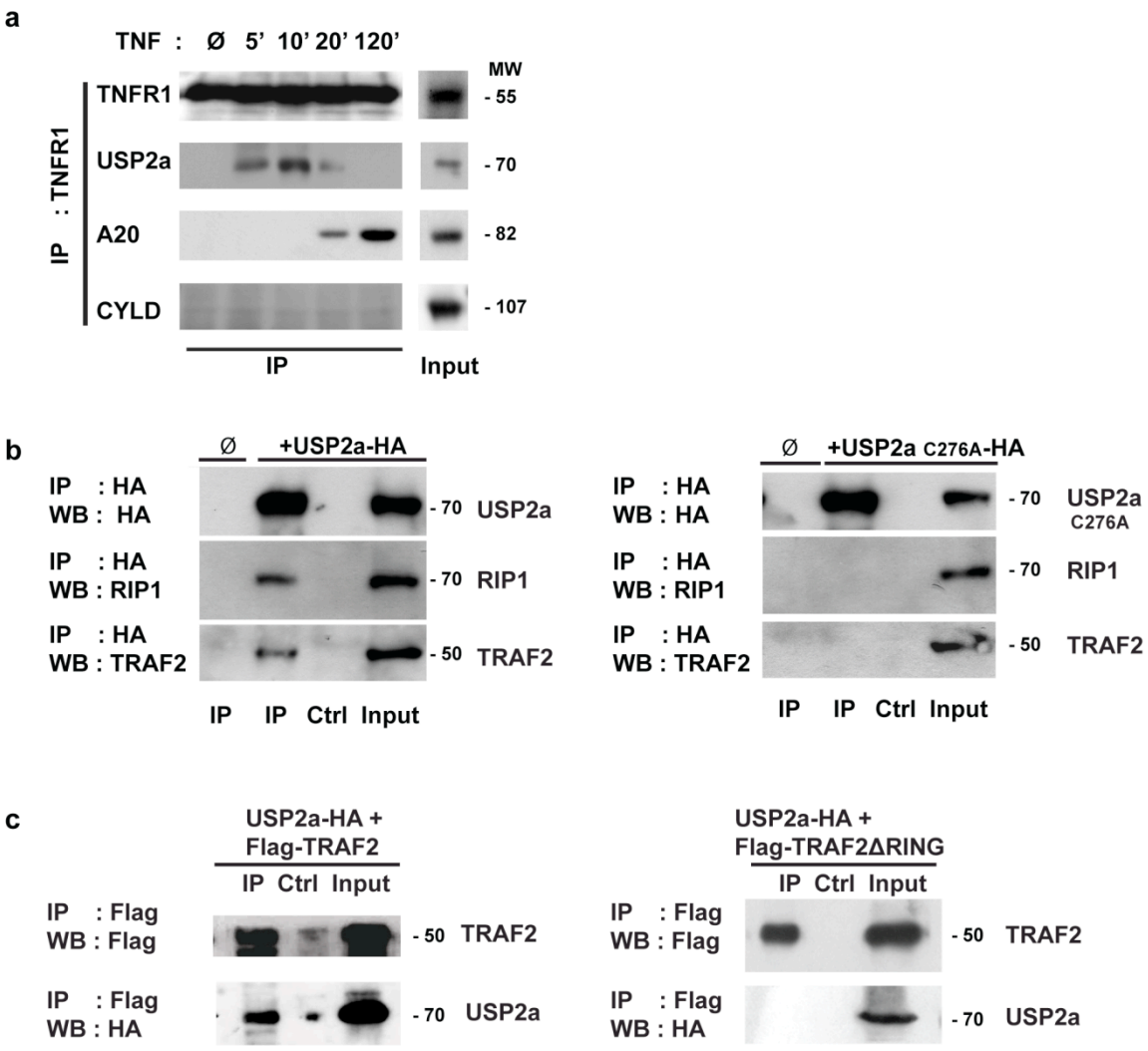
Supplementary Figure 1



Supplementary figure 1. Effects of USP2a expression levels on the TNF response. (**a**) MCF7 cells were transfected with a shRNA construct against USP2a or with a control scrambled shRNA. The downregulation of the USP2a protein was confirmed by Western blot (left panels). The cells were left untreated or the indicated reagents were added. Apoptosis was measured 24 hours later using FACS analysis for sub-G1 cells. (**b**) Overexpression of USP2a inhibits NF-κB activation upon TNF-α

treatment. 293T cells were co-transfected with USP2a and a NF-κB reporter plasmid or with β-Galactosidase and the NF-κB reporter plasmid as negative control. Cells were treated with zVAD to inhibit cell death induced by USP2a. After 24 hours, TNF (20 ng/ml) was added and GFP readings, reflecting NF-κB activation, were taken by FACS at 0hr, 2hrs, 4hrs, 6hrs and 8hrs post treatment. (**c**) Effect of TNF on the transcriptional upregulation of the NF-κB target gene TNF. 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 TNF at the indicated time points after addition of TNF and the results presented as the fold induction in comparison to the respective levels in untreated cells. Means and standard deviation of triplicates are shown from one representative experiment. Bars are means +/- SD. *t-test, $p<0.05$. (d) USP2a does not affect TWEAK signalling. Hela cells were transfected with siRNA against USP2a or with a control scrambled siRNA construct (sc). 48 hours post transfection, cells were transfected with a reporter plasmid pNF-κB-hrGFP. 24 hours later the cells were treated with TNF (40 ng/ml) or with human recombinant TWEAK (2μg/ml) for the time indicated. The activity of the reporter plasmid was quantified by FACS for the GFP fluorescence at the indicated time points. Bars represent means +/- SD of a representative experiment performed in triplicate; *** t-test $p<0.005$; n.s. non significant $p>0.05$.

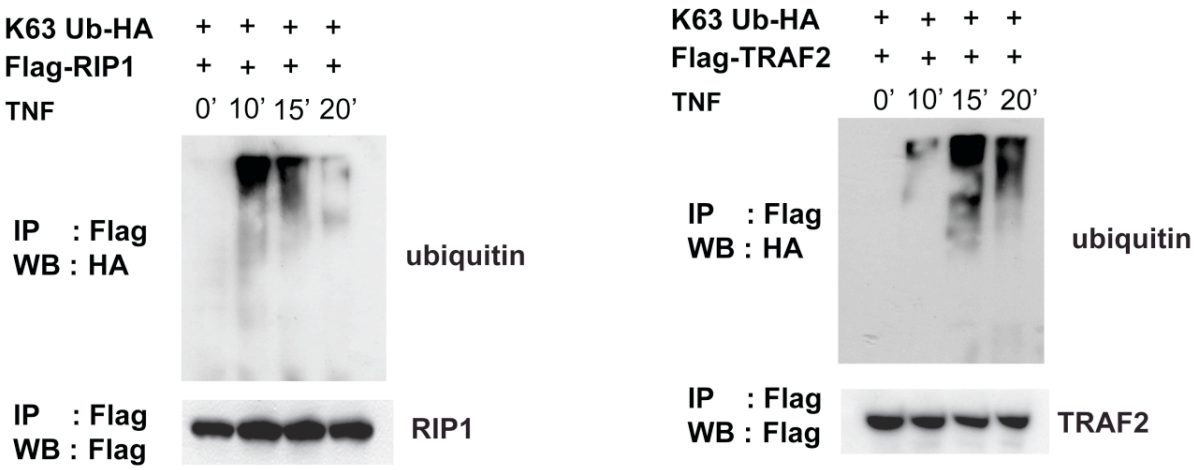
Supplementary Figure 2



Supplementary figure 2. Interaction of USP2a with components of the TNFR1 complex. (**a**) USP2a is recruited to the TNFR1 complex before A20 and CYLD. MCF7 were treated with TNF (20 ng/ml) for 5, 10, 20 or 120 minutes after which immunoprecipitations against the endogenous TNFR1 were performed and the co-precipitated proteins detected on a Western blot as indicated. (**b**) USP2a co-immunoprecipitates with endogenous RIP1 and TRAF2. MCF7 cells were transfected with plasmids coding WT USP2a-HA (left panels) or USP2a C276A-HA (right panels). Total cell lysates were left untreated (Ctrl, Input) or immunoprecipitated with an anti-HA antibody and blots probed with the indicated

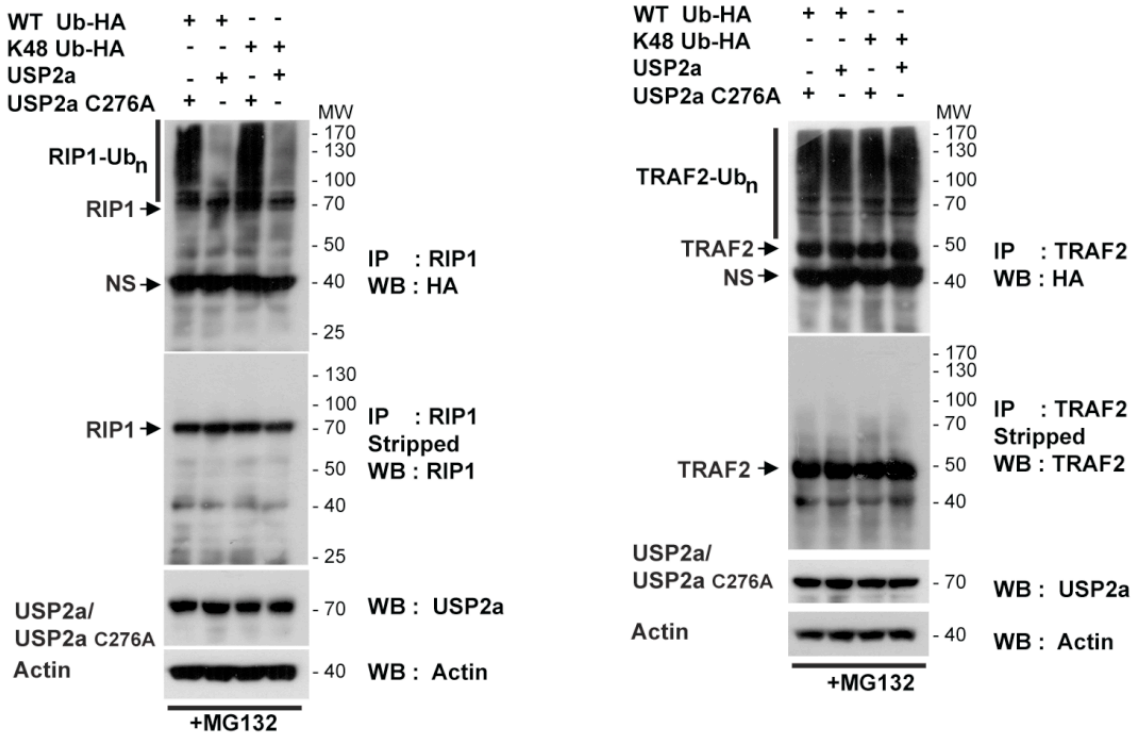
antibodies. (**c**) USP2a immunoprecipitates with TRAF2 via its RING domain. MCF7 cells were co-transfected with plasmids coding for Flag-TRAF2 or Flag-TRAF2ΔRING and USP2a-HA. Total cell lysates were immunoprecipitated with an anti-Flag antibody and blots probed with the indicated antibodies (lower panels).

Supplementary Figure 3



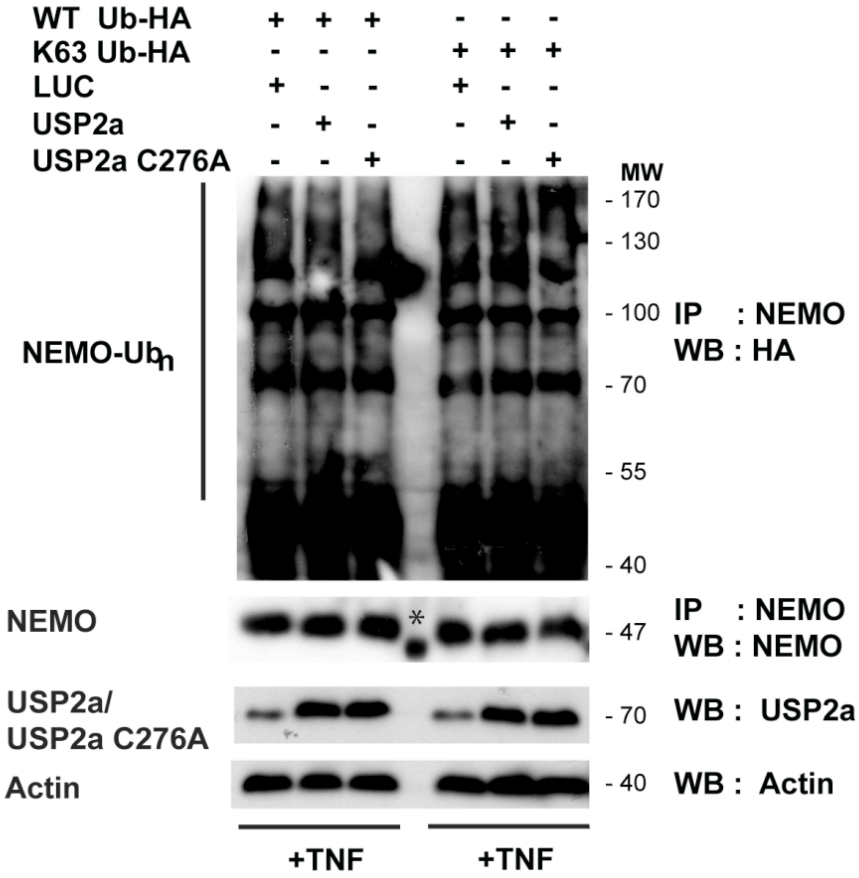
Supplementary figure 3. Setting up conditions to induce RIP1 and TRAF2 ubiquitination. The indicated expression constructs were transfected into MCF7 cells and TNF was added over a time course. An immunoprecipitation against the Flag-tagged protein was performed, and the presence of ubiquitin on the respective protein assessed by a protein blot with a suitable antibody against ubiquitin.

Supplementary Figure 4



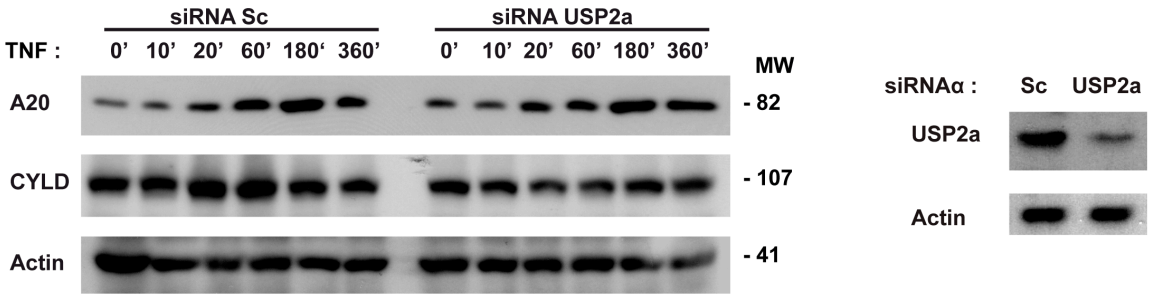
Supplementary figure 4. USP2a is able to disassemble K48 ubiquitin chains from endogenous RIP1 but not from TRAF2. 293T cells were transfected with expression plasmids for USP2a or its catalytically inactive mutant C276A together with a plasmid coding for WT ubiquitin-HA or K48 ubiquitin-HA. 24 hours post-transfection, cells were treated with MG132 for 4 hours, lysed in SDS 1% and then diluted in the dissociation buffer. Endogenous RIP1 and TRAF2 were pulled-down and blots probed with the indicated antibodies.

Supplementary Figure 5



Supplementary figure 5. USP2a is not able to disassemble K63 ubiquitin chains from endogenous NEMO/IKKγ. 293T cells were transfected with the expression plasmids for the negative control Luciferase (LUC), USP2a or its catalytically inactive mutant C276A together with a plasmid coding for WT ubiquitin-HA or K63 ubiquitin-HA mutants. 24 hours post-transfection, cells were treated with TNF (20ng/ml) for 15 minutes. Cells were lysed in SDS 1% and then diluted in the dissociation buffer. Endogenous NEMO was pulled-down and blots probed with the indicated antibodies. * Non specific.

Supplementary Figure 6



Supplementary figure 6. A20 and CYLD stability in USP2a KD cells. 293T cells were transfected with siRNA against USP2a or with a control scrambled siRNA construct (sc) (right-hand blots). 48 hours post transfection, the stability of A20 and CYLD was monitored at different time points in a Western blot after TNF addition to both control-transfected cells as well as cells transfected with siRNA against USP2a. Actin was used as a loading control (left-hand blots).