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Supplemental Information

Direct, Noncatalytic Mechanism of IKK Inhibition by A20

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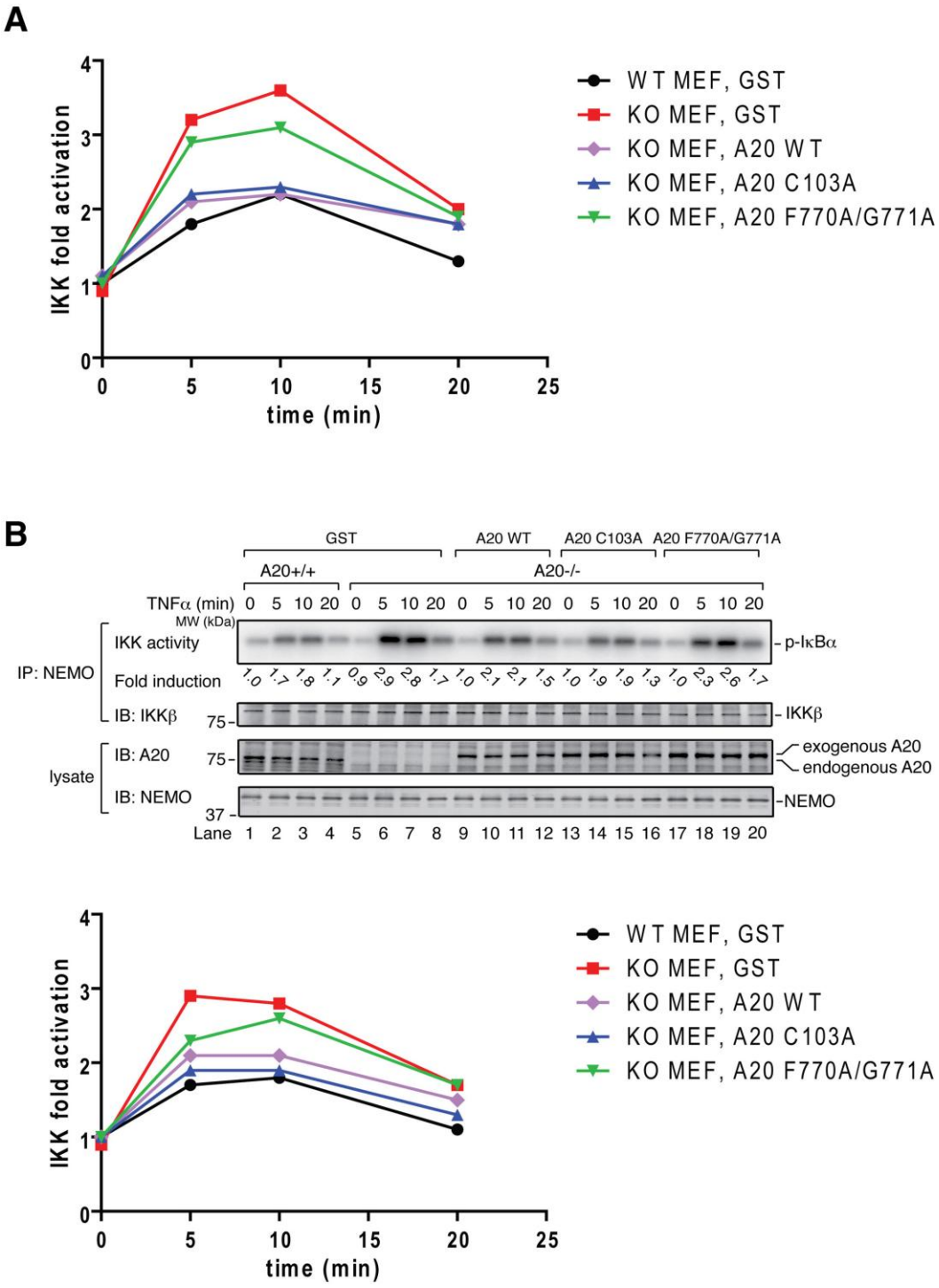

Figure S1. IKK Suppression in MEFs by A20 Does Not Require Cys 103, but Requires ZnF7-Dependent Polyubiquitin Binding

(A) Quantification of IKK activation by IL-1β based on data shown in Figure 2G of main text.

(B) Experiment was performed as in Figure 2G except that TNFα was used to stimulate cells. Graphs are representative of data from two independent experiments. This figure is related to Figure 2.

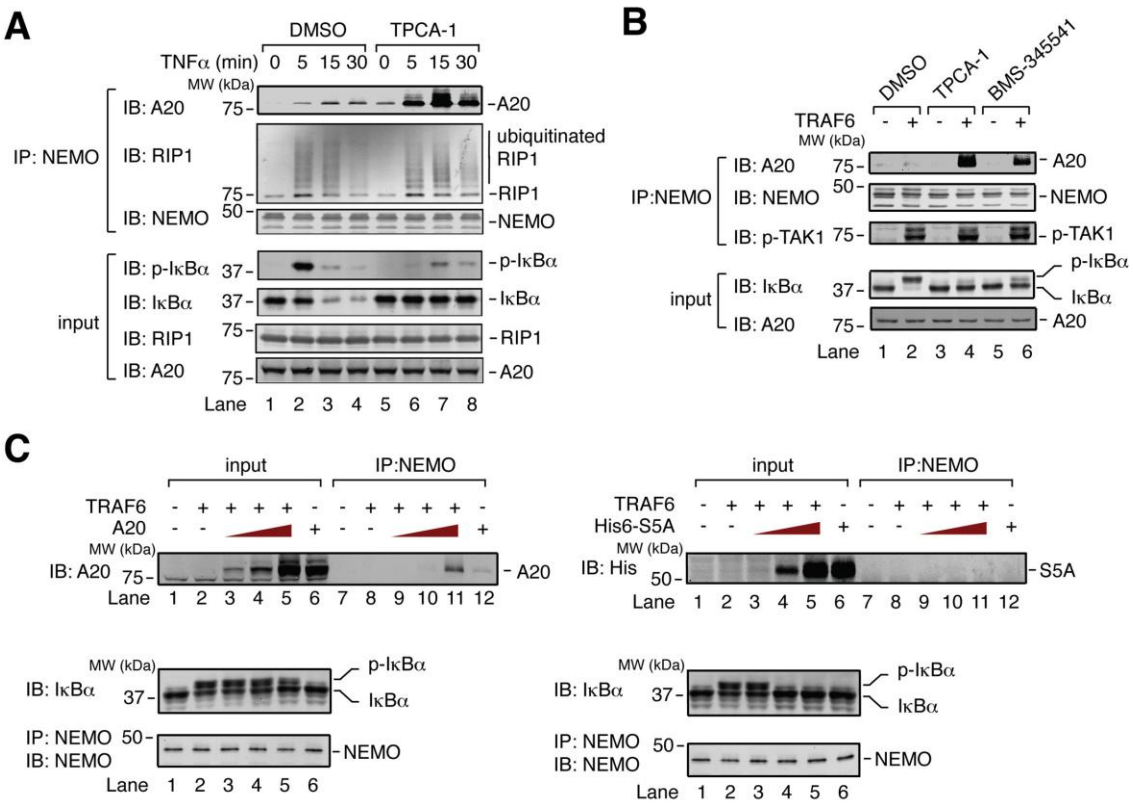

Figure S2. Regulation and Specificity of NEMO-A20 Interaction

(A) HEK293 cells were treated with TPCA-1 (10 μM) or an equivalent volume of DMSO for 3 hr. Cells were then treated with TNFα for the indicated time points, and a NEMO antibody was used for immunoprecipitation. Cell lysate and immunoprecipitate were immunoblotted with the indicated antibodies.

(B) TPCA-1 (10 μM final), BMS-345541 (100 μM final), or an equivalent volume of DMSO was added to S100 supplemented with ATP, -/+ TRAF6 as indicated. After incubation for 1 hr, a NEMO antibody was used for immunoprecipitation. S100 and immunoprecipitate were immunoblotted with the indicated antibodies.

(C) Flag-A20 (left panel) or His_6-S5A (right panel) was added to S100, followed by His_6-TRAF6, then incubated for 1 hr. 5% of the S100 was immunoblotted with the indicated antibodies. The remaining S100 was used for immunoprecipitation with a NEMO antibody. As a control for detection sensitivity, input and immunoprecipitate were run on the same gel. The failure of TRAF6 to induce IκBα phosphorylation in the presence of high S5A concentrations likely indicates that S5A bound to the polyubiquitin chains synthesized by TRAF6, thereby competing with NEMO for polyubiquitin binding. Related to Figure 4.

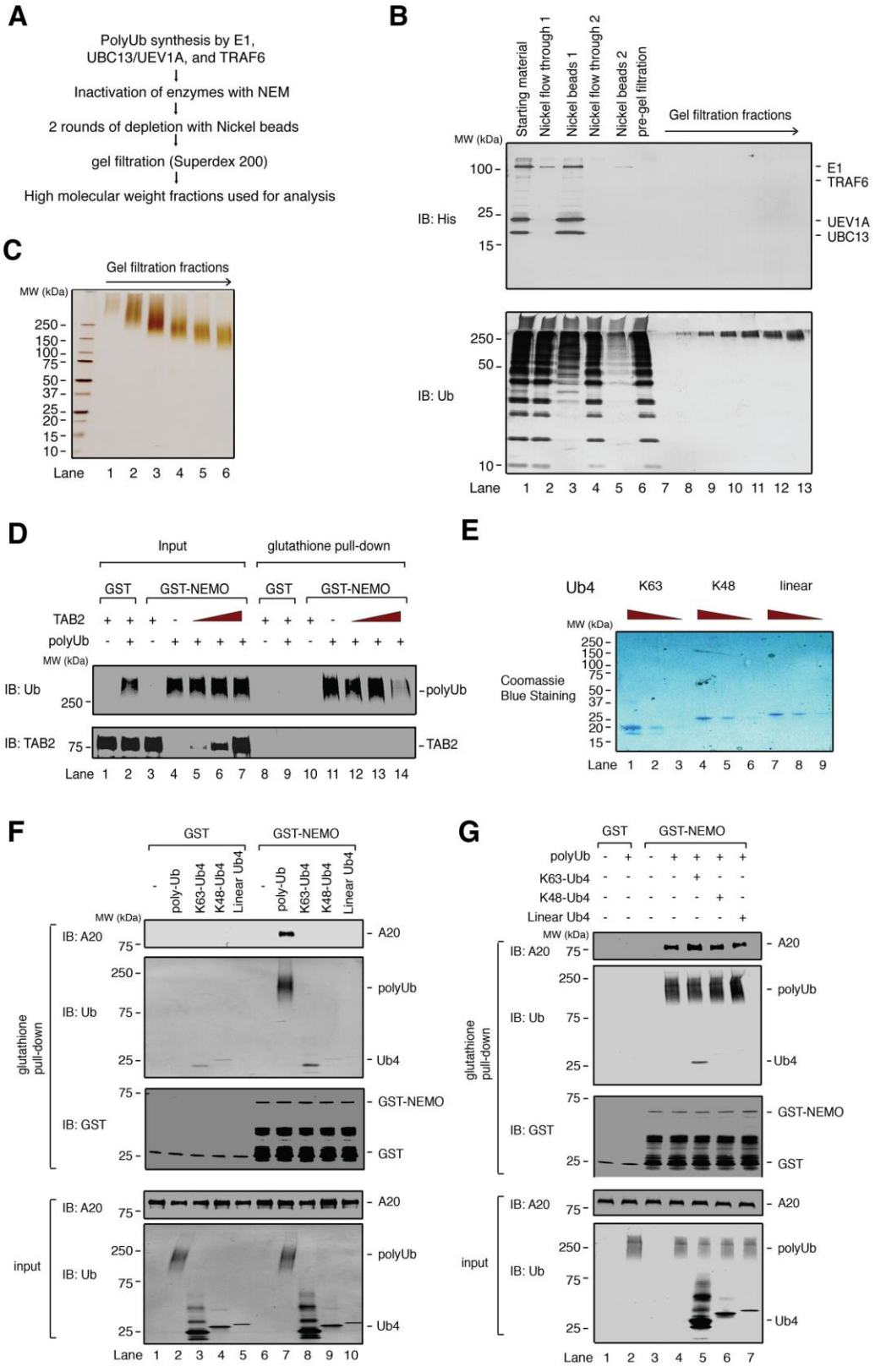

Figure S3. Polyubiquitin Chains Induce NEMO-A20 Interaction

(A) Flow chart summarizing method for synthesis and purification of polyubiquitin chains.
(B) An aliquot from each step of the polyUb purification was analyzed by immunoblotting with the indicated antibodies.
(C) High molecular weight fractions from gel filtration were analyzed by silver staining.
(D) Recombinant GST or GST-NEMO was incubated -/+ polyubiquitin chains, -/+ His_6-TAB2 from Sf9 cells. 10% of the input, and the glutathione pull-down, were run on the same SDS-PAGE and immunoblotted with the indicated antibodies.
(E) Coomassie stain of K63-, K48-, or linear polyUb.
(F) GST or GST-NEMO was incubated with A20, -/+ polyUb of various lengths and linkages as indicated. Samples were then incubated with glutathione Sepharose. 10% of input and glutathione pull-down were immunoblotted with the indicated antibodies.
(G) As in (F), except in lanes 5-7 Ub4 was added in addition to polyUb synthesized by TRAF6 and Ubc13/Uev1A. Related to Figure 5.

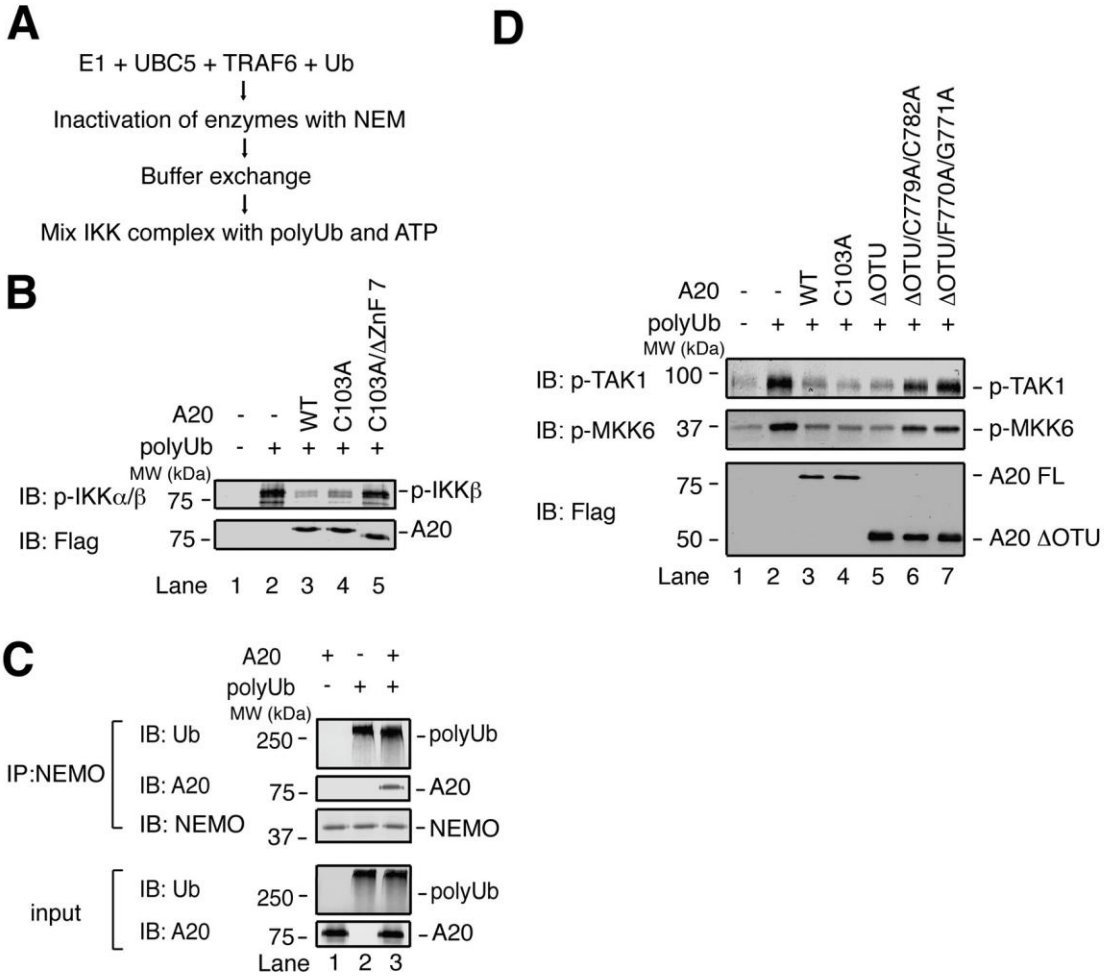

Figure S4. A20 Can Directly and Noncatalytically Impair IKK and TAK1 Autophosphorylation

(A) Method to generate polyUb that can directly induce IKK autophosphorylation.

(B) IKK complex was incubated with ATP, -/+ polyubiquitin chains, -/+ A20 WT, C103A, or C103A/ΔZnF7. Samples were immunoblotted with the indicated antibodies.

(C) IKK complex was incubated with or without affinity-purified A20, with or without polyUb. After 15 min, 10% of input was withdrawn, and the remaining mixture was used for immunoprecipitation with an antibody against NEMO. Input and immunoprecipitate were immunoblotted with the indicated antibodies. Related to Figure 6.

(D) TAK1 complex was incubated with MKK6 (K82A) and ATP, -/+ polyubiquitin chains that had been synthesized by TRAF6 and UBC13/UEV1A, -/+ A20 WT or mutant as indicated. The reaction mixtures were immunoblotted with the indicated antibodies.

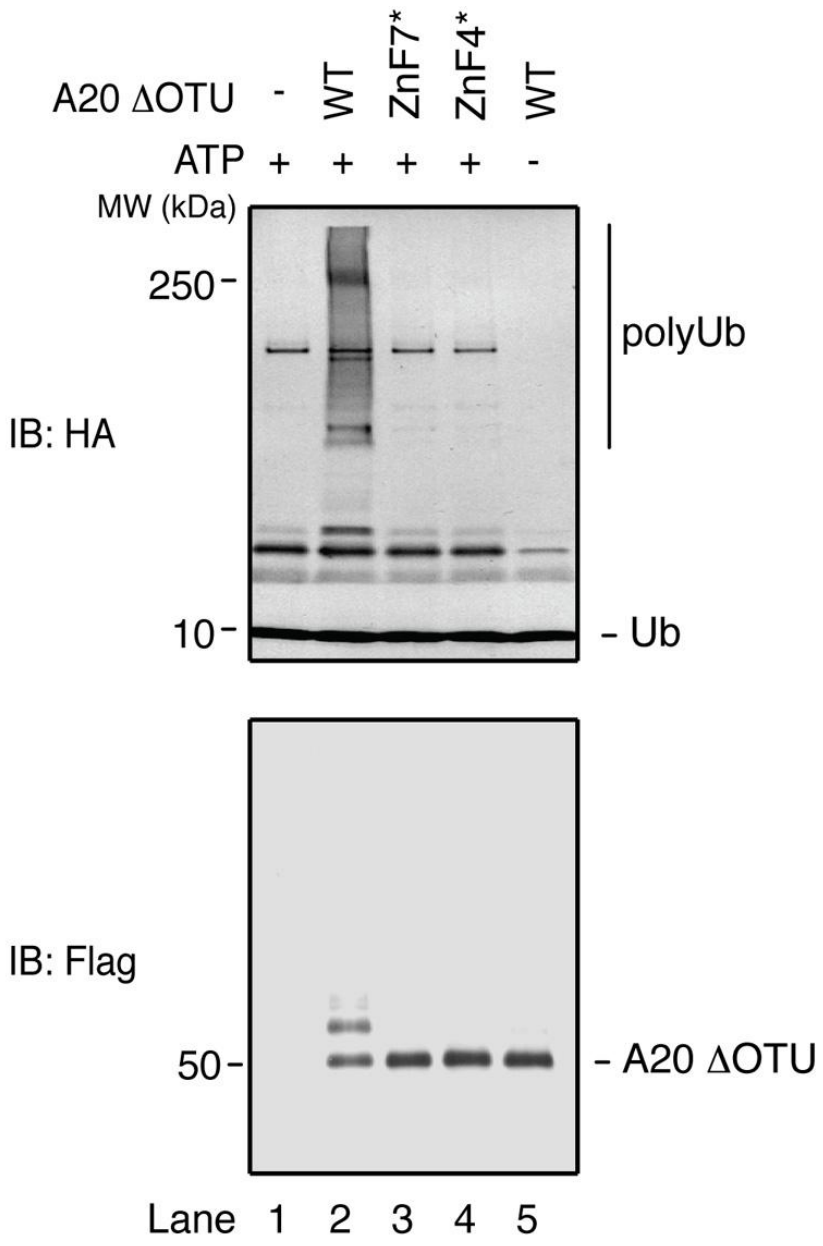

Figure S5. A20 ZnF7 and ZnF4 PolyUb Binding Mutants Have Impaired E3 Ligase Activity

HA-ubiquitin, E1, UBC5, and A20 ΔOTU proteins were incubated together for 30 min at 30°C. The A20 proteins include WT, F770A/G771A (ZnF7*), and Y614A/F615A (ZnF4*). Samples were immunoblotted with the indicated antibodies.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Plasmids

Expression plasmids for E1, UBC13, UEV1A, UBC5, TRAF6, TAB2, MKK6 (K82A), MEKK1, and TAK1 were described previously (Deng et al., 2000; Lee et al., 1997; Xia et al., 2009). N-terminally Flag-tagged A20 for expression in HEK293 cells was constructed in pcDNA3.1 or pEAK. N-terminally TAP-tagged, C-terminally Flag-tagged A20 was constructed in pEF-IRES-P. Deletion and point mutagenesis were performed by PCR using standard methods. All constructs were verified by DNA sequencing. GST-NEMO ΔN was constructed in Gateway pDEST 15 (Invitrogen). The bacterial expression plasmid for the viral OTU was provided by A. Garcia-Sastre (Frias-Staheli et al., 2007).

Cell Culture, Antibodies, Reagents

HEK293 cells were maintained in DMEM (Cellgro) supplemented with 10% calf serum (Hyclone) and antibiotics (penicillin G [100 mg/ml) and streptomycin [10 mg/ml]). MEF cells and HeLa cells were maintained in DMEM supplemented with 10% fetal bovine serum (Hyclone) and antibiotics. U2OS cells were maintained in DMEM supplemented with 10% tetracycline-free fetal bovine serum (Hyclone) and antibiotics.

A20-overexpression cell line was prepared by infecting HEK293 cells with retrovirus expressing Flag-A20 and selecting with puromycin (1 mg/ml). Single colonies were scaled up and tested for A20 expression by immunoblotting.

Antibodies used in this study and their sources and catalog numbers are as follows: Santa Cruz Biotech: IκBα (371) Ub (8017), NEMO (8330), IKKα/β (7607), p-

MKK6 (7994); Imgenex: A20 (161A); Cell Signaling: IκBα (4814), phospho-IκBα (9246), p-IKKα/β (2078), p-TAK1 (4536), TNF-R1 (3736); BD Bioscience: IKKβ (611254), RIP1 (610458), NEMO (557383), TRADD (610572); Zymed: UBC13 (37-1100); Covance: GST (MMS-112P), HA (MMS-101P); Sigma: Flag (F3165 and F4042); Qiagen: penta-His (34660). For detection of mouse A20, a homemade antibody was used (Boone et al., 2004).

Other commonly used reagents: M2 (anti-Flag) sepharose (Sigma A2220), A/G sepharose (Pierce #53133), Glutathione Sepharose 4B (GE Healthcare), Ni-NTA agarose (Qiagen). TPCA-1 and BMS-345541 were purchased from Sigma and dissolved in DMSO.

Protein Expression and Purification

GST and GST-tagged NEMO, NEMO ΔN, IκBα 1-54 (NT), TNFα, IL-1β, and viral OTU (CCHFV-L [1-169]) were expressed in E. Coli BL21/pLys and purified using Glutathione Sepharose 4B. GST tag was removed from IL-1β and viral OTU by thrombin. E1, TRAF6, and TAB2 were expressed in Sf9 cells as His_6-tagged proteins and purified using Ni-NTA agarose. UBC13, UEV1A, UBC5, MKK6 (K82A), and TEV protease were expressed in E. Coli BL21/pLys as His_6-tagged proteins and purified using Ni-NTA agarose.

To purify the IKK complex, $NEMO^{-/-}$ MEF cells and $NEMO^{-/-}$ MEF cells reconstituted with Flag-NEMO were harvested in Buffer C. 20,000 x g supernatants were mixed at a 10:1 ratio for 30 min, then mixed with M2 agarose overnight. Beads

were rinsed with Buffer C, then Buffer F. IKK complex containing Flag-NEMO was eluted with 0.2 mg/ml Flag peptide.

Isolation of TNF Receptor Complex

For "0" timepoint, A20-overexpression or parental cells were cooled to 4°C, then treated with GST-TNFα for 10 min. For 5' and 15' timepoints, cells that were not cooled were treated with GST-TNFα as indicated. Cells were harvested in Buffer A, then centrifuged at 20,000 x g for 15 min. Supernatant was mixed with glutathione sepharose 4B. Beads were washed with Buffer A, then analyzed by immunoblotting as indicated.

Mass Spectrometry

Identification of proteins (Figure 2A of the main text) by mass spectrometry was performed as described previously (Chiu et al., 2009).

Depletion of UBC13 from S100

His_6-tagged UEV1A was coupled to NHS-activated sepharose 4 Fast Flow (GE Healthcare) according to manufacturer's instructions. Sepharose was incubated with HeLa S100 (~1 mg of UEV1A per 3 mg total protein) three times, rotating end-over-end at 4°C for 4 hr or overnight. The third flow-through was used for assays.

Rescue of A20 Function in $A20^{-/-}$ MEFs with Retrovirus

Constructs and protocol for preparation of retrovirus to express A20 were described previously (He and Ting, 2002). A20 mutants were subcloned into retroviral

expression vector by PCR using standard methods. $A20^{+/+}$ and $A20^{-/-}$ MEFs were prepared from the wild-type and mutant mice and immortalized with SV40 large T antigen. These cells were infected with retrovirus in the presence of polybrene (1.7 mg/ml final). 6 hr later, virus media was replaced with fresh media. 24 hr later (30 hr after infection), cells were stimulated as indicated and harvested. 20,000 x g supernatant was analyzed for A20 expression using a polyclonal antibody that can recognize both human and mouse A20 (Boone et al., 2004). Remaining supernatant was used for NEMO immunoprecipitation and IKK assay. Kinase activity was quantitated using ImageQuant software (Molecular Dynamics).

Generation of Stable Cell Lines Using Lentivirus

Coding sequence for GFP or A20 was inserted downstream of the puromycin-resistance gene and foot-and-mouth disease virus 2A segment, which enables multicictronic expression of transgenes in mammalian cells using a single promoter. Virus was prepared as described in (He et al., 2011). $A20^{+/+}$ or $A20^{-/-}$ MEFs were infected with lentivirus in the presence of polybrene (1.7 mg/ml final). 24 hr later, puromycin was added to 1 mg/ml final. After selection, pools of cells with stable expression were used for further analysis.

Generation of Constitutively Active TAK1 (TAK1-ca)

TAK1 complex (~1 nM) was mixed with ubiquitination enzymes as described (Xia et al., 2009), for 30 min. K63R ubiquitin (Boston Biochem) was added to 25 μM, and viral OTU was added to ~3 μM, and reaction was incubated at room temperature for

30 min. Reaction was diluted 1:2 in Buffer D (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM DTT, 0.5% NP-40), and anti-Flag (M2) agarose beads were added. Sample was rotated end-over-end overnight at 4°C. Beads were rinsed in Buffer D, then Buffer E (20 mM Tris-HCl [pH 7.5], 50 mM NaCl, 1 mM DTT, 0.05% NP-40, 5% glycerol). TAK1 complex was eluted with 0.2 mg/ml Flag peptide.

Protein Phosphatase and Deubiquitination Treatment of IKK Complex

HEK293 cells were unstimulated or stimulated for 10 min with GST-TNFα. Cells were harvested in Buffer A, and 20,000 x g supernatant was incubated with anti-NEMO antibody and Protein A/G beads. Beads were washed in Buffer A, then with Buffer H (20 mM Tris-HCl [pH 7.5], 1 mM DTT, 0.05% NP-40). For deubiquitination treatment, beads were re-suspended in 15 μl of Buffer I (20 mM Tris-HCl [pH 7.5], 0.5 mM DTT, 0.2 mg/ml BSA) and viral OTU (~ 30 μM), then incubated at 30°C for 30 min. For phosphatase treatment, buffer I was removed and replaced with Lambda protein phosphatase buffer (New England Biolabs) and 400 U of lambda phosphatase. After 10 min at 30°C, phosphatase buffer was removed, beads were rinsed with Buffer A, then with Buffer B. Kinase assay was then performed as described above.

SUPPLEMENTAL REFERENCES

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