Online Methods

Plasmids and Cell Lines

Expression plasmids for E1, ubiquitin, TRAF6, Ubc13, Uev1A, TAK1, TAB2 and MKK6(K82A) have been described previously\(^4,16\). N-terminal FLAG-tagged IsoT, HOIP, HOIL-1L and TRIM25 were constructed in pcDNA3.1. TAK1(K63W), TAK1(T187A), TRAF6-7KR(K>R substitutions at K91, 96, 104, 124, 133, 137 and 142), TAB2(C670A/C673A), and IsoT(C335A) were generated using QuikChange® II XL kit (Stratagene). Bacterial expression plasmid for CCHFV viral OTU domain (L1-169) was provided by Dr. Adofo Garcia-Sastre (Mount Sinai School of Medicine). All constructs were verified by DNA sequencing. IRAK1-deficient HEK293/IL-1R (I1a) and its parental cell line were provided by Dr. Xiaoxia Li (Cleveland Clinics)\(^5\).

Protein Expression and Purification

Chromatography columns were from GE Healthcare unless otherwise indicated. E1 and TRAF6 were expressed in Sf9 cells as His\(_6\)-tagged proteins and purified using Ni-NTA agarose beads (QIAGEN). His\(_6\)-tagged Ubc13, Uev1A, MKK6 (K82A) and MBP-Ub-RIG-I(N) were expressed in E.coli BL21/pLys and affinity purified. GST-tagged NEMO and viral OTU(L1-169) were similarly expressed in BL21/pLys and affinity purified followed by removal of GST with thrombin. Ubiquitin and its lysine mutants (no affinity tag) were expressed in a modified \textit{E.coli} strain BL21(DE3)-pJY2\(^17\), and purified through sequential chromatography on HiTrap-Q, HiTrap-SP and Superdex-75. FLAG-tagged TRIM25, NEMO (WT and Y308S) were transiently expressed in HEK293 cells and purified using anti-FLAG (M2) agarose beads (Sigma). GST-CYLD and its mutant
(C601S) were expressed in Sf9 cells and purified by glutathione-Sepharose chromatography.

**Purification of the TAK1 complex**

The TAK1 kinase complex was purified from a HEK293T cell line stably expressing TAK1, in which the N-terminus of mouse TAK1 was fused to a tandem affinity tag consisting of protein A and calmodulin-binding peptide (CBP) separated by a Tev protease cleavage site, and the C-terminus was fused to a FLAG tag. The cells were grown in DMEM media supplemented with 10% FBS. Cell extracts were prepared in Buffer A [50mM Tris-Cl, pH7.5, 150mM NaCl, 10% glycerol, 0.5% NP-40, 1mM DTT, 1mM PMSF and protease inhibitor cocktail (Roche)]. The TAK1 complex, which contains endogenous TAB1 and TAB2, was purified using IgG-Sepharose and eluted by cleavage with Tev. The complex was further purified using anti-FLAG (M2) agarose beads and eluted with the FLAG peptide. The TAK1 complex containing wild type TAB2 (WT) or its C>A mutant (C670A/C673A) was purified from HEK293T cells stably expressing the FLAG-tagged TAB2 proteins using anti-FLAG (M2) agarose beads. TAK1(T187A) was transiently expressed in HEK293 cells and then purified using its N-terminal FLAG tag.

**Purification of the IKK complex**

Hela S100 was loaded onto a HiTRAP Q column and fractions containing the IKK activity were eluted with 200 mM to 300 mM NaCl. After precipitation with ammonium sulfate (15-30%) and dialysis, proteins were loaded onto a Heparin column. Fractions
eluted with 100 mM to 220 mM of NaCl were pooled and mixed with ATP-Sepharose beads\textsuperscript{18}. After washing with 1 M NaCl, bound materials were eluted with 100 mM ATP, and then loaded onto Superdex-200. Fractions containing IKK were used for assays.

For purification of endogenous IKK lacking NEMO, S100 from a NEMO-deficient murine pre-B cell line (1.3E2) was loaded onto a HiTRAP Q column and fractions containing IKK\(\alpha/\beta\) were eluted with 200 - 300 mM NaCl. After precipitation with ammonium sulfate (15-30%) and dialysis, proteins were loaded onto Superdex-200. Fractions containing IKK\(\alpha/\beta\) were used for assays.

**Polyubiquitin Chain Synthesis and Purification**

K63-linked polyUb chains were synthesized in a reaction mixture containing 0.2 \(\mu\)M E1, 1 \(\mu\)M Ubc13/Uev1A, 0.4 \(\mu\)M TRAF6 and 12.5 \(\mu\)M Ub in Buffer B (50 mM Tris-HCl (pH7.5), 2 mM ATP, 5 mM MgCl\(\text{2}\)). After incubation at 30°C for 1 hour, the reaction was terminated by EDTA (10 mM). DTT (5 mM) was then incubated with the reaction mixture at room temperature for 15 minutes to release Ub from E1 and E2, which were subsequently alkylated with 20 mM NEM (N-ethylmaleimide, Sigma) for 15 minutes at room temperature. Finally, NEM was quenched with excess DTT (10 mM) and the reaction mixture was exchanged to storage buffer (20 mM HEPES-KOH, pH7.4, 10% glycerol, 1 mM DTT, 0.4 mM PMSF) by repeated dilution and concentration using Amicon concentrators (Millipore). This mixture was incubated with nickel-agarose beads to deplete His\(6\)-tagged proteins and then polyUb chains were further purified by
Superdex-200 followed by Mono-S chromatography using the SMART purification system.

PolyUb chains synthesized by Ubc5 and TRAF6 were prepared as above except that Ubc5 (E2, 0.1 µM) and TRAF6 (0.4 µM) were used. To test polyubiquitination of TRAF6, SDS was added to the reaction mixture to a final concentration of 1% and the sample was boiled for 4 minutes. After a 10-fold dilution of SDS with Buffer C (50 mM Tris-Cl, pH7.5, 150 mM NaCl, 0.5% NP40), a TRAF6 antibody (0.2 µg, Santa Cruz Biotech) and protein A/G agarose beads (5 µl, Pierce) were added. The mixture was incubated for 2 hours at room temperature and spun at 1000 RPM to collect protein A/G beads. The beads were extensively washed with Buffer C plus 0.1% SDS, and then proteins were eluted with 1% SDS and analyzed by immunoblotting with a mouse antibody against TRAF6 or ubiquitin.

K48-linked polyUb chains were synthesized as above except that Ubc3 (E2, 1µM) and Skp1-Cul1-Roc1-βTrcP complex (E3, 0.4 µM) were used to replace Ubc13/Uev1A and TRAF6, respectively\(^\text{19}\). Linear polyUb chains were synthesized in reactions containing Ubc5 (0.2 µM), HOIP (1 µM) and HOIL-1L (1 µM). K63-linked Ub3 and Ub4 were synthesized as previously described\(^\text{20}\). Linear Ub2 and Ub3 were expressed as GST fusion proteins in E.coli (expression constructs kindly provided by Dr. Jonathan Ashwell\(^\text{8}\)). After affinity purification, the GST tag was removed by digestion with thrombin, followed by absorption on a second glutathione column. Linear Ub2 and Ub3 were further purified by cation exchange chromatography using a HiTrapS column.
**TAK1 and IKK Activation Assays**

The TAK1 activation assay was carried out in 20 µl of reaction mixture containing 0.1 µM E1, 0.5 µM Ubc13/Uev1A, 0.2 µM TRAF6, 12.5 µM Ub and the TAK1 complex (~1 nM) in Buffer B. The mixture was incubated for 1 hour at 30°C and analyzed by immunoblotting with an antibody specific for phosphorylated Thr-187 of TAK1 (p-TAK1; Cell Signaling). In some assays, the reaction mixture contained the TAK1 substrate MKK6 (K82A; 1.6 µM) and the phosphorylation of MKK6 was analyzed by immunoblotting using an antibody that recognizes phosphorylated Ser-207 of MKK6 (p-MKK6; Santa Cruz Biotech). To measure activation of TAK1 by purified polyUb chains, the TAK1 complex (~1 nM) was incubated with polyUb (ranging from 1.2 to 300 µg/ml) in a reaction mixture (20 µl) containing 1.6 µM MKK6 (K82A) in Buffer B. After incubation at 30°C for 1 hour, activation of TAK1 was analyzed by immunoblotting with an antibody against p-TAK1 or p-MKK6.

To measure IKK activation by polyUb chains, the IKK complex (~10 nM) was incubated with polyUb (~150 µg/ml) in a reaction mixture (10 µl) containing ~50 nM FLAG-IκBα in Buffer B. After incubation at 30°C for 1 hour, IKK activation was analyzed by immunoblotting with an antibody against p-IKKα/β (Cell Signaling) or IκBα (Santa Cruz Biotech).

**Protein Phosphatase and Deubiquitination Enzyme Treatment**
After TAK1 activation by TRAF6, ATP was removed using Zeba Desalt Spin column (Pierce), and the reaction mixture (20 µl) was incubated with lamda protein phosphatase (10 units/µl; New England Biolabs) at 37°C for 30 minutes. Phosphorylation of TAK1 was then analyzed by immunoblotting with a TAK1 antibody. For deubiquitination experiments, polyUb chains (3 µg) were incubated with CYLD (200 nM) or isopeptidase T (100 nM; Boston Biochem) in a reaction mixture (10 µl) containing 20 mM Tris-HCl (pH7.5) and 0.5 mM DTT for 1 hour at 30°C.

**Ubiquitination and deubiquitination of TRAF6, NEMO and RIG-I**

Polyubiquitination of TRAF6 was carried out in a reaction mixture containing 0.2 µM E1, 1 µM Ubc5, 0.4 µM TRAF6, and 12.5 µM Ub in Buffer B at 30°C for 1 hour. Linear polyubiquitination of NEMO was carried out in a similar reaction except that NEMO (0.4 µM) and HOIP/HOIL-1L (0.4 µM) were used in lieu of TRAF6. K63 polyubiquitination of RIG-I was also carried out in a similar reaction except that 0.3 µM of MBP-Ub-RIG-I(N), 0.4 µM TRIM25 and 1 µM Ubc13/Uev1A were used to replace TRAF6 and Ubc5. All reactions were terminated by EDTA (10 mM) before incubation with deubiquitination enzymes (IsoT, CYLD or viral OTU) at 37 °C for 1 hour.

**Isolation and Detection of Polyubiquitin Chains Induced by IL-1β**

HEK293T/IL-1R cells were stimulated with IL-1β for different lengths of time and then extracted with a lysis buffer containing 20 mM Tris-Cl, pH7.5, 100 mM NaCl, 10% glycerol, 0.5% NP-40, 20 mM 2-glycerolphosphate, 1 mM sodium orthovanadate, 0.5 mM DTT, and protease inhibitor cocktail. After centrifugation at 13,000 x g for 5
minutes, cell lysates (~1 mg protein) were incubated with an antibody against TAB2 or NEMO on ice for 1 hour, followed by additional 2-hour incubation with protein A/G-Sepharose (Pierce) at 4°C. Beads were washed extensively in lysis buffer and then resuspended in a buffer (10 μl) containing 20 mM Tris, pH7.5, 10 mM DTT, 0.1mg/ml BSA, and 10 μg/ml of IsoT. After incubation at 30°C for 1 hour, the proteins on the beads were analyzed by immunoblotting.