

Cell Reports

Supplemental Information

LUBAC-Recruited CYLD and A20 Regulate Gene Activation and Cell Death by Exerting Opposing Effects on Linear Ubiquitin in Signaling Complexes

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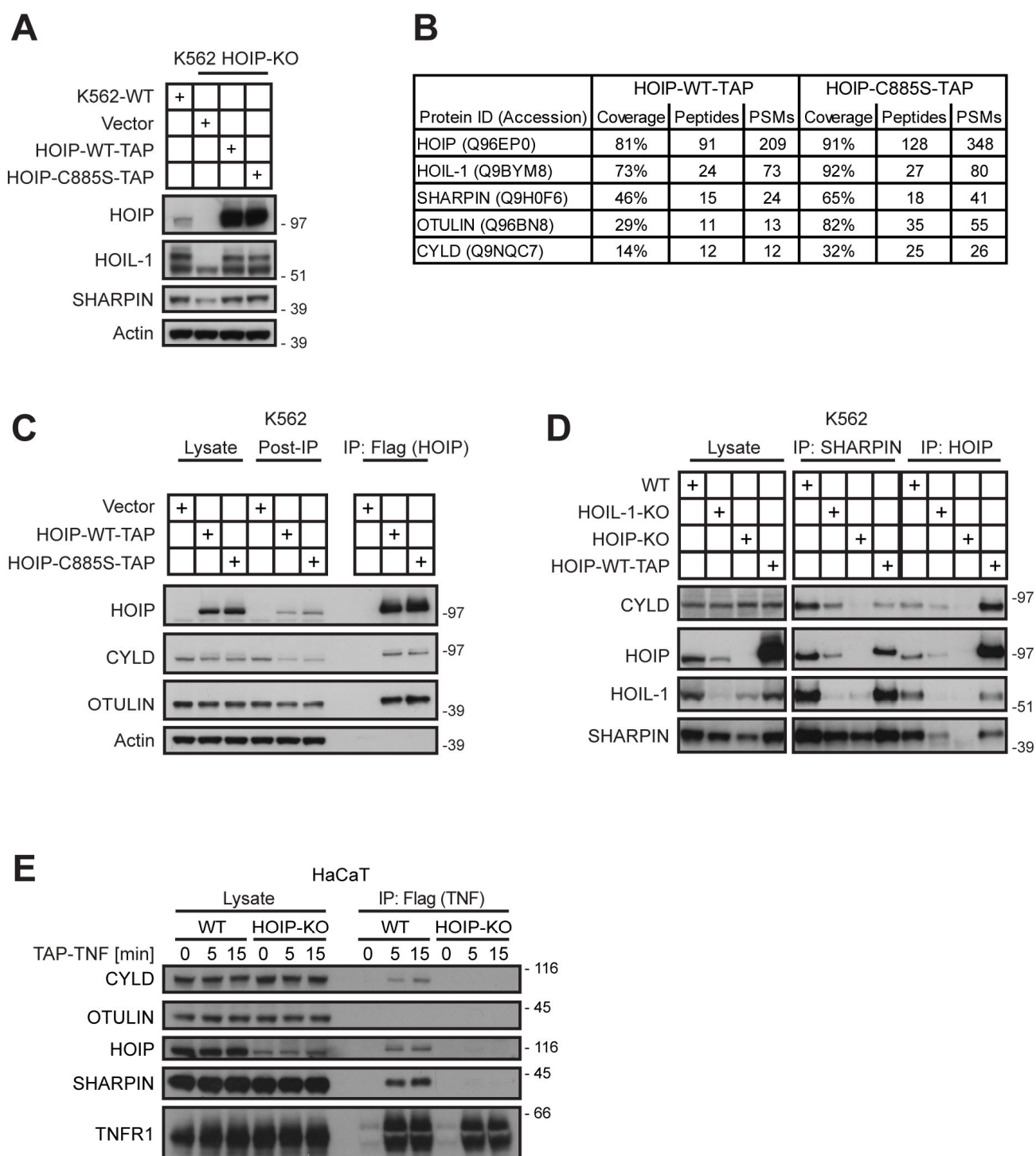


Figure S1: Identification of the DUBs CYLD and OTULIN as constitutive interaction partners of HOIP; related to Figure 1.

(A) Lysates of K562 proficient or deficient in HOIP were virally reconstituted with HOIP constructs and analyzed by western blot.

(B) K562 cells virally reconstituted with HOIP-WT-TAP or HOIP-C885S-TAP were subjected to immunoprecipitation via Flag followed by subsequent StrepTactin purification. The purified

complexes were then employed for mass spectrometric analysis. Identified proteins in samples are shown.

(C) HOIP-WT-TAP or HOIP-C885S-TAP from reconstituted K562 cells were immunoprecipitated via Flag and analyzed by western blot.

(D) LUBAC was immunoprecipitated either via SHARPIN or HOIP in K562 cells which were either WT, HOIL-1-, HOIP-deficient or HOIP-deficient and reconstituted with HOIP-WT-TAP. Precipitates were analyzed by western blot.

(E) Cells were stimulated with TAP-TNF [500ng/ml] for indicated times and the TNF-RSC was isolated from HaCaT WT or HOIP-deficient cells via Flag immunoprecipitation. Samples were analyzed by western blot.

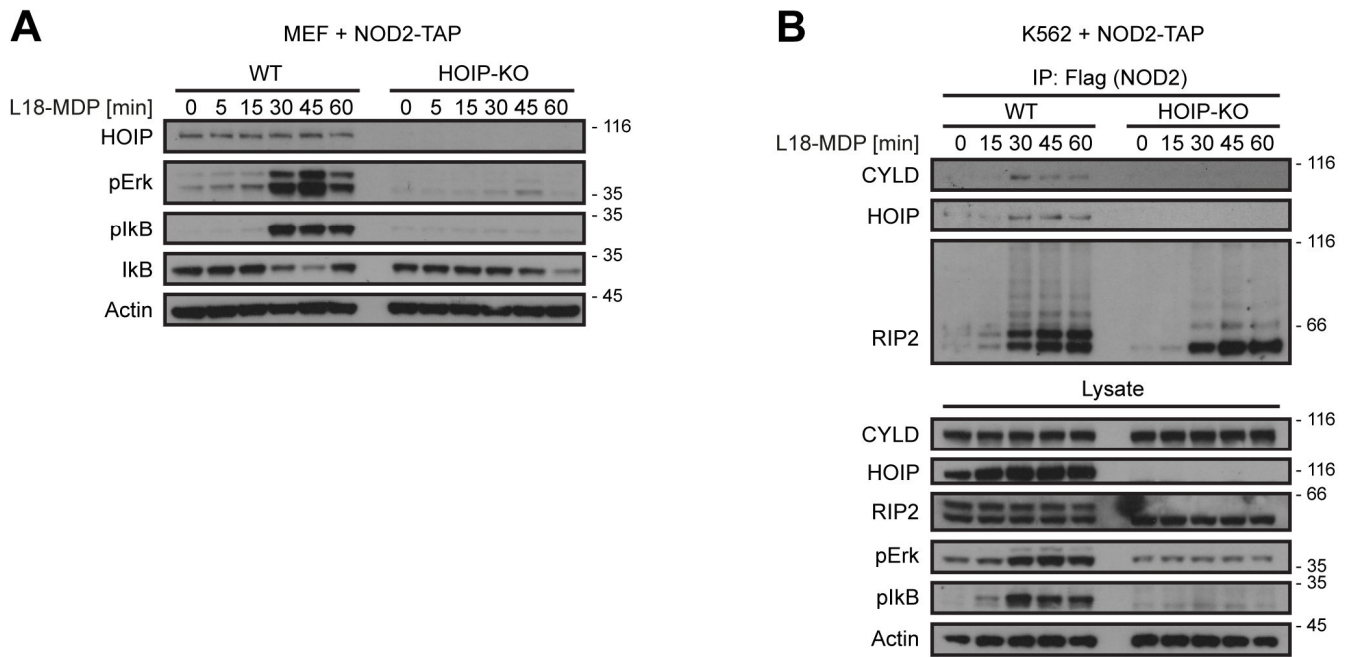


Figure S2: HOIP deficiency abrogates CYLD recruitment to the NOD2-SC and results in diminished gene activatory signaling emanating from this complex; related to Figure 2.

(A) Mouse embryonic fibroblasts (MEFs) from wild-type or HOIP-deficient mice were stably transfected with NOD2-TAP, stimulated with L18-MDP [200ng/ml] for the indicated times and analyzed by western blot.

(B) K562 cells pro- or deficient in HOIP were virally transfected with NOD2-TAP and stimulated with L18-MDP [200ng/ml] for the indicated times. The NOD2-SC was isolated by Flag-tag immunoprecipitation.

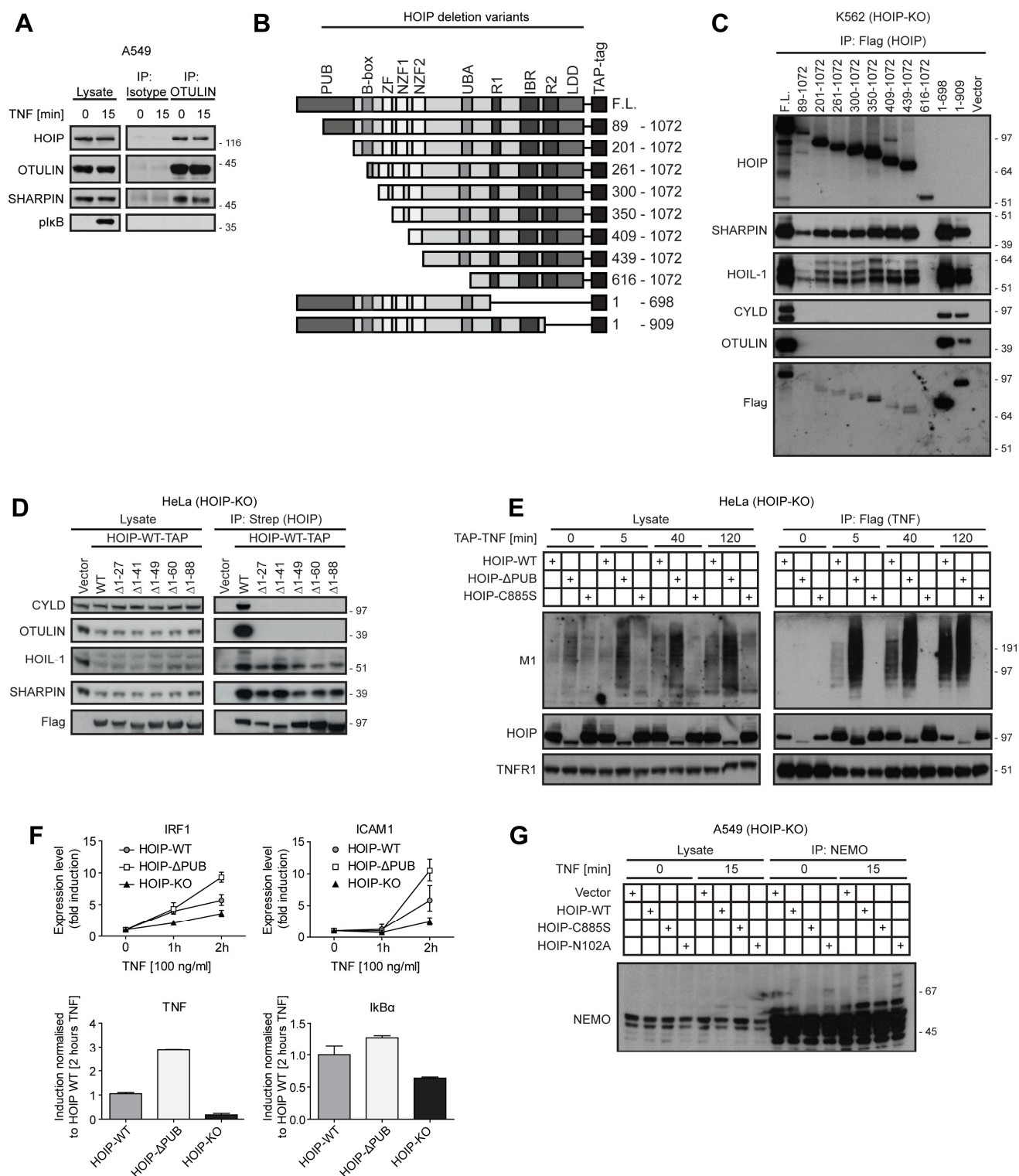


Figure S3: Loss of HOIP's interaction with CYLD and OTULIN results in stabilization of linear ubiquitin chains in signaling complexes; related to Figure 3.

(A) A549 cells were stimulated with TNF [500ng/ml] for 15 minutes or left untreated. Immunoprecipitation of OTULIN was performed and samples were subsequently analyzed by western blot.

(B) Schematic representation of TAP-tagged HOIP truncations employed in Figure S2C.

(C) The indicated truncations of HOIP-TAP were used to reconstitute HOIP-deficient K562 cells. HOIP was immunoprecipitated via Flag and the interaction of HOIP with CYLD and OTULIN was assessed by western blot.

(D) HOIP-KO HeLa cells reconstituted with either WT or truncated versions of HOIP-TAP lacking the indicated amino acids were subjected to Flag immunoprecipitation.

(E) HeLa cells expressing HOIP-WT, HOIP- Δ PUB (lacking amino acids 1-88) or HOIP-C885S were stimulated with TAP-TNF [500ng/ml] for indicated times and the TNF-RSC was isolated via Flag immunoprecipitation.

(F) HeLa cells expressing HOIP-WT, HOIP- Δ PUB or vector control were either left untreated or treated with TNF [100ng/ml] for one or two hours and fold change of mRNA levels of the indicated genes as compared to unstimulated cells were determined by qPCR. Data is presented as mean \pm SD (n = 3).

(G) A549 cells deficient in HOIP were reconstituted with HOIP-WT, HOIP-C885S, HOIP-N102A or vector control. Cells were treated with TNF [200ng/ml] and NEMO was subsequently immunoprecipitated from lysates and samples were analyzed by western blot.

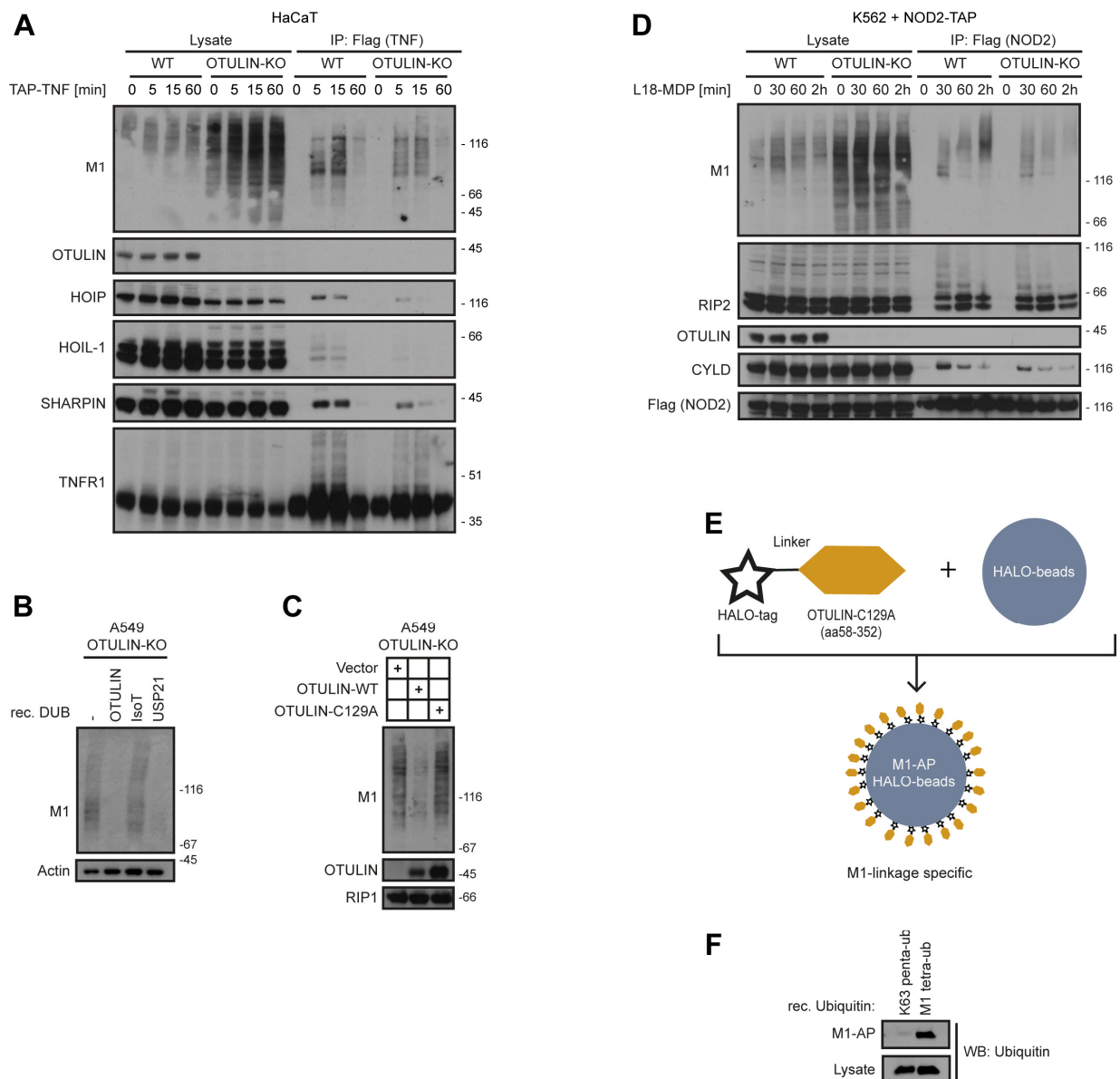


Figure S4: OTULIN deficiency leads to aberrant, anchored linear ubiquitination in the cytosol but not at receptor complexes; related to Figure 4.

(A) The TNF-RSC was isolated from either WT or OTULIN-KO HaCaT cells stimulated with TAP-TNF [500ng/ml] for the indicated times and subjected to western blot analysis.

(B) Lysates of A549 cells deficient in OTULIN were subjected to treatment with recombinant OTULIN, IsoT, USP21 or left untreated. OTULIN cleaves only linear ubiquitin chains. IsoT can only hydrolyze ubiquitin chains starting at their C-terminus which would only be accessible in unattached ubiquitin chains. USP21 can cleave all ubiquitin linkage types irrespectively of their C-terminus. Changes of linear ubiquitination were analyzed by western blot.

(C) A549 cells deficient in OTULIN were transfected with plasmids encoding for OTULIN-WT, OTULIN-C129A or vector control. Subsequently, linear ubiquitination in cellular lysates was analyzed by western blot.

(D) K562 cells pro- or deficient in OTULIN were virally transfected with TAP-NOD2 and stimulated with L18-MDP [200ng/ml] for the indicated times. The NOD2-SC was isolated by Flag-tag immunoprecipitation.

(E) Schematic representation of M1-linkage specific ubiquitin affinity purification (AP) resin preparation.

(F) M1-AP was performed on recombinant K63 penta-ubiquitin or M1 tetra-ubiquitin.

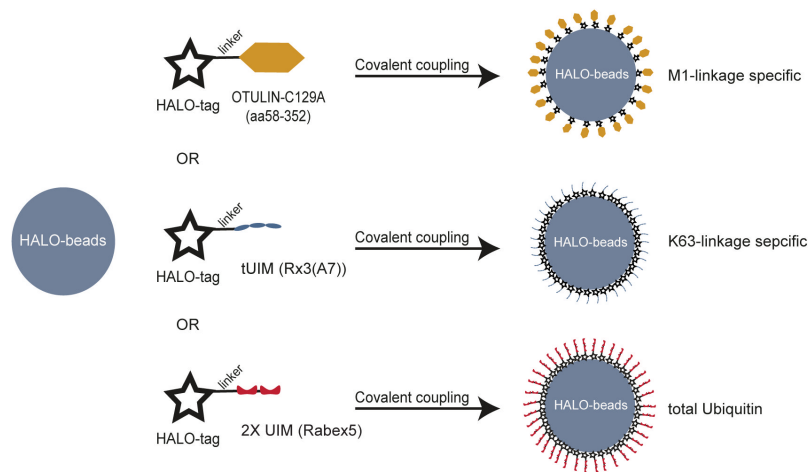
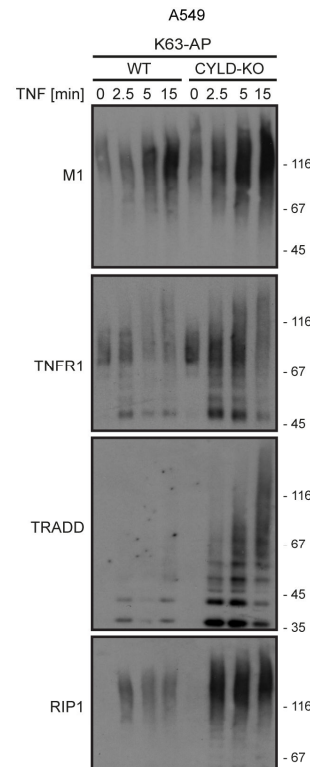
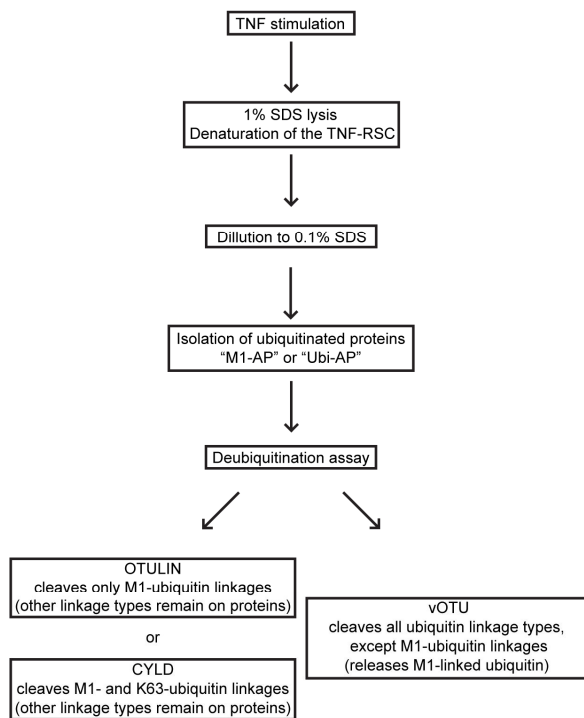
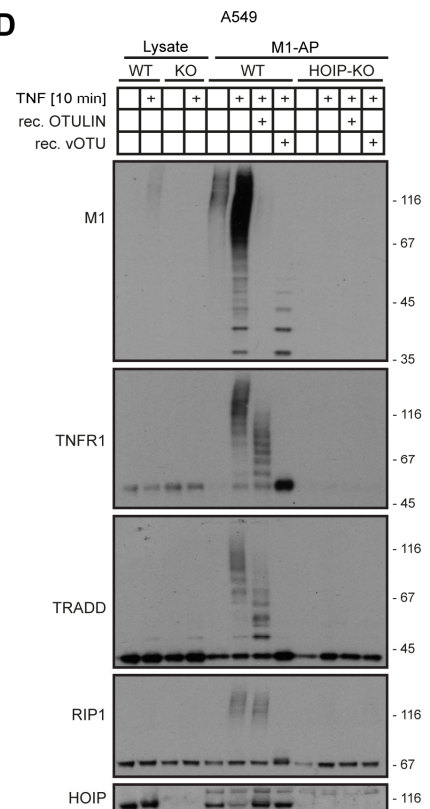
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Figure S5: Specific purification of M1- and K63- ubiquitinated proteins using the affinity purification principle; related to Figure 5.

(A) Schematic representation of M1-, K63-, or total Ubiquitin-linkage specific ubiquitin affinity purification (AP) resin preparation.

(B) A549 pro- or deficient in CYLD expression were stimulated with TNF [200ng/ml] for the indicated times and samples subjected to K63-AP and analyzed by western blot.

(C) Schematic representation of the isolation of ubiquitinated proteins followed by treatment with recombinant DUBs.

(D) A549 pro- or deficient in HOIP expression were stimulated with TNF [200ng/ml] for the indicated times and subjected to M1-AP followed by treatment with either recombinant OTULIN or vOTU.

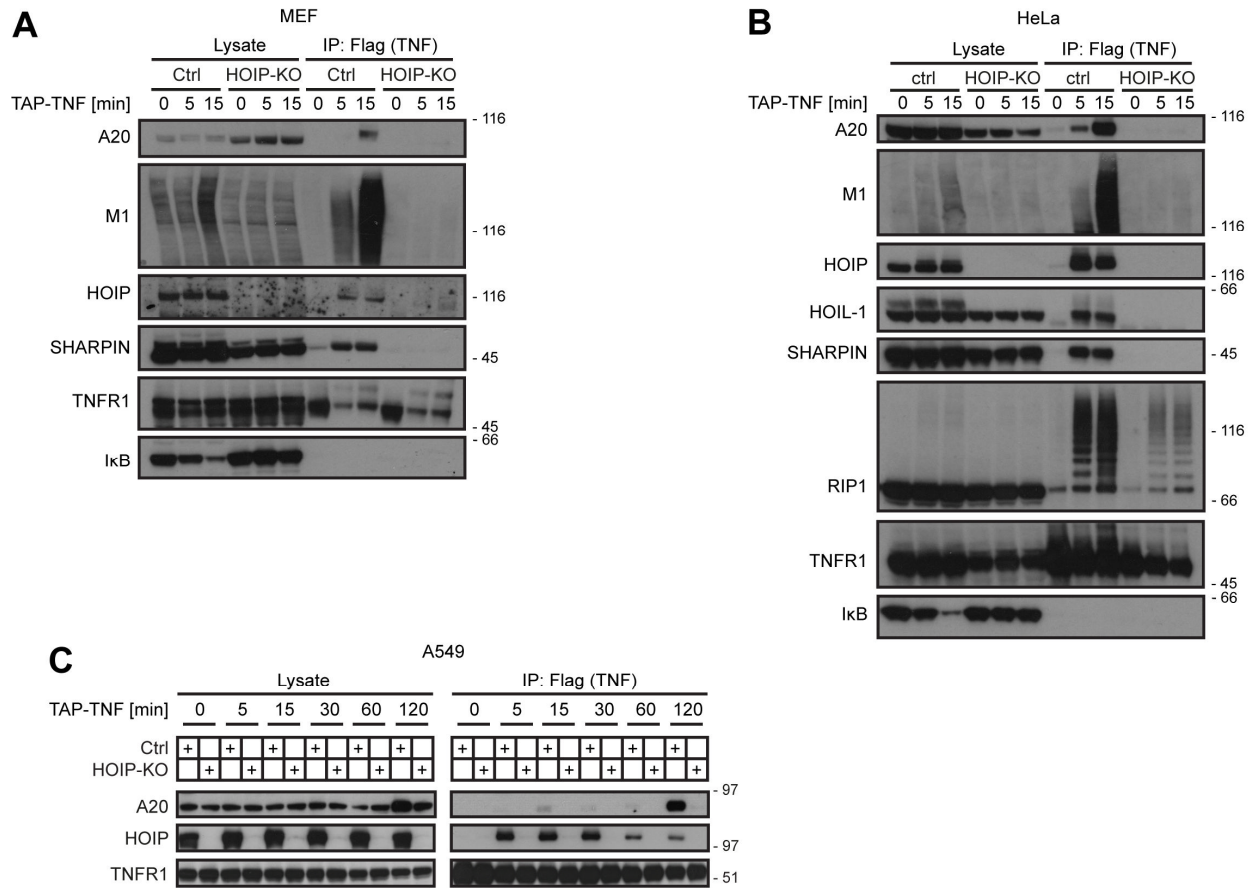


Figure S6: HOIP deficiency leads to loss of A20 recruitment to the TNF-RSC; related to Figure 6.

(A, B, C) MEFs (A), HeLa (B), and A549 (C) pro- or deficient in HOIP expression were stimulated with TAP-TNF [500ng/ml] for the indicated times and the TNF-RSC was immunoprecipitated via Flag-tag and analyzed by western blot.

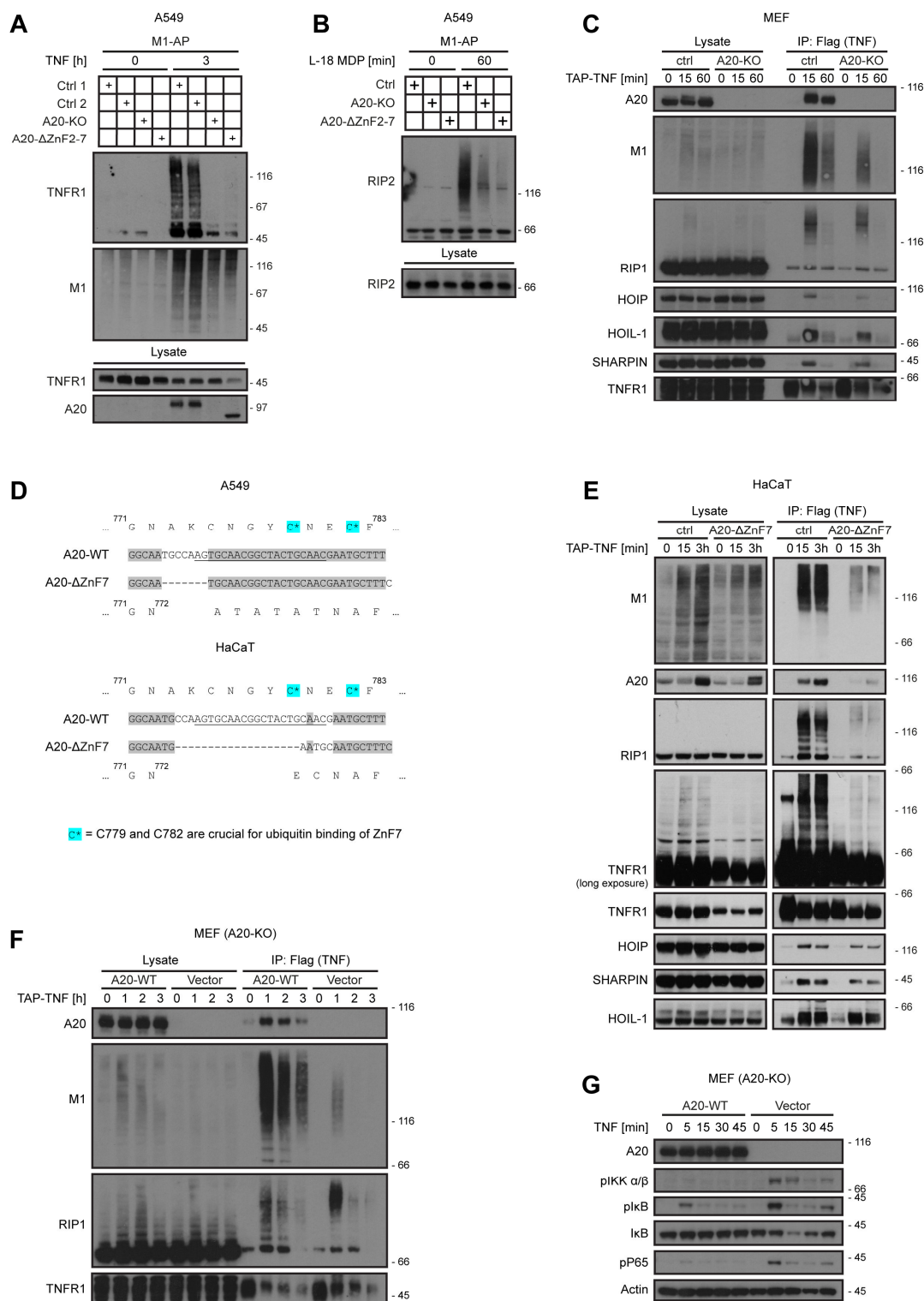


Figure S7: Zinc finger 7 of A20 stabilizes linear ubiquitin chains in the context of TNF and NOD2 receptor signaling, specifically on TNFR1 itself, RIP1 and RIP2; related to Figure 7.

(A) A549 cells pro- or deficient in A20 or lacking A20's zinc fingers 2-7 (A20-ΔZnF2-7) were left untreated or stimulated for 3 hours with TNF [200ng/ml], subjected to M1-AP and analyzed by western blot.

(B) A549 cells pro- or deficient in A20 or lacking A20's zinc fingers 2-7 (A20- Δ ZnF2-7) were virally transfected with TAP-NOD2, left untreated or stimulated with L18-MDP [200ng/ml] for 60 minutes and subjected to M1-AP.

(C) MEFs pro- or deficient in A20 expression were stimulated with TAP-TNF [500ng/ml] for the indicated times and the TNF-RSC was immunoprecipitated via Flag-tag and analyzed by western blot.

(D) Amino acid sequences of A20's ZnF7 region in CRISPR-Cas9 generated A549 and HaCaT cell lines deficient for ZnF7 only. C779 and C782 are crucial for ubiquitin binding of ZnF7 and are absent in both Δ ZnF7 cell lines (sequence underlined corresponds to guide RNA targeting sequence).

(E) HaCaT control cells or HaCaT cells lacking only zinc finger 7 of A20 (A20- Δ ZnF7) were analyzed as in (C)

(F) MEFs deficient in A20 expression were reconstituted with A20-WT or vector control and analyzed as in (C)

(G) MEFs deficient in A20 expression were reconstituted with A20-WT or vector control and stimulated with TNF [500ng/ml] for indicated times and lysates were analyzed by western blot.

Supplemental Experimental Procedures

Production and coupling of ubiquitin affinity proteins

The M1-AP tool compound is based on the coding sequence of OTULIN (aa58-352; C129A), cloned from cDNA isolated from K562 cells. The coding sequence of the K63-AP tool compound is based on the tUIM(Rx3(A7)) construct described previously (Sims et al., 2012) and was synthesized by Life Technologies. Coding sequences were cloned into pH6HTC His6HaloTag® T7 Vector (Promega) using XhoI and XbaI restriction sites. Total ubiquitin binding protein is derived from the UIM (aa147-198) of Rabex5 and synthesized (Life Technologies) as tandem repeat and integrated into pH6HTC His6HaloTag® T7 Vector (Promega) using XhoI and XbaI restriction sites. Expression vectors were transformed into competent BL21 (DE3) bacteria. Halo-tagged proteins were expressed using 2YT Broth auto induction medium (ForMedium) according to the manufacturer's recommendation. Bacteria were resuspended in bacteria lysis buffer (50 mM HEPES, pH 7.6, 150 mM NaCl, 100 µg/ml Lysozyme) and sonicated for one minute. Lysates were then cleared by centrifugation at 25,000 rpm for 45 minutes. Cleared lysates were aliquoted and frozen at -20°C. Aliquots were then used to freshly couple HALO-tagged ubiquitin affinity proteins to HALO-beads (Promega) according to the manufacturer's manual prior to experiment.

Production of deubiquitinases

Coding sequences for OTULIN or vOTU were cloned in frame after GST sequence in GEX6-P2 vector. Expression vectors were transformed into competent BL21 (DE3) bacteria. GST-tagged proteins were expressed as described above. GST-tagged deubiquitinases were purified using Glutathione agarose beads (Sigma) according to manufacturer's manual. Beads were washed three times with DUB-buffer (50mM HEPES, pH 7.6, 150mM NaCl, 5mM DTT). PreScission protease (GE Healthcare) was added (160 U per 1ml of beads, 4°C, overnight) in order to remove the GST-tag and to elute the proteins. Protein concentration was measured with a Nanodrop 2000 (Thermo Scientific), glycerol was added to final concentration 20% and samples were aliquoted and stored at -80°C. Recombinant human CYLD (aa583-956) was produced essentially as described previously (Komander et al., 2008).

Production of recombinant TNF

The coding sequences of TAP-TNF, consisting of a His-tag followed by 3x Flag-tag, a PreScission cleavage site and 2x Strep-tag II and the extracellular portion of TNF (aa78-233), or His-TNF (aa78-233) were inserted in pQE30 vector. Expression vectors were transformed into competent BL21 (DE3) bacteria and expression of proteins was induced by

1mM IPTG overnight. Bacterial pellets were resuspended in bacterial lysis buffer (50 mM Tris, pH 7.4, 0.5 % Triton X-100, 200 mM NaCl, 100 mM KCl, 10% glycerol, 40 mM imidazole, 200 µg/ml lysozyme, 70 µM AEBSF, 5 µM mM E-64, 1.2 µg/ml aprotinin, 1 µM pepstatin A, 5 U/ml benzonase, 2 mM β-mercaptoethanol), sonicated, cleared by centrifugation at 13,300 rpm, 30 min, 4°C, purified on His GraviTrap TALON columns (GE Healthcare), eluted with 500 mM imidazole and dialysed against storage buffer (50 mM Tris, pH 7.4, 100 mM NaCl, 0.02% Tween, 2 mM DTT, 0.5 M arginine). Protein concentration was determined with a Nanodrop 2000 (Thermo Scientific), samples were aliquoted and stored at -20°C.

Isolation of ubiquitin conjugates from cell lysates

Cells were lysed in denaturing SDS containing AP-lysis buffer (30 mM Tris-HCl, pH 7.4, 120 mM NaCl, 2 mM EDTA, 2 mM KCl, 0.5% CHAPS, 1%SDS, 50 mM NaF, 5 mM Na₃VO₄, 1x COMPLETE protease-inhibitor cocktail (Roche)). After incubation of lysates on ice for 10 min, samples were sonicated for 10 s and cleared by centrifugation at 13,500 rpm for 30 minutes. Samples were then diluted with AP-lysis buffer without SDS in order to reduce SDS concentration down to 0.1%. HALO-beads (10 µl per sample) freshly pre-coupled with either total ubiquitin, M1- or K63-linkage specific recombinant affinity protein were then added. Samples were incubated overnight at 4°C, beads were washed three times with AP-lysis buffer without SDS, protease- and phosphatase-inhibitors and proteins were either eluted from beads by boiling of the beads in LDS sample buffer or subjected to '*in vitro* deubiquitination assay'.

***In vitro* deubiquitination assay**

Isolated ubiquitin conjugates from cell lysates were prepared as described above. Beads were resuspended in DUB-buffer (50 mM HEPES, pH7.6, 150 mM NaCl, 5 mM DTT) and 1 µM of recombinant deubiquitinase was added; untreated samples served as controls. The assays were carried out at 37°C for 1 hour. The reaction was stopped and proteins were eluted by boiling of the beads in reducing sample buffer.

Cell activation and immunoprecipitation

For TNF-RSC preparation, cells were washed with PBS, resuspended in serum-free medium and stimulated with TAP-TNF for the indicated times. Cells were lysed in IP-lysis buffer (30 mM Tris-HCl, pH 7.4, 120 mM NaCl, 2 mM EDTA, 2 mM KCl, 10% Glycerol, 1% Triton X-100, 50 mM NaF, 5 mM Na₃VO₄, 1x COMPLETE protease-inhibitor cocktail (Roche)) at 4°C for 1 hour. TAP-TNF (500ng) was added to the lysates of non-stimulated control samples. Subsequently, the lysates were centrifuged at 13,300 rpm for 20 min and the TNF-RSC was

immunoprecipitated using M2 beads (Sigma) overnight at 4°C. The following day, the beads were washed three times with 1 ml IP-lysis buffer and proteins were eluted by boiling in reducing sample buffer. Samples were analyzed by western blotting. For isolation of the NOD2-SC, cells stably transfected with NOD2-TAP were stimulated with 200 ng/ml MDP-L18, lysed and subjected to M2 immunoprecipitation as above. For isolation of retrovirally expressed TAP-tagged proteins, cells were stimulated with untagged TNF as indicated and subjected to immunoprecipitation with M2 beads. Immunoprecipitation with specific antibodies for HOIP (custom made by Eurogentec), OTULIN (Abcam, ab151117), CYLD (Cell Signalling, 4495) or NEMO (Santa Cruz, SC-8330) was performed by antibody coupling to protein A/G-Agarose beads (Santa Cruz) for 3 hours on room temperature.

Cell death analysis

Cells were treated with 200ng/ml TNF or in combination with 20μM zVAD-fmk (Abcam) and/or 10μM Nec-1s (Biovision) for 24 hours. Supernatant was collected and remaining live cells were trypsinized. Supernatant and detached cells were combined and centrifuged at 2,500rpm for 10 minutes. The pellet was then resuspended in PBS containing 5μg/ml Propidium iodide (Sigma). The cell staining was analyzed by FACS (BD Accuri C6). Data is presented as mean ± SEM (n = 3), statistics were performed using T-test.

Real-time RT-PCR analysis

HeLa cells were left untreated or treated with 100 ng/ml TNF for the indicated times. Total RNA was isolated from 1×10^6 cells using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Subsequently, cDNA was generated from total RNA using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific). The real-time RT-PCR analysis was performed using FastStart Universal Probe Master (Rox) (Roche) in combination with the Mastercycler ep realplex (Eppendorf). The RNA levels of the genes of interest were measured by real-time RT-PCR according to the Universal Probe Library system (Roche) using the following primers and probes: IRF1: 5' gagctgggccattcacac, 3' ttggccttcacgtcttg with Probe #36; ICAM1: 5' ccttcctcaccgtgtactgg, 3' agcgtagggttaaggttcttgc with Probe #71; TNF: 5' cagcctctctccttctgat, 3' gccagagggtgattagaga with Probe #29; IkB α : 5' gtcaaggagctgcaggagat, 3' gatggccaagtgcaggaa with Probe #38. The expression of HPRT1 was used as an internal control/housekeeping gene and measured with the primers 5' tgaccttgattatgttgcatacc, 3' cgagcaagacgttcagtct and Probe #73. Data is presented as mean ± SD (n = 3).

Transfection

For transient transfection, the DNA fragments encoding the indicated HOIP deletion mutants fused at the C-terminus to a TAP-tag were ligated into pcDNA3.1 before transfection of HeLa cells using Lipofectamine 2000 (Life Technologies). Samples were analyzed two days after transfection.

Retroviral transduction of cells

For tagging of exogenously expressed proteins we used a C-terminal TAP-tag consisting of 2x Strep-tag II sequence followed by a PreScission cleavage site and 1x Flag-tag. Coding sequences of HOIP-WT, HOIP-C885S, deletion mutants of human HOIP fused or not at the C-terminus to a TAP-tag or NOD2 (aa28-1040)-TAP were inserted into the retroviral MSCV vector, followed by an internal ribosome entry site (IRES) and the open reading frame of EGFP. These vectors were transfected using Lipofectamine 2000 in Phoenix cells. One day after transfection, the medium was replaced and viral supernatants were collected at day three. Viral supernatants were passed through 0.45 µm filter, added to cells in the presence of polybrene at 6 µg/ml before cells were subjected to spinfection (2500 rpm, 45 min, 30°C). EGFP-positive cells were isolated using MoFlo FACS (Beckman Coulter) to more than 95% purity.

Production of knockout cell lines

Messenger RNA encoding zing finger nucleases targeting exon 4 of RNF31 (HOIP) (target sequences were cactggagcagctgt and gacaagggtgaagatgat) or exon 3 of RBCK1 (HOIL-1) (target sequences were ccaccagtcttcagcag and gattgggcagcggctggca) from Sigma were electroporated in cells using AMAXA (Lonza) according to manufacturer instruction. Single cell cloning was achieved by limiting dilution and HOIP- and HOIL-1-deficient cells were validated by western blotting.

For production of Lentiviral knockout vectors, LentiCRISPR version 2 vector targeting the following sequences were used: CYLD(KO) 5'-CACATCAATGATATCATCCC; OTULIN(KO) 5'-GAATTGCTTATACATGAAAG; A20(KO) 5'-GGCGCTGTTTCAGCACGCTCA; A20(ΔZnF2-5) 5'-GTGAACGTTGCCACAACGCC; A20(ΔZnF7) 5'-GTTGCAGTAGCCGTTGCACT; HOIP(KO) 5'-TTGACACCACGCCAGTACCG. Lentiviral particles were produced in HEK293FT cells upon co-transfection of LentiCrispr vector together with packaging vectors psPAX2 and pMD2G. Cells were infected as in the case of retroviral transduction and subjected to puromycin (2 µg/ml) selection for one week. Cells were used as mixed population or subcloned.

Isolation of Bone Marrow Derived Macrophages (BMDMs)

For preparation of BMDMs, 8 week old mice were sacrificed. Hind legs were removed and bones were separated from muscle tissue. Femur and tibia were opened on each site and bone marrow was flushed out using a 25-gauge needle and syringe. Cells were then resuspended in RPMI medium containing 10%FCS, 1% penicillin/streptomycin (Invitrogen) and 10% conditioned medium from L929 cells and passed through a cell strainer. Subsequently, cells were plated in a 12-well plate. The conditioned medium was replaced every two days and cells were incubated for seven days before the experiment.

Tandem affinity purification and mass spectrometry analysis

K562 cells expressing HOIP-WT-TAP or HOIP-C885S-TAP (7.5×10^8 cells each) were lysed in IP-lysis buffer, cleared by centrifugation (13,000 rpm, 30 min, 4°C), incubated overnight with 100 µl of anti-Flag M2 beads (Sigma), washed three times with IP-lysis buffer and proteins were eluted overnight in IP lysis buffer containing 150 µg/ml 3x Flag peptide (Sigma) and 20 U/ml PreScission Protease (GE Healthcare). Samples were subsequently subjected to a second affinity precipitation using Strep-Tactin resin (QIAGEN) overnight at 4°C and eluted with 5 mM biotin. Proteins were precipitated using 2-D Clean-Up Kit (GE Healthcare), resuspended in ice cold denaturation buffer (100 mM ammonium bicarbonate, 8M urea), reduced with 4mM dithiothreitol at 56°C for 30 min, alkylated with 8mM iodoacetamide at 22°C in the dark, and 4mM dithiothreitol was used to neutralize the excess of iodoacetamide. Proteins were digested first with endoproteinase Lys-C (Wako Chemicals) for 4 hours at 37°C. Subsequently samples were diluted to 2M urea, and further digested with sequencing grade trypsin (Promega) for 15 hours at 37°C. Samples were desalted with microspin columns filled with SEM SS18V silica (The Nest Group), eluted with 50% acetonitrile 0.1% TFA, evaporated to dryness at 30°C, and resolubilized in 20 µL water containing 10% formic acid. 1 µL of the resulting peptidic solution was used for LC-MS analysis. nLC-MS/MS was performed on a Q Exactive Orbitrap interfaced to a NANOSPRAY FLEX ion source and coupled to an Easy-nLC 1000 (Thermo Scientific). Peptides were separated on a 15 cm fused silica emitter, 75 µm diameter, packed in-house with Reprosil-Pur 200 C18-AQ, 3 µm resin (Dr. Maisch) using a linear gradient from 5% to 30% acetonitrile/0.1% formic acid over 30 min, at a flow rate of 300 nL/min. Precursor ions were measured in a data-dependent mode in the orbitrap analyzer at a resolution of 70,000 and a target value of 1×10^6 ions. The ten most intense ions from each MS1 scan were isolated, fragmented in the HCD cell, and measured in the orbitrap at a resolution of 17,500. Data were searched with SEQUEST HT against the human UniProt database (downloaded 03/04/2013) in Proteome Discoverer 1.4.0.288 (Thermo Scientific). The precursor mass

tolerance was set to 10 ppm, the fragment ion mass tolerance was set to 0.05 Da. Enzyme specificity was set to trypsin with maximally 2 missed cleavages allowed. Oxidation of methionines was set as variable modification and carbamidomethylation of cysteines was set as fixed modification. The percolator node was used to filter the PSMs at less than 1% false discovery rate (FDR).

SDS-PAGE, Western blot and Antibodies

Proteins were separated using 4 – 12% Bis-Tris-NuPAGE-gels (Invitrogen) with NuPAGE® MOPS running buffer. Proteins were transferred from gels onto ECL- Membrane Hybond 0.45 µm nitrocellulose membrane (GE-Healthcare). Proteins were detected with antibodies against HOIP (Sigma, SAB2102031), HOIL-1 (Haas et al., 2009), SHARPIN (Proteintech, 14626-1-AP), OTULIN (Abcam, ab151117), CYLD (Santa Cruz, E10), A20 (Santa Cruz, A-12), RIP1 (BD, 610459), TRADD (BD, 610572), TNF-R1 (Santa Cruz, SC-8436), RIP2 (Santa Cruz, SC-22763), NEMO (Santa Cruz, FL-419 or Cell Signaling, DA10-12), M1-Ub (Merck Millipore, MABS199), K63-ubiquitin (Biomol HW A4C4), total ubiquitin (Biomol, PW8805), IKKα (Cell Signaling, 2682), pIKKα/β Cell Signaling, 16A6), IκBα (Cell Signaling, 9242), pIκBα (Cell Signaling, 9246S), pP65 (Cell Signaling, 7F1), pErk (Santa Cruz, SC-7383), pP38 (Cell Signaling, D3F9), pJNK (Cell Signaling, 98F2), phospho tyrosine (p-Tyr-100 Cell Signalling 9411S), Actin (Sigma, A1978), GAPDH (Abcam, ab8245), and Flag (Sigma, M2).

ELISA

A549 cells were stimulated with TNF [50ng/ml]. After 24h, chemo-/cytokine concentrations in the cell supernatants were measured via ELISA (R&D). Error bars represent the mean +/- SEM of experiments performed in triplicates.

Supplemental References

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