Supplementary Information

LUBAC PUB domain interactions restrict Met1-linked ubiquitination to prevent embryonic lethality and immune pathology

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Materials and Methods

Antibodies

Target & clone	Supplier	Cat. nr.	Application	Dilution
HOIP (E6M5B)	Cell Signaling Technology	99633	WB	1:1000
HOIP	abcam	ab46322	WB	1:1000
HOIP	abcam	ab315162	WB	1:2000
HOIP	MRC PPU	S174D	IP	2 µg; 4.5 µg
HOIL-1 (EPR28157-53)	abcam	ab309104	WB	1:1000
SHARPIN (3A9E2)	proteintech	67177-1-Ig	WB, IP	1:3000, 1 µg
SHARPIN	proteintech	14626-1-AP	WB, IP	1:1500, 1 µg
CYLD (D1A10)	Cell Signaling Technology	8462	WB	1:1000
CYLD (E10)	Santa Cruz	sc-74435	WB	1:200
OTULIN	abcam	ab151117	WB	1:1000
OTULIN	Cell Signaling Technology	74125		
Ubiquitin (P4D1)	Cell Signaling Technology	3936	WB	1:2000
Met1-Ubiquitin (1E3)	Merck	ZRB2114	WB	1:10000
TNFR1 (C25C1)	Cell Signaling Technology	3736	WB	1:1000
TNFR1 rodent (D3I7K)	Cell Signaling Technology	13377	WB	1:1000
RIPK1 (D94C12)	Cell Signaling Technology	3493	WB	1:1000
Phospho-RIPK1 (Ser166) (D1L3S)	Cell Signaling Technology	65746	WB	1:1000
Phospho-RIPK1 (Ser166)	Cell Signaling Technology	31122	WB	1:1000
RIPK1	BD Biosciences	51-6559GR	WB	1:1000
RIPK2 (D10B11)	Cell Signaling Technology	4142	WB	1:1000
NEMO (EPR16629)	abcam	ab178872	WB	1:1000
IKKβ	Cell Signaling Technology	8943	WB	1:1000

Phospho-IKKα/β (Ser176/180) (16A6)	Cell Signaling Technology	2697	WB	1:1000
IκBα (44D4)	Cell Signaling Technology	4812	WB	1:1000
IκBα (L35A5)	Cell Signaling Technology	4814	WB	1:1000
Phospho-IκBα (Ser32) (14D4)	Cell Signaling Technology	2859	WB	1:1000
P65 (D14E12)	Cell Signaling Technology	8242	WB	1:1000
Phospho-P65 (Ser536) (93H1)	Cell Signaling Technology	3033	WB	1:1000
P38 (D13E1)	Cell Signaling Technology	8690	WB	1:1000
Phospho-P38 (Thr180/Tyr182) (28B10)	Cell Signaling Technology	9216	WB	1:1000
Phospho-JNK (Thr183/Tyr185) (81E11)	Cell Signaling Technology	4668	WB	1:1000
TBK1 (E8I3G)	Cell Signaling Technology	38066	WB	1:1000
Phospho-TBK1 (Ser172) (D52C2)	Cell Signaling Technology	5483	WB	1:1000
A20/TNFAIP3	Cell Signaling Technology	5630	WB	1:1000
Abin-1	proteintech	15104-1-AP	WB	1:2000
Abin-2	proteintech	15459-1-AP	WB	1:2000
Abin-2	abcam	ab205925	WB	1:2000
TRADD	Cell Signaling Technology	3694	WB	1:1000
TRAF2 (C192)	Cell Signaling Technology	4724	WB	1:1000
TAK1	Cell Signaling Technology	4505	WB	1:1000
FADD	Santa Cruz	SC-271748	WB	1:200
FADD	abcam	ab108601	IP	1.35 µg
p105	abcam	ab32360	WB	1:1000
cRel	Thermo	14-6111-82	WB	1:1000
cRel	abcam	EPR25178-58	WB	1:1000
cReb	Cell Signaling Technology	9104S	WB	1:1000
ATF2	Cell Signaling Technology	35031S	WB	1:1000
c-Jun	Santa Cruz	SC-74543	WB	1:200
Lamin A/C	Cell Signaling Technology	4777T	WB	1:3000
Caspase-3 (D3R6Y)	Cell Signaling Technology	14220	WB	1:1000
Cleaved Caspase-3	Cell Signaling Technology	9661	WB	1:1000
Caspase-8 (D35G2)	Cell Signaling Technology	4790	WB	1:1000
Cleaved Caspase-8 (18C8)	Cell Signaling Technology	9496	WB	1:1000
Cleaved Caspase-8 rodent (D5B2)	Cell Signaling Technology	8592	WB	1:1000
FLIP (D5J1E)	Cell Signaling Technology	56343	WB	1:1000
Phospho-MLKL rodent (Ser345) (D6E3G)	Cell Signaling Technology	37333	WB	1:1000
Vinculin (hVIN-1)	Merck	V9131	WB	1:3000
V5 tag (SV5-Pk1)	Thermo Fisher Scientific	R960-25	WB	1:5000
HA tag (3F10)	Roche	11867431001	WB	1:5000
Mouse IgG2a isotype control	Cell Signaling Technology	61656	IP	1 µg

Rabbit IgG isotype control	proteintech	3000-0-AP	IP	1 µg
Goat anti-rabbit HRP	Southern Biotech	4030-05	WB	1:10000
Goat anti-mouse HRP	Southern Biotech	1030-05	WB	1:10000
Goat anti-rat HRP	Southern Biotech	3030-05	WB	1:5000
anti-Rabbit IgG light chain HRP	abcam	ab99697	WB	1:5000
Veriblot	abcam	ab131366	WB	1:3000

Target & clone	**Conjugate**	**Supplier**	**Cat. nr.**	**Application**	**Dilution**
FC-block (2.4g2)	unconjugated	BD Biosciences	553142	Flow cytometry	1:100
CD45 (30F11)	BV510 & BV480	BD Biosciences	566095 & 563891	Flow cytometry	1:200
Ly6G (1A8)	BUV737	BD Biosciences	741813	Flow cytometry	1:200
Ly6C (AL21)	APC	AL-21	560595	Flow cytometry	1:200
CD11c (N418)	Pe-Cy7	Thermo Fisher Scientific	25-0114-81	Flow cytometry	1:400
MHC-II (M5/114.15.2)	AF488	Biolegend	107616	Flow cytometry	1:200
Xcr1 (ZET)	BV650	Biolegend	148220	Flow cytometry	1:200
CD172 (P84)	BV421	BD Biosciences	740071	Flow cytometry	1:200
CD11b (M1/70)	APC-eFlour780	Thermo Fisher Scientific	47-0112-82	Flow cytometry	1:400
CCR2 (475301)	BV786	BD Biosciences	747966	Flow cytometry	1:200
CD64 (X54-5/7.1)	PE	Thermo Fisher Scientific	12-0641-82	Flow cytometry	1:100
F4/80 (T45-2342)	BUV395	BD Biosciences	565614	Flow cytometry	1:200
SiglecF (E50-2440)	BV711	BD Biosciences	740764	Flow cytometry	1:200
CD19 (1D3)	BB700	BD Biosciences	566494	Flow cytometry	1:200
CD3e (145-2C11)	Pe-CF594	BD Biosciences	562286	Flow cytometry	1:200
B220 (RA3-6B2)	AF700	Thermo Fisher Scientific	56-0452-82	Flow cytometry	1:100
NK1.1 (PK136)	AF700	BD Biosciences	560515	Flow cytometry	1:100
Ter119 (Ter-119)	AF700	Thermo Fisher Scientific	56-5921-80	Flow cytometry	1:100
CD8 (53-6.7)	FITC	BD Biosciences	553030	Flow cytometry	1:200

TCRβ (H57-597)	APC-eFlour780	Thermo Fisher Scientific	47-5961-80	Flow cytometry	1:200
CD4 (GK1.5)	BUV395	BD Biosciences	563790	Flow cytometry	1:200
Foxp3 (FJK-16a)	PE	Thermo Fisher Scientific	12-5773-82	Flow cytometry	1:100
CD31 (MEC13.3)	Biotin	Biolegend	102504	IF & whole tissue imaging	1:250
Streptavidin	Alexa fluor 488	Biolegend	405235	IF & whole tissue imaging	1:300
Cleaved Caspase-3	unconjugated	Cell Signaling Technology	9661	IF & whole tissue imaging	1:250
Anti-rabbit	Alexa fluor 555	Thermo Fischer Scientific	A32732	IF & whole tissue imaging	1:500

Mice breeding and *in vivo* treatments

Mice were bred and maintained at the animal facilities of the department of experimental medicine (AEM) at the University of Copenhagen, Denmark. Breeding of mice and experiments involving mice were performed under breeding and experimental licenses approved by the Danish Animal Experiments Inspectorate. Cyagen generated the *Rnf31*$^{N101D/N101D}$ mice by CRISPR/CAS-mediated genome engineering. Briefly, the gRNA to mouse *Rnf31*, the donor oligo containing N101D (AAC to GAT) mutation and two synonymous mutations P102 (CCC to CCT) and V103 (GTC to GTT), and Cas9 were co-injected into fertilised mouse eggs. F0 founder animals were identified by sequencing and were bred to wildtype mice to establish germline transmission and F1 progeny. The mouse line C57BL/6N-Rnf31<tm1c(KOMP)Wtsi>/Tcp was made as part of the NorCOMM2 project with C57BL/6N-Rnf31<tm1a(KOMP)Wtsi>/Tcp made from KOMP ES cells[1] at the Toronto Centre for Phenogenomics and was obtained from the Canadian Mouse Mutant Repository. The t(ROSA)26Sor<tm1(cre/Esr1)Tyj strain (JAX Strain #:008463) were rederived from the core facility for transgenic mice (TCF) repository. TNF-receptor knockout mice were generously provided by Geert van Loo, VIB, University of Ghent, Belgium.

For Temporal ablation of *Rnf31*^{f} allele, mice were injected intraperitoneal (i. p.) with either 100 mg/Kg body weight Tamoxifen (Merck, T5648) dissolved in vehicle of 10%

absolute ethanol and 90% sunflower seed oil (Merck, S5007) or an equal volume of vehicle only for 5 days. To neutralise TNF *in vivo*, mice were i. p. injected with anti-TNF neutralising antibody (BioXcell InVivoPlus anti-mouse TNF, BP0058) at a dose of 10 mg/Kg body weight every 48 hours starting at day 1 of the experiment. For the TNF-induced systemic inflammatory response syndrome (SIRS), mice were i. p. injected with murine TNF at a dose of 250 µg/Kg body weight (5 µg / 20 g) diluted in PBS. Rectal temperatures were measured before the injections and at 3 hours intervals afterwards. Mice were euthanized when their rectal temperature was at or below 27 °C or at 8 hours post-challenge.

Retroviral plasmids and cloning

Retroviral plasmids were constructed using In-Fusion cloning (Takara) as per the manufacturer's instructions. The coding sequence for mouse *Rnf31* was PCR-amplified from cDNA isolated from MEFs and inserted into a pcDNA3 backbone linearised with BamHI and EcoRI to add an N-terminal 2xHA-2xStrep tag. The CDS was then PCR-amplified from the pcDNA3 backbone and inserted into the pBABE-Neo backbone. The C879A and N101D mutants were generated by overlap extension PCR and re-insertion into the empty pBABE-Neo backbone. Plasmids were transformed into Stellar competent *E. coli* and successful plasmid assembly was verified by Sanger sequencing (Macrogen). Finally, plasmid DNA was purified using the Nucleobond Xtra Midi kit (Macherey-Nagel) as per the manufacturer's instructions.

Plasmid	**Primer sequences 5' – 3'**
pcDNA3-2xHA-2xStrep-mmHOIP-WT	ATCACACTGGCGGCCGCAAATGCCGGGAGACGAGG CGAAGGGCCCTCTAGACTACTTTCTTCTGCGGGCAATACTC
pBABE-Neo-2xHA-2xStrep-mmHOIP-WT	GGCGCCGGCCGGATCCATGGTTTACCCATACGACGTTCC CTGTGCTGGCGAATTCATGGTGATGGTGATGATGACCGG
pBABE-Neo-2xHA-2xStrep-mmHOIP-C879A	GGAGGCGCCATGCACTTCCACTGCACG CGTGCAGTGGAAGTGCATGGCGCCTCC
pBABE-Neo-2xHA-2xStrep-mmHOIP-N101D	TCAGTGAAGTTTAATGACCCCGTCTTTCGC GCGAAAGACGGGGTCATTAAACTTCACTGA

Retrovirus production and infection

Phoenix ECO cells were plated in a 10 cm dish and grown to 70% confluence. Then, the cells were transfected with respective plasmid constructs. The next day, the cells were washed once with PBS and fresh DMEM without phenol-red was added to the

cells. Viral supernatants were harvested every 24 h starting at 48 h post transfection to 96 h post transfection, filtered through a 0.45 µm PES filter and mixed with an equal volume of fresh media. Polybrene was added to 8 µg/mL to complete the infection mix, after which it was added to MEFs at 20% confluence in a 10 cm dish. Infections were carried out for 24 h and repeated once for a total duration of 48 h. Infected cells were recovered in fresh complete DMEM for 24 h, after which infected cells were selected with 0.4 mg/mL G-418 (Gibco) for 5 days. Afterwards, individual clones were isolated as described for CRISPR knockout clones and successful integration was verified by anti-HA immunoblot. Selected clones were maintained in phenol-red-free DMEM supplemented with 0.2 mg/mL G-418.

Generation and maintenance of immortalised MEFs

Mouse embryos were dissected at 13.5 days post coitum (dpc) and MEFs were generated using the trypsin digestion method as previously described[2]. Primary MEFs were seeded at a confluency of 8000 cells/cm^2 in D10 medium, which was made from DMEM (Thermo Fisher Scientific, 10569010) supplemented with 20 mM HEPES (Thermo Fischer Scientific, 15630056), 1x Penicillin-Streptomycin (P/S, 10,000 U/mL, Thermo Fischer Scientific, 15140122) and 10% FBS (Thermo Fisher Scientific, 16000044), and were subsequently transfected with a retrovirus co-expressing SV40T antigen, eGFP and puromycin resistance genes. The transfected cells were puromycin-selected by culturing for 3 passages in D10 supplemented with 2 µg/mL puromycin (Merck, P7255). Recombination in MEFs was induced using 1 µM 4-hydroxytamoxifen (4-OHT) (Merck, H6278) for another 3 passages.

Phoenix ECO cells were cultured in DMEM supplemented with 10% FBS and 1x P/S. Inducible OTULIN KO MEFs were described previously[3] and cultured in DMEM without phenol red and supplemented with 10% FBS and 1% P/S. Acute OTULIN KO in inducible MEFs was induced by treating the cells with 100 nM 4-OHT for 5 days.

Generation and maintenance of human cell lines

All HCT-116 cells were cultured in McCoy's 5A (Modified) Medium (Thermo Fischer Scientific, 36600021) supplemented with 10% FBS and 1x P/S at 37 °C and 5% CO_2 in a humidified incubator and regularly checked for mycoplasma infection. Monoclonal HCT-116 HOIP KO cells were previously described[4] and used for stable reconstitution of wild type or PUB mutant HOIP using the PiggyBac transposon system. Cells were

seeded 24 hours prior to transfection and were co-transfected with either pPB-HOIP, pPB-HOIP-N102D or pPB-HOIP-N102A and pRP-hyPBase plasmids (Vectorbuilder) using jetOPTIMUS transfection reagent (Polyplus, 101000051). Transfected cells were positively selected by the addition of 5 µg/mL of puromycin until only resistant cells remained. Both pools, consisting of multiple clones with different expression levels of HOIP, and single cell clones were generated and used in experiments.

siRNA-mediated knockdown of target genes

One 10 cm dish per condition of HCT-116 HOIP-reconstituted cells was seeded 24 hours before transfection. On the following day, cells were transfected with 147.5 pmol of either siCTRL, siOTULIN #1 and #2 (mixed 1:1), or siCYLD using 44.2 µL Lipofectamine RNAi MAX (Thermo Fisher Scientific, 13778075) following the manufacturer's instructions. Cells were incubated with the transfection mix for 72 hours before the plates were washed once with PBS and lysed to analyse target protein levels.

siRNA	**Sense 5' - 3'**	**Antisense 5' - 3'**
siOTULIN #1	(GACUGAAAUUUGAUGGGAA)TT	(UUCCCAUCAAAUUUCAGUC)TT
siOTULIN #2	(CAAAUGAGGCGGAGGAAUA)TT	(UAUUCCUCCGCCUCAUUUG)
siCYLD	(GAACAGAUUCCACUCUUUA)TT	UAAAGAGUGGAAUCUGUUC

Cell death assays

Cells were seeded at least 24 hours before start of treatment on 48-well plates to allow full attachment to the surface. After confluency of 60-80% was reached, cells were treated with either 10 ng/mL recombinant human or mouse TNF (PeproTech, 300-01A or 315-01A), or with TNF and 20 µM zVAD-fmk (MedChemExpress, HY-164388) and transferred to an Incucyte S3 Live-Cell Analysis Instrument (Sartorius) for observation of cell death. Four pictures were taken of different parts of each well at a rate of one picture per hour to a total of 24 hours. Dead or dying cells were stained with SYTOX Green (Thermo Fisher Scientific, S7020), Propidium iodide (Thermo Fisher Scientific, P3566)or DiYo-3 (equivalent to YoYo-3; AAT bioquest; ABD-17581)nucleic acid stains and were captured in the green (ex. 441-481 nm, em. 503-544 nm) or red (ex. 567-

607 nm, em. 622-704 nm) channel, respectively. The number of green or red objects was counted per picture and divided by cell confluency for comparison.

Immunoprecipitation of stably expressed HOIP constructs

One 10 cm dish per condition of HCT-116 HOIP KO and HOIP-reconstituted cells was seeded at least 24 hours before start of treatment to allow full attachment to the surface. Samples used to investigate HOIP catalytic activity were pre-treated with 3 µM HOIPIN-8 (MedChemExpress, HY-122882) for 30 min. Cells were treated at ~90% confluency with 10 ng/mL recombinant human TNF for 15 min and were subsequently washed with cold PBS. Afterwards, cells were lysed by adding 500 µL TNF lysis buffer (30 mM Tris HCl (pH 7.4), 120 mM NaCl, 2 mM EDTA, 2 mM KCl, 1% Triton X-100, supplemented with1 mM DTT, 0.5 mg/mL NEM and 1 tablet each of cOmplete protease inhibitor and PhosSTOP phosphatase inhibitor (Merck, 5892970001 and 4906837001) added per 10 mL of buffer) and scraped from the dishes using a cell lifter. Samples were incubated on ice for 30 min and afterwards centrifuged at 21000 x g and 4 °C for 10 min. Input fractions were taken and mixed with 6x Laemmli Sample Buffer (LSB) and the remaining supernatants were incubated with 25 µL per sample of pre-washed anti-V5 agarose affinity gel (Merck, A7345) overnight at 4 °C on a rotating wheel. On the following day, samples were washed four times with TNF lysis buffer and proteins were eluted from the affinity gel by addition of 60 µL LSB and incubation at 95 °C for 10 min. Eluted proteins were transferred to clean reaction tubes and separated by SDS-PAGE or stored at -20 °C.

Immunoprecipitation of endogenous SHARPIN or HOIP

One 15 cm dish per condition of HOIP-reconstituted HCT-116 HOIP KO cells or immortalised MEFs was seeded as mentioned before. Cells were treated at ~90% confluency with 10 ng/mL recombinant human or mouse TNF for various time points and were subsequently washed with cold PBS. Cells were lysed with 1 mL of Co-IP buffer (25 mM HEPES pH 7.5, 150 mM NaCl, 0.5% IGEPAL CA-630, 10% glycerol, supplemented with1 mM DTT, 0.5 mg/mL NEM and 1 tablet each of cOmplete protease inhibitor and PhosSTOP phosphatase inhibitor added per 10 mL of buffer), incubated on ice and centrifuged as mentioned before. Input fractions were taken and the remaining supernatants were incubated with 1-3 µg of SHARPIN antibody (proteintech, 67177-1-Ig for human samples, 14626-1-AP for mouse samples) , or

HOIP antibody (MRC PPU, S174D, 2 µg for mouse samples) or the respective species isotype control overnight at 4 °C on a rotating wheel. On the next day, 12-25 µL per sample of pre-washed Protein G magnetic beads (Thermo Fisher Scientific, 88848) were added and the samples were incubated at 4 °C on a rotating wheel for 4 hours. Afterwards, samples were washed five times with Co-IP buffer and proteins were eluted from the beads by addition of 50 µL LSB and incubation at 95 °C for 10 min. Eluted proteins were transferred to clean reaction tubes and separated by SDS-PAGE or stored at -20 °C.

For immunoprecipitation of endogenous SHARPIN from embryos, each E10.5 embryo was finely chopped in a total volume of 300 µL of Co-IP buffer and incubated at 4 °C rotating for 1 hour. The total lysate was cleared by centrifugation and 7.5% of lysate volume was aliquoted as the input fraction. The remainder of the lysate was incubated with 0.5 µg of SHARPIN antibody or IgG isotype control overnight at 4 °C on a rotating wheel. On the next day, 10 µL per sample of pre-washed Protein G magnetic beads (Thermo Fisher Scientific, 88848) were added and the samples were incubated at 4 °C on a rotating wheel for 4 hours. Afterwards, samples were washed three times with Co-IP buffer and proteins were eluted from the beads by addition of 50 µL LSB and incubation at 95 °C for 10 min. Eluted proteins were transferred to clean reaction tubes and separated by SDS-PAGE or stored at -20 °C.

TNFR1 pulldown assay and

One 15 cm dish per condition of HOIP-reconstituted HCT-116 HOIP KO cells or immortalised MEFs was seeded as mentioned before. Cells were treated at ~90% confluency with 50 ng/mL (500 ng total per dish) biotinylated recombinant human or mouse TNF (ACROBiosystems, TNA-H8211 or TNA-M82E9) for various time points and subsequently washed with cold PBS. Cells were lysed with 1 mL of TNF lysis buffer, incubated on ice and centrifuged as mentioned before. Input fractions were taken and 250 ng of biotinylated TNF was added to the unstimulated control samples. The remaining supernatants were incubated with 20 µL per sample of pre-washed streptavidin magnetic beads (Thermo Fisher Scientific, 88817) overnight at 4 °C on a rotating wheel. On the following day, samples were washed five times with TNF lysis buffer and proteins were eluted from the beads by addition of 50 µL LSB and

incubation at 95 °C for 10 min. Eluted proteins were transferred to clean reaction tubes and separated by SDS-PAGE or stored at -20 °C.

Ubiquitin Chain Restriction (UbiCRest) assay

Two 15 cm dishes per condition of HOIP-reconstituted HCT-116 HOIP KO cells were seeded and treated with biotinylated recombinant human TNF for the indicated time points as described above. Subsequently, each plate was lysed with 1 mL of TNF lysis buffer and identical duplicates were combined, incubated on ice and centrifuged as mentioned before. Input fractions were taken and 100 ng of biotinylated TNF was added to the unstimulated control samples. The remaining supernatants were incubated with 40 µL per sample of pre-washed streptavidin magnetic beads overnight at 4 °C on a rotating wheel. On the following day, samples were washed five times with PBST. Each sample was then split into 4 equal fractions. The beads from each fraction were collected and resuspended in 40 µL of either DUB buffer alone (50 mM HEPES 7.5, 100 mM NaCl, 2 mM DTT, 1 mM $MnCl_2$, 0.01% BRIJ35), DUB buffer + OTULIN (1 µM recombinant OTULIN), DUB buffer + AMSH* (0.5 µM recombinant AMSH*) or DUB buffer + USP21 (0.25 µM recombinant USP21). The samples were then incubated at 37 °C for 1 hour while shaking at 1200 rpm. Subsequently, the reactions were neutralised by addition of 50 µL LSB and incubation at 95 °C for 10 min. Eluted proteins were transferred to clean reaction tubes and separated by SDS-PAGE or stored at -20 °C.

Met1-Ub pulldown assay

One 10 cm dish per condition of HOIP-reconstituted HCT-116 HOIP KO cells or one 15 cm dish per condition of immortalised MEFs was seeded as mentioned before. Samples used to investigate HOIP catalytic activity were pre-treated with 3 µM HOIPIN-8 for 30 min. Cells were treated at ~90% confluency with 10 ng/mL recombinant human or mouse TNF or with 200 ng/mL L18-MDP (Invivogen, tlrl-lmdp) for various time points and were subsequently washed with cold PBS. Afterwards, cells were lysed by adding 500 µL (10 cm dishes) or 1 mL (15 cm dishes) of TUBE lysis buffer (20 mM Na_2HPO_4, 20 mM NaH_2PO_4, 1% IGEPAL CA-630, 2 mM EDTA, supplemented with 1 mM DTT, 0.5 mg/mL NEM and 1 tablet each of cOmplete protease inhibitor and PhosSTOP phosphatase inhibitor added per 10 ml of buffer) and scraped from the dishes using a cell lifter. The cells were incubated on ice,

centrifuged and input fractions were taken as mentioned before. The remaining supernatants were supplemented with 5 µg per sample of biotinylated Met1-Ub TUBE binder (LifeSensors, UM306) and 10 µL of pre-washed streptavidin magnetic beads were incubated overnight at 4 °C on a rotating wheel. Alternatively, in some experiments the supernatants were supplemented with 40 µg Halo-OTULIN 80-348 C129A Met1-Ub binder (M1-Trap)[5] and 20 µL of pre-washed Halo-beads instead. On the following day, samples were washed four times with TUBE lysis buffer before eluting with 50 µL of LSB and incubation at 95 °C for 10 min. Samples used for on-bead ubiquitin chain digestion were incubated in 50 µL DUB buffer supplemented with 5 µM OTULIN 80-352 for 10 min at 37 °C prior to elution. Eluted proteins were transferred to clean reaction tubes and separated by SDS-PAGE or stored at -20 °C.

In vitro LUBAC ubiquitination assay

One 15 cm dish per condition of immortalised MEFs was seeded as mentioned before and 4.5 µg of HOIP antibody (R&D Systems, AF8039) was incubated with 45 µL Protein G magnetic beads in 500 µL Co-IP buffer overnight at 4 °C on a rotating wheel. On the following day, cells were washed once with PBS and lysed with 1 mL of Co-IP buffer supplemented with 1 mM DTT and 1 tablet of PhosSTOP phosphatase inhibitor. Lysates were incubated on ice, centrifuged and input fractions were taken from supernatants as mentioned before. The remaining supernatants were incubated with the HOIP antibody pre-incubated Protein G magnetic beads for 2 hours at 4 °C on a rotating wheel. Afterwards, the beads were washed three times with Co-IP buffer and resuspended in 1 mL of DUB buffer. Bead solutions were split into two equal fractions and resuspended in 30 µL of DUB buffer alone or DUB buffer + 1 µM USP21 and incubated at 37 °C for 30 min. Then, beads were washed six times with Co-IP buffer and two times with Ub reaction buffer (20 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 2 mM DTT) and were subsequently split into four equal fractions. Ubiquitination reactions were initiated by addition of 30 µL Ub reaction buffer supplemented with 10 µg/mL UBE1 (Enzo, BML-UW9410), 7.5 µg/mL UbcH7 (Bio-Techne, E2-640), 100 µg/mL ubiquitin, and 2 mM ATP and the samples were incubated at 30 °C for different time points. To test for potential residual USP21 activity, 30 µL Ub reaction buffer supplemented with 1 µg of Met1-linked tetra-Ub (Enzo, BML-UW0785) was used as a control sample. Reactions were stopped immediately after incubation by addition of

10 µL 6x LSB and incubation at 95 °C for 10 min. Eluted proteins were transferred to clean reaction tubes and separated by SDS-PAGE or stored at -20 °C.

Immunoprecipitation of endogenous FADD

One 10 cm dish per condition of HCT-116 HOIP KO and HOIP-reconstituted cells was seeded at least 24 hours before start of treatment to allow full attachment to the surface. Cells were pre-treated at ~90% confluency with 20 µM zVAD-fmk for 30 min and subsequently stimulated with 10 ng/mL recombinant human TNF for 0, 3 or 6 hours. Plates were washed once with PBS and afterwards lysed with 500 µL Co-IP buffer, incubated on ice and centrifuged as mentioned before. Input fractions were taken and the remaining supernatants were incubated with 1.35 µg of FADD antibody (abcam, ab108601) or isotype control overnight at 4 °C on a rotating wheel. On the next day, 15 µL per sample of pre-washed Protein G magnetic beads were added and the samples were incubated at 4 °C on a rotating wheel for 4 hours. Afterwards, samples were washed five times with Co-IP buffer and proteins were eluted from the beads by addition of 50 µL LSB and incubation at 95 °C for 10 min. Eluted proteins were transferred to clean reaction tubes and separated by SDS-PAGE or stored at -20 °C.

Expression and immunoprecipitation of exogenous NEMO constructs

One 10 cm dish per condition of HCT-116 HOIP-reconstituted cells was seeded 24 hours before transfection. On the following day, cells were transfected with 7 µg of either pcDNA3.1 vehicle, HA-tagged NEMO or HA-tagged NEMO R316E + R319E + V414E using 9 µL JetOPTIMUS transfection reagent following the manufacturer's instructions. After 24 hours, plates were washed once with PBS and subsequently lysed with 500 µL TNF lysis buffer, incubated on ice and centrifuged as mentioned before. Input fractions were taken, and the remaining supernatants were incubated with 15 µL per sample of pre-washed HA-beads overnight at 4 °C on a rotating wheel. On the following day, samples were washed four times with TNF lysis buffer before eluting with 50 µL of LSB and incubation at 95 °C for 10 min. Eluted proteins were transferred to clean reaction tubes and separated by SDS-PAGE or stored at -20 °C.

Nuclear translocation assay

HCT-116 HOIP-reconstituted cells or immortalised MEFs were seeded on 24-well plates at least 24 hours before start of treatment to allow full attachment to the surface and treated at ~90% confluency with either 10 ng/mL recombinant human or mouse TNF. At the indicated timepoints the cells were trypsinised using 1x TrypLE Select (Thermo Fisher Scientific, A1217702). The cells were then washed once with ice-cold PBS and the nuclear or cytoplasmic fractions were prepared using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, 78833) following the manufacturer's instructions assuming a packed cell volume of 15 µL.

RNA isolation, reverse transcription and RT-qPCR

Reconstituted HCT-116 or immortalised MEFs cells were seeded on 24-well plates and treated at ~90% confluency with either 10 ng/mL recombinant human or mouse TNF, 200 ng/mL of L18-MDP, or 10 ng/mL of recombinant human or mouse Il-1β. At the indicated timepoints the cells were directly lysed in 350 µL of PureLink™ RNA Mini Kit lysis buffer supplemented with β-mercaptoethanol and the total RNA was isolated using PureLink™ RNA Mini Kit (Thermo Fisher Scientific, 12183025) following the manufacturer's instructions. cDNA was prepared from approximately 500 ng of isolated total RNA using iSCRIPT cDNA synthesis kit (BioRad, 1708891) following the manufacturer's protocol. SSoAdvanced Universal SYBR Green (BioRad, 1725272) was used for RT-qPCR reactions, which were analysed using the BioRad CFX 384 OPUS system. Relative gene expression was calculated using the 2^{-dCt} method relative to *Actb* for mouse genes or *HPRT* for human genes. The following primers were used for the RT-qPCR reactions:

Species	**Gene**	**Forward primer 5' - 3'**	**Reverse Primer 5' - 3'**
Mouse	*Actb*	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT
Mouse	*Nfkbia*	TGAAGGACGAGGAGTACGAGC	TTCGTGGATGATTGCCAAGTG
Mouse	*Tnfaip3*	ACCATGCACCGATACACGC	AGCCACGAGCTTCCTGACT
Mouse	*Tnf*	GACGTGGAACTGGCAGAAGAG	TTGGTGGTTTGTGAGTGTGAG
Mouse	*Cxcl2*	CCAACCACCAGGCTACAGG	GCGTCACACTCAAGCTCTG
Human	*HPRT*	AGCCAGACTTTGTTGGATTTGA	TTTACTGGCGATCTCAATACG
Human	*TNF*	TGCTGCAGGACTTGAGAAGA	GAGGAAGGCCTAAGGTCCAC
Human	*TNFAIP3*	ATGCACCGATACACACTGGA	GGATGATCTCCCGAAACTGA
Human	*NFKBIA*	GCTGATGTCAATGCTCAGGA	CCCCACACTTCAACAGGAGT

Human	*CXCL8*	TCTGGCAACCCTAGTCTGCT	AAACCAAGGCACAGTGGAAC
Human	*CCL20*	AAGTTGTCTGTGTGCGCAAATCC	CCATTCCAGAAAAGCCACAGTTTT

Measurement of serum cytokines

At the indicated time points, mice were euthanised and blood was collected in reaction tubes that were left at room temperature for 30 minutes and then centrifuged at 15,000 x g for 10 min to isolate the serum fraction, which was then stored at -20 °C. The collected serum was used for cytokine measurements using custom U-Plex assays from MSD and following the manufacturer's guidelines. Undetected measurements were set to limit of detection concentrations for each assay.

Organ isolation and tissue processing

At the indicated time points, mice were euthanized by cervical dislocation and the organs were immediately isolated. Liver lobes were directly fixed in 4% PFA (Thermo Fisher Scientific, J19943.K2), small or large intestines were flushed once with ice-cold 1x HBSS (Thermo Fisher Scientific, 14180046) and fixed in 4% PFA. Spleens were collected in R10, which was made from RPMI 1640 (Thermo Fisher Scientific, 11554516) supplemented with 20 mM HEPES, 1x Penicillin-Streptomycin and 10% FBS. Splenocytes were prepared by finely chopping the spleens in 5 mL of R10 containing 0.5 mg/mL Collagenase IV (Merck, C5138) and 20 µg/mL DNase I (Merck, 10104159001) and incubating at 37 °C for 45 min with magnetic stirring at 800 rpm. The isolated total splenocytes were collected in equal volume of cold R10, filtered through a 70 µm filter and then pelleted by centrifugation at 500 x g and 4 °C for 5 min. Red blood cells were then lysed by resuspending the splenocyte pellet in 1x ice-cold RBC lysis buffer (Biolegend, 420301) at 1 mL/spleen and incubating on ice for 5 min. The reaction was neutralized by mixing with 4 mL R10 and the cells were pelleted by centrifugation at 500 x g and 4 °C for 5 min. The cells were resuspended in 5 mL R10, filtered through a 40 µm filter and then counted using a cell counter (Sysmex). The isolated splenocytes were then stained and analysed by flow cytometry.

Flow cytometry staining and acquisition

Unless otherwise indicated, 3 million splenocytes were used for flow cytometry staining and analysis. Briefly, the cells were collected in 96-well U-bottom plates and

incubated with FC-block (BD Biosciences, 553141) in PBS at 1:100 dilution for 15 min on ice. For the staining of myeloid cells, a 100 µL antibody mix in FACS buffer (3% FBS in PBS) was then added to the cells and incubated for 30 min on ice. The cells were then washed once and resuspended in 250 µL FACS buffer. For the discrimination of dead cells, 30 µL of 10 mg/mL DAPI solution in PBS was added to cells 5 min prior to analysis by flow cytometry. For the staining of lymphoid cells and transcription factors, the cells were first stained with Fixable Viability Stain 700 (BD Biosciences, 564997, 1:1000) together with FC-block as stated above. The cells were washed once and resuspended in 100 µL of the surface antigen antibody mix in FACS buffer. The cells were incubated for 30 min on ice and washed once. Subsequently, the cells were fixed for 2 hours on ice using 100 µL of 1x fixation/permeabilisation buffer from the eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set (Thermo Fisher Scientific, 00-5523-00). The cells were then washed once using 1x permeabilisation buffer from the same kit and stained overnight at 4 °C in 100 µL of the transcription factor antibody mix in 1x permeabilisation buffer. The cells were then washed once using the permeabilisation buffer and resuspended in 250 µL FACS buffer followed by flow cytometry analysis. The samples were analysed using BD LSRFortessa™ X-20 and analysed using FlowJo V10.10.

Yolk sac staining

Yolk sacs from E10.5 embryos were dissected, and each yolk sac was placed in a well of a 24-well plate containing 0.5 mL of 4% PFA (Thermo Fisher Scientific, J19943.K2) and incubated overnight at 4 °C shaking. The fixed yolk sacs were washed 3 times with PBS and afterwards permeabilised for 1 hour shaking at room temperature in 0.2% TritonX-100 (Merck, T8787) in PBS supplemented with 3% FBS. The yolk sacs were then blocked for 2 hours shaking at room temperature in 0.2% TritonX-100 (Merck, T8787) in PBS supplemented with 10% goat serum. Subsequently, the samples were stained with primary antibodies overnight at 4 °C shaking. The yolk sacs were then washed 3 times with PBS and stained with secondary antibodies for 2 hours shaking at room temperature then washed 3 times with PBS. Afterwards, the samples were incubated with DAPI (Merck, MBD0015) at 1 µg/mL for 15 min at room temperature and washed once with PBS. The stained yolk sacs were transferred to glass slides and carefully opened and spread on the slide and excess liquid was blotted away. The yolk sacs were then overlayed with ProLong™ Glass Antifade

Mountant (ThermoFisher, P36980), covered with coverslips and let to cure. Slides were imaged using a LSM 900 confocal microscope (Zeiss).

Tissue clearing and whole embryo imaging

E10.5 embryos were fixed in 4% PFA overnight at 4 °C and subsequently washed three times with PBS. The embryos were cleared and stained following the iDISCO protocol[6] using and incubation (n) number of 1 (https://idisco.info/idisco-protocol/). For the staining of nuclei, SYTOX™ Deep Red Nucleic Acid Stain (Thermo Fisher Scientific, S11380) was used at a concentration of 2 µM. The stained embryos were embedded in 1.5% low melt agarose (Thermo Fischer Scientific, 16520050) solution in water and then cleared as written in the protocol. The embedded and cleared embryos were imaged using a Lightsheet 7 microscope (Zeiss). Images were deconvoluted, stitched and corrected for chromatic aberration using Huygens software.

Isolation of RNA, DNA and protein from embryos and RNA-sequencing analysis

Embryos were collected in Qiagen Allprotect tissue reagent (Qiagen, 76405) and total RNA, DNA and Protein were subsequently prepared using Qiagen AllPrep DNA/RNA/Protein Mini Kit (Qiagen, 80004) following the manufacturer's instructions. DNA was eluted in EB Buffer; RNA was eluted in water and the precipitated protein was redissolved in 5% SDS solution. The isolated total RNA was then used for RNA sequencing library prep by BGI, Hong Kong. The amplified library was paired end sequenced on a DNBSEQ platform at a read depth of 40 million reads/sample and 150 bases read length. The raw fastq files were processed to remove adapters and low-quality reads to achieve a Phred quality score of at least 33. The data was then aligned using STAR[7] aligner and mapped to the reference mouse genome "Mus_musculus.GRCm39.107.gtf". The aligned reads were counted using Rsubreads and analysed using Deseq2[8] on R version 4.4.1. Genes were considered as differentially expressed genes (DEGs) based on a cutoff of adjusted p value lower than 0.05 and Log2 fold change of above 1 or below -1. The Entrez numbers for the DEGs were then used to for analysis of promoter regions by HOMER[9] enriching for motifs from 500 bases upstream to 100 bases downstream of the transcriptional start site.

Expression and purification of HOIP variants

Rosetta 2 (DE3) competent cells (Merck, 71400-M) were transformed with either human HOIP 1-184 in expression vector pOPINB or mouse HOIP 1-184 WT, N101D or N101A in expression vector pET (Vectorbuilder), respectively. Overnight cultures were prepared from the transformed cells which were then used to inoculate one litre per construct of LB media containing 100 µg/mL ampicillin and 34 µg/mL chloramphenicol. Bacterial cultures were grown in a shaking incubator at 37 °C and 180 rpm until OD_{600} of 0.8 was reached. Temperature was reduced to 18 °C and overnight protein expression was induced by the addition of 400 µM IPTG (Merck, I6758). Cells were harvested on the following day by centrifugation at 3400 x g for 45 min and bacterial cell pellets were frozen at -80 °C.

For the purification of the expressed protein constructs, bacterial cell pellets were thawed and solubilized in 25 mL protein lysis buffer (20 mM Tris pH 8.0, 300 mM NaCl, 20 mM imidazole pH 7.5, 5% glycerol, 2 mM DTT) supplemented with DNAse I, lysozyme and 1 tablet of cOmplete protease inhibitor. The cell suspensions were further lysed by sonication for 10 min and subsequently centrifuged at 21000 x g and 4 °C for 60 min. The supernatants were additionally filtered with a 0.22 µm filter and afterwards transferred to 50 mL Superloops (Cytiva, 18111382) and loaded on a 5 mL HisTrap FF column (Cytiva, 17525501), which was operated through an ÄKTA go protein purification system (Cytiva). The expressed proteins were eluted in fractions from the HisTrap column with elution buffer (20 mM Tris pH 8.0, 300 mM NaCl, 250 mM imidazole, 5% glycerol, 2 mM DTT) and fractions containing the target proteins were pooled after identification by SDS-PAGE and Coomassie InstantBlue (abcam, ab119211) staining. The pooled fractions were supplemented with 2.5 µg/mL of 3C protease for 6His-tag removal (Merck, SAE0045) and dialysed against dialysis buffer (20 mM Tris pH 8.0, 300 mM NaCl, 5% glycerol, 2 mM DTT) overnight at 4 °C using Slide-A-Lyzer dialysis cassettes (Thermo Fisher Scientific, 66810).

To remove the cleaved 6His-tag, the protein solutions were loaded again on 5 mL HisTrap FF columns from which the flow-through fractions were collected that contained the target proteins. Peak protein fractions were identified through SDS-PAGE and Coomassie InstantBlue staining and pooled respectively. The protein solutions were concentrated to 5 mL volume and further purified on a HiLoad 16/600 Superdex 75 pg column (Cytiva, 28989333) using size-exclusion buffer (20 mM Tris pH 8.0, 200 mM NaCl, 5% glycerol, 4 mM DTT). Peak protein fractions containing highly purified proteins were identified by SDS-PAGE and Coomassie InstantBlue

staining and subsequently concentrated to 1-2 mL volumes using Amicon concentration tubes with 10 kDa MWCO (Merck, UFC801008). Protein concentrations were measured by absorbance at 280 nm and flash frozen in liquid nitrogen for storage at -80 °C.

Expression and purification of Halo-OTULIN 80-348 C129A Met1-Ub binder

Rosetta 2 (DE3) competent cells were transformed with OTULIN 80-348 C129A Met1-Ub binder (M1 Trap) in expression vector pGEX. Overnight cultures were prepared from the transformed cells which were then used to inoculate four litres of LB media containing 50 µg/mL kanamycin. Bacterial cultures were grown in a shaking incubator at 37 °C and 180 rpm until OD_{600} of 0.8 was reached. Temperature was reduced to 18 °C and overnight protein expression was induced by the addition of 400 µM IPTG. Cells were harvested on the following day by centrifugation at 3400 x g for 45 min and bacterial cell pellets were frozen at -80 °C.

For the purification of the Met1-Ub binder, bacterial cell pellets were thawed and solubilized in 40 mL protein lysis buffer supplemented with DNAse I, lysozyme and 1 tablet of cOmplete protease inhibitor. The cell suspensions were further lysed by sonication for 10 min and subsequently centrifuged at 21000 x g and 4 °C for 60 min. The supernatants were additionally filtered with a 0.22 µm filter and afterwards transferred to 50 mL Superloops and loaded on a 5 mL HisTrap FF column which was operated through an ÄKTA go protein purification system. The expressed proteins were eluted in fractions from the HisTrap column with elution buffer and fractions containing the target proteins were pooled after identification by SDS-PAGE and Coomassie InstantBlue staining. The pooled fractions were dialysed against dialysis buffer overnight at 4 °C using Slide-A-Lyzer dialysis cassettes. Dialyzed protein fractions were concentrated to 3 mL volume using Amicon concentration tubes with 30 kDa MWCO (Merck, UFC203024). Protein concentrations were measured by absorbance at 280 nm and flash frozen in liquid nitrogen for storage at -80 °C.

AlphaFold multimer modelling

The amino acid sequences of murine SHARPIN, HOIP, HOIL-1 and OTULIN were retrieved from uniprot.org and used as input for modelling LUBAC folding in complex with OTULIN using AlphaFold multimer on Google Colab (Available at https://colab.research.google.com/github/deepmind/alphafold/blob/main/notebooks/A

lphaFold.ipynb). The model was run using A100 GPU runtime with “run_relax“ (amber relax) and “relax_use_gpu” options selected. The multimer model recycling was set to max_num 10 to avoid session timeout. The predicted model was downloaded and visualized locally using ChimeraX software.

Fluorescence polarisation assay

Serial dilutions were prepared of the different HOIP PUB domains (1-184) using dilution buffer (20 mM Tris pH 8.0, 200 mM NaCl, 5% glycerol, 4 mM DTT) and were mixed in equal volumes with 100 nM of either 5(6)-carboxyfluorescein-labelled OTULIN (48-67) or SPATA2 (334-344) PIM peptides (JPT Peptide Technologies). Fluorescence polarisation was measured on a CLARIOstar Plus plate reader using bandpass filters at 482 and 530 nm (BMG LABTECH). Detected values were exported from MARS and the respective binding parameters were calculated with a one-site binding model in GraphPad Prism 10.

Isothermal titration calorimetry

HOIP PUB domains (1-184) were dialyzed overnight at 4 °C against PBS (1.5 mM KH_2PO_4, 155 mM NaCl, 2.7 mM Na_2HPO_4) supplemented with 1% DMSO to match the dilution buffer of unlabelled OTULIN PIM peptides (48-67) (JPT Peptide Technologies). PUB domains and OTULIN peptides were degassed for 20 min to remove residual air from the solutions. 300 µL of OTULIN peptide solution was loaded into the sample cell and 300 µL of degassed ultrapure water was loaded into the reference cell of a Nano ITC Low Volume isothermal titration calorimeter (TA Instruments). The injection syringe was loaded with 50 µL of 200 µM HOIP PUB domain. A total of 25 injections with 2.02 µL injection volume each were monitored per reaction. Thermodynamical parameters were determined for each reaction with an independent sample model in NanoAnalyze (TA Instruments).

Cell lysis, protein extraction and digestion for mass spectrometry

One 10 cm dish per condition and repeat of HOIP-reconstituted HCT-116 HOIP KO cells was seeded at least 24 hours before start of treatment to allow full attachment to the surface. Cells were treated at ~90% confluency with either fresh media or 10 ng/mL recombinant human TNF diluted in fresh media for 5 and 15 min and were subsequently washed with cold PBS. The cells were then lysed with 500 µL of boiling

lysis buffer (5% SDS, 100 mM Tris pH 8.5, 5 mM TCEP, and 10 mM CAA) and incubated at 95 °C for 10 minutes with mixing (1000 rpm). Lysates were sonicated with a probe sonicator (2 min, 1 second on, 1 second off, 80% amplitude, probe #423A for Fisherbrand™ Model 120 Sonic Dismembrator). Protein concentration was calculated by BCA assay. 200 µg of protein was digested using the Protein Aggregation Capture protocol[10] in the King-fisher Robot[11]. Briefly, proteins were resuspended with acetonitrile to a final 70% concentration. MagReSyn® Hydroxyl beads were added in a proportion 1:2 (protein:beads). Protein aggregation was performed in two steps of 1 min mixing at 1000 rpm, followed by 10 min pause each. Beads were subsequently washed three times with 1 ml 95% ACN and two times with 1 mL 70% EtOH. 300 µL of digestion buffer (50 mM Ammonium Bicarbonate) and proteases were added in the following proportions: trypsin 1:250 (enzyme:protein) and lysC 1:500 (enzyme:protein). Digestion was carried out overnight at 37 °C with looping mixing. Digested peptides were acidified after digestion with TFA to a final 1% concentration. An aliquot corresponding to an estimated amount of 0.3 µg was loaded on evotips for proteome analysis. The rest was loaded into a Sep-Pak tC18 96-well Plate (40 mg Sorbent per Well, Waters) for desalting. Peptides were eluted with 150 µL 40% ACN, followed by 150 µL 40% ACN. The combined eluate was reduced by SpeedVac (Eppendorf, Germany) and the final peptide concentration was estimated by measuring absorbance at 280 nm on a NanoDrop 2000C spectrophotometer (Thermo Fisher Scientific).

Enrichment of phosphorylated peptides

For phospho-enrichment, 60 µg of peptides were resuspended with 200 µL of Loading buffer (80% ACN; 5% TFA, 0.1 M Glycolic Acid). Phospho-enrichment was performed in the Kingfisher robot using 5 µL of MagReSyn® Zr-IMAC HP beads (20 mg/ml)[12]. Briefly, the 96-well comb is stored in plate #1, 10 µL of Ti-IMACHP beads in 100% ACN in plate #2 and loading buffer in plate #3. Plates 5-7 are filled with 500 µL washing solutions; WB1 (Loading buffer), WB2 (80% ACN, 5% TFA), and WB3 (10% ACN, 0.2% TFA) respectively. Plate #8 contains 200 µL of 1% ammonia for elution. The beads were washed in loading buffer for 5 min at medium mixing speed, followed by binding of the phospho-peptides for 20 minutes and medium speed. The sequential washes were performed in 2 min and fast speed. Phosphopeptides were eluted in 10

minutes at medium mixing speed. Enriched phosphopeptides were acidified with 10% TFA until pH < 3 and loaded into Evotips for subsequent MS analysis.

LC-MS/MS Analysis

The samples were eluted online using an Evosep One system (Evosep Biosystems)[13] and separated using an Evosep 8 cm (Evosep, EV1109) performance column connected to a steel emitter (Evosep, EV1086) and heated to 40 °C. The gradient method “110 SPD” was used for proteome analysis, and the “60 SPD” (21 min) was used for phosphoproteome analysis.

The samples were analysed on an Orbitrap Astral Mass Spectrometer (Thermo Fisher Scientific)[14] applying a spray voltage of 1800 (proteome) or 2000 V (phosphoproteome), with funnel radio frequency level at 40, and a heated capillary temperature set to 280 (proteome) or 275 °C (phosphoproteome). The mass spectrometer was operated in positive mode. Full scan precursor spectra (proteome: 380-980 m/z; phosphoproteome: 480–1080 m/z) were recorded in profile mode using a resolution of 240,000 at m/z 200, a normalized automatic gain control (AGC) target of 500%, and a maximum injection time of 3 (proteome) or 30 ms (phosphoproteome). Isolated precursors were fragmented in the HCD cell using 25% normalized collision energy, a normalized AGC target of 500%, and a maximum injection time of 3 (proteome) or 4 ms (phosphoproteome). The fragment spectra were acquired in data-independent acquisition (DIA) mode, with a fragment mass range of 150 to 2000 m/z. The isolation window was set to 3 (proteome) or 4 m/z (phosphoproteome) without overlap.

Mass spectrometry raw data processing

Proteome raw data were analysed on DiaNN[15] with standard settings, except the ones described below. The predicted library was generated in DiaNN version 1.9. The Human Uniprot fasta file (downloaded in May 2024; 20,434 entries) was supplemented with a contaminant fasta file containing 370 entries[16], downloaded from: https://github.com/HaoGroup-ProtContLib. “FASTA digest for library-free search / library generation” and “Deep learning-based spectra, RTs and IMs prediction” were selected. Precursor charge range was set to 2-4. Precursor mass range was set to 380-980 m/z. Fragment ion mass range was set to 150-2000 m/z. Raw MS data were searched in DiaNN version 1.9.2 using the predicted library with MBR.

Phosphoproteome raw data were analysed on Spectronaut[17] version 19 in directDIA mode with the standard settings besides the ones described below. The same fasta files discussed above were used. Phosphorylation of serine, threonine and tyrosine as variable modification, together with N-terminal acetylation and oxidation of methionine. Quantification was performed using the automatic setting, which was Quant 2.0 in this Spectronaut version. Cross-run normalisation and imputation were not enabled.

Bioinformatic analysis of proteomic data

Bioinformatic analysis of proteomics data was performed using R (version 4.4.1) with R studio (version 2024.09.0+375).

For proteome analysis, the "pr_matrix.tsv" was used for the analysis of RNF31 precursors. For protein expression analysis, the "pg_matrix.tsv" was used. Data were log2-transformed and filtered by removing contaminants and IDs without gene name. As 11 runs were run separately from the other 61 due to technical issues, the batch effect was removed through the Limma R package[18]. Significance analysis was performed using Limma-trend[19, 20], after removing rows with more than 50% missing values. P values were corrected using the Benjamini–Hochberg procedure.

For phosphoproteome analysis, the PTM site report pivot was exported from the Spectronaut "sne" file. Data were log2-transformed and filtered by removing contaminants, IDs without gene name and other modifications than phosphorylation. Negative quantification values were replaced by NA. Only one row per protein group was kept. The run "KO_Ctrl_05min_d1_1" was removed as it held fewer identification compared to the others. Data were filtered by removing rows with more than 50% missing values and rows with less than 4 values in at least one of the following experimental groups: WT Ctrl, WT TNF, KO Ctrl, KO TNF, N102D Ctrl, N102D TNF. Data were median normalised by using the R package DAPAR[21]. Significance analysis was performed using Limma-trend. P values were corrected using the Benjamini–Hochberg procedure. Heatmaps were performed by using the R package pheatmap. Proteins belonging to TNF and NF-κB pathways were retrieved using the R package KEGGREST.

Data availability statement

Raw mass spectrometry files have been uploaded to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner

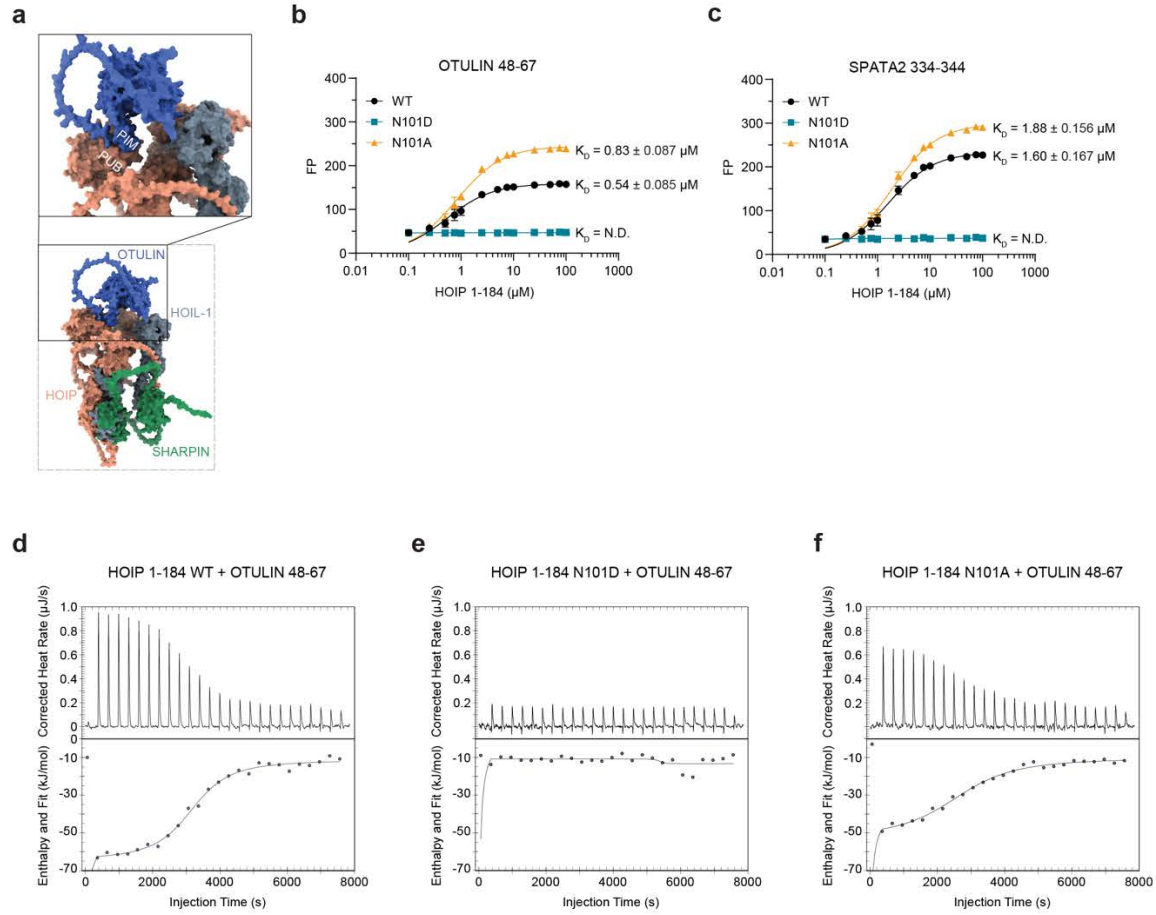
repository[22] with the dataset identifier PXD060649 (Username: reviewer_pxd060649@ebi.ac.uk; Password: 1AWmHrY4sa8a). All the scripts utilised for data analysis and plotting are available at the following GitHub repository: https://github.com/Giu-F/TNF_project.

Statistical analysis

Statistical significances were determined using Mixed-effects ANOVA with Fisher's LSD multiple comparison, Mann-Whitney U-test, Gehan-Breslow-Wilcoxon test, or Mixed-effects ANOVA with Tukey's multiple comparisons test. All statistical tests were analysed using Graphpad Prism 10, except for statistical analysis of RNAseq data that was tested using DEseq2 or fgsea packages for differential gene expression or pathway enrichment analysis, respectively.

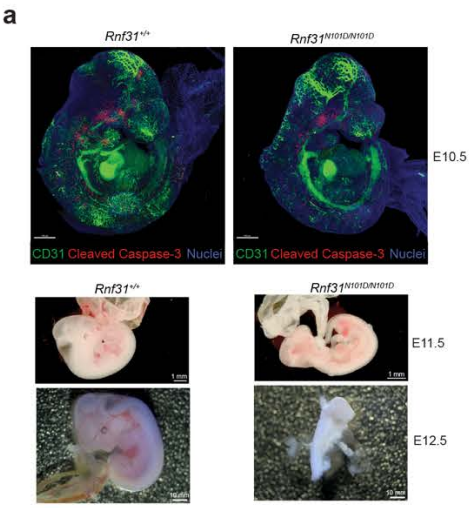
Supplementary Figures and Figure legends

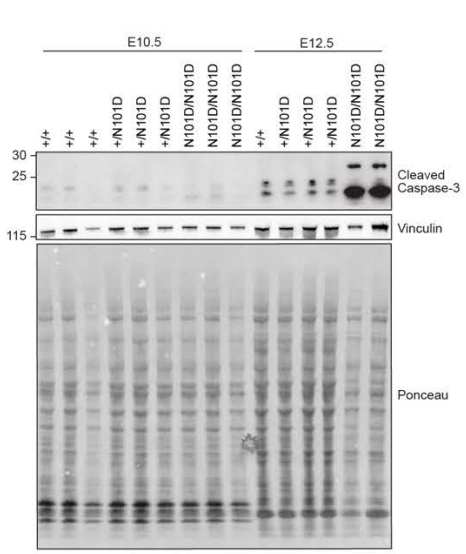
Supplementary Figure 1



Supplementary Figure 1: Characterisation of human and murine HOIP PUB domain mutations.
(a) AlphaFold multimer modelling of LUBAC components HOIP (orange), HOIL-1 (grey) and SHARPIN (green) in complex with OTULIN (blue). Close-up view of the interaction of OTULIN PIM with HOIP PUB domain. **(b** and **c)** Binding affinities of HOIP (1-184) WT, N101D and N101A to 5(6)-carboxyfluorescein-labelled OTULIN 48-67 **(b)** and SPATA2 334-344 **(c)** PIM peptides determined by fluorescence polarisation. Error bars and calculated dissociation constants K_D are shown as mean ± SEM (n = 3). **(d-f)** Thermograms (upper plots) and binding isotherms (lower plots) of HOIP (1-184) WT **(d)**, N101D **(e)**, and N101A **(f)** with OTULIN 48-67 determined by isothermal titration calorimetry (n = 4 for WT and N101D; n = 3 for N101A).

Supplementary Figure 2





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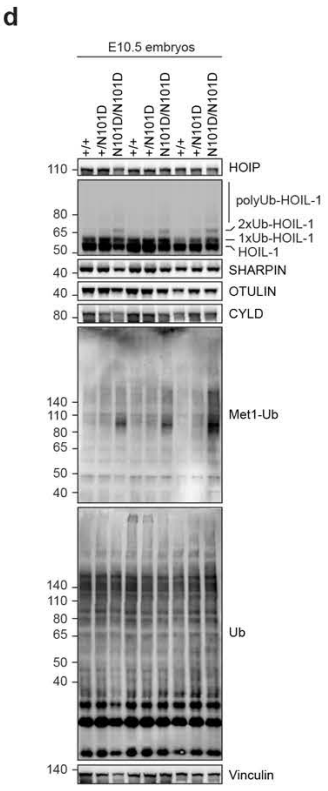
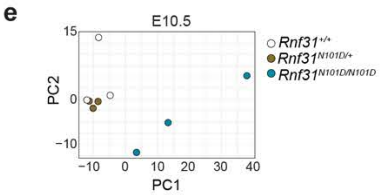
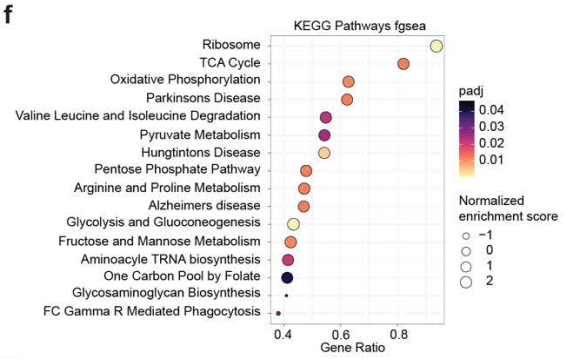
Otulin$^{Y56A/+}$ *Spata2*$^{-/-}$ X *Otulin*$^{Y56A/+}$ *Spata2*$^{+/-}$			
Genotype	Expected frequency (%)	Observed frequency (%)	Number of mice
Otulin$^{+/+}$ *Spata2*$^{+/-}$	12.5	14.8±6.4	10
Otulin$^{+/+}$ *Spata2*$^{-/-}$	12.5	11.0±4.3	8
Otulin$^{Y56A/+}$ *Spata2*$^{+/-}$	25	33.4±9.0	19
Otulin$^{Y56A/+}$ *Spata2*$^{-/-}$	25	20.2±5.5	14
Otulin$^{Y56A/Y56A}$ *Spata2*$^{+/-}$	12.5	13.3±4.0	9
Otulin$^{Y56A/Y56A}$ *Spata2*$^{-/-}$	12.5	7.2±3.1	5
litters: 9, breeding pairs: 2			65







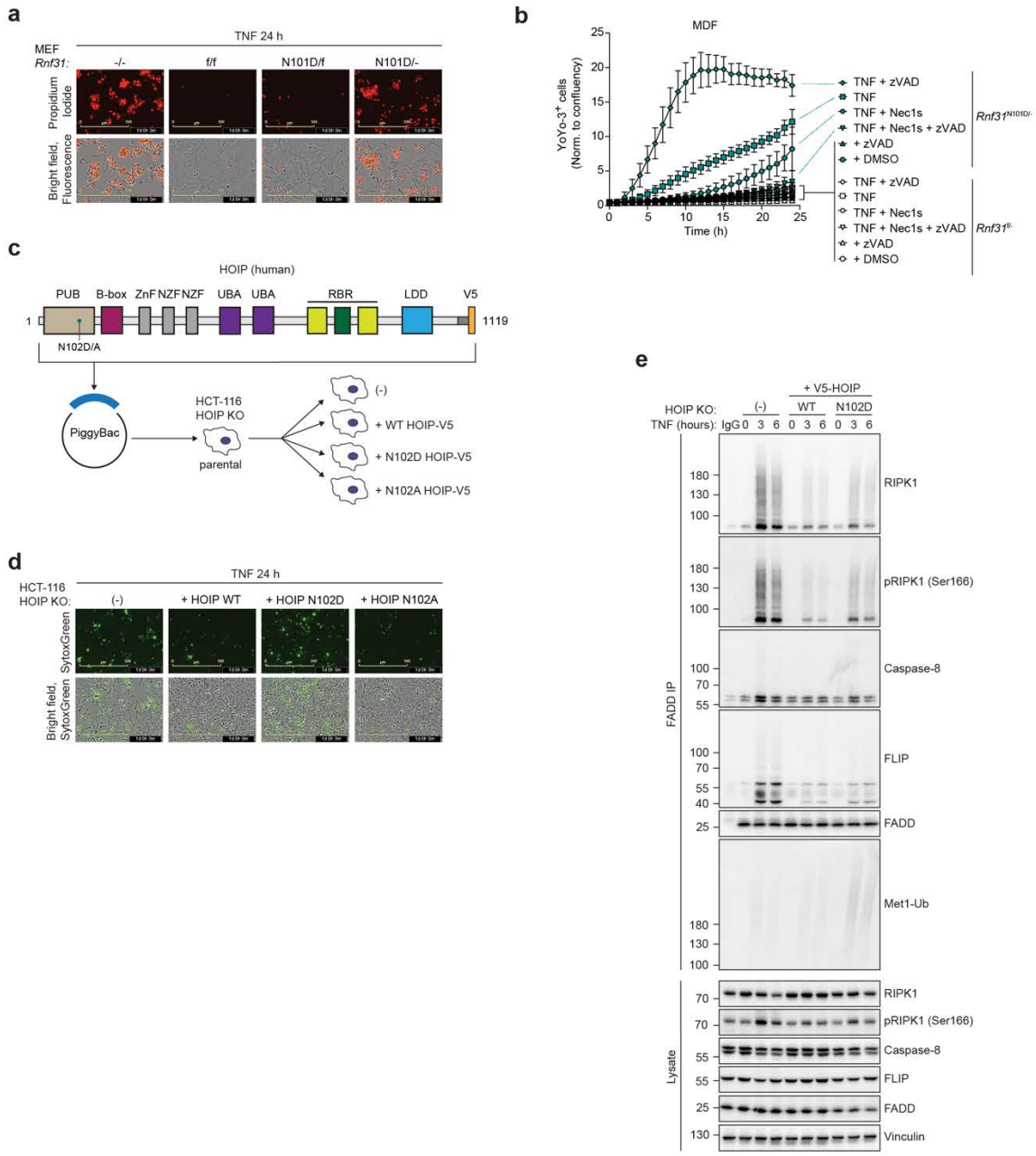
g

Rank	Motif	Name	P value	targets sequences with motif	% targets sequences with motif	% background sequences with motif
1		HIF2a(bHLH)/785_O-HIF 2a-ChIP-Seq(GSE34871)	1E-9	170	17.59%	10.35%
2		HIF-1a(bHLH)/MCF7-HIF 1a-ChIP-Seq(GSE28352)	1E-4	97	12.19%	7.77%
3		HIF-1b(HLH)/T47D-HIF1b -ChIP-Seq(GSE59937)	1E-4	311	39.07%	31.87%
4		TATA-Box(TBP)/Promoter	1E-3	269	33.79%	28.37%

Supplementary Figure 2: HOIP N101D alters the metabolic signature in embryos.

(a) Representative light-sheet immunofluorescence imaging of E10.5 embryos stained for Nuclei (blue), CD31 (green) and cleaved Caspase-3 (red) (n = 2 per genotype) and representative light-microscopy imaging of E11.5 and E12.5 embryos.. **(b)** Western blots of protein lysates from *Rnf31*$^{+/+}$, *Rnf31*$^{+/N101D}$ and *Rnf31*$^{N101D/N101D}$ embryos at E10.5 and E12.5 indicating cell death (n = 3 per genotype for E10.5; n = 1 *Rnf31*$^{+/+}$, n = 3 *Rnf31*$^{+/N101D}$, n = 2 *Rnf31*$^{N101D/N101D}$ for E12.5). **(c)** Frequencies from crossings of *Otulin*$^{Y56A/Y56A}$ *Spata2*$^{-/-}$ mice from total of 9 litters and 2 breeding pairs. **(d)** Western blots of protein lysates from *Rnf31*$^{+/+}$, *Rnf31*$^{+/N101D}$ and *Rnf31*$^{N101D/N101D}$ embryos at E10.5 (n = 3 per genotype). **(e** and **f)** PCA **(e)** and KEGG-pathway functional gene set enrichment analysis (fgsea) **(f)** of RNA sequencing data from *Rnf31*$^{+/+}$, *Rnf31*$^{+/N101D}$ and *Rnf31*$^{N101D/N101D}$ embryos at E10.5; normalised enrichment score of pathways enriched in *Rnf31*$^{N101D/N101D}$ vs. *Rnf31*$^{+/+}$. **(g)** Top 4 promoter motifs enriched by Homer analysis of DEGs from *Rnf31*$^{N101D/N101D}$ vs. *Rnf31*$^{+/+}$ RNA sequencing data.

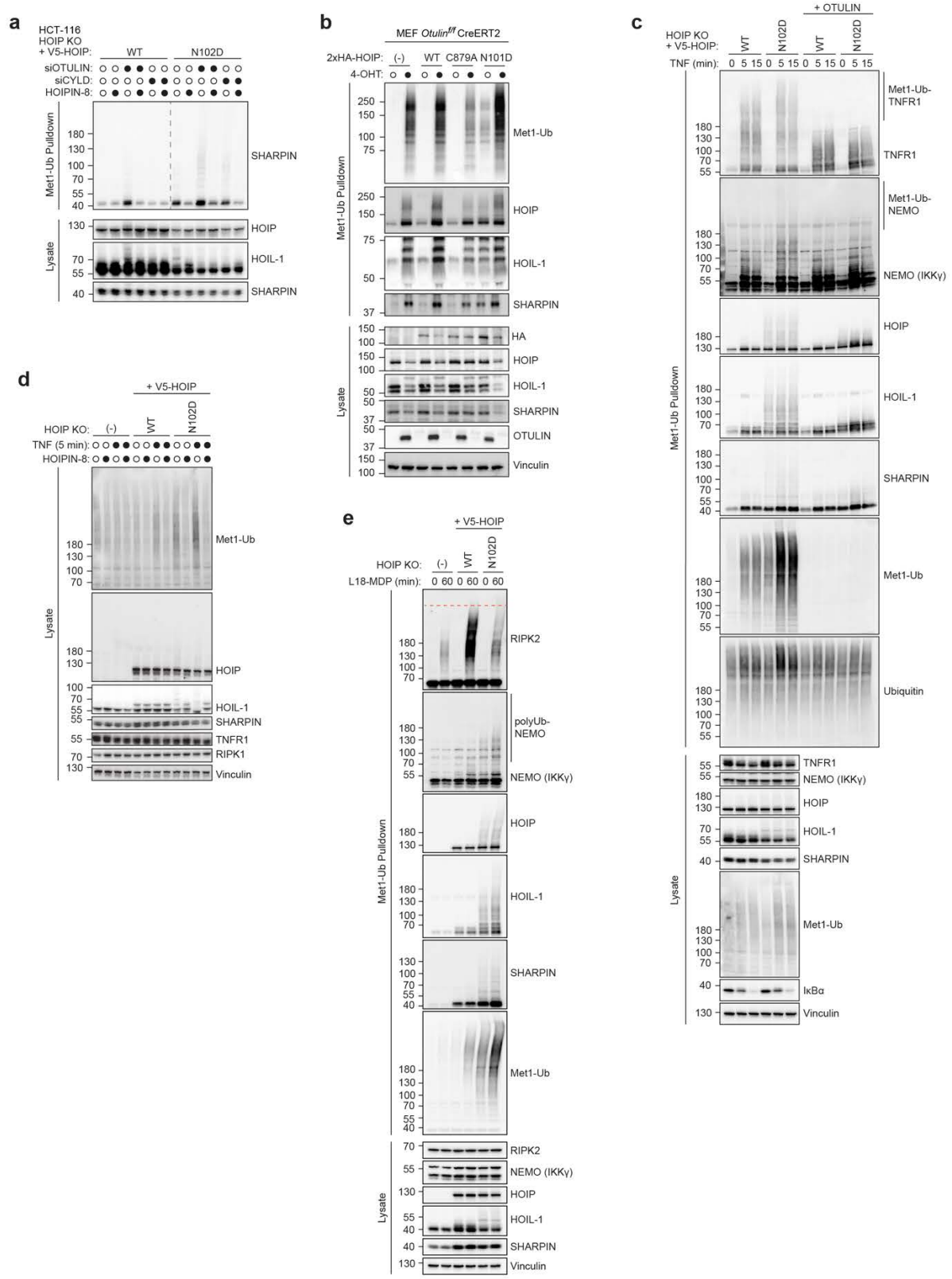
Supplementary Figure 3



Supplementary Figure 3: Disrupted LUBAC PUB-PIM interactions sensitise to TNF-induced cell death in MEFs, MDFs and HCT-116 cells.
(a) Cell death visualised by Propidium Iodide staining in immortalised MEFs after stimulation with recombinant murine TNF for 24 hours. Images are representative of four pictures total taken from different parts of the well in each condition (n = 3). **(b)** Graph indicating YoYo-3^{+} cells normalised to confluency after indicated treatments. Data is presented as mean ± SEM and *P* values by Mixed-effects ANOVA with Tukey's multiple comparisons test (n = 3). **(c)** Schematic workflow of HOIP WT, N102D and N102A reconstitution in HCT-116 HOIP KO cells. Coloured boxes indicate functional domains in human HOIP. **(d)** Cell death visualised by SytoxGreen staining in HOIP-reconstituted HCT-116 HOIP KO cells after stimulation with recombinant human TNF for 24 hours. Images are representative of four pictures from different parts of the well

in each condition (n = 6 for HOIP KO (-), +WT, +N102D; n = 3 for +N102A). **(e)** Immunoprecipitation (IP) of endogenous FADD from HCT-116 HOIP KO cells (-) reconstituted with HOIP WT or N102D, after pre-treatment with zVAD-fmk and stimulation with recombinant human TNF for indicated time points.

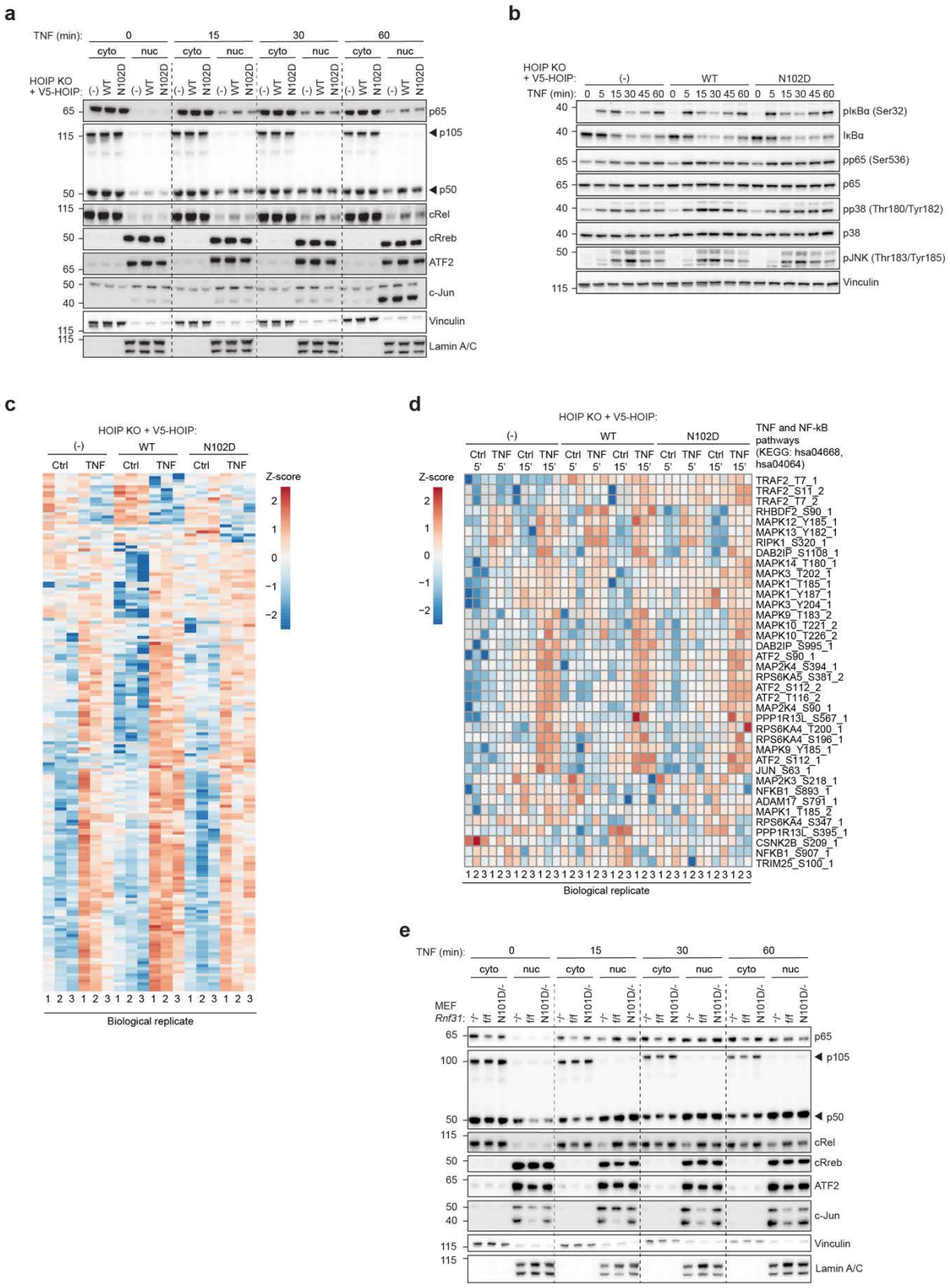
Supplementary Figure 4



Supplementary Figure 4: Ablation of LUBAC PUB-PIM interactions leads to autoubiquitination but retains LUBAC activity.
(a) Met1-Ub Pulldown of LUBAC after siRNA-mediated knockdown of OTULIN and CYLD and HOIPIN-8 treatment from HCT-116 HOIP KO cells (-), reconstituted with

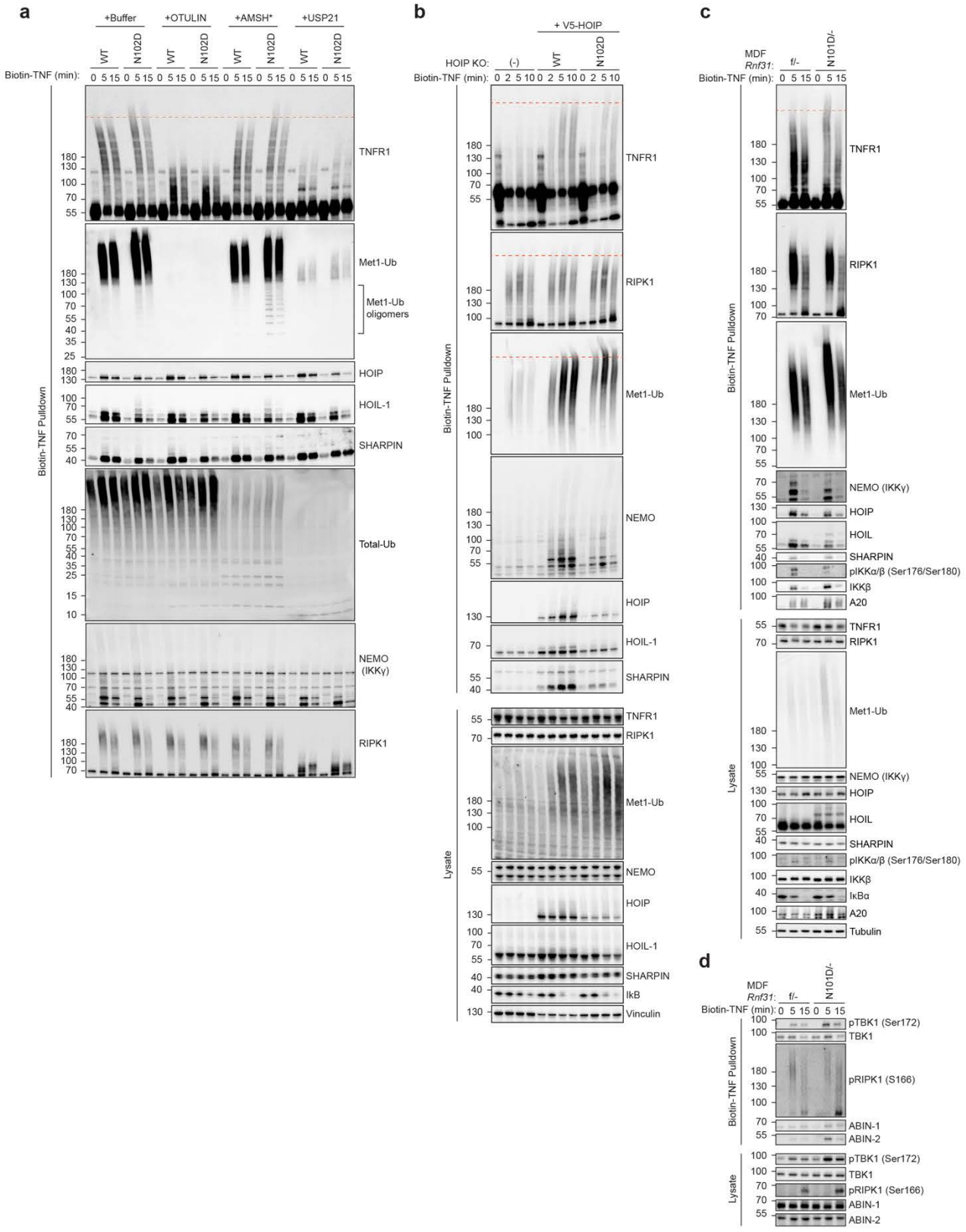
HOIP WT or N102D (n = 3). **(b)** Met1-Ub Pulldown of LUBAC after transfection with HOIP WT, C879A, or N101D constructs and 4-OHT-induced KO of *Otulin* in *Otulin*$^{f/f}$ MEFs (n = 3). **(c)** Met1-Ub Pulldown of LUBAC and TNF RSC proteins following stimulation with recombinant human TNF and treatment with recombinant OTULIN 80-352 in HOIP-reconstituted HCT-116 HOIP KO cells (n = 3). **(d)** Lysates of Met1-Ub Pulldowns of LUBAC and TNFR1 from HOIP-reconstituted HCT-116 HOIP KO cells pre-treated with HOIPIN-8 and stimulated with recombinant human TNF (n = 2). **(e)** Met1-Ub Pulldown of LUBAC and RSC proteins following stimulation with L18-MDP in HOIP-reconstituted HCT-116 HOIP KO cells (n = 3).

Supplementary Figure 5



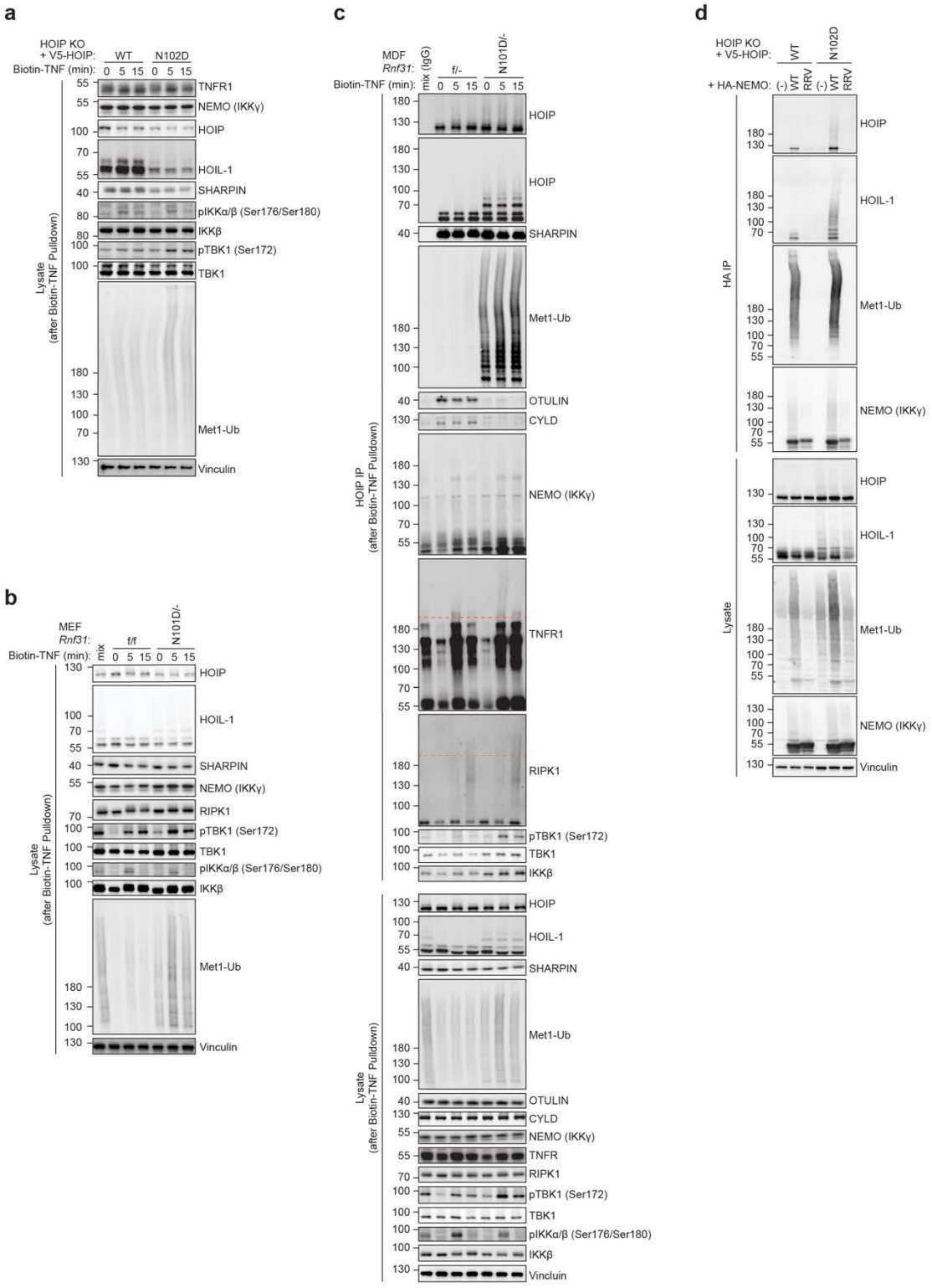
Supplementary Figure 5: Disrupted LUBAC PUB-PIM interactions impair immune signalling and nuclear translocation of NF-κB.
(a and **e)** Western Blots of lysates from HOIP-reconstituted HCT-116 HOIP KO cells **(a)** and immortalised MEFs **(e)** showing nuclear translocation of canonical NF-κB pathway proteins following stimulation with recombinant human TNF with their respective cytosolic (cyto) and nuclear (nuc) fractions (n = 3 for HCT-116; n = 2 for MEFs). **(b)** Western Blots of lysates from HOIP-reconstituted HCT-116 HOIP KO cells stimulated with recombinant human TNF for indicated time points (n = 4). **(c)** Phosphoproteome analysis of lysates from HOIP-reconstituted HCT-116 HOIP KO cells following stimulation with human recombinant TNF for 15 min. Phospho-site inclusion criteria: Log2 FC >1 or < -1, Padj < 0.05 for Ctrl vs TNF in at least one genotype. **(d)** as in **(c)** but stimulated with human recombinant TNF for 5 min or 15 min. Phospho-site inclusion criteria: Padj < 0.05 for Ctrl vs TNF in at least one genotype and KEGG-annotated TNF and NF-κB pathway genes. Samples in **(c** and **d)** were collected from three independent experiments (biological replicates).

Supplementary Figure 6



Supplementary Figure 6: HOIP N102D alters ubiquitin chain composition at the TNF RSC causing dysregulated signalling.
(a) Western blots of UbiCRest analysis of TNF RSC proteins after biotin-TNF pulldown from HOIP-reconstituted HCT-116 HOIP KO cells for indicated time points (n = 2). **(b)** Western blots of TNF RSC proteins after biotin-TNF pulldown from HOIP-reconstituted HCT-116 HOIP KO cells for indicated time points (n = 2). **(c** and **d)** Western blots of TNF RSC proteins after biotin-TNF pulldown from immortalised MDFs for indicated time points (n = 3).

Supplementary Figure 7

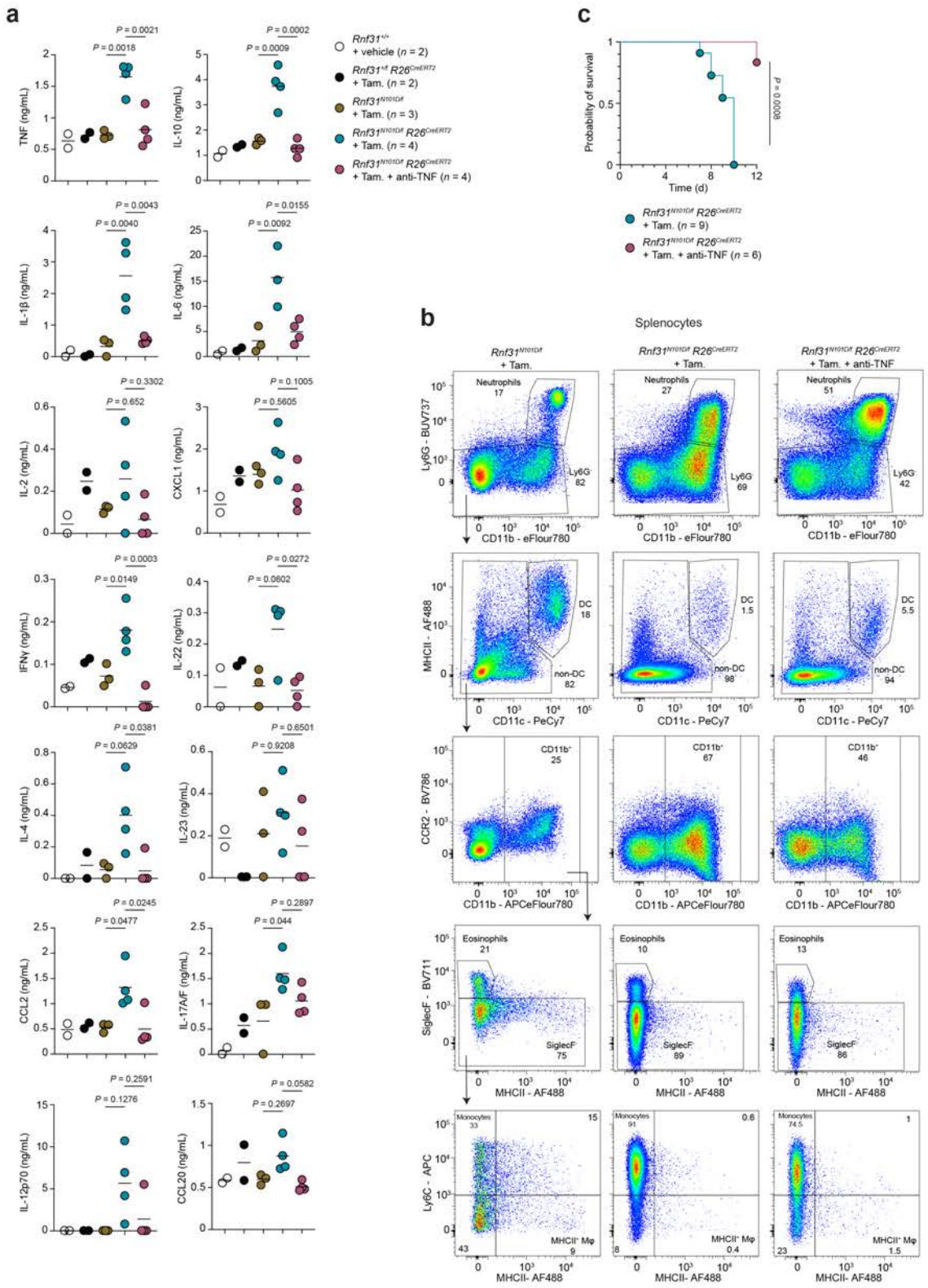


Supplementary Figure 7: Disrupted LUBAC PUB-PIM interactions destabilise LUBAC and NEMO at the TNF RSC.

(a and **b)** Western Blots of lysates from (**a**) sequential V5 IPs from HOIP-reconstituted HCT-116 HOIP KO cells or (**b**) sequential HOIP IPs from immortalised MEFs following initial biotin-TNF pulldown (n = 2). **(c)** Western Blots from sequential HOIP IPs from

immortalised MDFs following initial biotin-TNF pulldown (n = 2). **(d)** IPs of exogenous NEMO constructs from HCT-116 HOIP KO cells reconstituted with HOIP WT or N102D after transfection with HA-tagged NEMO WT or RRV (R316A, R319A, V414A) (n = 3).

Supplementary Figure 8



Supplementary Figure 8: HOIP N101D induces lethal cytokine storm and widespread immune dysregulation.

(a) Chemokine and cytokine levels in serum of endpoint euthanised mice. Each dot represents a mouse; the line is the mean and *P* values by ANOVA with Tukey's multiple comparisons test. **(b)** Representative flow cytometry plots of myeloid cell populations

in the spleens of endpoint euthanized mice. Cells were pre-gated on DAPI$^-$ Lin$^-$ (Ter119$^-$ NK1.1$^-$ B220$^-$ CD3$^-$ CD19$^-$) CD45$^+$ populations. Arrows indicate sequential gating. **(c)** Kaplan-Meier probability of survival analysis of Tam. or Tam. and anti-TNF treated *Rnf31*$^{N101D/f}$ *R26*^{CreERT2} mice (n = 9 Tam.; n = 6 Tam. and anti-TNF). *P* values by Gehan-Breslow-Wilcoxon test.

References

1. Bradley, A. *et al*. The mammalian gene function resource: the International Knockout Mouse Consortium. *Mamm Genome* **23**, 580-586 (2012).

2. Xu, J. Preparation, culture, and immortalization of mouse embryonic fibroblasts. *Curr Protoc Mol Biol* **Chapter 28**, Unit 28 21 (2005).

3. Damgaard, R.B. *et al*. OTULIN deficiency in ORAS causes cell type-specific LUBAC degradation, dysregulated TNF signalling and cell death. *EMBO molecular medicine* **11** (2019).

4. Hrdinka, M. *et al*. CYLD Limits Lys63- and Met1-Linked Ubiquitin at Receptor Complexes to Regulate Innate Immune Signaling. *Cell Rep* **14**, 2846-2858 (2016).

5. Elbaek, C.R. *et al*. M1-linked ubiquitination by LUBAC regulates AMPK signalling and the response to energetic stress. *Cell Death Differ* (2026).

6. Renier, N. *et al*. iDISCO: a simple, rapid method to immunolabel large tissue samples for volume imaging. *Cell* **159**, 896-910 (2014).

7. Dobin, A. *et al*. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* **29**, 15-21 (2013).

8. Love, M.I., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* **15**, 550 (2014).

9. Heinz, S. *et al*. Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. *Mol Cell* **38**, 576-589 (2010).

10. Batth, T.S. *et al*. Protein Aggregation Capture on Microparticles Enables Multipurpose Proteomics Sample Preparation. *Mol Cell Proteomics* **18**, 1027-1035 (2019).

11. Bekker-Jensen, D.B. *et al*. A Compact Quadrupole-Orbitrap Mass Spectrometer with FAIMS Interface Improves Proteome Coverage in Short LC Gradients. *Mol Cell Proteomics* **19**, 716-729 (2020).

12. Martinez-Val, A. *et al*. Hybrid-DIA: intelligent data acquisition integrates targeted and discovery proteomics to analyze phospho-signaling in single spheroids. *Nature communications* **14**, 3599 (2023).

13. Bache, N. *et al*. A Novel LC System Embeds Analytes in Pre-formed Gradients for Rapid, Ultra-robust Proteomics. *Mol Cell Proteomics* **17**, 2284-2296 (2018).

14. Guzman, U.H. *et al*. Ultra-fast label-free quantification and comprehensive proteome coverage with narrow-window data-independent acquisition. *Nat Biotechnol* **42**, 1855-1866 (2024).

15. Demichev, V., Messner, C.B., Vernardis, S.I., Lilley, K.S. & Ralser, M. DIA-NN: neural networks and interference correction enable deep proteome coverage in high throughput. *Nature methods* **17**, 41-44 (2020).

16. Frankenfield, A.M., Ni, J., Ahmed, M. & Hao, L. Protein Contaminants Matter: Building Universal Protein Contaminant Libraries for DDA and DIA Proteomics. *J Proteome Res* **21**, 2104-2113 (2022).

17. Bruderer, R. *et al*. Extending the limits of quantitative proteome profiling with data-independent acquisition and application to acetaminophen-treated three-dimensional liver microtissues. *Mol Cell Proteomics* **14**, 1400-1410 (2015).

18. Ritchie, M.E. *et al*. limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res* **43**, e47 (2015).

19. Phipson, B., Lee, S., Majewski, I.J., Alexander, W.S. & Smyth, G.K. Robust Hyperparameter Estimation Protects against Hypervariable Genes and Improves Power to Detect Differential Expression. *Ann Appl Stat* **10**, 946-963 (2016).

20. Law, C.W., Chen, Y., Shi, W. & Smyth, G.K. voom: Precision weights unlock linear model analysis tools for RNA-seq read counts. *Genome Biol* **15**, R29 (2014).

21. Wieczorek, S. *et al*. DAPAR & ProStaR: software to perform statistical analyses in quantitative discovery proteomics. *Bioinformatics* **33**, 135-136 (2017).

22. Perez-Riverol, Y. *et al*. The PRIDE database at 20 years: 2025 update. *Nucleic Acids Res* **53**, D543-D553 (2025).