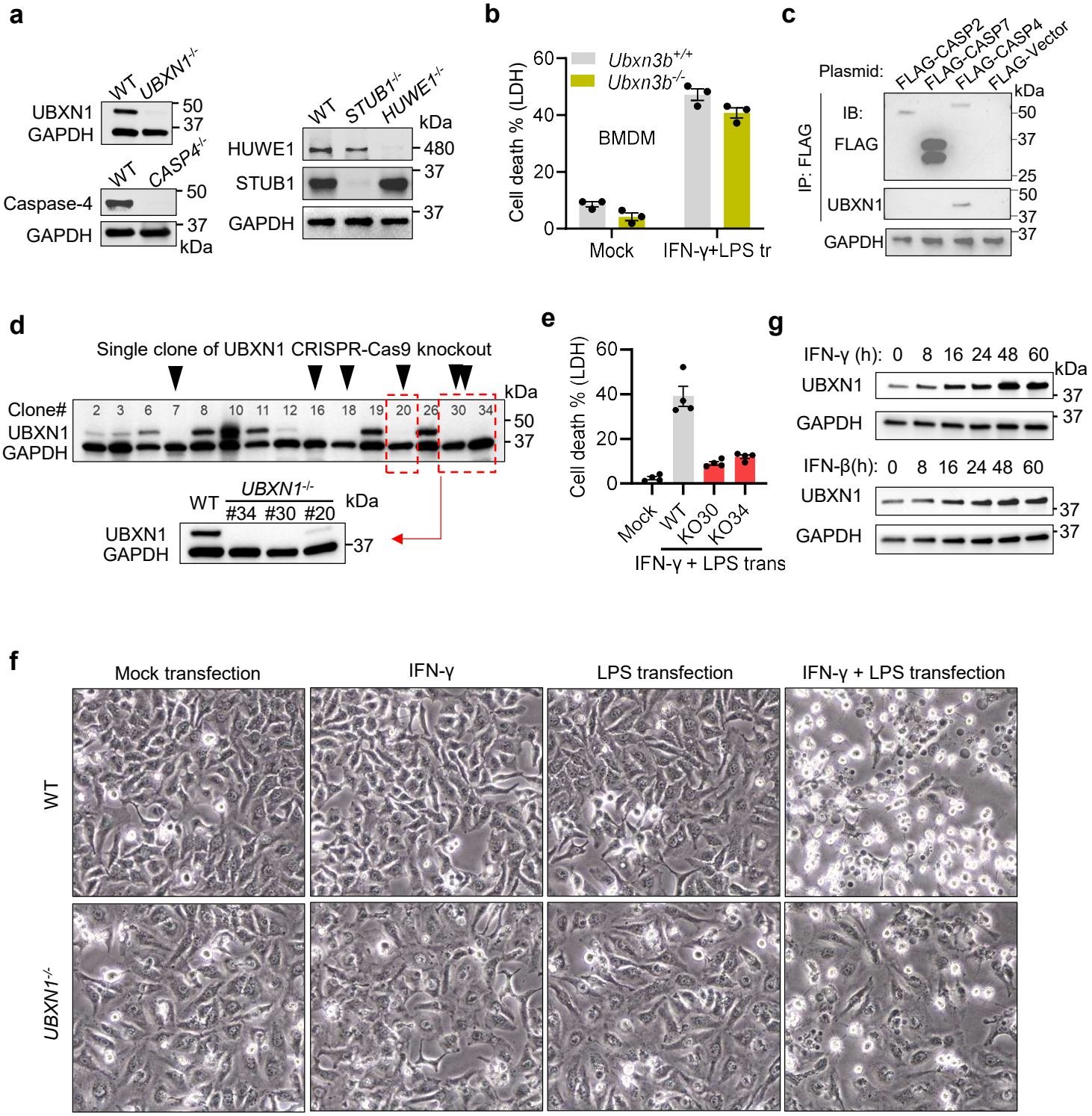
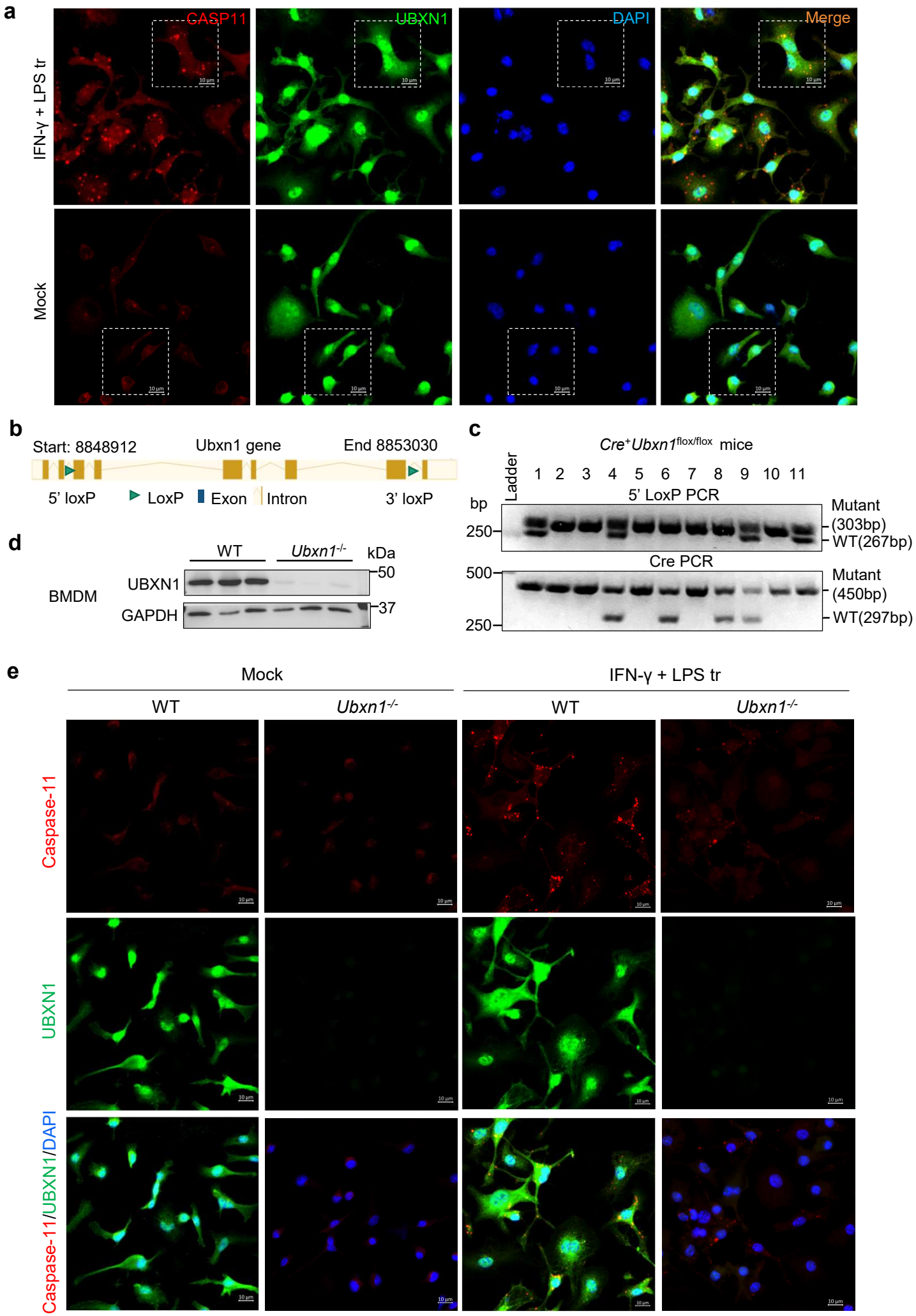
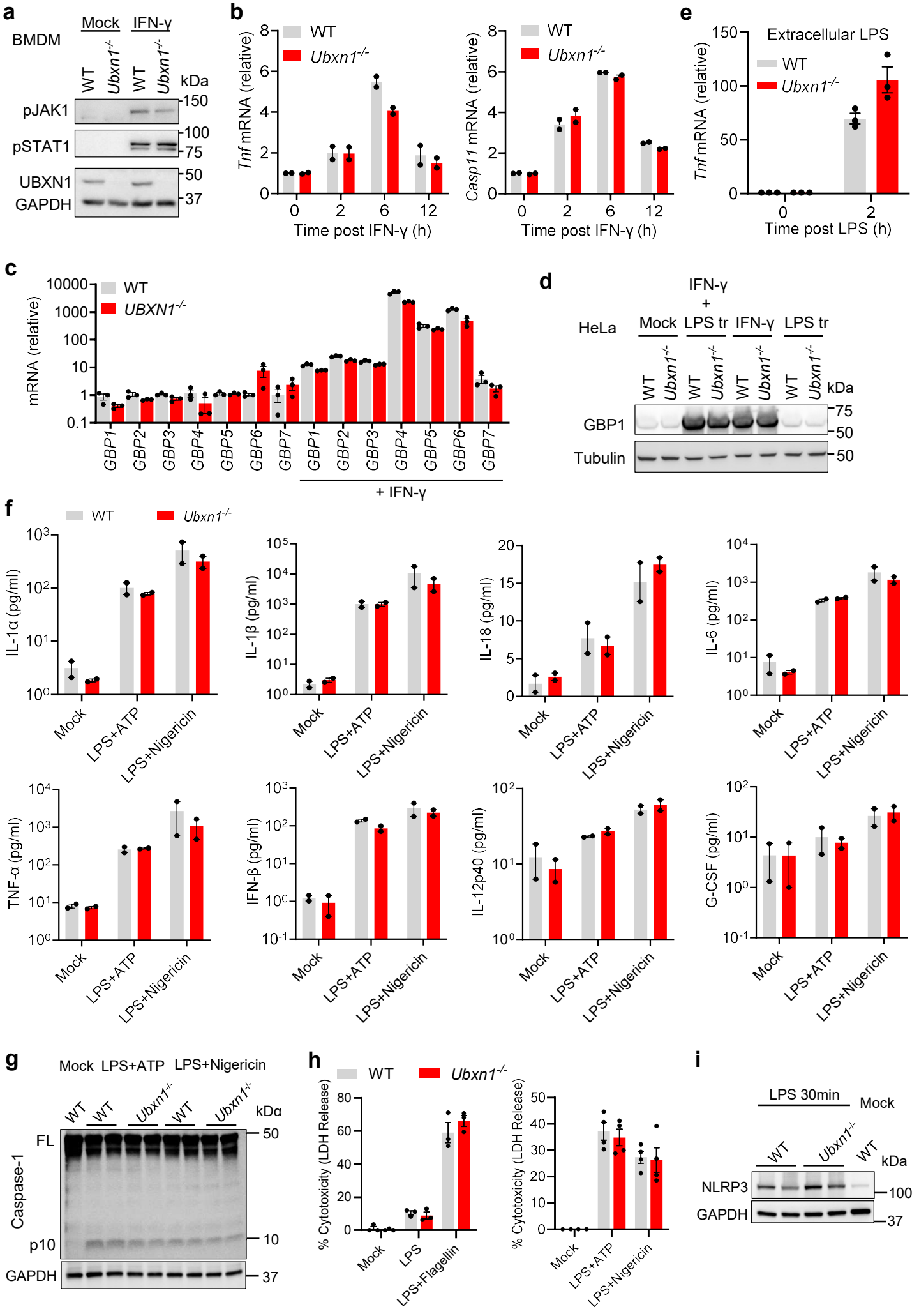


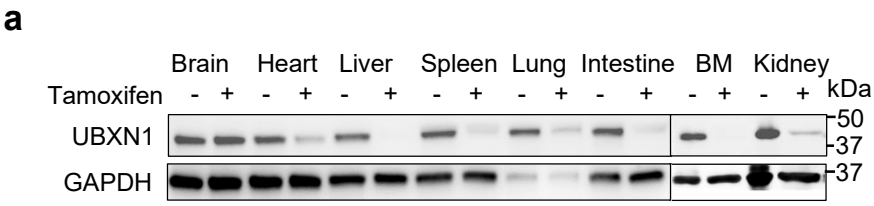
Supplemental Fig.S1. **UBXN1 is critical for the non-canonical inflammasome signaling in human cells.** **a)** Validation of *UBXN1, HUWE1*, *STUB1* and *CASP4* knockout HeLa cell by immunoblotting. **b)** Percent cell death (based on the release of LDH, lactate dehydrogenase) of *Ubxn3b*$^{+/+}$ and *Ubxn3b*$^{-/-}$ bone marrow-derived macrophages (BMDMs) primed with 10 ng/mL of mouse IFN-γ for 3 h followed by LPS (1 μg/mL) transfection for 16 h. **c)** Co-immunoprecipitation (IP) of FLAG-caspases and endogenous UBXN1 with an anti-FLAG antibody from HeLa cells transfected with FLAG-CASP plasmids for 24 h. IB, immunoblotting. **d)** Validation of monoclonal UBXN1 knockout HeLa cells by immunoblotting. **e)** Percent cell death (based on the release of LDH, lactate dehydrogenase) of cells primed with human IFN-γ (10 ng/mL) overnight and transfected with 1 μg/mL of LPS for 5 h. **f)** Differential interference contrast (DIC) microscopy of HeLa cells treated as in **(e)**. Bright, round cells are typical dying cells. Scale bar: 20 μM. **g)** The immunoblots for UBXN1 protein in HeLa cells treated with IFN-γ (20 ng/mL) or IFN-β (40 ng/mL). **Related to Fig.1**

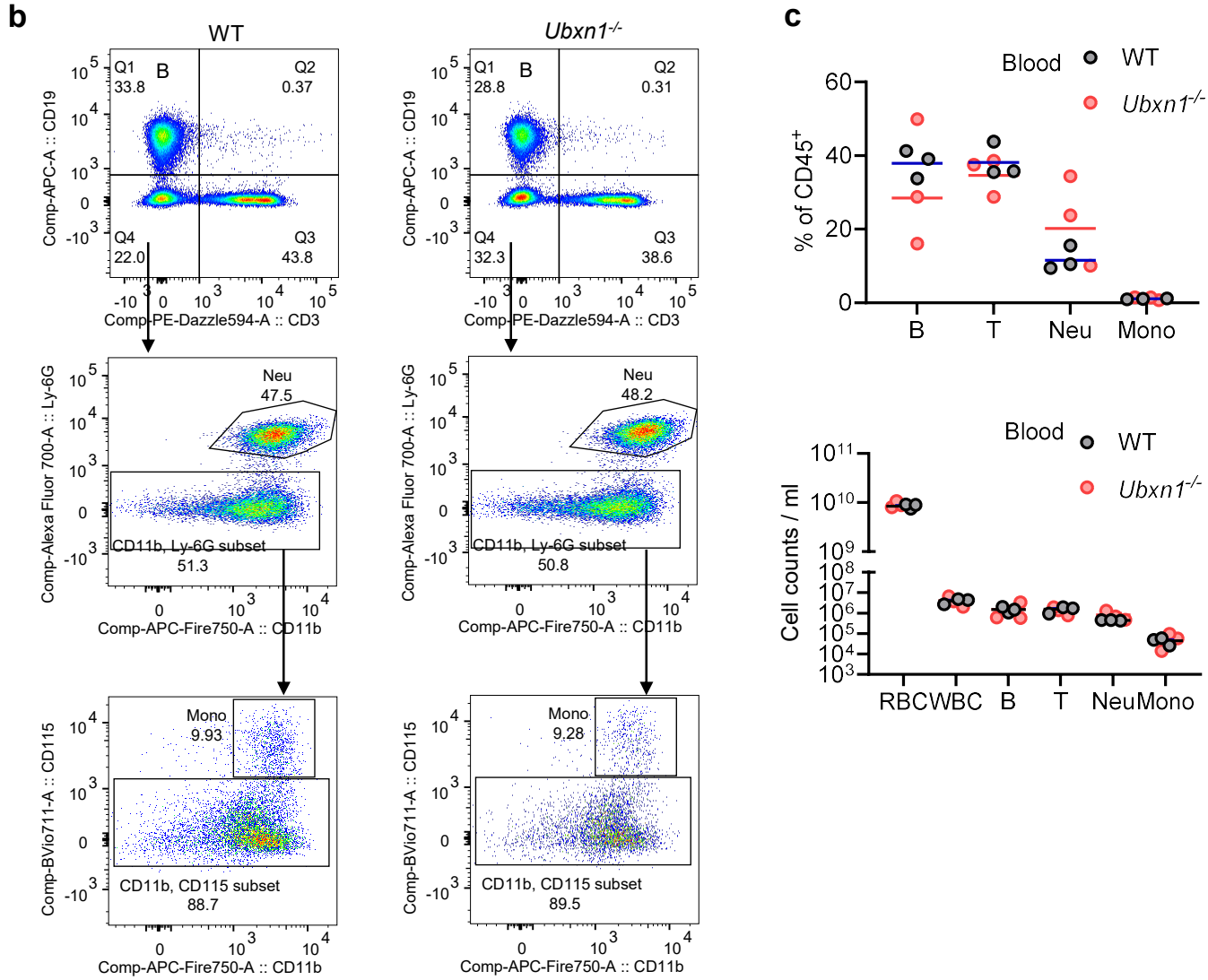

a
CASP11
UBXN1
DAPI
Merge
IFN-γ + LPS tr
Mock
b
Start: 8848912
Ubxn1 gene
End 8853030
5' loxP
LoxP
Exon
Intron
3' loxP
c
Cre+Ubxn1flox/flox mice
Ladder
1 2 3 4 5 6 7 8 9 10 11
5' LoxP PCR
bp
250
Mutant
(303bp)
WT(267bp)
Cre PCR
500
250
Mutant
(450bp)
WT(297bp)
d
WT
Ubxn1-/-
kDa
BMDM
UBXN1
GAPDH
50
37
e
Mock
IFN-γ + LPS tr
WT
Ubxn1-/-
WT
Ubxn1-/-
Caspase-11
UBXN1
Caspase-11/UBXN1/DAPI

Supplemental Fig.S2. **UBXN1 is crucial for the non-canonical inflammasome activation in mouse macrophages. a)** Original microscopic view for Fig.1g. Immunofluorescent staining of the indicated proteins in BMDM that were primed with IFN-γ and transfected with LPS. Mock, transfection reagent only. DAPI stains DNA. Scale bar: 10 μm. **b)** A graph depicting *Ubxn1* gene structure and insertion of loxP. **c)** Genotyping PCR of Cre^{+}*Ubxn1*$^{flox/flox}$ mice. **d)** Immunoblots of UBXN1 protein expression in bone marrow-derived macrophages (BMDMs). **e)** Immunofluorescence staining of caspase-11 in WT and *Ubxn1*$^{-/-}$ BMDMs treated with 10 ng/mL of mouse IFN-γ for 3 h plus LPS (transfection,1μg/mL) for 16 h. LPS tr: LPS transfection, Mock: transfection reagent only. Scale bar, 10 μM. **Related to Fig.2**

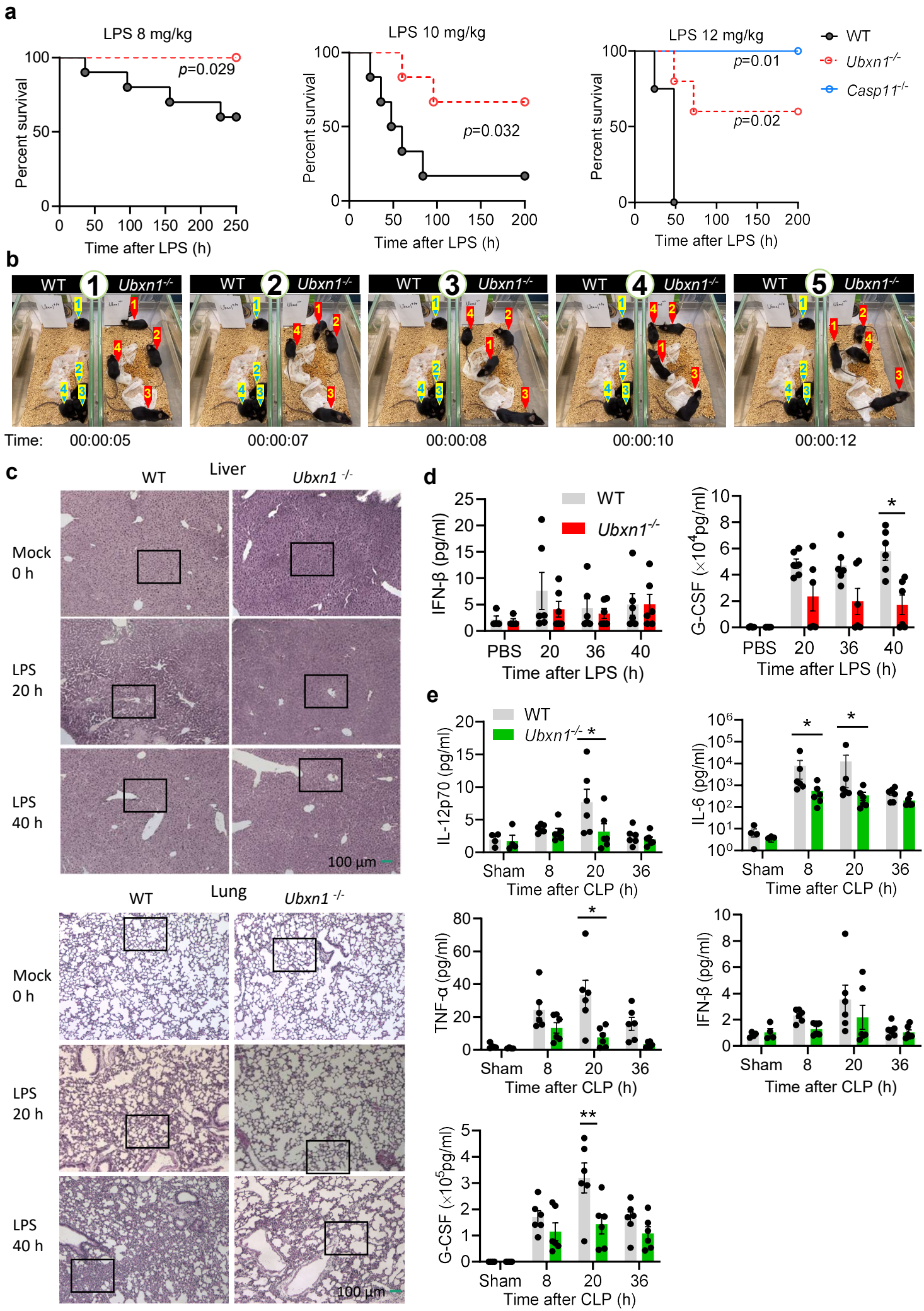

a
BMDM
Mock
IFN-γ
WT
Ubxn1-/-
kDa
pJAK1
pSTAT1
UBXN1
GAPDH
150
100
75
50
37
b
WT
Ubxn1-/-
Tnf mRNA (relative)
Casp11 mRNA (relative)
Time post IFN-γ (h)
e
Extracellular LPS
Time post LPS (h)
c
UBXN1-/-
mRNA (relative)
GBP1
GBP2
GBP3
GBP4
GBP5
GBP6
GBP7
+ IFN-γ
d
HeLa
IFN-γ + LPS tr
LPS tr
GBP1
Tubulin
f
IL-1α (pg/ml)
IL-1β (pg/ml)
IL-18 (pg/ml)
IL-6 (pg/ml)
TNF-α (pg/ml)
IFN-β (pg/ml)
IL-12p40 (pg/ml)
G-CSF (pg/ml)
Mock
LPS+ATP
LPS+Nigericin
g
Mock LPS+ATP LPS+Nigericin
Caspase-1
FL
p10
GAPDH
kDα
h
% Cytotoxicity (LDH Release)
LPS
LPS+Flagellin
i
LPS 30min
NLRP3

Supplemental Fig.S3. UBXN1 is dispensable for the IFN-γ-JAK-STAT, TLR4 and the canonical NLRP3 inflammasome signaling. **a)** Phosphorylation (p) of JAK1 and STAT1 in primary bone marrow-derived macrophages (BMDMs) treated with 10 ng/mL of mouse IFN-γ for 30 min. **b)** Quantitative RT-PCR of mRNA expression for the indicated genes in BMDMs treated with 10 ng/mL of mouse IFN-γ. **c)** Quantitative RT-PCR of mRNA expression of GBPs in WT and *UBXN1*$^{-/-}$ HeLa cells treated with human IFN-γ for 12 h. **d)** The immunoblots of GBP1 protein in HeLa cells primed with 10 ng/mL of IFN-γ for 12 h, and/or transfecetd with 1 μg/mL of LPS for 5 h. **e)** Quantitative RT-PCR of *Tnf* mRNA expression in BMDMs stimulated with 0.5 μg/mL of LPS. Bar: mean ± SEM. Each symbol represents one mouse. Mock: transfection reagent only. **f)** Primary bone marrow-derived macrophages (BMDMs) were primed with LPS (1 μg/mL) for 3 h and then stimulated by ATP (5 mM) or Nigericin (10 μg/mL) for 30 min. The concentrations of cytokines/growth factors in the cell culture medium were analyzed by a bead-based multiplex ELISA. Bar: mean ± SEM. N=2 mice/group. **g)** The immunoblots of caspase-1 in BMDMs treated as in **f)**. Each lane = one mouse. FL: full-length. **h)** Percent cell death (based on the release of LDH, lactate dehydrogenase) of BMDMs treated as in **f)** except for the flagellin conditions at 20 μg/mL for 4 h. **i)** The immunoblots of NLRP3 in BMDMs primed with 1 μg/mL of LPS for 30 min. Mock: phosphate buffered saline. **Related to Fig.1 and Fig.2**

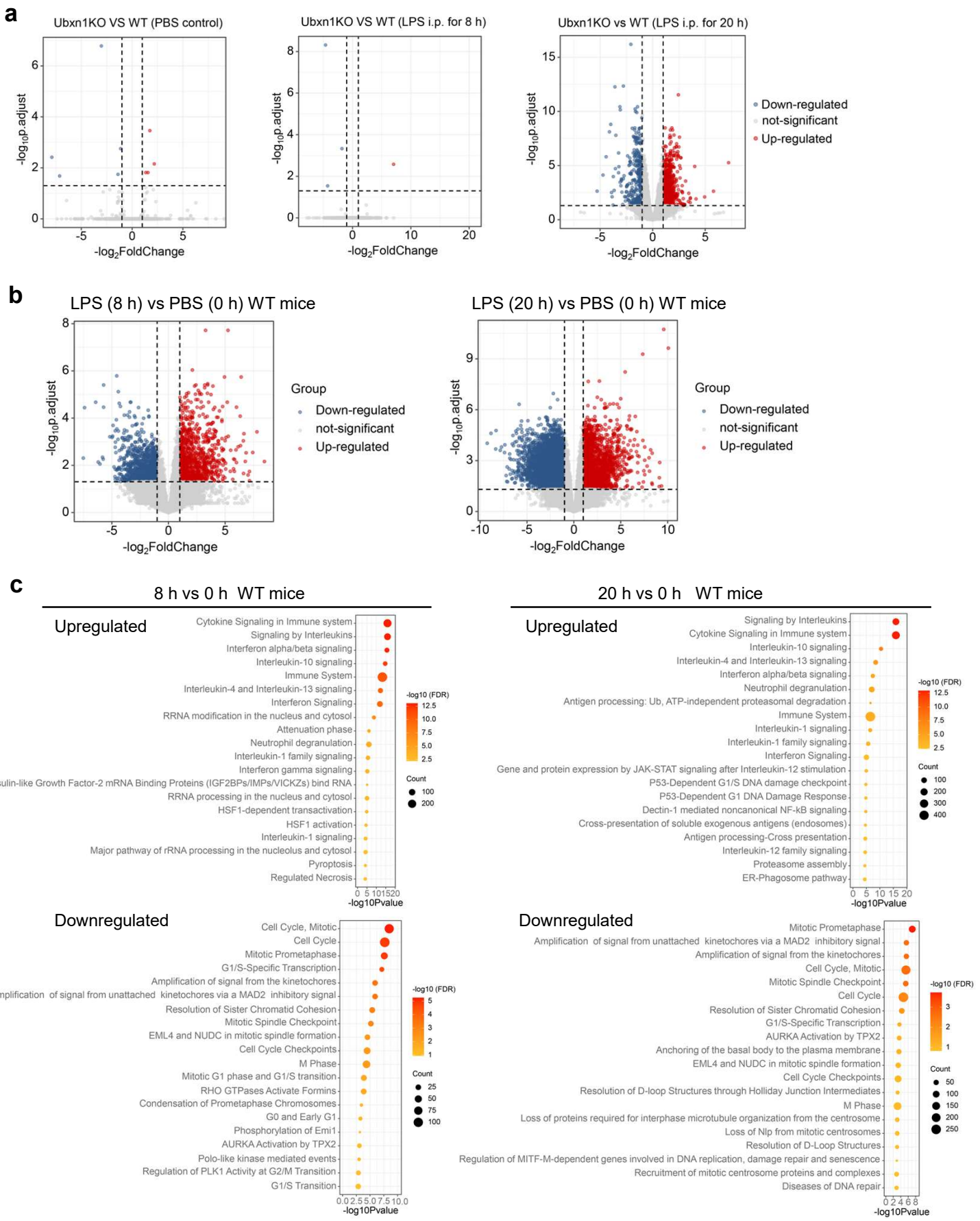




Supplemental Fig.S4. Normal peripheral immune populations in *Ubxn1*$^{-/-}$ mice. **a)** The immunoblots of UBXN1 protein in various tissues from the Cre^{+}*Ubxn1*$^{flox/flox}$ mice treated without (-) or with tamoxifen (+). BM: bone marrow. **b)** The gating strategy and **(c)** ratios and counts of major blood immune populations to total leukocytes (CD45^{+}). The blood immune populations in aged- and sex-matched mice were quantitated by flow cytometry with specific surface markers. RBC: red blood cell , WBC: white blood cell CD45, T cell: CD3, B cell: CD19, Neutrophil: Ly6G, Monocyte: CD115/CD11b. Bar: mean ± SEM, N=3 mice/group. **Related to Fig.3**


a
LPS 8 mg/kg
LPS 10 mg/kg
LPS 12 mg/kg
Percent survival
Time after LPS (h)
p=0.029
p=0.032
p=0.01
p=0.02
WT
Ubxn1-/-
Casp11-/-
b
WT
Ubxn1-/-
Time: 00:00:05 00:00:07 00:00:08 00:00:10 00:00:12
c
Liver
Lung
WT
Ubxn1 -/-
Mock 0 h
LPS 20 h
LPS 40 h
100 μm
d
IFN-β (pg/ml)
G-CSF (×10^4 pg/ml)
PBS 20 36 40
Time after LPS (h)
e
IL-12p70 (pg/ml)
IL-6 (pg/ml)
TNF-α (pg/ml)
IFN-β (pg/ml)
G-CSF (×10^5 pg/ml)
Sham 8 20 36
Time after CLP (h)

Supplemental Fig.S5. *Ubxn1*$^{-/-}$ mice are resistant to lethal LPS sepsis with attenuated systemic inflammatory responses. a) The survival curves of age- and sex-matched littermates administered with the indicated dose of LPS intraperitoneally. For 8 mg/kg of LPS, N=10/group; for 10 mg/kg of LPS, N=6/group; for 12 mg/kg of LPS, N=4 for WT and *Casp11*$^{-/-}$, N=5 for *Ubxn1*$^{-/-}$; Log-Rank test. **b**) Time-lapse morbidity of age- and sex-matched littermates injected with LPS (10 mg/kg) intraperitoneally for 36 h. Related to Supplemental video. **c**) The hematoxylin and eosin staining (H&E) of tissues from mice administered 10 mg/kg LPS. The boxed regions are shown in Fig.3d. Scale bar, 100 μm. **d, e**) Age- and sex-matched littermates were injected with 10 mg/kg of LPS intraperitoneally or subjected to a cecal-ligation-and–puncture (CLP) procedure. The concentrations of cytokines/growth factors in the **d**) sera (at 20, 36 and 40 h after LPS), and **e**) sera at 8, 20, 36 h post CLP were analyzed by a bead-based multiplex ELISA. Bar: mean ± s.e.m. Each dot = one animal. *p<0.05, **p<0.01 (Two way-ANOVA, Dunnett comparisons). **Related to Fig.3 and Fig.4**



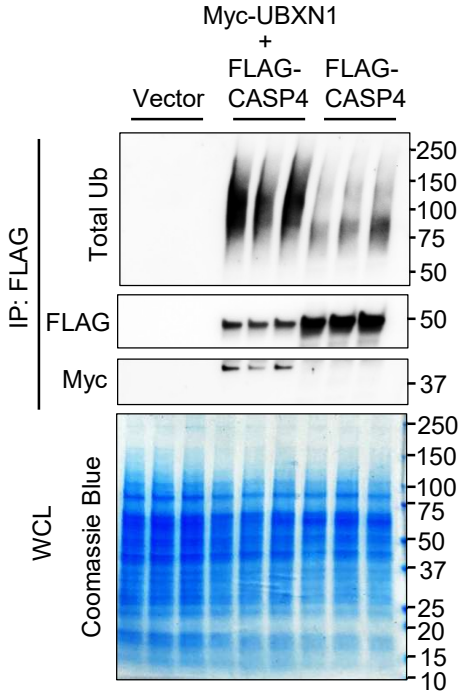
Supplemental Fig.S6. **UBXN1 enhances inflammation but suppresses cell cycle during LPS sepsis**. Age- and sex-matched mice (n=2 for 0 h, 3 for 8 and 20 h per group) were administered 10 mg/kg of LPS intraperitoneally. Splenic macrophages were purified and subjected to RNA-sequencing. **a, b)** The volcano plots of differentially expressed genes (DEGs) **a)** in *Ubxn1*$^{-/-}$ vs WT at 0, 8, and 20 h after LPS, **b)** in WT mice at 8 vs 0 h, 20 vs 0 h after LPS. Red/blue symbols represent significantly up/down-regulated DEGs, respectively (Log_2fold change ≥ 1 or ≤ −1, *padj* < 0.05). **c)** Top Reactome pathways enriched from **b)** ($p < 0.05$, right-tailed Fisher's exact *t* test with Benjamini & Hochberg). **Related to Fig.4**

a

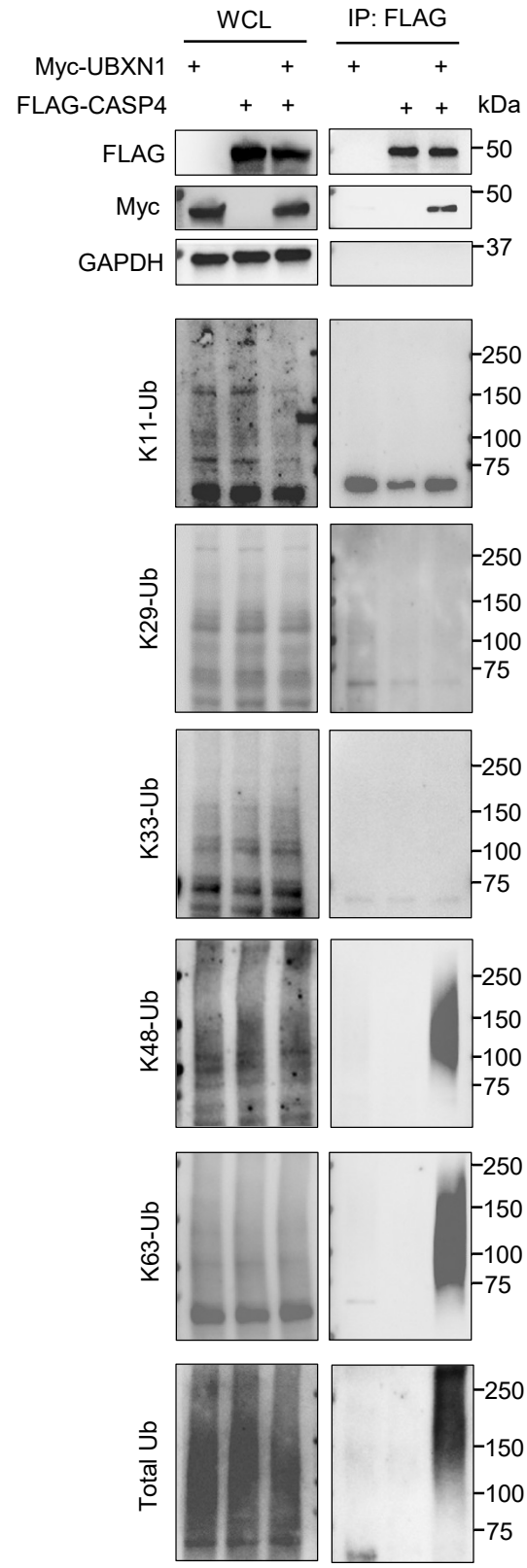
Kn-Ub mutants

	6	11	27	29	33	48	63
HA-Ub	K	K	K	K	K	K	K
HA-K6	K	R	R	R	R	R	R
HA-K11	R	K	R	R	R	R	R
HA-K27	R	R	K	R	R	R	R
HA-K29	R	R	R	K	R	R	R
HA-K33	R	R	R	R	K	R	R
HA-K48	R	R	R	R	R	K	R
HA-K63	R	R	R	R	R	R	K

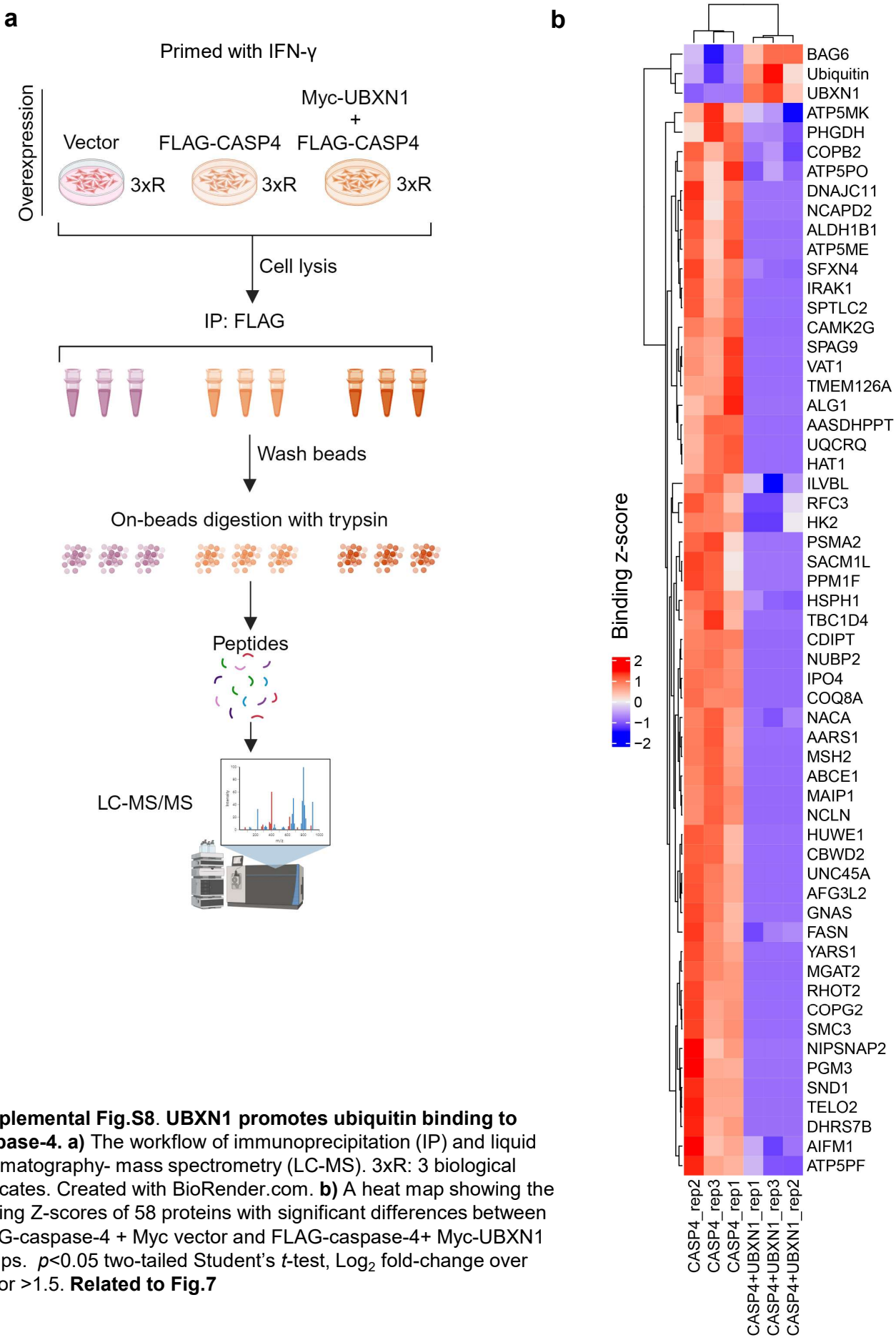
b



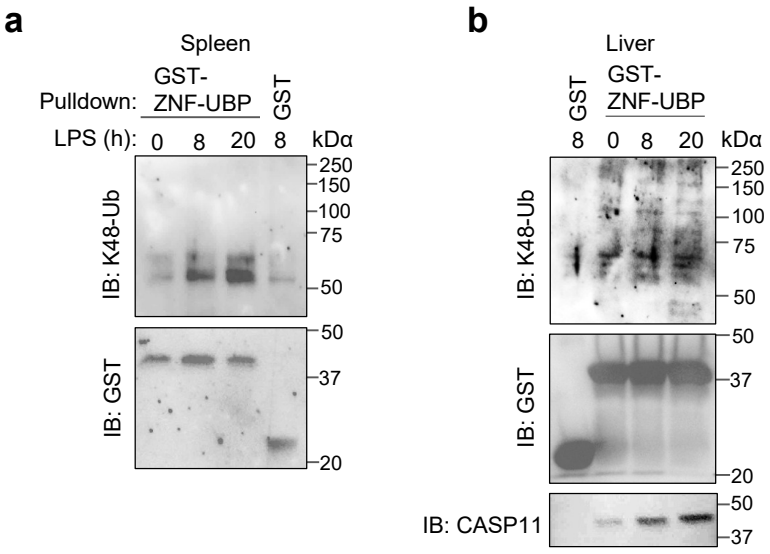
c



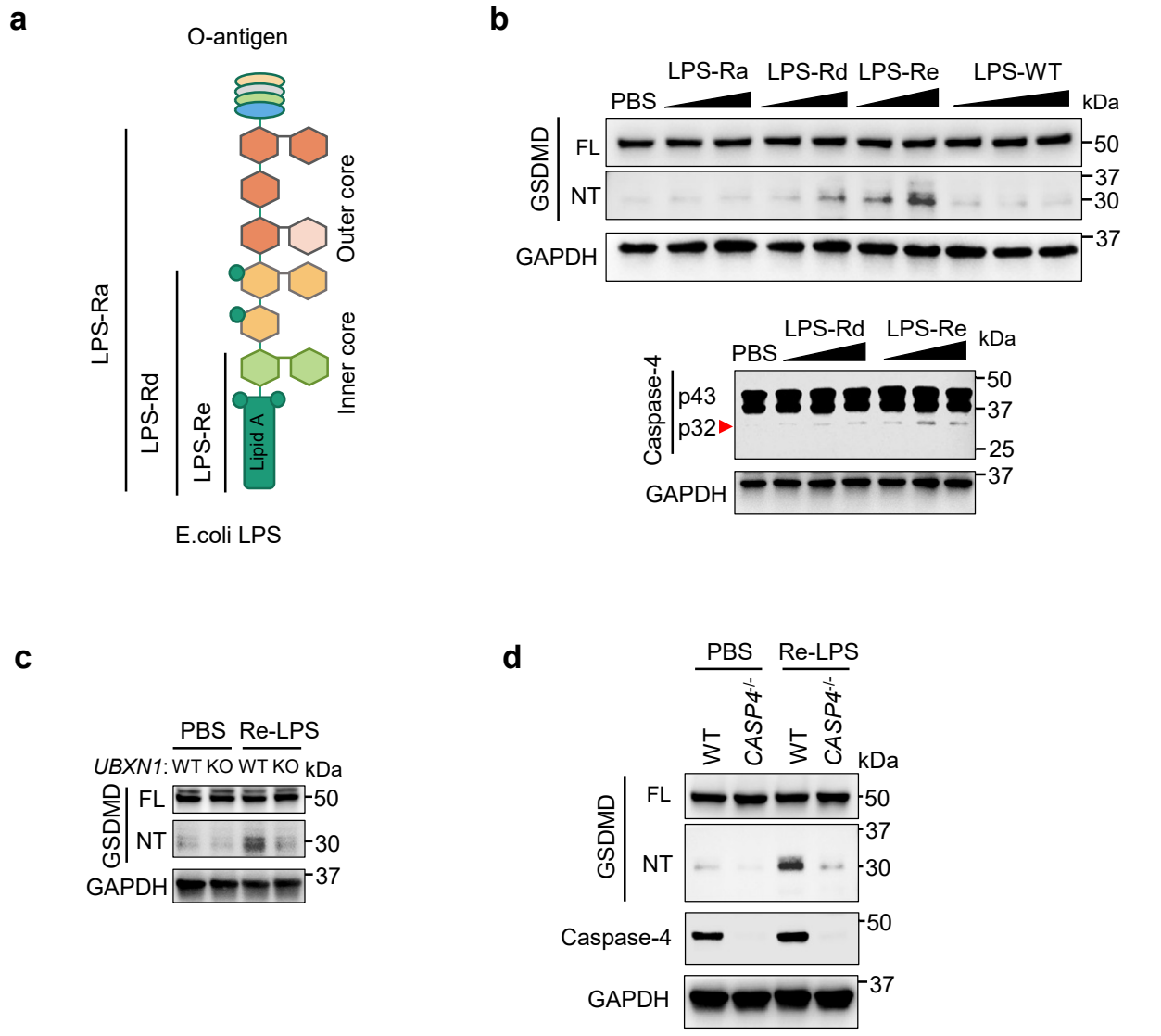
Supplemental Fig.S7. **UBXN1 enhances K48- and K63- linked ubiquitination of caspase-4**. **a)** A graph illustrating mutant Kn-Ub. **b, c)** Co-IP of FLAG-CASP4 and bound Myc-UBXN1 with an anti-FLAG antibody from HEK293T cells transfected with different combinations of FLAG-CASP4, Myc-UBXN1 and vector plasmids. Shown are the immunoblots (IB) and Coomassie blue staining. Endogenous Ub is detected with a polyubiquitin (total Ub) or linkage specific Ub antibody as indicated. WCL, whole cell lysate. **Related to Fig.6**



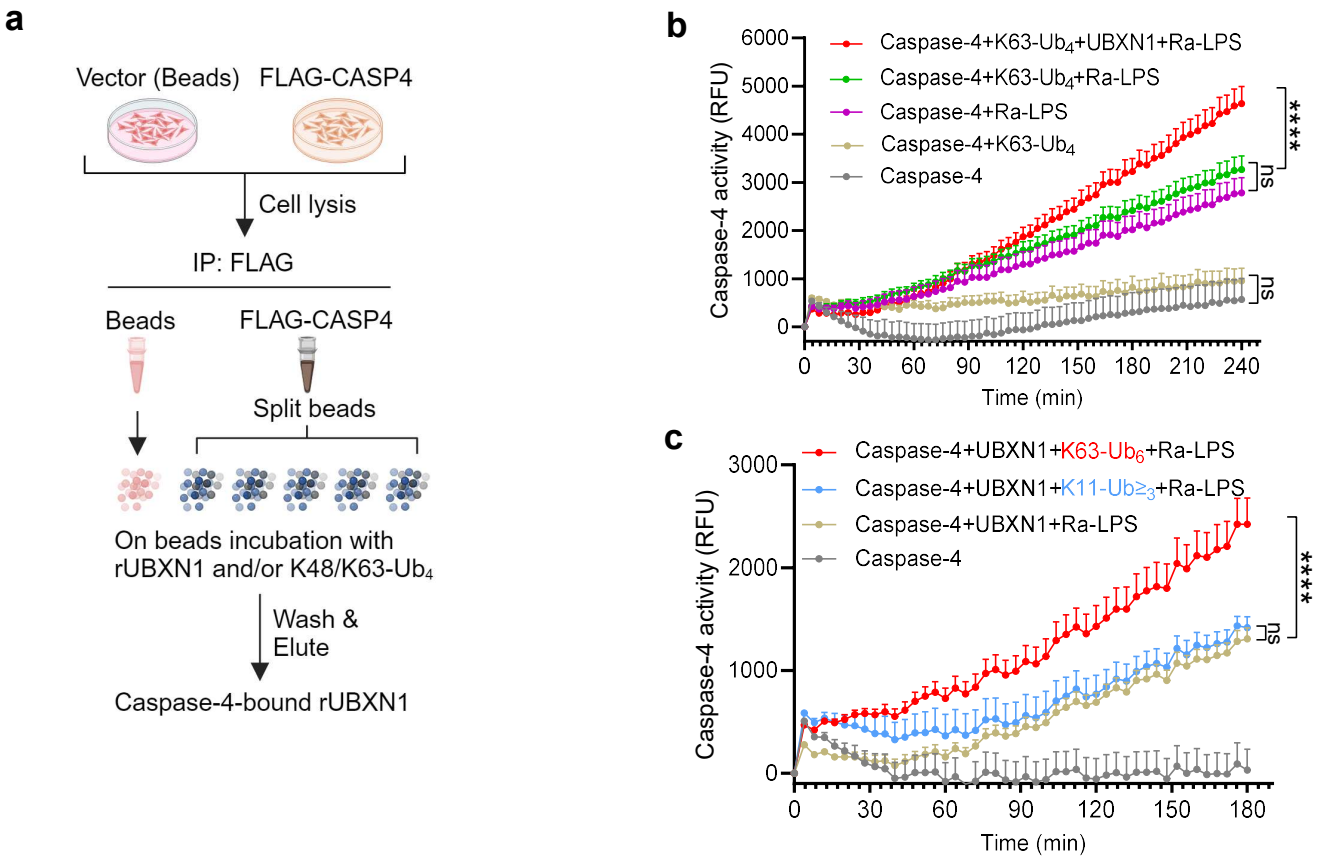
Supplemental Fig.S8. **UBXN1 promotes ubiquitin binding to caspase-4. a)** The workflow of immunoprecipitation (IP) and liquid chromatography- mass spectrometry (LC-MS). 3xR: 3 biological replicates. Created with BioRender.com. **b)** A heat map showing the binding Z-scores of 58 proteins with significant differences between FLAG-caspase-4 + Myc vector and FLAG-caspase-4+ Myc-UBXN1 groups. $p<0.05$ two-tailed Student's *t*-test, Log_2 fold-change over vector >1.5. **Related to Fig.7**



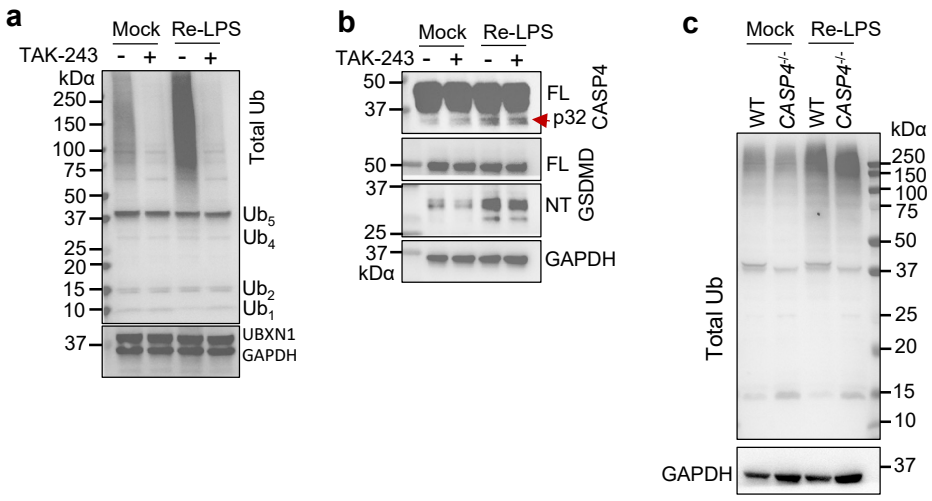
Supplemental Fig.S9. **Caspase-11 binding to unanchored K48-Ub chains increases with LPS stimulation *in vivo***. C57BL/6 mice were administered 10 mg/kg of LPS intraperitoneally. Unanchored ubiquitin chains were precipitated with GST-ZNF-UBP from the spleens **a**) and livers **b**) (pooled from 3 mice per group) at 0, 8 and 20 h after LPS. GST serves as a negative control. Shown are the immunoblots (IB) for K48-Ub, GST and caspase-11. **Related to Fig.7**



Supplemental Fig.S10. **Short LPS mutants activate the noncanonical inflammasome in a cell-free system. a)** A schematic illustration of *E.coli* WT, Ra, Rd and Re mutant LPS structures. **b,d)** The immunoblots of GSDMD and caspase-4 in the IFN-γ-primed HeLa cell lysates from (**Fig.7h)** that were incubated with LPS for 4 h at 37°C. The concentrations of LPS-Ra: 250 and 500 μg/mL, LPS-Rd: 325, 625 and 1250 μg/mL, LPS-Re: 125, 250 and 500 μg/mL, LPS-WT: 625, 1250 and 2500 μg/mL. The concentration of LPS-Re in **d)** is 500 μg/mL. **c)** The immunoblots of GSDMD in HeLa cells that were primed with IFN-γ, lysed, treated with Re-LPS. KO, knockout; PBS, phosphate-buffered saline; FL, full-length; NT, N-terminal fragment. **Related to Fig.7**



Supplemental Fig.S11. **UBXN1 and free K63-Ub chains interact with and promote caspase-4 activity *in vitro***. **a)** The schematic workflow for *in vitro* protein-protein interaction assay. Created with BioRender.com. K48-, K63-Ub4 are *E. coli*-derived recombinant tetra ubiquitin chains. Recombinant 6xHis-UBXN1 (rUBXN1) is *E. coli*-derived too. **b, c)** *In vitro* caspase-4 activity assay with a fluorogenic substate. Data are presented as mean + S.E.M., N=3 biological replicates, ****p<0.0001 by two way-ANOVA with repeated measures and Sidak's multiple comparisons test. 6xHis-caspase-4 is derived from insect cells, 6xHis-UBXN1 and K63-Ub_n from *E. coli*. **Related to Fig.8**



Supplemental Fig.S12. **Inhibiting *de novo* synthesis of ubiquitin chains has little impact on the caspase-4 inflammasome** in a cell free system. **a, b)** IFN-γ-primed HeLa lysates were incubated with a UBA1 (ubiquitin activating enzyme E_1) inhibitor, TAK-243, at 2 μM for 1 h then together with 500 μg/mL of LPS-Re for 4 h, at 37°C. **c)** IFN-γ-primed HeLa lysates were incubated only with Re-LPS. Shown are the immunoblots for the indicated proteins. In **a)**, several unanchored ubiquitin chains ($Ub_{1, 2,...}$) are marked on the right. The abundances of existing unanchored polyUb chains are not affected by TAK-243; while, the smeared bands, mostly polyUb that are covalently conjugated to the other proteins, are reduced by TAK-243. FL, full-length; NT, N-terminal. **Related to Fig.8**