Supplementary Materials for

Functional degradation: a mechanism of NLRP1 inflammasome activation by diverse pathogen enzymes

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Materials and Methods

Plasmids and constructs

The coding sequence of *Nlrp1b* allele 1: 129S1/SvimJ (129) *Nlrp1b* (DQ117584.1), allele 2: C57BL/6J (B6) *Nlrp1b* (BC141354) or allele 3: AKR/J, and variants thereof, were cloned into either pCMSCV-IRES-GFP or pAcSG2, with an N-terminal maltose binding protein (MBP) tag followed by a 3C protease cleavage site and a C-terminal HA or FLAG tag, respectively, or into pQCXIP with N-terminal GFP and C-terminal HA tags. The Fla-NLRP1B hybrid was constructed by replacing the first 45 N-terminal amino acids of NLRP1B with residues 431-475 of *Legionella pneumophila* flagellin (ANN95373) followed by the TEV cleavage sequence. CASP1, IL-1β, TEV and LF producing constructs were described previously (*16*). The *Nlrp1b* coding sequence was subcloned in-frame with AID-GFP (Addgene #80076). GFP-fused IpaH producing plasmids were constructed as follows: the *ipaH* coding sequences from the *S. flexneri* 2a str. 2457T virulence plasmid were transferred using the Gateway™ vector conversion system (ThermoFisher) from Gateway entry clones (*57*) into the SmaI restriction site of the Gateway-compatible destination vector pC1-eGFP (Clontech) via LR reactions. The *ipaH7.8* coding sequence was also subcloned into pQCXIP with an N-terminal mCherry tag. For protein expression in *E. coli* the *ipaH* coding sequences were subcloned into pET28a with a C-terminal 6X-HIS tag. Mutations were engineered by overlapping PCR.

Cell culture

293T and RAW264.7 cells were grown in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamine. Primary bone marrow-derived macrophages (BMDMs) were cultured in RPMI supplemented with 5% FBS, 5% mCSF, 100 U/ml penicillin, 100 mg/ml Streptomycin and 2 mM L-glutamine. BMDM immortalization was performed as previously described (*58*).

Bacterial strains and infections

2457T *S. flexneri*-derived *ipaH* deletion strains were constructed using the λ red recombinase-mediated recombination system (*59*), as previously described (*60*). To construct complemented strains, the coding sequence of *ipaH7.8* and 407 basepairs upstream, representing the endogenous promoter, were Gateway cloned into the pCMD136 plasmid and transformed into the Δ*ipaH7.8* mutant strain. *S. flexneri* was grown at 37°C on tryptic soy agar plates containing 0.01% Congo red, supplemented with 100 μg/ml spectinomycin for growth of complemented strains. For infections, 5 ml of tryptic soy broth (TSB) was inoculated with a single Congo red-positive colony and grown overnight shaking at 37°C. Saturated cultures were back-diluted 1:100 in 5 ml of fresh TSB and incubated for 2-3 hours shaking at 37°C. Bacteria were washed in cell culture medium and spun onto cells for 10 minutes at 300×*g*. Infected cells were incubated at 37°C for 20 minutes and then washed twice with cell culture medium containing 25 μg/ml gentamicin, then returned to 37°C for further incubation (30 minutes

to 2 hours). Cells were infected at an MOI of 30 unless otherwise specified. Cell death was assessed by LDH activity in clarified culture supernatants as previously described (*61*). Protein in supernatants was TCA precipitated for anti-CASP1 immunoblotting.

Reconstituted NLRP1B activity assays

To reconstitute inflammasome activity in 293T cells, constructs producing NLRP1B (or mutants), CASP1 and IL-1β were co-transfected with constructs producing TEV, LF, IpaHs or empty vector (MSCV2.2 or pcDNA3) using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. For experiments using recombinant proteins, fresh media containing 10 μg/ml PA and 2.5 μg/ml LF, supplemented with or without 10 μM MG132, 1 μM Bortezomib, or 0.5 μM NMS-873, was added to cells for 2-4 hours. For auxin-inducible degradation, AID–NLRP1B and TIR1-producing constructs (TIR1, Addgene #80073) were co-transfected and treated with 500 μM indole-3-acetic acid sodium salt (IAA) (Sigma) for 3-6 hours in the presence or absence of 10 μM MG132. In all experiments, cells were lysed in RIPA buffer with protease inhibitor cocktail (Roche) 24 hours post-transfection.

Endogenous NLRP1B activity assays

Immortalized 129 (i129) BMDMs ($2.5x10^6$) were plated in 6-well plates. Two hours prior to challenge, cells were primed with 1.0 μg/ml Pam3CSK4 (Invivogen). Cells were washed with PBS and media was replaced with 0.5 ml of Opti-MEM (Gibco) with or without 20 μg/ml PA, 10 μg/ml LF and/or 10 μM MG132. Cells and media were lysed by addition of 120 μl of 10 X RIPA buffer with protease inhibitor cocktail 2.5 hours post-treatment.

Immunoblotting and antibodies

Lysates were clarified by spinning at ~16,000×*g* for 10 minutes at 4°C. Clarified lysates were denatured in SDS loading buffer. Samples were separated on NuPAGE Bis–Tris 4%-12% gradient gels (ThermoFisher) following the manufacturer's protocol. Gels were transferred onto Immobilon-FL PVDF membranes at 35 V for 90 minutes and blocked with Odyssey blocking buffer (Li-Cor). Proteins were detected on a Li-Cor Odyssey Blot Imager using the following primary and secondary antibodies: 100 ng/ml anti-HA clone 3F10 (Sigma), 200 ng/ml anti-IL-1β (R&D systems, AF-401-NA), 1 μg/ml anti-GFP (Clontech, JL8), 2 μg/ml anti-mCherry (ThermoFisher, 16D7), 1 μg/ml anti-CASP1 (Adipogen, AG-20B-0042-C100). Anti-MBP (NEB, E8032S) and anti-Ubiquitin (Cell Signaling, P4D1) antibodies were used at 1:1000 dilution of manufacturer's stock. Alexfluor-680 conjugated secondary antibodies (Invitrogen) were used at 0.4 μg/ml. Band intensities were quantified with Image Studio Lite software v5.2.5. The complete gel images are shown in Fig. S7.

To produce the 2A12 mouse anti-NLRP1B monoclonal antibody, the pAcSG2-*Nlrp1b* construct was co-transfected with BestBac linearized baculovirus DNA (Expression Systems) into SF9 cells following the manufacturer's protocol to generate

NLRP1B expressing baculovirus. Primary virus was amplified in SF9 cells. NLRP1B was produced by infecting 4 l of High Five cells with 1 ml of amplified virus per l cells. Cells were harvested 48 hours after infection by centrifugation at 300×*g* for 15 minutes. Cell pellets were resuspended in lysis buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 1% NP-40, 5% glycerol) and lysed on ice using a dounce homogenizer. Homogenized samples were clarified at 24,000x*g* for 30 minutes and supernatants were batch bound to 1 ml of amylose resin for 2 hours at 4°C. Samples were column purified by gravity. Resin was washed with 50 ml of wash buffer (20 mM Hepes pH 7.4, 150 mM NaCl, 0.02% NP-50, 5% glycerol). Samples were eluted with 1-ml elution buffer (20 mM Hepes pH 7.4, 150 mM NaCl, 0.02% NP-50, 5% glycerol, 20 mM maltose) fractions. Peak elutions were pooled and MBP was cleaved by treatment overnight with 3C protease. Free MBP was removed by passing the sample over amylose resin. BALB/c mice were immunized with 10 μg of NLRP1B in 100 μl of Sigma adjuvant on day 0, 21, and 42, and with 10 μg of NLRP1B without adjuvant on day 60. Mice were sacrificed on day 63. Splenocytes were fused the with the P3X63-Ag8.653 parental line. Clones were screened via ELISA against recombinant NLRP1B protein or recombinant FLAG-tagged MBP protein to identify clones specifically reactive to NLRP1B. Clarified supernatant from the hybridoma clone 2A12 was used for immunoblotting.

In Vitro Ubiquitylation Assay

Recombinant 129 or B6 NLRP1B was produced in insect cells and purified as described above prior to 3C treatment. Recombinant IpaH7.8, IpaH7.8 C357A (catalytic mutant), and IpaH9.8 were expressed in BL21 *E. Coli*. BL21. 1 l of cells were grown to ~0.7 OD600 and induced with 1 M IPTG (Sigma) for 4 hours at 37 °C. Pellets were resuspended in 50 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40 and sonicated to lyse. Samples were clarified at 24,000×*g* for 30 minutes. The NaCl concentration of the supernatants was increased to 400 mM and 20 mM imidazole pH8.0 was then added to samples. Supernatants were batch bound to 1 ml of Ni resin (Qiagen) at 4°C for 2 hours. Samples were purified by gravity, washed with 50 ml of 20 mM Tris pH 7.4, 400 mM NaCl, 20 mM imidazole pH 8.0. Protein was eluted in 1-ml fractions of 20 mM Tris pH 7.4, 150 mM NaCl, 250 mM imidazole pH 8.0. Elution peaks were pooled and desalted into 20 mM HEPES pH 7.4, 150 mM NaCl and 2 mM DTT.

In vitro ubiquitylation assays were performed in 25 mM Tris pH 7.4, 50 mM NaCl, 10 mM $MgCl_2$, 5 mM ATP, 0.1 mM DTT with 60 nM Ubiquitin E1 (Boston Biochemistry), 200 nM UbcH5c (Boston Biochemistry), 10 μM Ubiquitin (Boston Biochemistry), 300 nM IpaH and 270 nM NLRP1B. Reaction was run for 1 hour at 37 °C. Solutions were then batch bound to anti-FLAG M2 agarose gel (Sigma) at 4°C for 2 hours. Bound samples were column-purified and washed with 5 ml of HBS (20 mM Hepes, 150 mM NaCl). Final samples were eluted in 150 μl HBS+150 μg/ml of FLAG peptide.

Native gel oligomerization assay

Samples were transfected into 293T cells with constructs as above in a six-well plate. After 24 hours, samples were harvested by removing media and washing cells off plate with cold PBS. Harvested cells were centrifuged at 300x*g* for 10 minutes at 4°C. Cells were lysed in lysis buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 1% NP-40, 5% glycerol) and samples were clarified by spinning at 16,000x*g* for 10 minutes at 4°C. Samples were run on NativePAGE Bis–Tris gels (ThermoFisher) according to manufacturer's protocols.

Detection of UPA–CARD and ASC speck formation by immunofluorescence

293T cells were grown on fibronectin-coated coverslips. Constructs producing NLRP1B and ASC were co-transfected with constructs producing TEV or an empty vector. The NLRP1B construct was designed with a C-terminal FLAG and N-terminal HA tag, where the HA sequence was inserted following the P1′ position of the TEV cleavage site. Twenty hours post-transfection, cells were fixed with 4% PFA/PBS (20 minutes) and permeabilized in 0.5% saponin in PBS (5 minutes). Blocking and antibody staining was performed at room temperature in 5% BSA/0.1% saponin in PBS. Primary antibodies: 0.5 μg/ml rabbit anti-ASC (Santa Cruz, N-15), 0.5 μg/ml rat anti-HA (Roche, 3F10), 1 μg/ml mouse anti-FLAG (Sigma, M2). Secondary antibodies: 3 ug/ml AMCA-labeled goat anti-rabbit IgG (Jackson Laboratories), 4 ug/ml Alexa Fluor 647-labeled goat anti-rat IgG, 4 ug/ml Alexa Fluor 555-labeled goat anti-mouse (Molecular Probes). Coverslips were mounted onto slides using Vectashield medium (Vector Laboratories, Inc., H-1000), and imaged on a ZEISS LSM 710 with a W Plan-Apochromat 40x/1.0 DIC oil immersion objective. Fluorophores were excited at 405, 543 and 633 nM. To quantify the presence of FLAG or HA per ASC speck, the Imaris imaging software (Bitplane) was used to first identify ASC specks as objects, then to count FLAG- and/or HA-positive objects based on a fluorescence intensity threshold set manually for each channel. Thresholds were determined manually using a training set of samples and controls (i.e., no primary antibody), and applied in batch to all samples.

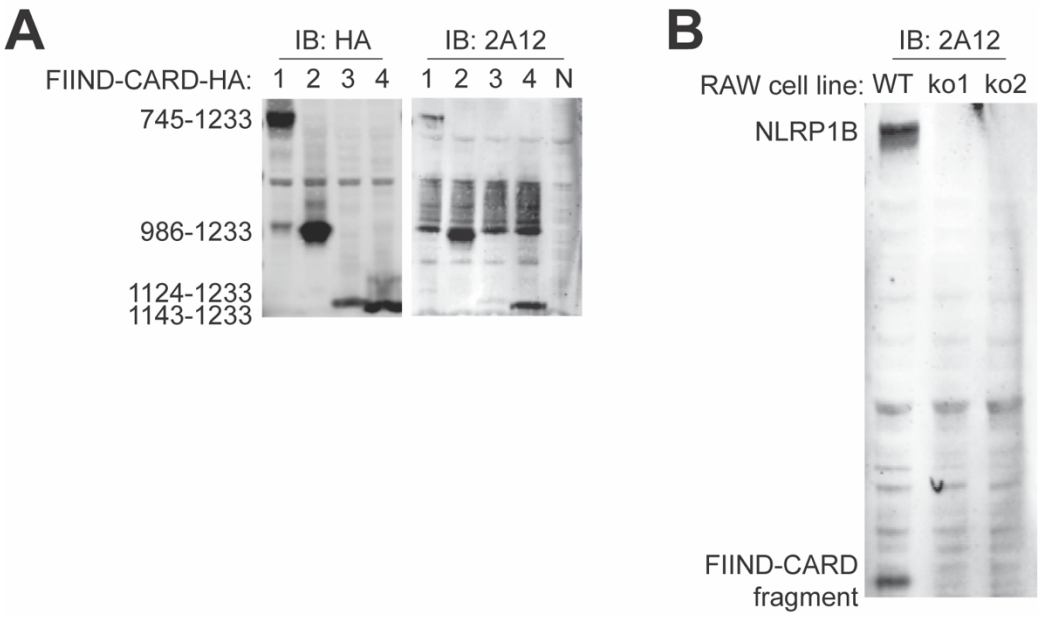

Figure S1. The 2A12 monoclonal antibody recognizes the CARD domain of NLRP1B. NLRP1B was detected using the anti-NLRP1B monoclonal Ab 2A12 in lysates from WT or *Nlrp1b*$^{-/-}$ RAW264.7 cells. ko1 and ko2, CRISPR-Cas9 *Nlrp1b*$^{-/-}$ cells clone 1 and 2 (*47*).

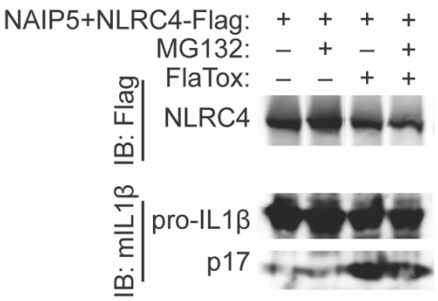

Figure S2. Proteasome inhibitors do not block NAIP/NLRC4 inflammasome activation.
293T cells were transfected with expression constructs for NAIP5, NLRC4, CASP1 and pro-IL-1β and then stimulated with FlaTox (PA + LFn–FlaA) in the presence or absence of MG132 (10 μM). Gel images are representative of experiments performed at least three times.

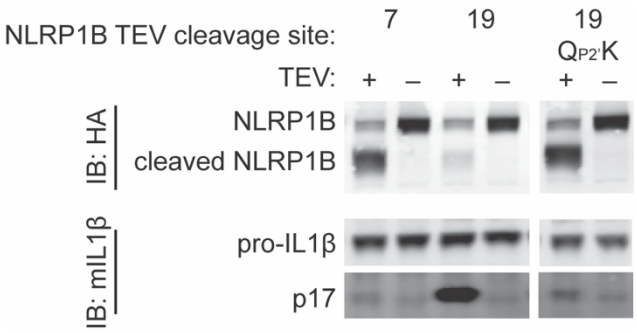

Figure S3. P2′ residue identity modulates TEV-induced proteasomal degradation and activation of NLRP1B.
The amino acid from the P2′ position from a relatively inactive TEV cleavage site (site 7 (see Fig. 1E), P2′=K) was introduced into the P2′ position of a relatively active TEV cleavage site (site 19, P2′=Q). The activity of the resulting C-terminal HA-tagged constructs was tested in 293T cells as described in Fig. 1. The P2′ lysine is sufficient to both stabilize and prevent NLRP1B activation via cleavage at site 19, suggesting that residues beyond the P1′ position are important for determining the stability (and subsequent activation) of NLRP1B. Gel images are representative of experiments performed at least three times.

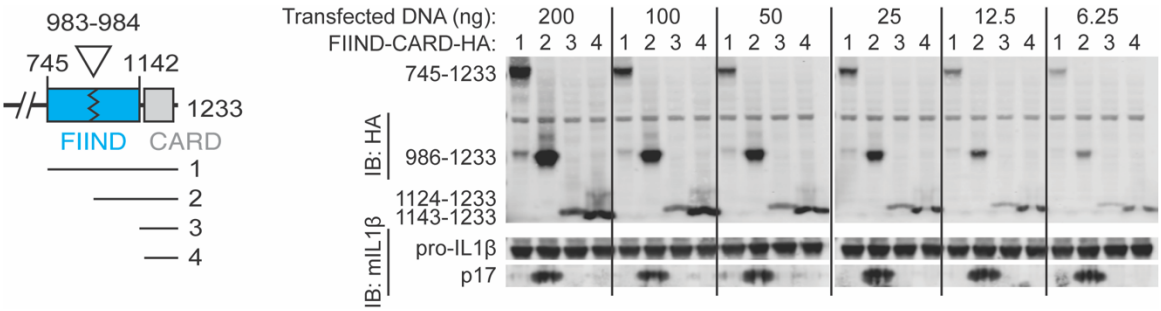

Figure S4. The FIIND(UPA)–CARD fragment is a potent activator of IL-1β processing.
The following C-terminal HA-tagged NLRP1B variants were transfected into 293T cells: full length FIIND(ZU5+UPA)–CARD [(1), 745-1233)], FIIND(UPA)–CARD fragment [(2), 986-1233)], FIIND(UPA)–CARD truncation [(3), 1124-1233)] or the CARD domain only [(4), 1143-1233)] as described for Fig. 3B. Gel images are representative of experiments performed twice.

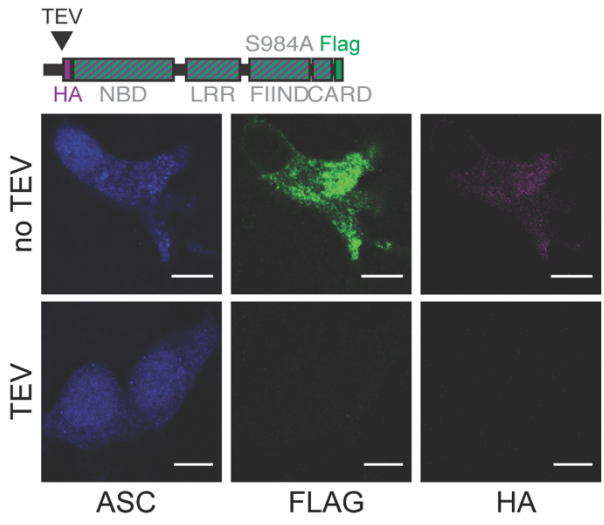

Figure S5. FIIND auto-processing is required for release of the FIIND(UPA)–CARD and its colocalization to the ASC speck.
293T cells were transfected with constructs producing ASC (blue) and an NLRP1B FIIND mutant (S984A) variant marked with a C-terminal FLAG (green) and an N-terminal HA (magenta). As schematized, the HA is inserted directly after the TEV cleavage site, allowing detection of the TEV-cleaved protein. Representative images depict cytosolic FLAG and HA signal in untreated and TEV-expressing cells. Unlike in Fig. 3G, where ASC colocalizes specifically with FLAG and not HA, no specific FLAG–ASC staining was observed for the FIIND mutant protein. Scale bar, 10 microns. Images are representative of experiments performed at least three times.

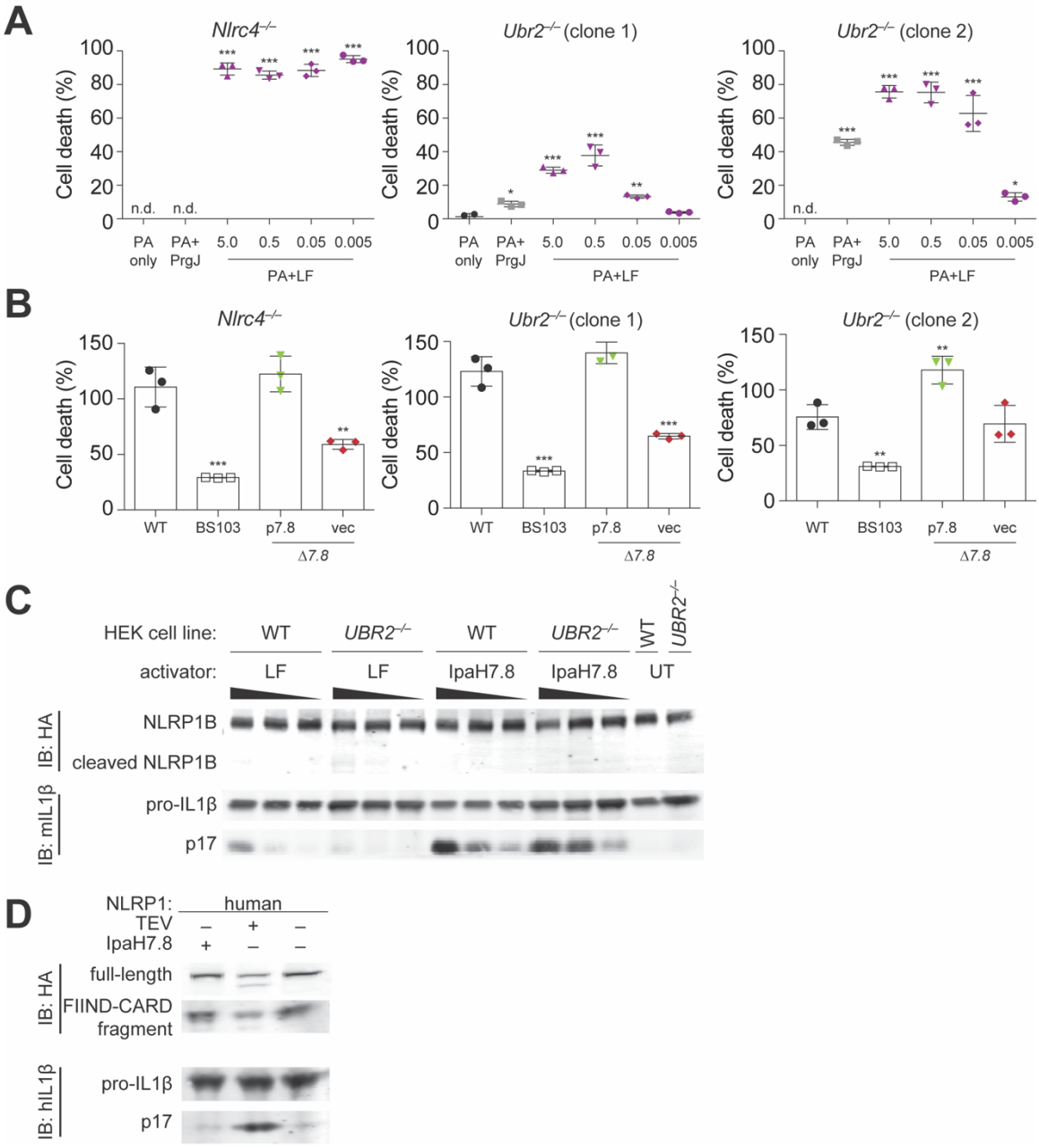

Figure S6. IpaH7.8 activates mouse NLRP1B, but not human NLRP1, independently of N-end rule ubiquitin ligases.

(A) NAIP2 inflammasome ligand (LFn-PrgJ) or varying amounts of NLRP1B agonist (lethal factor, LF) (μg/mL) was delivered into *Nlrc4*$^{-/-}$ or *Ubr2*$^{-/-}$ RAW264.7 cells via the PA channel. Inflammasome activation was measured by assessing LDH-release following CASP1-dependent pyroptosis. LF-mediated cell death remains saturated in *Nlrc4*$^{-/-}$ cells at all LF concentrations, but is reduced in two *Ubr2*$^{-/-}$ cell lines at lower concentrations. (**B**) RAW264.7 cells of the indicated genotypes were infected (MOI 10) with WT *Shigella flexneri* strain 2457T (black circle) or mutants strains. BS103, virulence plasmid-cured (grey box); vec, Δ*7.8* strain complemented with pCMD136 empty vector (red diamond); Δ*7.8* strain complemented with pCMD136 *ipaH7.8* (green inverted

triangle). (**C**) The NLRP1B inflammasome was reconstituted in wild-type (WT) or *UBR2*$^{-/-}$ 293T cells as described in Fig. 1 and was activated by co-transfection with expression constructs for lethal factor (LF) or IpaH7.8. Inflammasome activation was assessed by immunoblotting for CASP1-dependent processing of IL-1β to p17. UT, untransfected. (**D**) The human NLRP1 inflammasome (NLRP1, ASC, CASP1) was reconstituted in 293T cells, and inflammasome activation was monitored by immunoblotting for the processing of human pro-IL-1β to p17 by co-transfected human CASP1. A TEV-cleavable human NLRP1 variant was used to allow for TEV-mediated inflammasome activation. Images are representative of experiments performed at least three times.

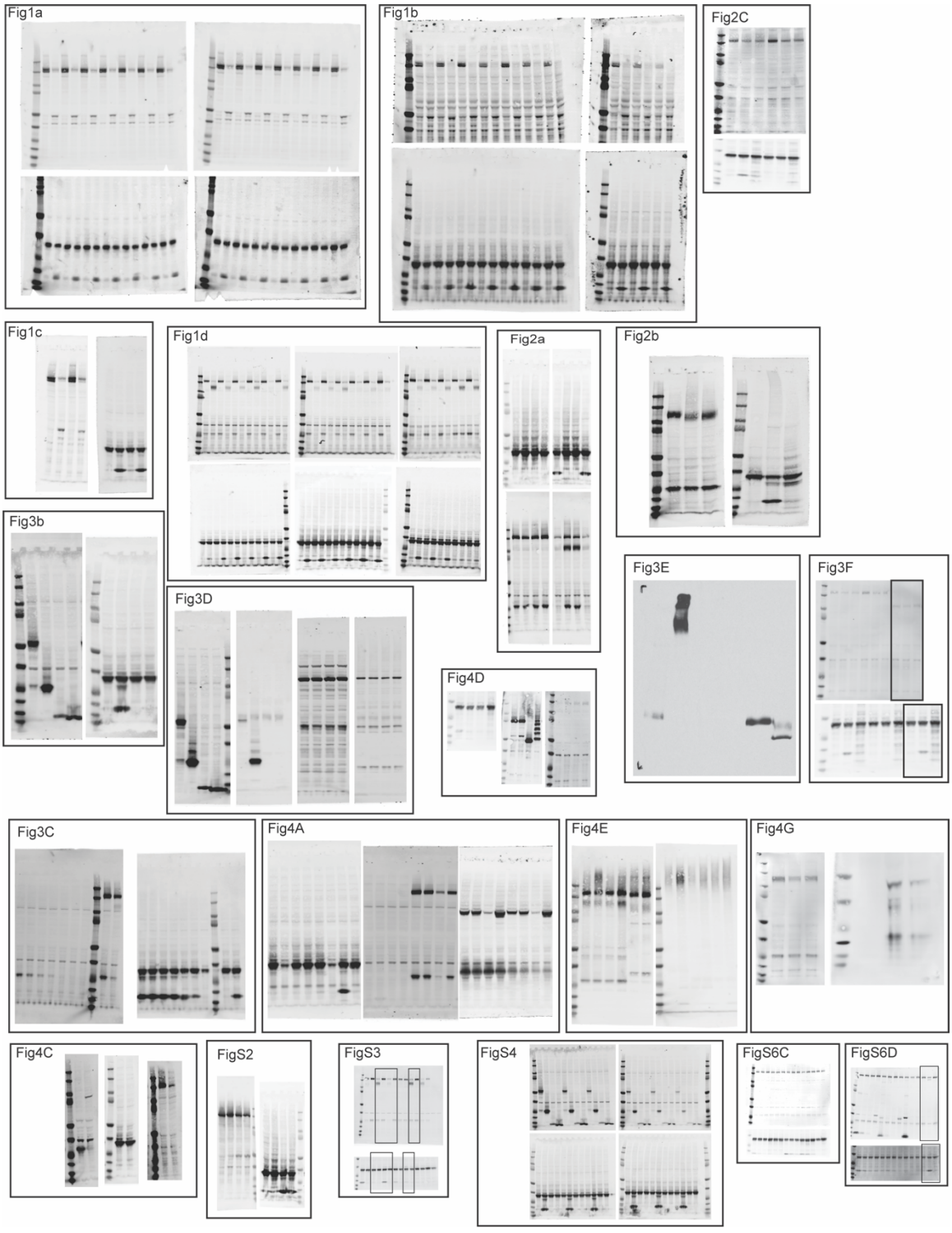

Figure S7. Complete images for all gels.
Above are shown the full images for each gel presented.