Supporting Information

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SI Materials and Methods

Cell Culture and Drug Treatment. Human colorectal cancer (CRC) cell lines, including HT29, SW1463, LoVo, RKO, HTC116, DLD1, SW48, SW480, SW1417, SW837, Lim1215, and Caco2, were purchased from American Type Culture Collection (ATCC). Vaco400 and Vaco425 were from Bert Vogelstein, Johns Hopkins University, Baltimore. These CRC cell lines were last tested and authenticated for genotypes, drug response, morphology, and absence of mycoplasma in February 2016. The CRC cell lines were cultured in McCoy's 5A modified medium (Invitrogen). *FADD* null Jurkat human leukemia cells (ATCC) were cultured in RPMI 1640 medium (Thermo Fisher Scientific). Immortalized WT cells, *p65* KO cells, *BAX*/*BAK* DKO cells, *PUMA* KO MEFs, 293T cells, and HeLa cells, were cultured in DMEM medium (Invitrogen). BMDMs were differentiated from WT or *PUMA* KO bone marrow cells for 7 d using DMEM medium supplemented with 30% L929-cell conditioned medium containing macrophage colony-stimulating factor secreted by L929 cells (1). To prepare L929-cell conditioned medium, L929 cells (ATCC) were cultured in DMEM for 7–10 d, and the medium was collected and passed through a 0.22-μm filter. All cells were maintained in a 37 °C incubator at 5% CO_2. Cell culture media were supplemented with 10% defined FBS (HyClone), 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen).

Cells were plated and treated with drugs at 40–50% density in 12-well plates. Chemicals used included LBW242 (Novartis); STS, NSA, and BAY 11–7082 (EMD Millipore); poly I:C (Tocris Bioscience); LPS (Sigma-Aldrich); Remicade and human TNF-α (R&D Systems); mouse TNF-α (Sino Biological); B/B homodimerizer (AP20187; Clontech); z-VAD (Bachem); and Nec-1 (Thermo Fisher Scientific). B/B homodimerizer was diluted in 100% ethanol. TNF-α, poly I:C, and LPS were diluted in sterile water. All other agents were diluted with DMSO (Sigma-Aldrich). For NF-κB inhibition, cells were pretreated with BAY 11–7082 for 1 h before other treatment.

Analysis of Cell Viability and Death. For analysis of cell viability, ATP levels were measured using the CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega) according to the manufacturer's instructions. Luminescence was measured using a Wallac Victor 1420 Multilabel Counter (PerkinElmer). Viable cells were stained with crystal violet solution (0.05% crystal violet in 3.7% paraformaldehyde diluted in distilled water). Apoptosis was measured by counting cells with condensed and fragmented nuclei after nuclear staining with Hoechst 33258 (Invitrogen) (2). Cell death was also analyzed by flow cytometry (Accuri C6; BD Biosciences) of cells stained with PI (Invitrogen) as described previously (3). TEM was performed as described previously (3). Images were acquired with a JEOL JEM 1011 transmission electron microscope at 80 V fitted with a bottom-mount AMT 2K digital camera (Advanced Microscopy Techniques).

Analysis of Protein and mRNA Expression. Western blot analysis was performed as described previously (2). Antibodies included those for PUMA, Bad, Bid, Bim, cleaved caspase 3, FOXO3a, STAT1, p65, and phospho-p65 (S536) (Cell Signaling Technology); cytochrome oxidase subunit IV (Invitrogen); AIF, Bax, p53, Mcl-1, cytochrome *c*, Lamin A/C, and E2F1 (Santa Cruz Biotechnology); DAI/Zbp1, Flag, and β-actin (Sigma-Aldrich); Bak, Noxa, Bcl-2, and α-tubulin (EMD Millipore); STING, RIP3, human phospho-RIP3 (S227), human phospho-MLKL (S358), mouse phospho-MLKL (S345), Drp1, HMGB1, and TNFR1 (Abcam); GFP, RIP1, and Bcl-X_L (BD Transduction); and p73 (Bethyl Labs).

For analysis of mRNA expression of target genes, total RNA was isolated using the Mini RNA Isolation II Kit (Zymo Research) according to the manufacturer's protocol. Total RNA (1 μg) was used to generate cDNA using SuperScript II reverse-transcriptase (Invitrogen). Real-time RT-PCR reactions were performed using the primers listed in Table S1 and under previously described conditions (4).

Transfection and siRNA/shRNA Knockdown. Transfection of plasmids and siRNA was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. PUMA-expressing adenovirus and V5-Bcl-X_L-pcDNA3.1 construct have been described previously (5). GFP-tagged full-length (WT) *DAI*/*Zbp1* vector was constructed by cloning a *DAI*/*Zbp1* cDNA fragment generated by RT-PCR into eGFP-N1 plasmid. Mutant *DAI*/*Zbp1* with a depletion of its N-terminal amino acid 6–150 residues containing the DNA-binding domain (6) was generated by site directed mutagenesis on WT eGFP-N1-DAI/Zbp1 vector. The SiRNA sequences used to knock down genes are listed in Table S2, except those for *IFNAR1* (sc-35637; Santa Cruz Biotechnology) and *p65* and *FOXO3a*, as described previously (7, 8).

Lentiviral shRNA vectors for stable knockdown are listed in Table S3. Lentiviral particles were generated by cotransfecting 293T cells with the shRNA lentiviral vectors pMD2.G (VSVG), pMDLg/pRRE, and pRSV-REV (Addgene). After lentiviral transduction, cells were plated in 96-well plates and selected by 5 μg/mL puromycin (EMD Millipore). Knockdown of *RIP1*, *RIP3*, *PUMA*, *DAI*/*Zbp1*, *p53*, and *Bcl-X_L* in the puromycin-resistant clones was verified by Western blot analysis.

Retroviruses were produced by cotransfecting 293T cells with the RIP3 dimerization construct pBabe RIPK3-2xFV (9) (from Andrew Oberst, University of Washington, Seattle, WA), or control pBabe GFP vector, along with the packaging plasmids VSVG and Gag-Pol (from Kathy H. Y. Shair, University of Pittsburgh, Pittsburgh, PA). Retroviruses were harvested at 48 h after transfection by collecting media followed by centrifugation at 1,000 × *g*. Filtered supernatants were used to infect WT and *PUMA* KO MEFs, followed by selection with 2.5 μg/mL puromycin for 5 d. RIP3 dimerization was induced by treating cells with B/B homodimerizer (Clontech), a synthetic, cell-permeable ligand that can induce homodimerization of FV fusion proteins, as described previously (9).

Analysis of NF-κB Nuclear Translocation. NF-κB nuclear translocation was analyzed by p65 Western blotting of nuclear and cytoplasmic fractions isolated from HT29 cells treated with 2 μM LBW242 along with 10 μM z-VAD (L+Z) for 3 h in 75-cm^2 flasks using the NE-PER Nuclear/Cytoplasmic Extraction Kit (Thermo Fisher Scientific) according to the manufacturer's instructions, and analyzed by p65 Western blotting. For p65 immunofluorescence, HT29 cells treated with L+Z for 3 h in chamber slides were stained with anti-p65 (Cell Signaling Technology) overnight at 4 °C, following by secondary staining with anti-rabbit Alexa Fluor 488-conjugated secondary antibody (Invitrogen) for 1 h at room temperature as described previously (7). Images were acquired with an Olympus IX71 microscope.

Luciferase Assays. Luciferase reporter constructs containing WT or mutant *PUMA* promoter sequences in pBV-Luc vector have been described previously (7). To measure reporter activities,

cells were transfected with WT or mutant *PUMA* reporters along with the transfection control β-galactosidase reporter pCMVβ (Promega). Cell lysates were collected, and luciferase activities were measured and normalized to those of pCMVβ as described previously (7). All reporter experiments were performed in triplicate and repeated three times.

TNF-α ELISA. To analyze TNF-α secretion, HT29 cells were lysed in 1% Triton X-100 in PBS supplemented with Roche Complete Protease Inhibitor. Total protein levels were determined by the Bradford protein assay (Bio-Rad). TNF-α levels in cell lysate were quantified using the hTNF-α ELISA Kit (R&D Systems) according to the manufacturer's instructions.

Targeting of *PUMA* and *RIP3* by CRISPR/Cas9. *PUMA* and *RIP3* in HT29 cells were targeted by CRISPR/Cas9 using guide RNAs designed based on previously published protocols (10), including *PUMA* sgRNA1 (5′-AGCTCCCCGGAGCCCGTAGA-3′), *PUMA* sgRNA2 (5′-CAGGGCTGCTTCCACGACGT-3′), and *RIP3* sgRNA (5′-CTCGTCGGCAAAGGCGGGTT-3′). DNA duplexes with sgRNA sequences were cloned into the pSpCas9-2A-GFP vector (48138; Addgene). For gene targeting, HT29 cells were transfected with sgRNA vectors. After 72 h, cells were harvested and subjected to FACS analysis to isolate single GFP-positive cells. After 2–3 wk in culture, single cell clones with *PUMA* or *RIP3* targeting were identified by Western blotting for PUMA or RIP3 and then verified by genomic sequencing of the targeted regions for identifying altered sequences. *PUMA* KO (KO1 and KO2) and *RIP3* KO HT29 cells used in the described experiments contain an insertion frame-shift mutation in coding sequences.

Analysis of Mitochondrial DNA, Proteins, Membrane Potential, and Morphology. Mitochondrial and cytosolic fractions were isolated by differential centrifugation as described previously (7). DNA was extracted from mitochondrial fractions, cytosolic fractions, and total cell lysates using the Quick-gDNA MiniPrep Kit (Zymo Research) according to the manufacturer's protocol. Genomic PCR was performed using the primer pairs listed in Table S1. Mitochondria and mitochondrial DNA were analyzed by confocal fluorescence microscopy after cell staining with 100 nM MitoTracker Red CMXRos (Thermo Fisher Scientific) and 3 μg/mL PicoGreen (Thermo Fisher Scientific) for 1 h at 37 °C. Colocalization of MitoTracker Red and PicoGreen was quantified by analysis of Pearson's correlation in more than 20 randomly selected cells using ImageJ software (https://imagej.nih.gov/ij/). Mitochondrial interconnectivity and elongation indices (inverse circularity) were quantified by analyzing more than 20 randomly selected cells using an ImageJ micro as described previously (11). Mitochondrial membrane potential was analyzed by flow cytometry after cell staining with MitoTracker Red CmxRos (Thermo Fisher Scientific) at room temperature for 15 min.

HT29 cells with mitochondria DNA depletion were generated by treating cells with EB (450 ng/mL; Sigma-Aldrich) for 4 d (12). After recovery in media without EB for 16 h, cells were replated and treated. Depletion of mitochondria DNA was verified by PCR analysis of mitochondrial *CytB*, with the nuclear *GAPDH* gene as a control.

ChIP. ChIP with anti-p65 (Santa Cruz Biotechnology) or anti-GFP (BD Biosciences) antibody was performed using the EMD Millipore Chromatin Immunoprecipitation Assay Kit as described previously (4). Binding of p65 to the *PUMA* promoter and of GFP-DAI/Zbp1 to mitochondrial DNA was analyzed using the ChIP PCR primers listed in Table S1.

Analysis of Mouse Embryos. All animal experiments were approved by the University of Pittsburgh's Institutional Animal Care and Use Committee. The previously described *FADD* knockout (13) and *PUMA* knockout (14) strains were backcrossed on the C57BL/6 background for at least 10 generations. Male heterozygous C57BL/6 $FADD^{+/-}$ mice were crossed with C57BL/6 $PUMA^{-/-}$ mice to generate $FADD^{+/-}$;$PUMA^{+/-}$ double-heterozygous mice. Mating of male and female $FADD^{+/-}PUMA^{+/-}$ mice was set up in an evening. Female mice were examined early the next morning, and embryos were designated E0.5 on the day at which a vaginal plug was detected. E11.5–E13.5 embryos were dissected from pregnant mice, and genomic DNA was extracted from embryonic tissues on digestion with protease K in an SDS-containing lysis buffer. Genotyping was performed by PCR using the allele-specific primers listed in Table S1. FADD and PUMA protein expression in embryonic tissues was analyzed by Western blotting using previously described antibodies (13, 15). For postnatal analysis, pregnant females were closely monitored around the time of delivery (after 18.5 d postcoitum) by checking at least twice daily. Neonates were killed at birth for genotyping.

Dissected embryos were fixed in 10% buffered formalin overnight and embedded in paraffin. Paraffin-embedded sections were analyzed with H&E, TUNEL (EMD Millipore), active caspase-3 (Cell Signaling Technology), and HMGB1 (Abcam) immunostaining with Alexa Fluor 488-conjugated secondary antibody (Invitrogen) for detection as described previously (7).

$FADD^{-/-}$ embryos at E9.5 and DKO embryos at E12.5 were used to prepare primary MEFs as described previously (13). MEFs were cultured in complete DMEM to 80% confluence. Images were acquired with an Olympus IX71 inverted fluorescent microscope.

Statistical Analysis. Statistical analyses were performed using GraphPad Prism 4 software. P values were calculated using the Student's t test or χ^2 test, and $P < 0.05$ was considered to indicate statistical significant. In the figures, data are expressed as mean ± 1 SD.

1. He S, Liang Y, Shao F, Wang X (2011) Toll-like receptors activate programmed necrosis in macrophages through a receptor-interacting kinase-3-mediated pathway. *Proc Natl Acad Sci USA* 108:20054–20059.
2. Chen D, et al. (2014) TAp73 promotes cell survival upon genotoxic stress by inhibiting p53 activity. *Oncotarget* 5:8107–8122.
3. Brown MF, et al. (2015) Loss of caspase-3 sensitizes colon cancer cells to genotoxic stress via RIP1-dependent necrosis. *Cell Death Dis* 6:e1729.
4. Wang P, et al. (2009) PUMA is directly activated by NF-kappaB and contributes to TNF-alpha-induced apoptosis. *Cell Death Differ* 16:1192–1202.
5. Ming L, Wang P, Bank A, Yu J, Zhang L (2006) PUMA dissociates Bax and Bcl-X(L) to induce apoptosis in colon cancer cells. *J Biol Chem* 281:16034–16042.
6. Thapa RJ, et al. (2016) DAI senses influenza A virus genomic RNA and activates RIPK3-dependent cell death. *Cell Host Microbe* 20:674–681.
7. Chen D, Wei L, Yu J, Zhang L (2014) Regorafenib inhibits colorectal tumor growth through PUMA-mediated apoptosis. *Clin Cancer Res* 20:3472–3484.
8. Dudgeon C, et al. (2010) PUMA induction by FoxO3a mediates the anticancer activities of the broad-range kinase inhibitor UCN-01. *Mol Cancer Ther* 9:2893–2902.
9. Orozco S, et al. (2014) RIPK1 both positively and negatively regulates RIPK3 oligomerization and necroptosis. *Cell Death Differ* 21:1511–1521.
10. Ran FA, et al. (2013) Genome engineering using the CRISPR-Cas9 system. *Nat Protoc* 8: 2281–2308.
11. Dagda RK, et al. (2009) Loss of PINK1 function promotes mitophagy through effects on oxidative stress and mitochondrial fission. *J Biol Chem* 284:13843–13855.
12. Rongvaux A, et al. (2014) Apoptotic caspases prevent the induction of type I interferons by mitochondrial DNA. *Cell* 159:1563–1577.
13. Zhang H, et al. (2011) Functional complementation between FADD and RIP1 in embryos and lymphocytes. *Nature* 471:373–376.
14. Qiu W, et al. (2011) PUMA-mediated apoptosis drives chemical hepatocarcinogenesis in mice. *Hepatology* 54:1249–1258.
15. Qiu W, et al. (2011) PUMA-mediated intestinal epithelial apoptosis contributes to ulcerative colitis in humans and mice. *J Clin Invest* 121:1722–1732.

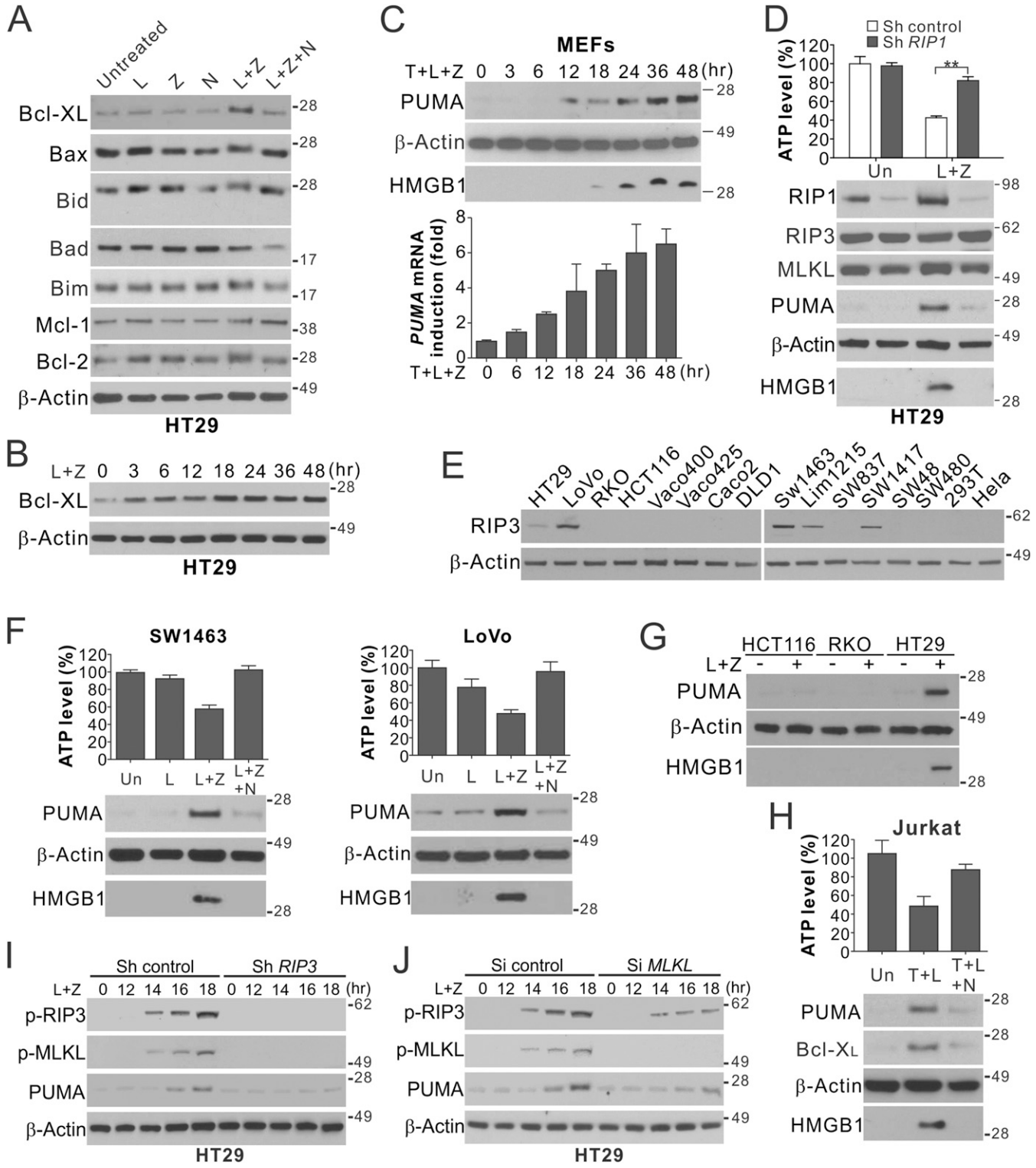

Fig. S1. PUMA induction in RIP3-expressing cells undergoing necroptosis. (*A*) Western blots of indicated Bcl-2 family proteins in HT29 cells treated with the SMAC mimetic LBW242 (L; 2 μM), the pan-caspase inhibitor z-VAD (Z; 10 μM), the RIP1 inhibitor Necrostatin-1 (N; 20 μM), or indicated combinations for 24 h. (*B*) Western blots of Bcl-X_L in HT29 cells treated with L+Z as in *A* at indicated time points. (*C*) WT MEFs were treated with 20 ng/mL TNF-α (T), 2 μM LBW242 (L), and 10 μM z-VAD (Z). (*Upper*) Western blots of PUMA in cell lysates and HMGB1 in 20 μL of cell culture medium at indicated time points. (*Lower*) *PUMA* mRNA expression at indicated time points. (*D*) HT29 cells stably expressing control or *RIP1* shRNA were treated with L+Z as in *A*. (*Upper*) ATP levels at 48 h. (*Lower*) Western blots of indicated protein expression and HMGB1 release in 20 μL of cell culture medium at 24 h. (*E*) Western blotting of RIP3 in 16 cell lines. (*F*) SW1463 (*Left*) and LoVo (*Right*) cells treated with L, L+Z, or L+Z+N as in *A* were analyzed for necroptosis and PUMA expression as in *D*. (*G*) PUMA expression and HMGB1 release in HCT116, RKO, and HT29 cells treated with L+Z as in *A* for 24 h were analyzed as in *D*. (*H*) *FADD* null Jurkat cells were treated with 10 ng/mL TNF-α and 2 μM LBW242 (T+L) for 24 h with or without Necrostatin-1 (N; 20 μM). (*Upper*) ATP levels after treatment. (*Lower*) Indicated proteins and HMGB1 release analyzed as in *D*. (*I*) Western blots of indicated proteins in HT29 cells stably transfected with control or *RIP3* shRNA and treated with L+Z as in *A* at indicated time points. (*J*) Western blots of indicated proteins in HT29 cells transfected with control scrambled or *MLKL* siRNA and treated with L+Z as in *A* at indicated time points. Values in *C*, *D*, *F*, and *H* are expressed as mean ± SD. $n = 3$. $^{**}P < 0.01$. Western blot data are representative of two independent experiments.

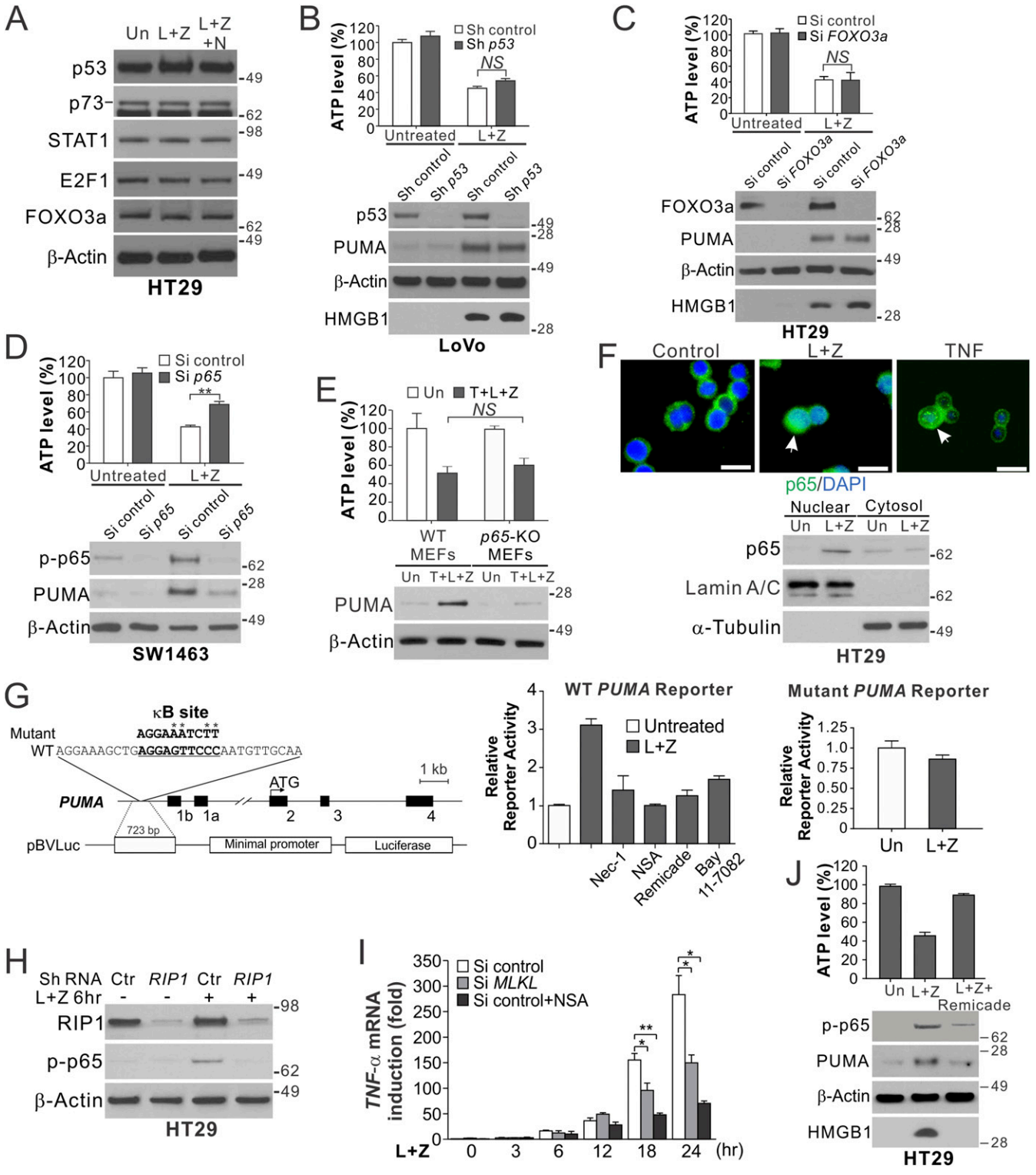

Fig. S2. *PUMA* is directly activated by p65 during necroptosis. (*A*) Western blots of indicated proteins in HT29 cells treated with L+Z (2 μM LBW242 and 10 μM z-VAD) with or without Necrostatin-1 (N; 20 μM) for 24 h. (*B*) *p53* WT LoVo cells stably transfected with control or *p53* shRNA were treated with L+Z. (*Upper*) ATP levels at 48 h. (*Lower*) Western blots of indicated proteins in cell lysates and HMGB1 in 20 μL of cell culture medium at 24 h. (*C*) HT29 cells transfected with control scrambled or *FOXO3a* siRNA were treated and analyzed as in *B*. (*D*) SW1463 cells transfected with control scrambled or *p65* siRNA and treated with L+Z. (*Upper*) ATP levels at 48 h. (*Lower*) Western blots of PUMA and phospho-p65 (p-p65; S536) at 24 h. (*E*) WT and *p65* KO MEFs treated with T+L+Z (20 ng/mL TNF-α, 2 μM LBW242, and 10 μM z-VAD) and analyzed as in *D*. (*F*) HT29 cells treated with L+Z for 6 h. (*Upper*) Analysis of p65 localization by immunostaining, with cells treated with TNF-α (10 ng/mL) for 6 h as a control. (Scale bars: 10 μm.) (*Lower*) Analysis of p65 localization by Western blotting of nuclear and cytosolic fractions from the treated cells. (*G*) HT29 cells transfected overnight with a luciferase reporter of the *PUMA* promoter containing either WT or mutant p65-binding sites. Cells were treated with L+Z for 24 h with or without pretreatment for 1 h with the RIP1 inhibitor Nec-1 (20 μM), the MLKL inhibitor NSA (1 μM), the anti-TNF Remicade (1 μg/mL), or the NF-κB inhibitor BAY 11–7082 (10 μM). (*Left*) Schematic representation of the p65-binding site in the *PUMA* promoter,

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with asterisks indicating the nucleotides changed in the mutant reporter. (*Right*) Luciferase activity of the WT and mutant *PUMA* promoter reporter after treatment. (*H*) Western blots of RIP1 and p-p65 in HT29 cells transfected with control or *RIP1* shRNA. (*I*) *TNF-α* mRNA expression at indicated time points in HT29 cells transfected with control or *MLKL* siRNA, or pretreated with NSA (1 μM), and then treated with L+Z. (*J*) HT29 cells treated with L+Z for 12 h were exposed to Remicade (1 μg/mL). Necroptosis and protein expression were analyzed as in *B*. Values in *B–E*, *G*, *I*, and *J* are expressed as mean ± SD. $n = 3$. NS, $P > 0.05$; $^{*}P < 0.05$; $^{**}P < 0.01$. Western blots are representative of two independent experiments.

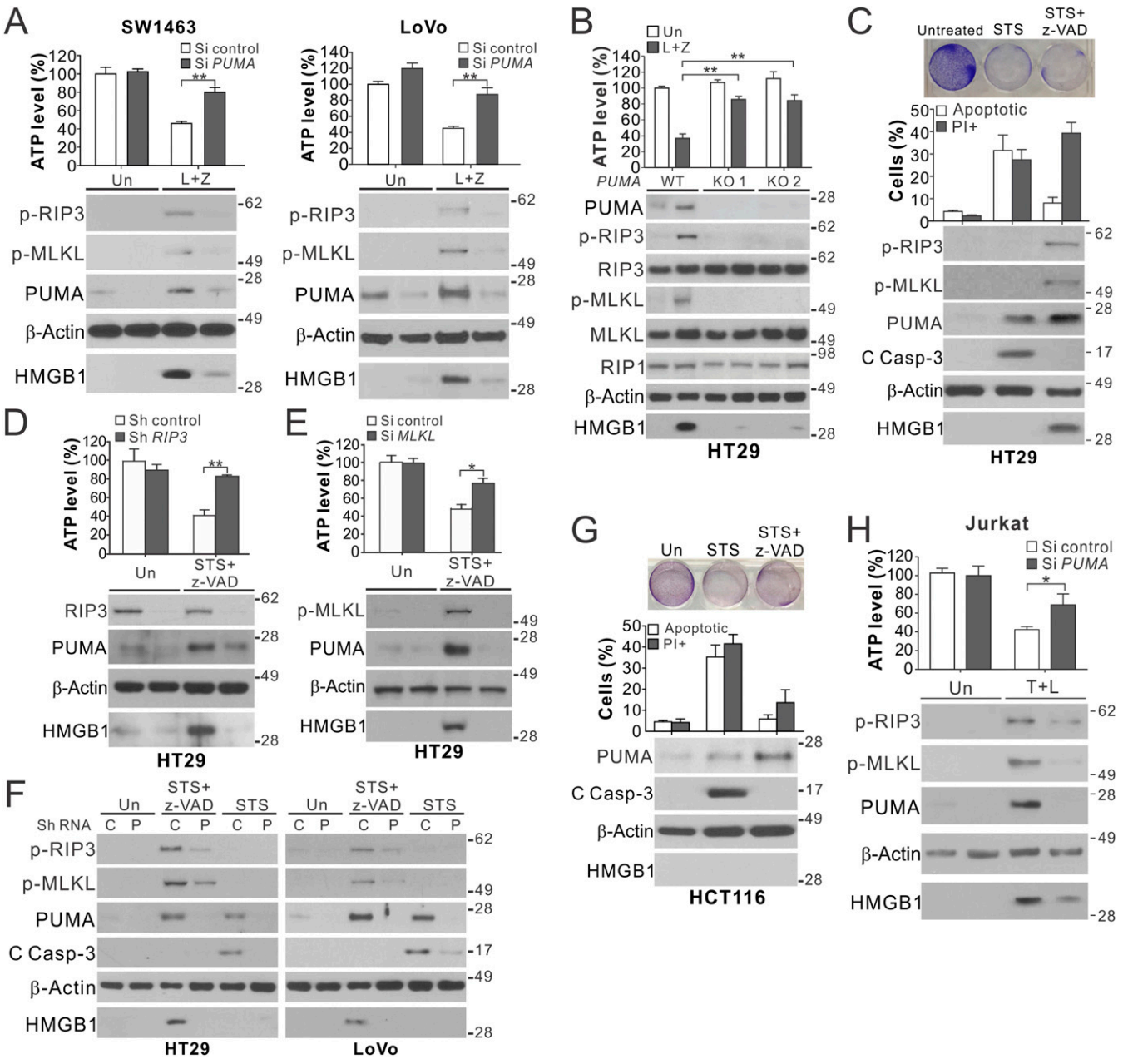

Fig. S3. PUMA induction contributes to induction of necroptosis in RIP3-expressing cells with caspase inhibition. (*A*) SW1463 (*Left*) and LoVo (*Right*) transfected with control scrambled or *PUMA* siRNA were treated with L+Z (2 μM LBW242 and 10 μM z-VAD). (*Upper*) ATP levels at 48 h. (*Lower*) Western blots of indicated proteins in cell lysates and HMGB1 in 20 μL of cell culture medium at 24 h. (*B*) Parental HT29 (WT) and two clones of isogenic *PUMA* KO (KO1 and KO2) cells generated by CRISPR/Cas9 using two different sgRNAs were treated with L+Z and analyzed as in *A*. (*C*) HT29 cells with or without pretreatment with z-VAD (Z; 10 μM) were treated with STS (200 nM). (*Upper*) Crystal violet staining at 48 h. (*Middle*) Analysis of apoptosis and PI staining at 48 h. (*Lower*) Indicated protein expression and HMGB1 release at 24 h, analyzed as in *A*. (*D*) HT29 cells stably transfected with control or *RIP3* shRNA were treated as in *C*. (*Upper*) ATP levels at 48 h. (*Lower*) Indicated proteins and HMGB1 release at 24 h analyzed as in *A*. (*E*) HT29 cells transfected with control scrambled or *MLKL* siRNA were treated and analyzed as in *D*. (*F*) HT29 and LoVo cells stably transfected with control (C) or *PUMA* shRNA (P) were treated with STS (200 nM for HT29; 50 nM for LoVo) with or without pretreatment with z-VAD (Z; 10 μM) for 24 h. Indicated proteins and HMGB1 release were analyzed as in *A*. (*G*) HCT116 cells with or without pretreatment with z-VAD (Z; 10 μM) were treated with 50 nM STS for 24 h. (*Upper*) Crystal violet staining of viable cells at 48 h after treatment. (*Middle*) Analysis of apoptosis by counting apoptotic nuclei after nuclear staining and nonapoptotic death at 48 h after treatment by flow cytometry of PI-stained cells. (*Lower*) Indicated proteins and HMGB1 release at 24 h after treatment analyzed as in *A*. C Casp-3, cleaved caspase-3. (*H*) *FADD* null Jurkat cells transfected with control scrambled or *PUMA* siRNA were treated with 10 ng/mL TNF-α and 2 μM LBW242 (T+L) for 24 h and analyzed as in *A*. Values in *A–E*, *G*, and *H* are expressed as mean ± SD. $n = 3$. $^{*}P < 0.05$; $^{**}P < 0.01$. Western blot data are representative of two independent experiments.

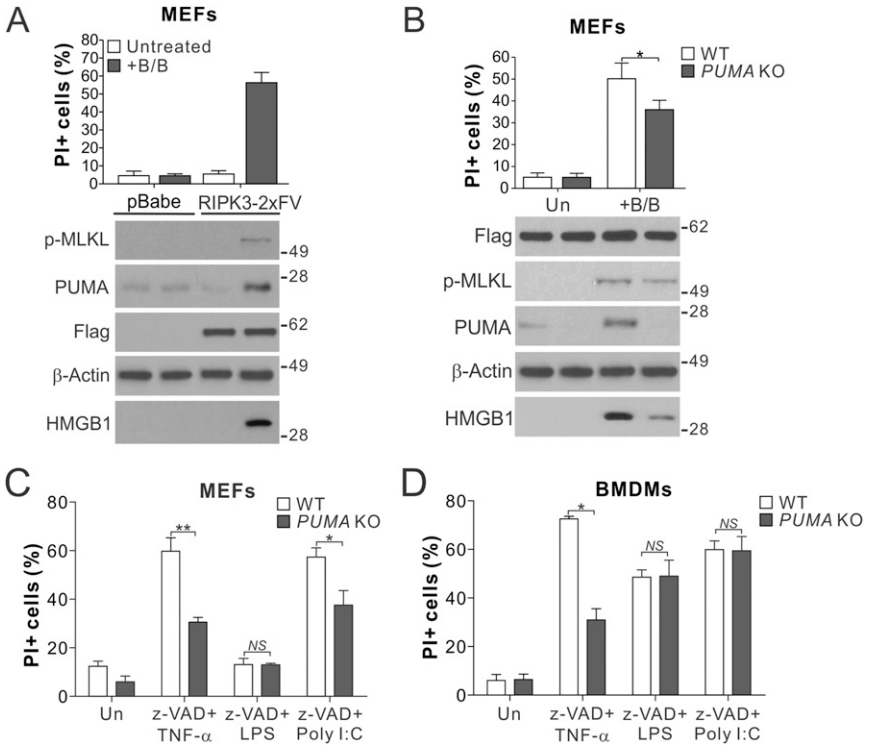

Fig. S4. Effects of *PUMA* KO on necroptosis in MEFs and BMDMs induced by different stimuli. (*A*) WT MEFs infected with a retroviral Flag-tagged RIP3 dimerization construct (pBabe RIPK3-2xFV) were treated with B/B homodimerizer (B/B) for 4 h to induce RIP3 dimerization. Cells infected with empty pBabe served as a control. (*Upper*) Analysis of cell death after treatment by PI staining. (*Lower*) Indicated proteins and HMGB1 release in 20 μL of cell culture medium were analyzed by Western blotting. (*B*) WT and *PUMA* KO MEFs infected with a retroviral Flag-tagged RIP3 dimerization construct (pBabe RIPK3-2xFV) were treated with B/B homodimerizer (B/B) for 4 h to induce RIP3 dimerization. (*Upper*) Analysis of nonapoptotic death by PI staining. (*Lower*) Indicated proteins and HMGB1 release analyzed as in *A*. (*C*) WT and *PUMA* KO MEFs were treated with z-VAD (20 μM) along with murine TNF-α (100 ng/mL), LPS (100 ng/mL), or poly I:C (50 μg/mL) for 24 h. Nonapoptotic cell death was analyzed by PI staining. (*D*) WT and *PUMA* KO BMDMs were treated and analyzed as in *C*. Values in *A*–*D* are expressed as mean ± SD. $n = 3$. NS, $P > 0.05$; *$P < 0.05$; **$P < 0.01$. Western blot data are representative of two independent experiments.

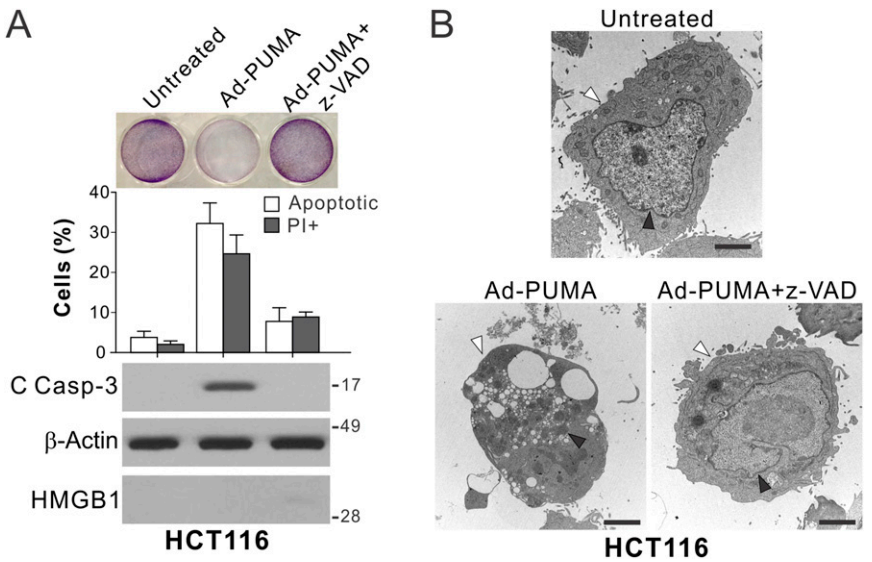

Fig. S5. PUMA does not induce necroptosis in HCT116 cells lacking RIP3. (*A*) HCT116 cells with or without pretreatment with z-VAD (Z; 10 μM) were infected with control or PUMA-expressing adenovirus (Ad-PUMA) for 24 h. (*Upper*) Crystal violet staining of viable cells after treatment. (*Middle*) Analysis of apoptosis by counting apoptotic nuclei after nuclear staining and nonapoptotic death by flow cytometry of PI-stained cells. (*Lower*) Western blots of cleaved caspase-3 (C Casp-3) in cell lysates and HMGB1 in 20 μL of cell culture medium at 24 h after treatment. Values are expressed as mean ± SD. $n = 3$. Western blot data are representative of two independent experiments. (*B*) Representative TEM pictures of HCT116 cells treated as in *A* for 24 h. Black arrowheads indicate nuclear fragmentation, and white arrowheads indicate plasma membranes. Cells treated with Ad-PUMA alone contain apoptotic and fragmented nuclei, whereas those treated with Ad-PUMA along with z-VAD lack signs of apoptosis and necroptosis. (Scale bars: 2 μm.)

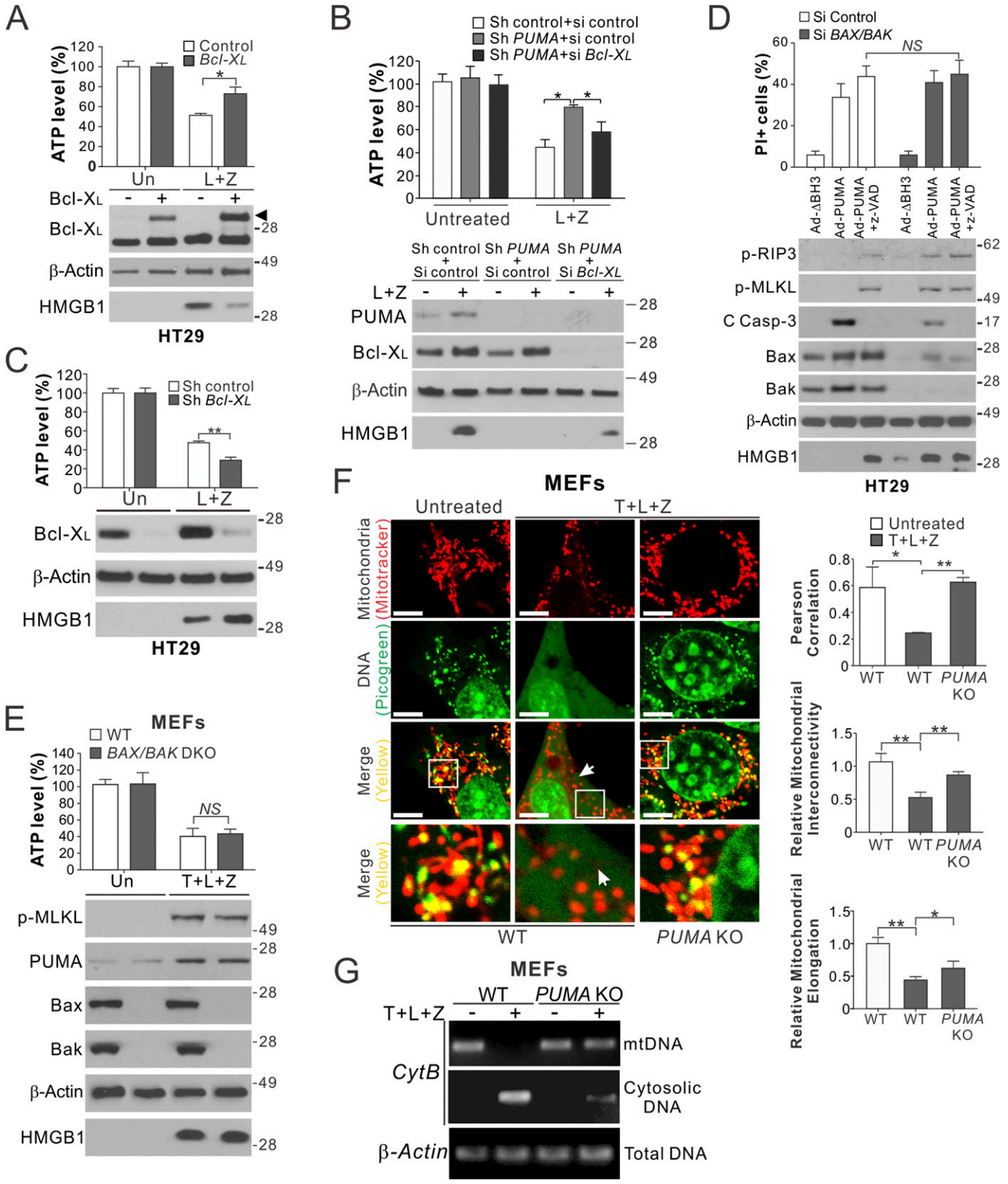

Fig. S6. PUMA promotes mitochondrial DNA release in necroptosis. (*A*) HT29 cells transfected with V5-Bcl-X_L were treated with L+Z (2 μM LBW242 and 10 μM z-VAD). (*Upper*) ATP levels at 48 h. (*Lower*) Western blots of Bcl-X_L in cell lysates and HMGB1 in 20 μL of cell culture medium at 24 h, with the arrowhead indicating transfected Bcl-X_L. (*B*) HT29 cells stably expressing control or *PUMA* shRNA were transfected with control scrambled or *Bcl-X*$_L$ siRNA and then treated with L+Z. (*Upper*) ATP levels at 48 h. (*Lower*) Western blots of indicated proteins and HMGB1 release at 24 h. (*C*) HT29 cells stably expressing control or *Bcl-X*$_L$ shRNA were treated with L+Z and analyzed for Bcl-X_L expression and necroptosis as in *A*. (*D*) HT29 cells transfected with *BAX* and *BAK* siRNAs or control scrambled siRNAs, with or without pretreatment with z-VAD (Z; 10 μM), were infected with PUMA-expressing adenovirus (Ad-PUMA) for 24 h. (*Upper*) Analysis of nonapoptotic death by PI staining followed by flow cytometry. (*Lower*) Indicated proteins and HMGB1 release analyzed as in *A*. (*E*) WT and *BAX/BAK* DKO

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MEFs were treated with T+L+Z (20 ng/mL TNF-α, 2 μM LBW242, and 10 μM z-VAD) and analyzed as in *A*. (*F*) WT and *PUMA* KO MEFs treated with T+L+Z for 24 h were analyzed by confocal microscopy after mitochondria staining with MitoTracker (red) and double-strand DNA staining with PicoGreen (green). (*Left*) Representative confocal pictures with arrows indicating increased cytoplasmic DNA content. (Scale bars: 5 μm.) (*Right*) Quantification of colocalization of MitoTracker (red) and PicoGreen (green) staining, and mitochondrial interconnectivity and elongation by ImageJ software in at least 20 randomly selected individual cells. (*G*) Mitochondrial and cytosolic fractions isolated from an equal number of MEFs treated as in *F* were analyzed by genomic PCR for murine *CytB*, a mitochondrial gene. To control for cell numbers, an equal fraction of total genomic DNA isolated from the same number of treated cells was analyzed by genomic PCR for the murine nuclear *β-actin* gene. Values in *A*–*E* are expressed as mean ± SD. $n = 3$. NS, $P > 0.05$; *$P < 0.05$; **$P < 0.01$. Western blot data are representative of two independent experiments.

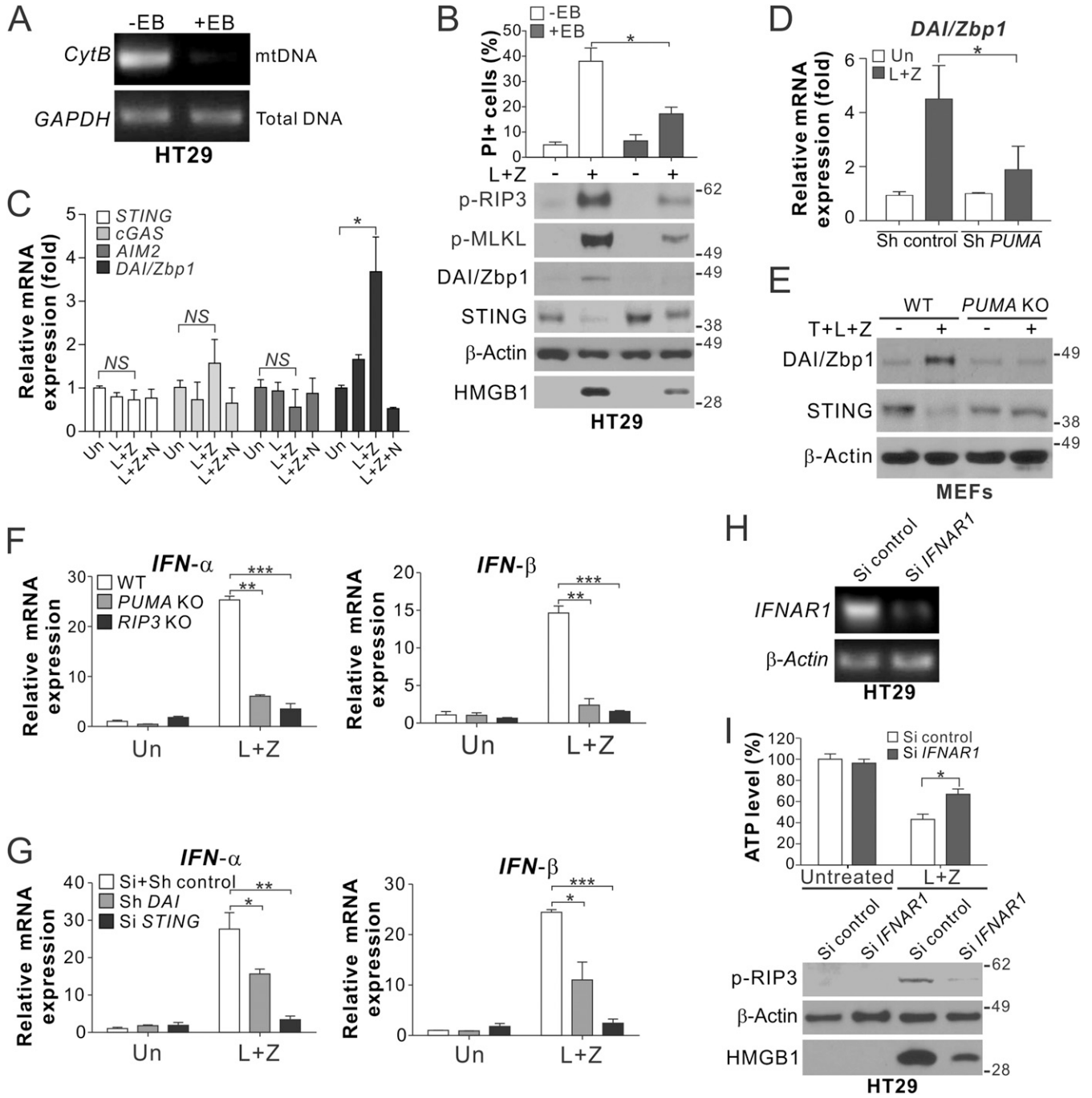

Fig. S7. PUMA-mediated mitochondrial DNA release activates DNA sensors to enhance RIP3/MLKL activation in necroptosis. (*A*) HT29 cells were treated with EB (450 ng/mL) for 4 d to deplete mitochondrial DNA (mtDNA), followed by culturing without EB for 16 h. Mitochondrial fractions isolated from an equal number of cells with or without EB treatment were analyzed by genomic PCR for *CytB* with nuclear *GAPDH* in total genomic DNA as a control. (*B*) HT29 cells with or without EB treatment from *A* were treated with L+Z (2 μM LBW242 and 10 μM z-VAD). (*Upper*) Analysis of nonapoptotic death by PI staining followed by flow cytometry. (*Lower*) Western blots of indicated proteins in cell lysates and HMGB1 in 20 μL of cell culture medium at 24 h. (*C*) mRNA expression of indicated genes in HT29 cells treated with L (2 μM LBW242), L+Z, or L+Z+N (N; 20 μM Necrostatin-1) was analyzed by real-time RT-PCR. The results were normalized to those of *GAPDH*. (*D*) HT29 cells stably expressing control or *PUMA* shRNA were treated with L+Z for 24 h. mRNA expression of *DAI/Zbp1* was analyzed by real-time RT-PCR. The results were normalized to those of *β-actin*. (*E*) Western blot analysis of DAI/Zbp1 and STING in WT and *PUMA* KO MEFs treated with T+L+Z (20 ng/mL TNF-α, 2 μM LBW242, and 10 μM z-VAD) for 24 h. (*F*) mRNA expression of *IFN-α* and *IFN-β* in WT, *RIP3* KO, and *PUMA* KO HT29 cells treated as in *B* was analyzed by real-time RT-PCR. The results were normalized to those of *β-actin*. (*G*) mRNA expression of *IFN-α* and *IFN-β* in HT29 cells expressing control or *DAI/Zbp1* shRNA or transfected with control scrambled or *STING* siRNA and treated as in *B* were analyzed as in *F*. (*H*) HT29 cells transfected with control scrambled or *IFN-α/β receptor 1* (*IFNAR1*) siRNA were analyzed for *IFNAR1* mRNA expression by real-time RT-PCR with *β-actin* as a control. (*I*) HT29 cells transfected with control scrambled or *IFNAR1* siRNA as in *H* were treated with L+Z. (*Upper*) ATP levels at 48 h. (*Lower*) Western blots of indicated proteins in cell lysates and HMGB1 in 20 μL of cell culture medium at 24 h. Values in *B–D*, *F*, *G*, and *I* are expressed as mean ± SD. $n = 3$. NS, $P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Western blot data are representative of two independent experiments.

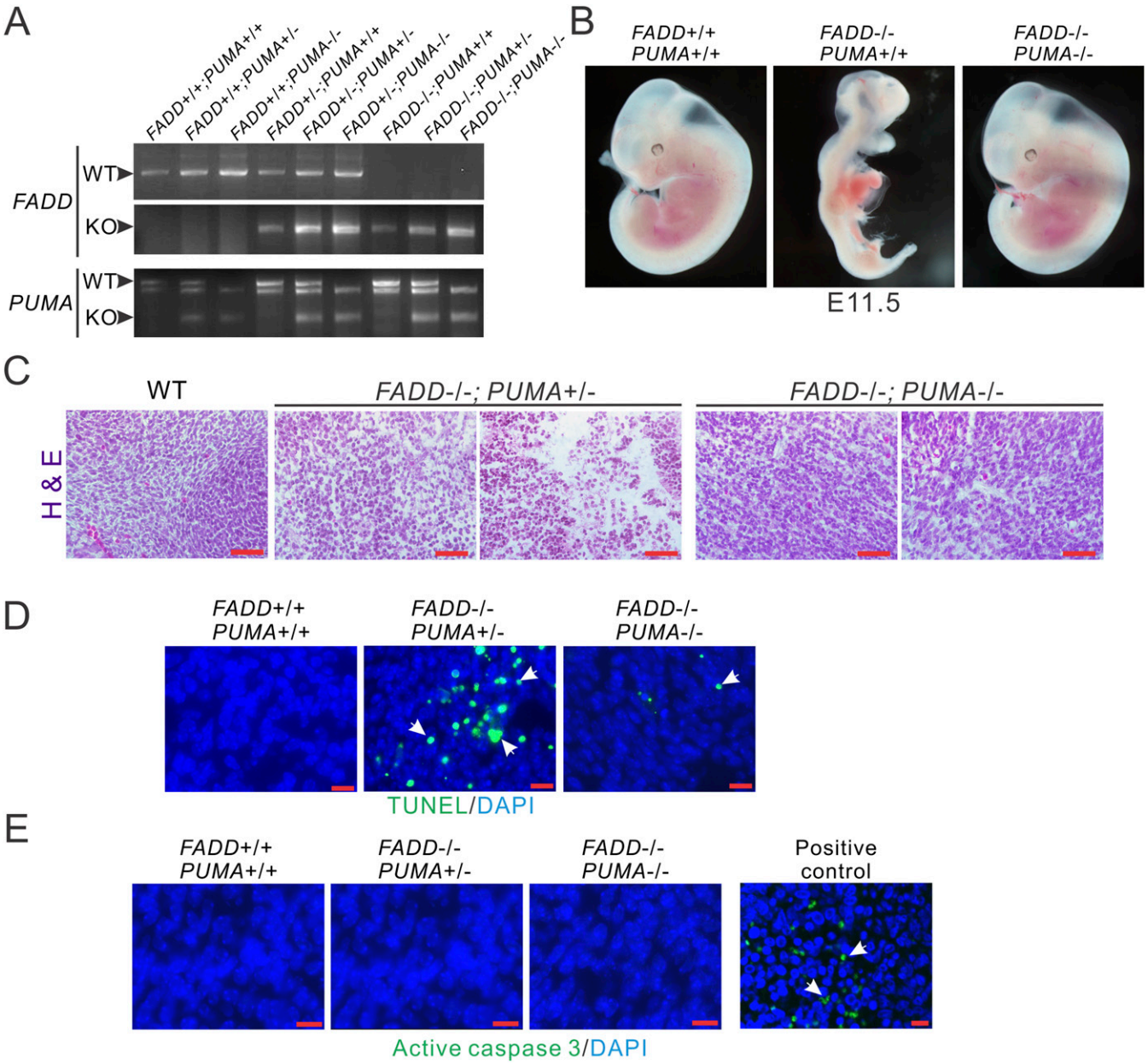

Fig. S8. PUMA deficiency rescues defective embryonic development of *FADD* KO mice. (*A*) Representative results of genotyping of embryos with indicated *FADD* and *PUMA* genotypes at E13.5. (*B*) Representative pictures of embryos with indicated genotypes at E11.5. (*C*) H&E staining of embryonic fibroblasts from E13.5 embryos with indicated genotypes. (Scale bars: 100 μm.) (*D*) TUNEL staining of tissues from E13.5 embryos with indicated genotypes. Arrows indicate example TUNEL signals. (Scale bars: 20 μm.) (*E*) Active caspase 3 staining of tissues from E13.5 embryos with indicated genotypes. A section from a xenograft tumor treated with 5-fluorouracil served as a positive control. Arrows indicate examples of active caspase 3 signals. (Scale bars: 20 μm.)

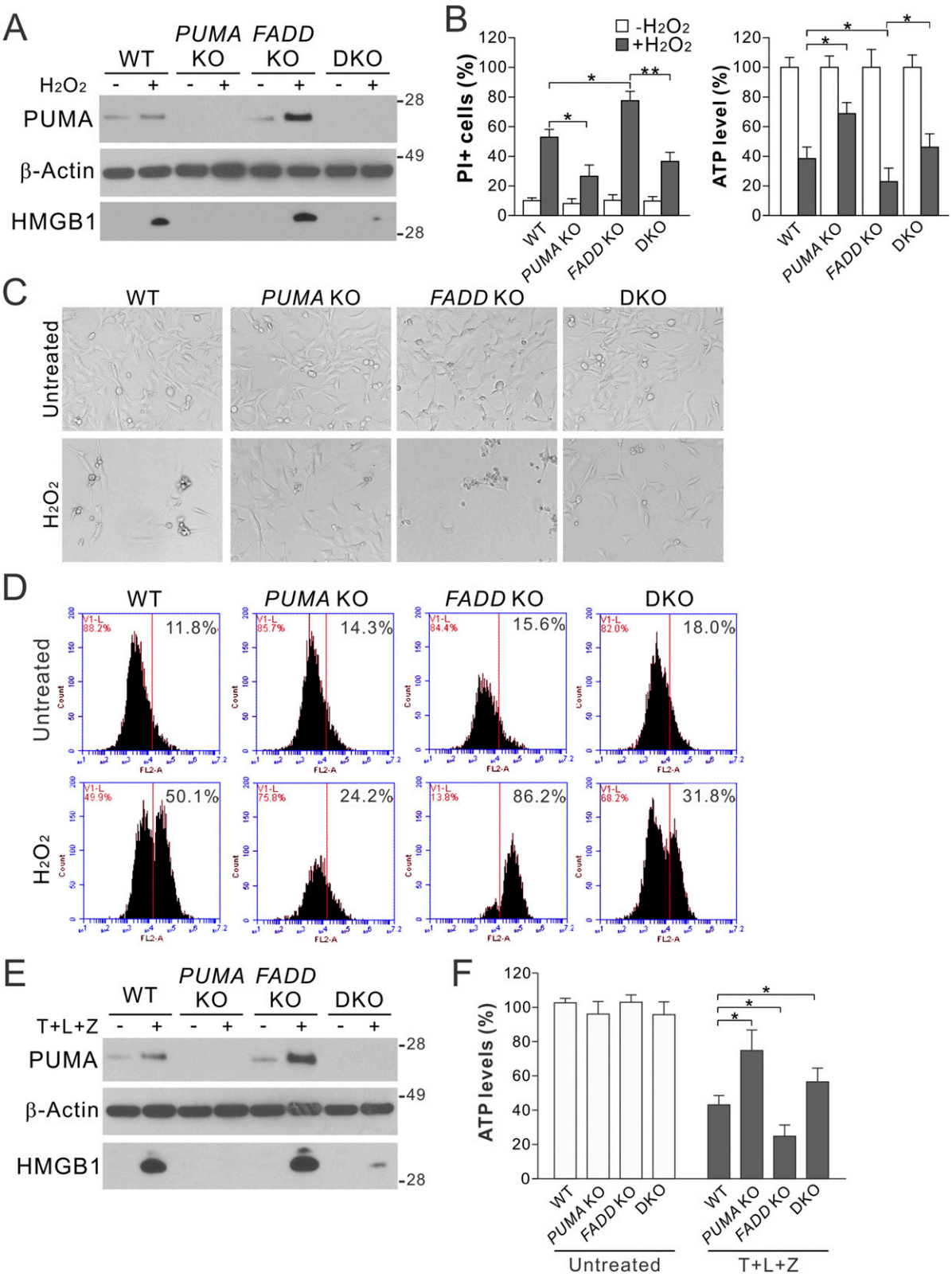

Fig. S9. PUMA contributes to necroptosis in *FADD* KO primary MEFs. (*A*) Primary MEFs with indicated genotypes were treated with 1 mM H_2O_2 for 24 h. PUMA expression in cell lysates and HMGB in 20 μL of cell culture medium were analyzed by Western blotting. (*B*) Cell death in primary MEFs with indicated genotypes treated as in *A* was analyzed by measuring ATP levels and flow cytometry of PI-stained cells. (*C*) Representative phase/contrast pictures of primary MEFs with indicated *PUMA* and *FADD* genotypes treated as in *A*. (*D*) Necroptosis in primary MEFs with indicated genotypes treated as in *A* was analyzed by flow cytometry of PI-stained cells. (*E*) Primary MEFs with indicated genotypes were treated with T+L+Z (2 μM LBW242, 20 ng/mL TNF-α, and 10 μM z-VAD). PUMA expression in cell lysates and HMGB1 in 20 μL of cell culture medium at 24 h after treatment were analyzed by Western blotting. (*F*) ATP levels in primary MEFs treated as in *E* for 48 h. Values in *B* and *F* are expressed as mean ± SD. $n = 3$. $^*P < 0.05$; $^{**}P < 0.01$. Western blot data are representative of two independent experiments.

Table S1. PCR primers

PCR primer	Forward (5′ to 3′)	Reverse (5′ to 3′)
RT-PCR		
h-*PUMA*	CGACCTCAACGCACAGTACGA	AGGCACCTAATTGGGCTCCAT
m-*PUMA*	ATGGCGGACGACCTCAAC	AGTCCCATGAAGAGATTGTACATGAC
TNF-α	CCCAGGCAGTCAGATCATCTTC	AGCTGCCCCTCAGCTTGA
IFN-α	GATGGCCGTGCTGGTGCTCA	TGATTTCTGCTCTGACAACCTCCC
IFN-β	TTGAATGGGAGGCTTGAATA	CTATGGTCCAGGCACAGTGA
IFNAR1	TATGCTGCGAAAGTCTTCTTGAG	TCTTGGCTAGTTTGGGAACTGTA
DAI/Zbp1	GAGGAGTCGCAGGGTCTG	GACTTCTGGATTGTGTGTCTGC
STING	CCTGAGCAGAACAACTGC	GGTCTTCAAGCTGCCCACAGTA
cGAS	GGGAGCCCTGCTGTAACACTTCTTAT	CCTTTGCATGCTTGGGTACAAGGT
AIM2	ATGTG AAGCCGTCCAGA	CATCATTTCTGA TGGCTGCA
β-actin	GACCTGACAGACTACCTCAT	AGACAGCACTGTGTTGGCTA
m-*GAPDH*	CTCTGGAAAGCTGTGGCGTGATG	ATGCCAGTGAGCTTCCCGTTCAG
Genomic PCR		
h-*CytB*	GCCTGCCTGATCCTCCAAAT	AAGGTAGCGGATGATTCAGCC
h-*ND2*	CCCTAAAACCCGCCACATCT	AGAGCGATGGTGAGAGCTAAGG
h-*GAPDH*	GTATTCCCCCAGGTTTACAT	TTCTGTCTTCCACTCACTCC
m-*CytB*	CCACTTCATCTTACCATTTATTATCGC	TTTTATCTGCATCTGAGTTTAA
m-*β-actin*	CTGCCTGACGGCCAGG	CTATGGCCTCAGGAGTTTTGTC
ChIP PCR		
PUMA promoter	CATGTAAGTGATGTCATATGTC	CTTCCTGGTCTTTTCCAAACT
h-*CytB*	GCCTGCCTGATCCTCCAAAT	AAGGTAGCGGATGATTCAGCC
h-*ND2*	CCCTAAAACCCGCCACATCT	AGAGCGATGGTGAGAGCTAAGG
Mouse genotyping		
FADD WT	TGCGCCGACACGATCTACTG	AGCTGTAGGCTTGTCAGGGTGTTTC
FADD KO	ACTGTAGTGCCCAGCAGAGACCAGC	CGCTCGGTGTTCGAGGCCACACGC
PUMA WT	TTATAGCCGGTGAGTCAGCA	CAGGCAGTTGTCAGCTGGG
PUMA KO	TTGACGAGTTCTTCTGAGGG	CAGGCAGTTGTCAGCTGGG

Table S2. siRNAs

siRNA	Sequence (5′ to 3′)	Custom synthesis
PUMA	ACGTGTGACCACTGGCATT and ACCTCAACGCACAGTACGA	Dharmacon
MLKL	CAAACTTCCTGGTAACTCA	Dharmacon
BAX	AAGGUGCCGGAACUGAUCAGA	Dharmacon
BAK	AACCGACGCUAUGACUCAGAG	Dharmacon
STING	GCATCAAGGATCGGGTTT	Dharmacon
Control	AACGTACGCGGAATACTTCGA	Dharmacon

Table S3. ShRNAs

ShRNA	Sequence (5′ to 3′)	Vendor/catalog no.
RIP1	TAAGCTGAAAGAACATGACCT	Open Biosystems/TRCN0000000705
RIP3	AGTTCATGCCTTGTCTCTCAG	Open Biosystems/TRCN0000002257
PUMA	TTGGCTCATTTGCTCTTCACG	Open Biosystems/TRCN0000033609
Bcl-X_L	ATCACTAAACTGACTCCAGCT	Open Biosystems/TRCN0000004685
DAI/Zbp1	ATCATGTTGATTGGATTGTGC	Open Biosystems/TRCN0000123050
p53	GACTCCAGTGGTAATCTACTG	Cloned into pLKO.1 puro (Addgene/8453)
Control	CCGCAGGTATGCACGCGT	Addgene/10879