Supplementary Materials for

The lysosomal Rag-Ragulator complex licenses RIPK1– and caspase-8–mediated pyroptosis by *Yersinia*

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Figs. S1 to S12

Other Supplementary Material for this manuscript includes the following:
(available at science.sciencemag.org/content//372/6549/eabg0269/suppl/DC1)

MDAR Reproducibility Checklist

Ranking	Gene	sgRNA	Sequence (5'-3')
1	*Tnfrsf1a*	Tnfrsf1a_2_B	AGGGGCTGCAGTCCACGCAC
2	*Lamtor4*	Lamtor4_3_A	CTGGGATTCGCTCCAGCCCT
3	*Fnip2*	Fnip2_2_A	ACCGTATGTAGTGTATCTTC
4	*Lamtor4*	Lamtor4_1_B	TAGACTTCCGCACTGACCCA
5	*Lamtor4*	Lamtor4_3_B	AGAGGGTGTTTGTAGTGAAG
6	*Lamtor1*	Lamtor1_1_B	CACCTGCTGCAAGTCAGAGA
7	*Lamtor1*	Lamtor1_1_A	GCTCTTCTTTCGCATCCACG
8	*Ndufa6*	Ndufa6_1_B	GACCAGCAGATCAACCACTC
9	*Rragc*	Rragc_3_A	TTTCTGTACCACCTTACTGA
10	*Fnip2*	Fnip2_1_B	ACTTTACTAATCATCAGTTG
11	*Flcn*	Flcn_1_A	GGCTGCCGGTCACTTGCCGT
12	*Lamtor1*	Lamtor1_2_A	TGACACTCACCTAGCTGTCT
13	*Tnfrsf1a*	Tnfrsf1a_3_B	CGGACAGTCACTCACCAAGT
14	*Rragc*	Rragc_2_A	TGACTTCCAGGACGACTACA
15	*Lamtor1*	Lamtor1_3_B	CTTACCTGTACTGCCTTGCC
16	*Tnfrsf1a*	Tnfrsf1a_1_A	CTGCAGACTGTATCCCGCCC
17	*Fnip2*	Fnip2_1_A	CATCCGCCTGCTAGTTTACC
18	*Ndufa13*	Ndufa13_1_A	TGCAAGTATGGCGGCGTCGA
19	*Tnfrsf1a*	Tnfrsf1a_2_A	GTGTCTCACTCAGGTAGCGT
20	*Ndufaf4*	Ndufaf4_3_A	GTCGCGGGTAGAAGGGTGCT
21	*Ndufaf4*	Ndufaf4_2_B	TTATCAAAGTGATTTCCTAT
22	*Ndufa13*	Ndufa13_3_A	GTGGCACCCATCGTGTGGTA
23	*Ndufa9*	Ndufa9_2_B	TATTCCTCGAGCAATAGCTC
24	*Ndufa9*	Ndufa9_3_B	CACAAGTGATCATACCATAT
25	*Flcn*	Flcn_1_B	CGATGATGCTGTACCAGCGC
26	*Flcn*	Flcn_3_B	GCCTGCTACCGCATGCCTTC
27	*Ndufa13*	Ndufa13_3_B	CCTCCAAGTCCTCAATCAGC

Fig. S1. List of sgRNA hits from the screening in iBMDMs treated with LPS/5z7.
Corresponding genes targeted with multiple sgRNAs and targeting sequences are shown.

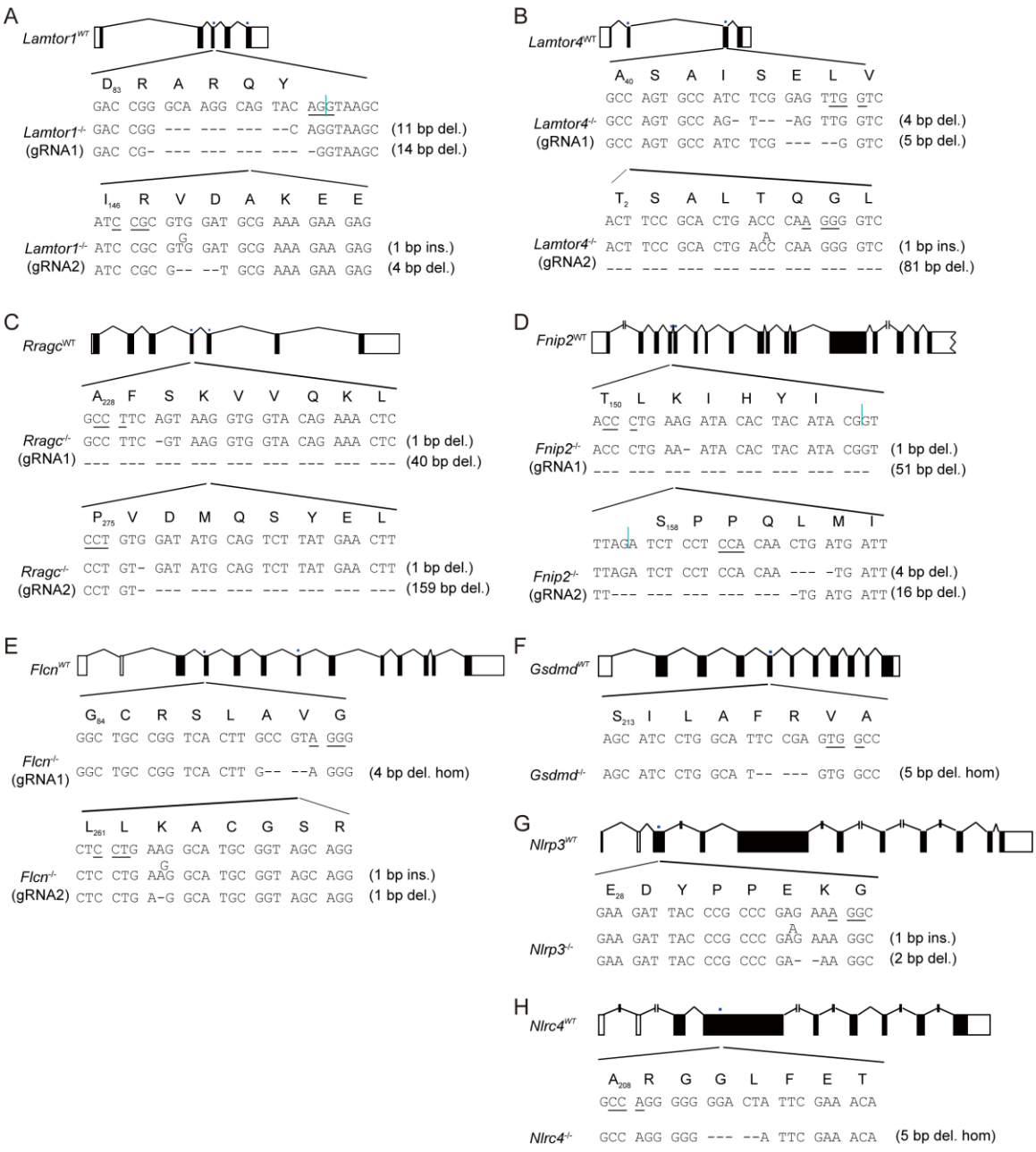

Fig. S2. Knockout iBMDMs used in this study. Gene coding sequences are represented as black boxes. Top sequence track is the gene wild-type allele. Location of gRNAs is indicated with blue bars and PAM sequences are underscored.

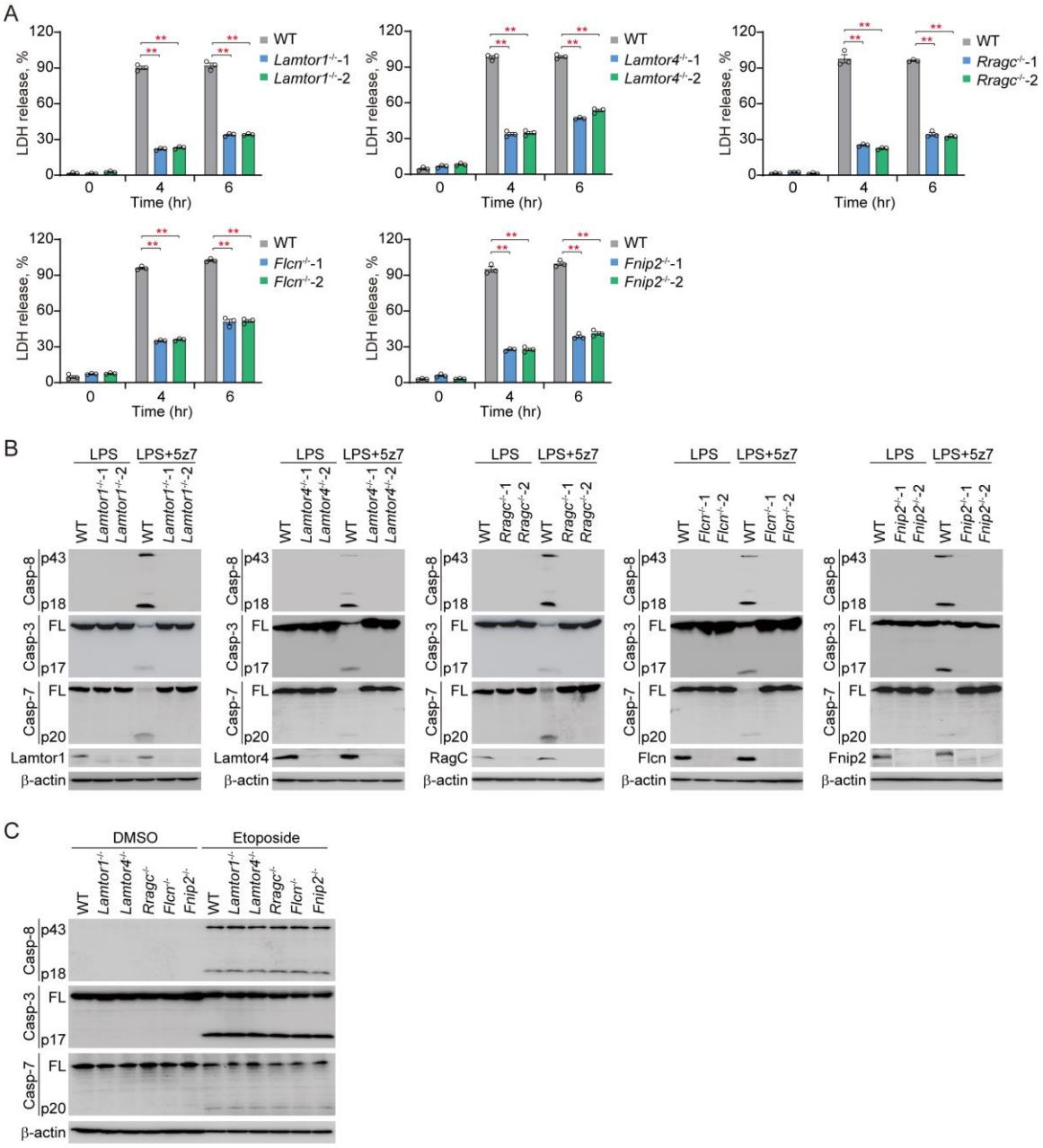

Fig. S3. FLCN-FNIP2-Rag-Ragulator gene deficient cells are resistant to LPS/5z7 treatment, but sensitive to etoposide-induced apoptosis. The indicated iBMDMs were treated with LPS, LPS/5z7 or etoposide. Cell death was measured by LDH release after 4 or 6 hr (A). Cleavage and activation of caspase-8, caspase-3 and caspase-7 in the indicated iBMDMs were analyzed by immunoblot 2.5 hr post LPS or LPS/5z7 treatment (B), or 16 hr post etoposide treatment (C). Graphs in A show mean ± SEM of triplicate wells. Data are representative of at least three independent experiments. Data were analyzed using a two-tailed Student's t test. $^{**}P < 0.01$.

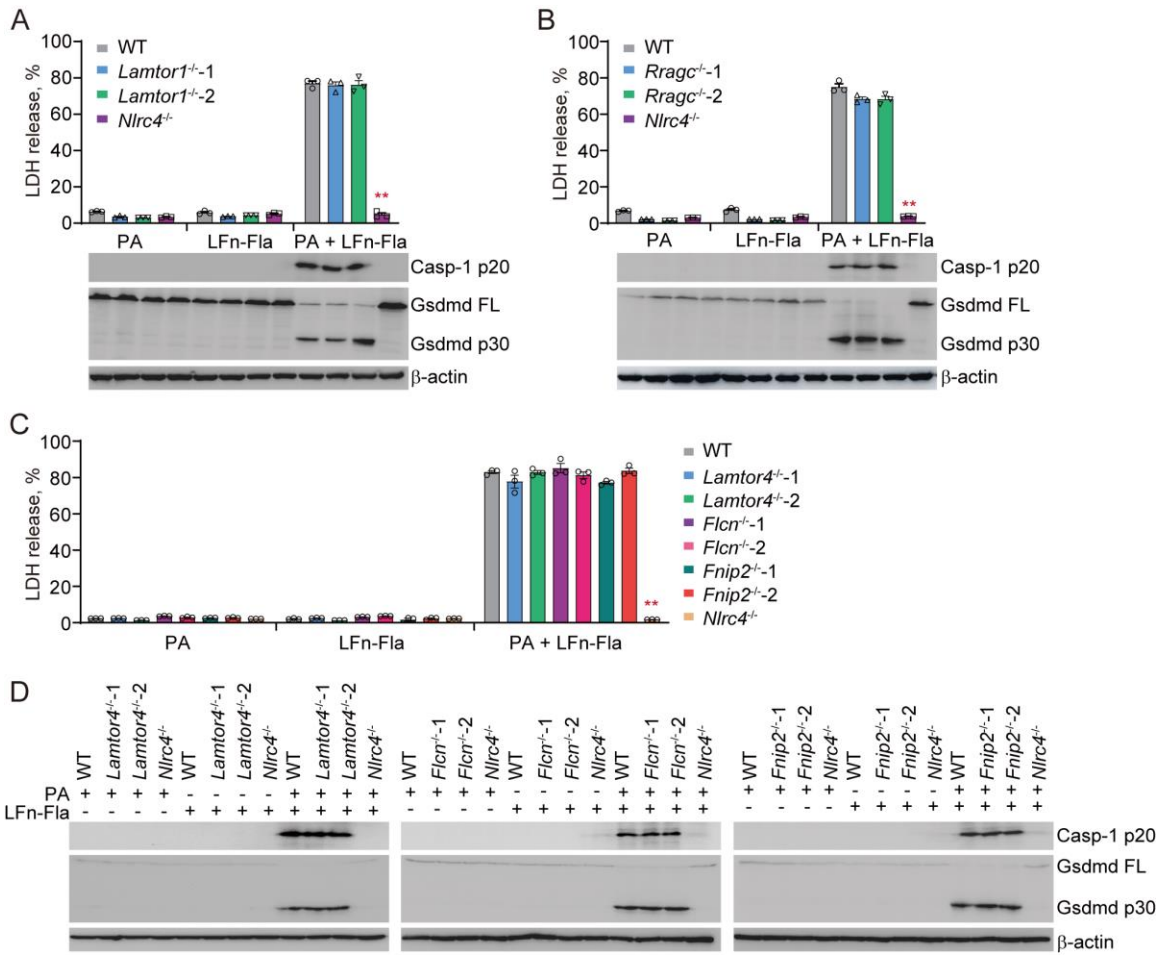

Fig. S4. FLCN-FNIP2-Rag-Ragulator had no effect on flagellin-triggered NLRC4 inflammasome-dependent pyroptosis. The indicated iBMDMs were incubated with PA and LFn-Flagellin individually or in combination. Cell death was measured by LDH release 1 hr later. Caspase-1 processing and Gsdmd cleavage were examined by immunoblot. Graphs in A-C show mean ± SEM of triplicate wells. Data are representative of at least three independent experiments. Data were analyzed using a two-tailed Student's t test. $^{**}P < 0.01$.

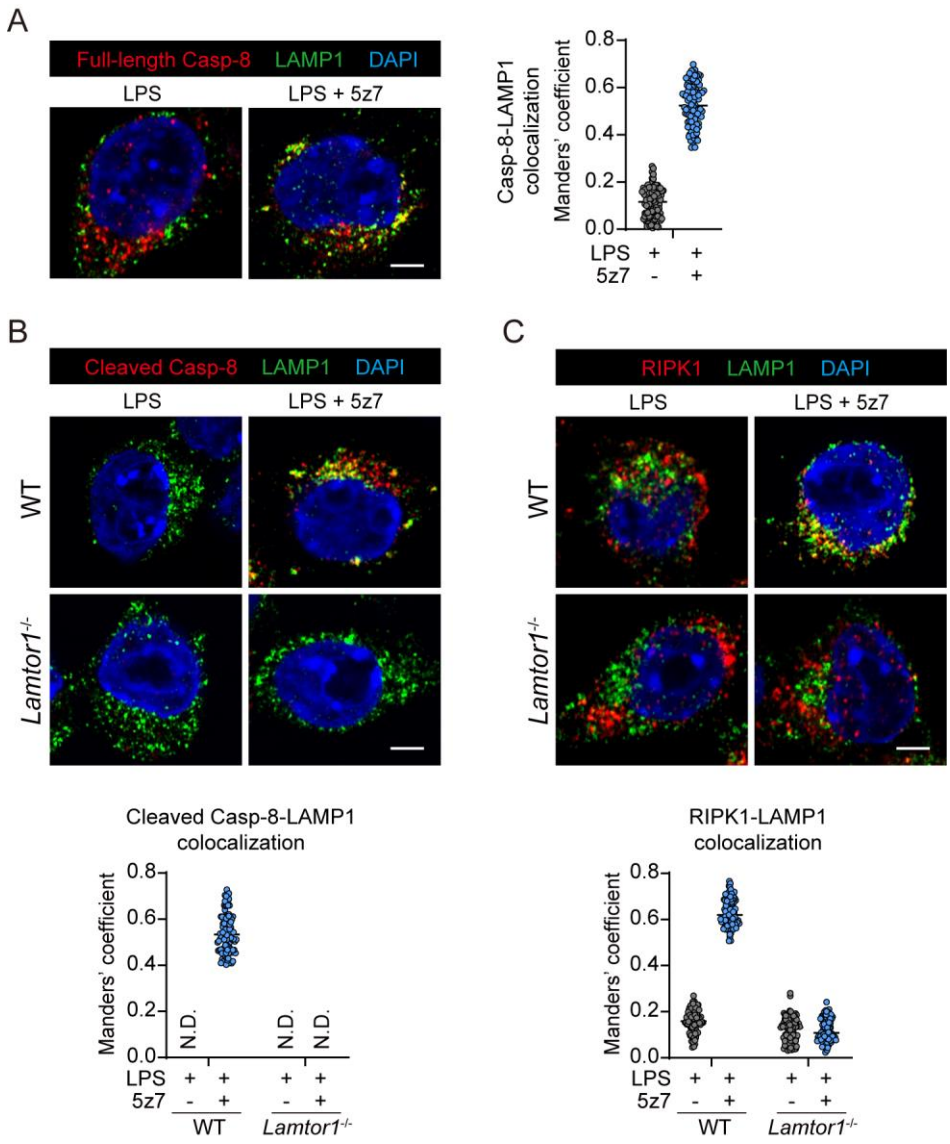

Fig. S5. Lamtor1 is required for lysosomal localization of cleaved caspase-8 and RIPK1 upon LPS/5z7 treatment. (**A**) Confocal fluorescence images of LPS or LPS/5z7 treated iBMDMs stained for full-length Casp-8, LAMP-1 and DAPI (left panel). Colocalization of Casp-8 with LAMP1 was analyzed by calculating the Manders' overlap coefficient (right panel). (**B-C**) Confocal fluorescence images of LPS or LPS/5z7 treated WT or *Lamtor1*$^{-/-}$ iBMDMs stained for cleaved Casp-8 (**B**, upper panel) or RIPK1 (**C**, upper panel) together with LAMP-1 and DAPI. Colocalization of cleaved Casp-8 (**B**, lower panel) or RIPK1 (**C**, lower panel) with LAMP1 was analyzed by calculating the Manders' overlap coefficient. Scale bars: 2 μm. Data are representative of at least three independent experiments. N.D., not detected.

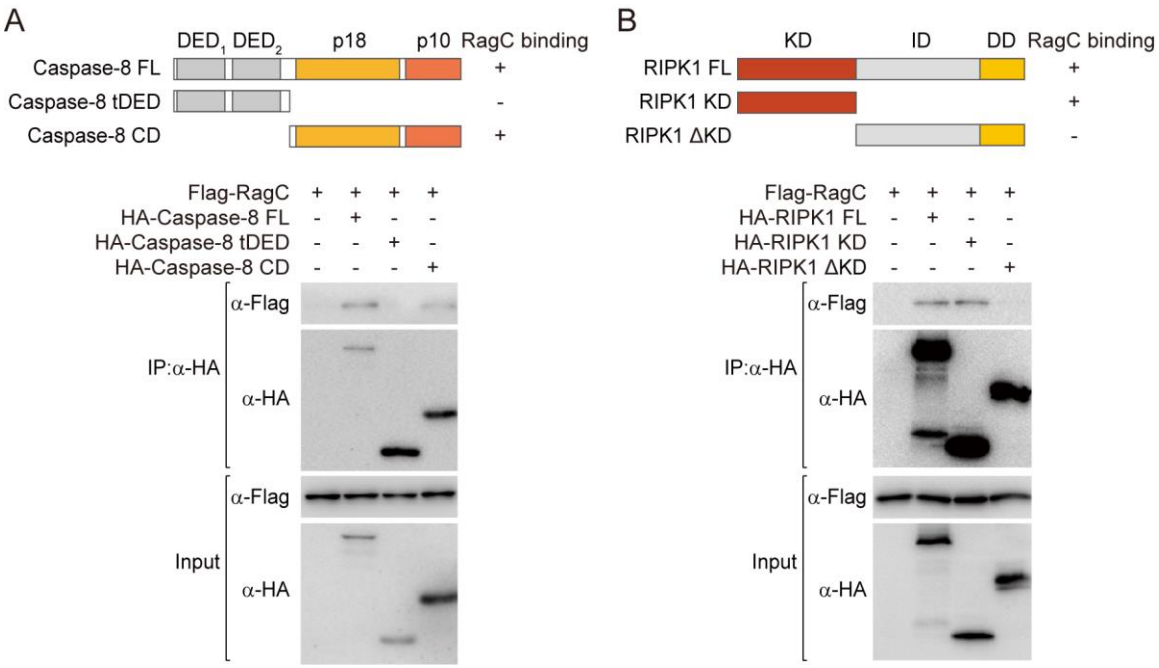

Fig. S6. RagC interacts with caspase-8 caspase domain (CD) and RIPK1 kinase domain (KD). Schematics show domain composition of caspase-8 (**A**, upper panel) and RIPK1 (**B**, upper panel). Lysates of HEK293T cells transfected with the indicated plasmids were assayed for immunoprecipitation with anti-HA antibody and analyzed by immunoblot probed with the indicated antibodies (**A** and **B** lower panels). Data are representative of at least three independent experiments. FL, full-length; tDED, tandem death effector domain; CD, caspase domain; KD, kinase domain; ID, intermediate domain; DD, death domain.

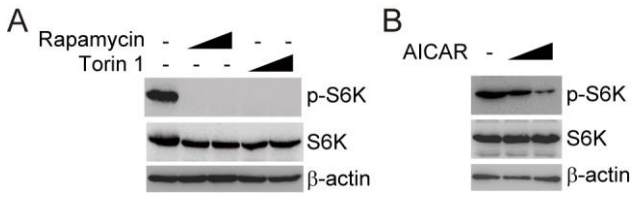

Fig. S7. The inhibitory effects on mTOR activity by Rapamycin, Torin 1 or AICAR were confirmed by assessing phospho-p70 S6 Kinase. iBMDMs were treated with increasing doses of Rapamycin (100 nM, 200 nM), Torin 1 (250 nM, 500 nM) or AICAR (1 mM, 3 mM) for 2 hr before whole cell lysates were collected for immunoblot analysis of the phosphorylation of p70 S6 kinase.

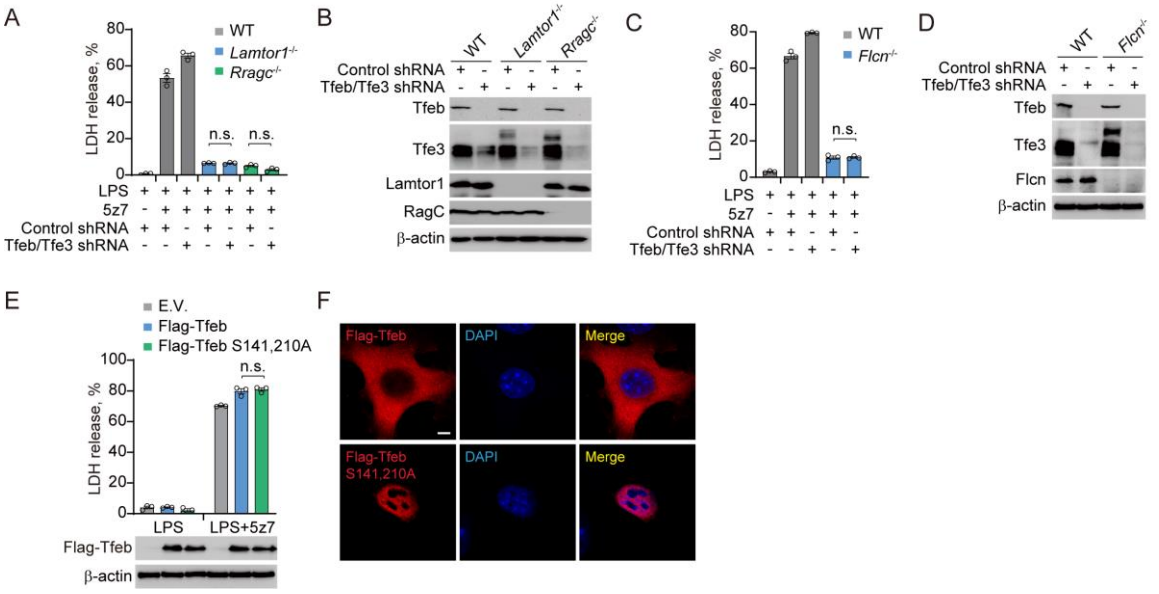

Fig. S8. Tfeb/Tfe3 is not involved in the blockade of LPS/5z7-induced pyroptosis by FLCN-Rag-Ragulator deficiency. iBMDMs were transduced with lentiviruses expressing the indicated shRNAs (**A**-**D**) or Flag-Tfeb (**E**-**F**) before being treated or not with LPS or LPS/5z7. Cell death was measured by LDH release 2.5 hr post LPS/5z7 treatment (**A**, **C** and **E**). Endogenous and exogenous Tfeb/Tfe3 protein levels were analyzed by immunoblot with the indicated antibodies (**B**, **D** and **E**). Confocal fluorescence images of indicated cells stained for Flag-Tfeb and DAPI (**F**). Scale bars: 5 μm. Graphs show mean ± SEM of triplicate wells. Data are representative of at least three independent experiments. Data were analyzed using a two-tailed Student's t test. n.s., not significant. E.V., empty vector.

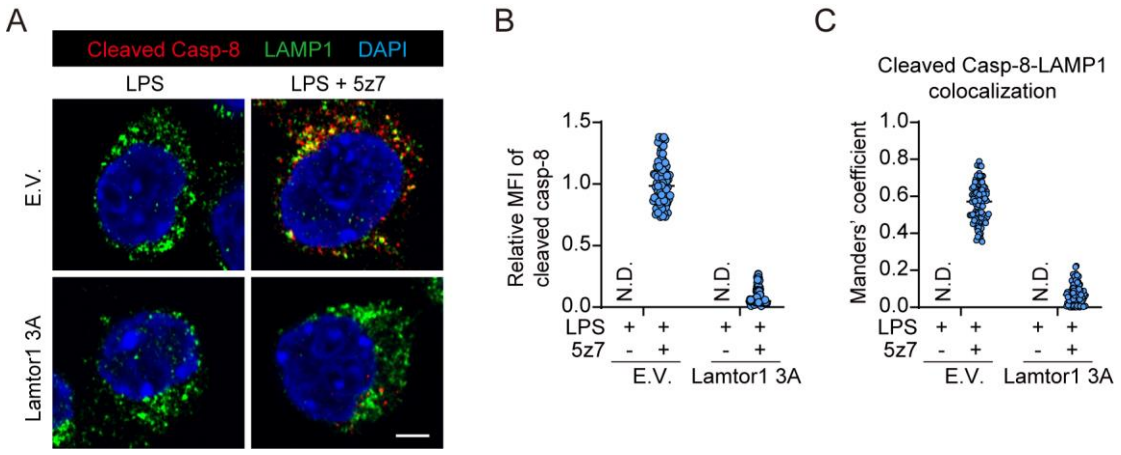

Fig. S9. Lamtor 3A acts as a competitive inhibitor of caspase-8 activation. (**A-C**) iBMDMs were transduced with lentiviral particles expressing the indicated plasmids before treated or not with LPS or LPS/5z7. Confocal fluorescence images of iBMDMs stained for cleaved Casp-8, LAMP1 and DAPI (**A**). Scale bars: 2 μm. Relative mean fluorescence intensity (MFI) of cleaved Casp-8 (**B**) and colocalization of cleaved Casp-8 with LAMP1 (**C**) were analyzed by ZEISS Zen software and by calculating Manders' overlap coefficient, respectively. Data are representative of at least three independent experiments. E.V., empty vector. N.D., not detected.

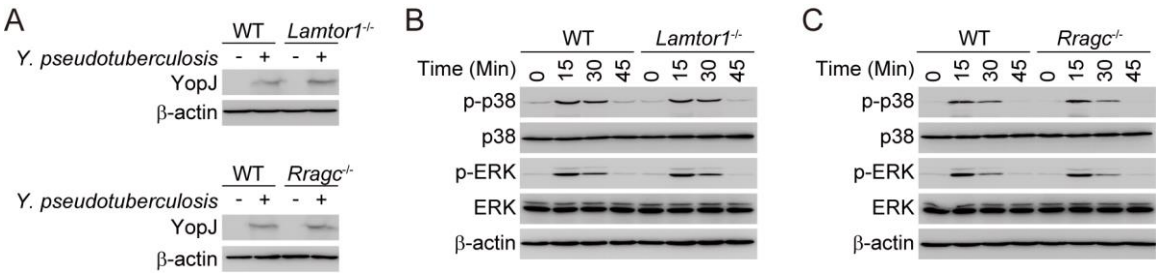

Fig. S10. Lamtor1 or RagC deficiency do not affect the secretion and activity of YopJ. (**A**-**C**) Lysates of the indicated iBMDMs infected with *Yersinia* were analyzed by immunoblot with the indicated antibodies. Data are representative of at least three independent experiments.

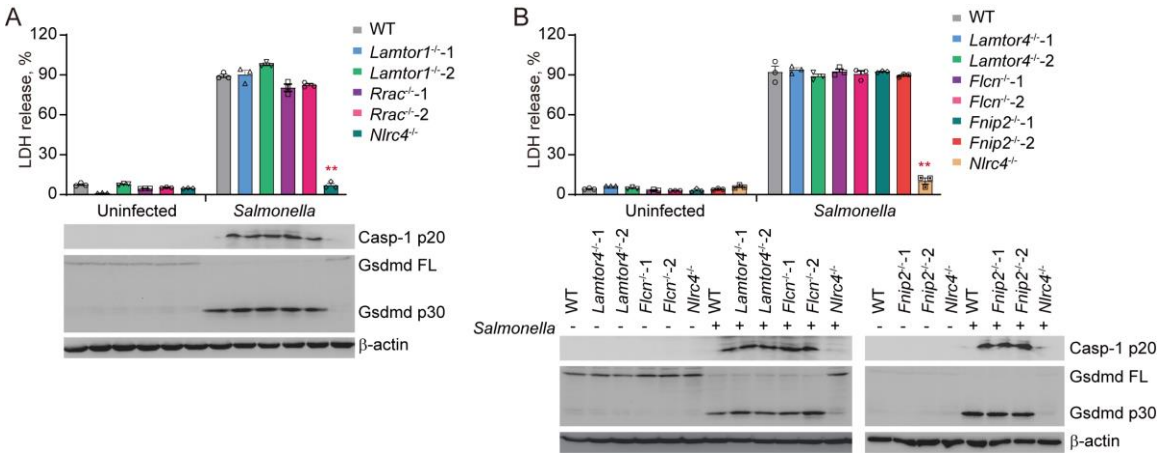

Fig. S11. FLCN-FNIP2-Rag-Ragulator is not involved in *Salmonella*-triggered pyroptosis. The indicated iBMDMs were infected with *Salmonella*. Cell death was measured by LDH release 1 hr later. Caspase-1 processing and Gsdmd cleavage were examined by immunoblot. Graphs in **A-B** show mean ± SEM of triplicate wells. Data are representative of at least three independent experiments. Data were analyzed using a two-tailed Student's t test. $^{**}P < 0.01$.

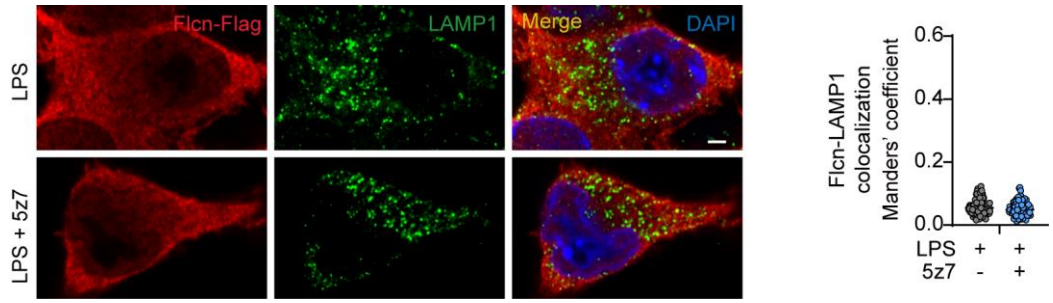

Fig. S12. Subcellular localization of Flcn is not changed by LPS/5z7 treatment. Confocal fluorescence images of LPS or LPS/5z7 treated iBMDMs stained for Flcn-Flag, LAMP1 and DAPI (left panel). Scale bars: 2 μm. Colocalization of Flcn with LAMP1 was analyzed by calculating Manders' overlap coefficient (right panel). Data are representative of at least three independent experiments.