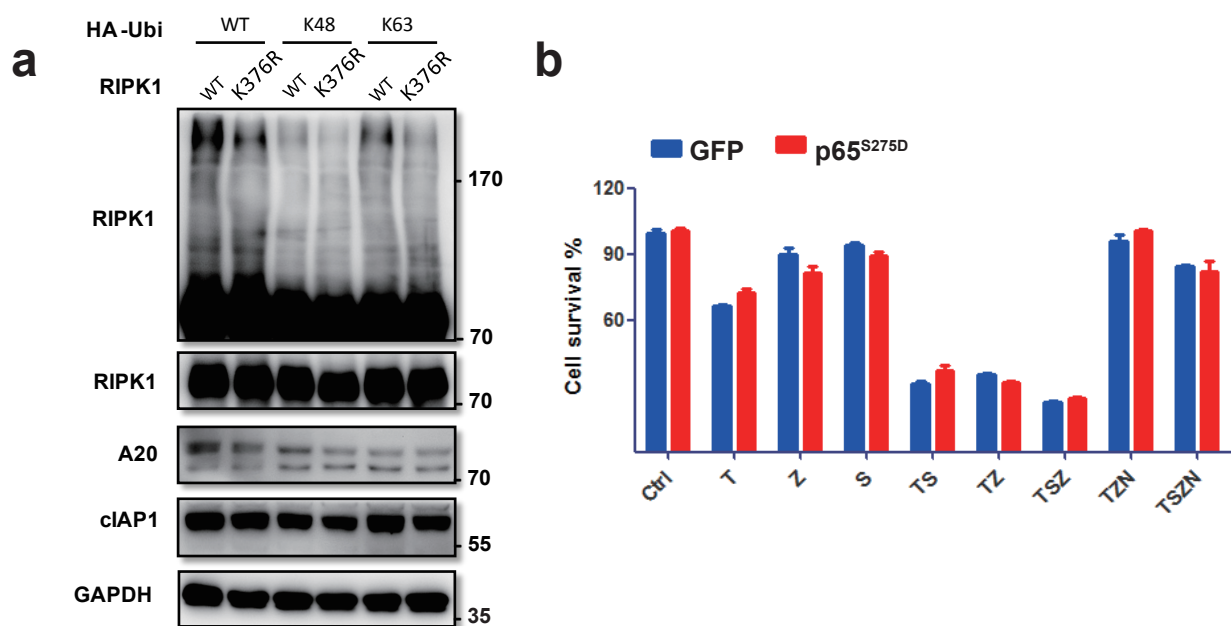


Supplemental Information

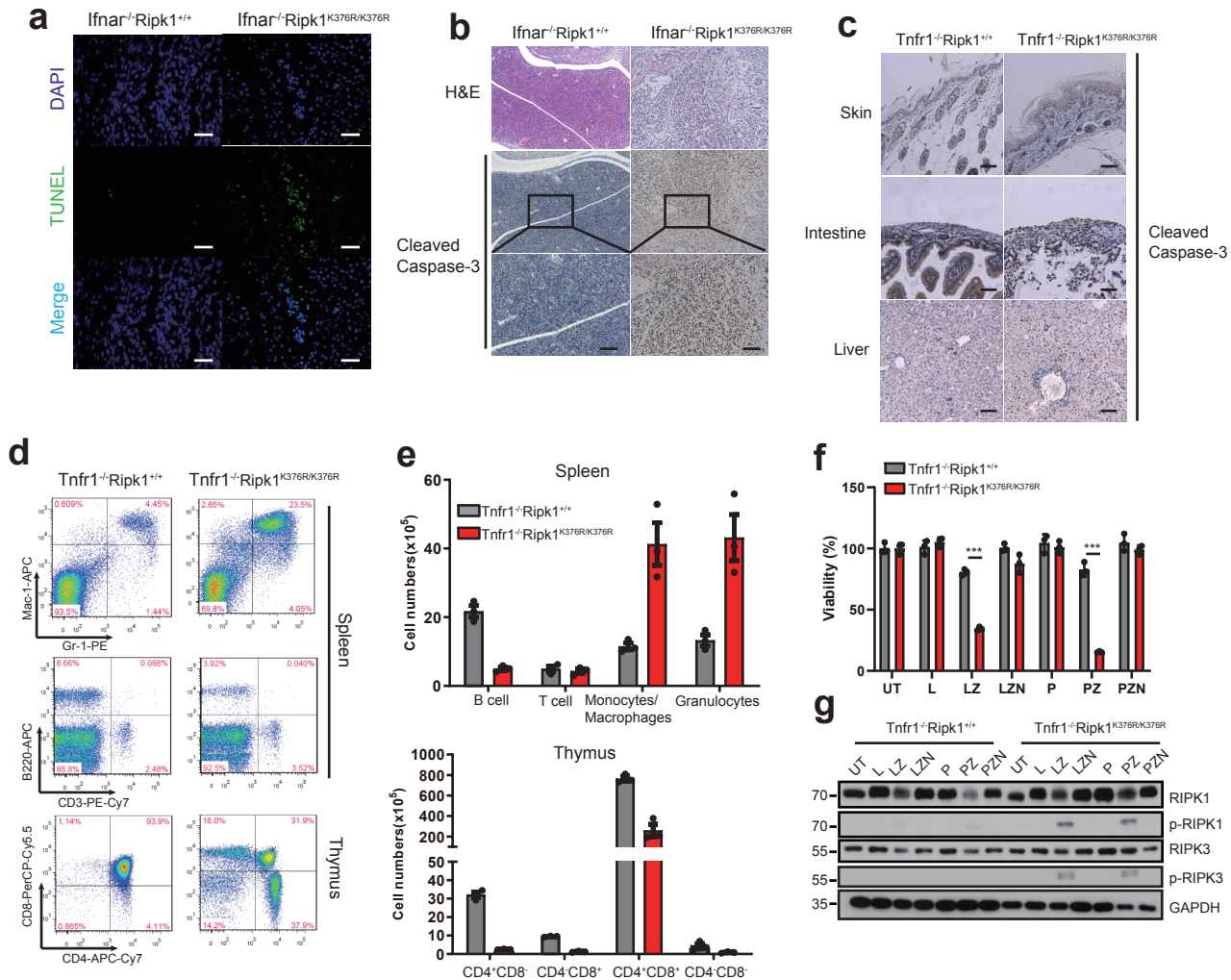
**Ubiquitination of RIPK1 suppresses programmed cell death by
regulating RIPK1 kinase activation during embryogenesis**

Zhang *et al.*



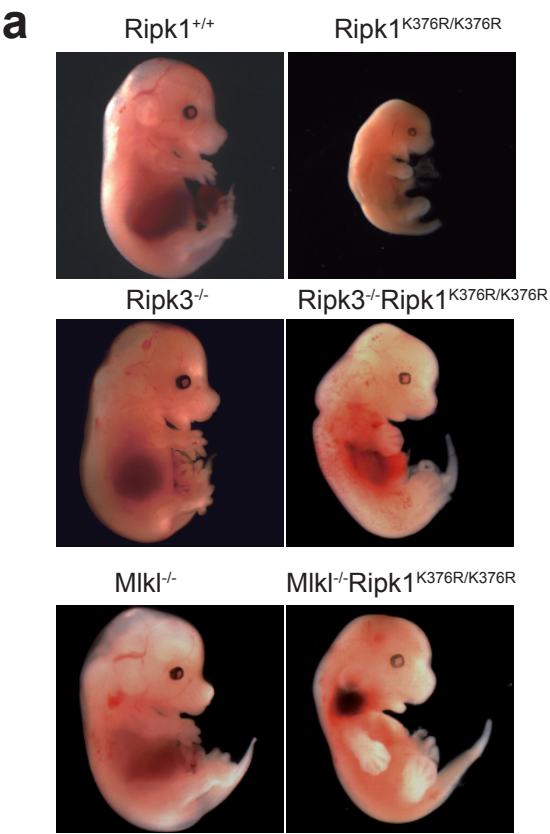
Supplementary Figure. 1 Defects of K63-mediated RIPK1 ubiquitination induced cell death is independent on impaired NF- κ B pathway. Related to Fig. 2

a HEK293T cells were transfected with vectors expressing HA-Ub and RIPK1 or RIPK1^{K376R}. The ubiquitinated RIPK1 was enriched by HA immunoprecipitation and detected using anti-RIPK1 antibody. **b** Ripk1^{K376R/K376R} MEFs infected with lentivirus to stably expressing GFP or p65^{S275D} were treated with indicated drugs for 24 hours. Cell viability was determined using the CellTiter-Glo kit. The data are represented as the mean $\pm$ SEM of three independent experiments. Abbreviations are as follows: Ctrl, untreated; T, TNF- α (20ng/ml); S, Smac mimetic(100nM); Z, zVAD(20 μ M); TS, TNF- α (20ng/ml)+Smac mimetic(100nM); TZ, TNF- α (20ng/ml)+zVAD(20 μ M); TSZ, TNF- α (20ng/ml)+Smac mimetic(100nM)+zVAD(20 μ M); TZN, TNF- α +zVAD+Necrostatin-1(20 μ M); TSZN, TNF- α +Smac mimetic +zVAD+Necrostatin-1(20 μ M). Source data are provided as a Source Data file.



Supplementary Figure 2. Ablation of *Ifnar* does not prevent the embryonic lethality of *Ripk1*^{K376R/K376R} mice. Related to Fig. 6

a TUNEL staining and **b** cleaved caspase-3 staining of fetal livers from mouse embryos with indicated genotypes. Scale bars, 50μm. **c** Cleaved caspase-3 stained tissues from *Tnfr1*^{-/-}*Ripk1*^{K376R/K376R} and littermate control. **a-c** Results are representative of three mice of each genotype (Scale bars, 100μm). **d** Representative flow cytometric images of myeloid cells (Gr-1⁺CD11b⁺), B cells (B220⁺) and T cells (CD3⁺) in spleens and CD4⁺, CD8⁺, CD4⁺CD8⁺ cells in thymus from 1-week-old *Tnfr1*^{-/-}*Ripk1*^{+/+} and *Tnfr1*^{-/-}*Ripk1*^{K376R/K376R} mice. **e** Cell counts in spleen and thymus from 1-week-old mice of indicated genotypes. Data are represented as mean ± SEM (n=3/genotype). **f** BMDMs were isolated from *Tnfr1*^{-/-}*Ripk1*^{+/+} and *Tnfr1*^{-/-}*Ripk1*^{K376R/K376R} mice and were treated with L, LZ, LZN, PZ and PZN for 6h, respectively. Cell viability was determined using the CellTiter-Glo kit. Data are represented as mean ± SEM of BMDMs derived from three mice. P value was determined by Student's t-test (***p<0.001). Abbreviations are as follows: UT, untreated; LZ, LPS (20ng/ml)+zVAD(20μM); LZN, LPS+zVAD+Nec-1(20μM); PZ, Poly(I:C) (50ug/ml)+zVAD; PZN, Poly(I:C)+zVAD+Nec-1. **g** *Tnfr1*^{-/-}*Ripk1*^{+/+} and *Tnfr1*^{-/-}*Ripk1*^{K376R/K376R} BMDMs were treated with LZ and PZ for 3h with or without Nec-1s. Immunoblottings of indicated proteins were shown.



b

Ripk3^{-/-}Ripk1^{K376R/+} intercrossing
Ripk3^{-/-}

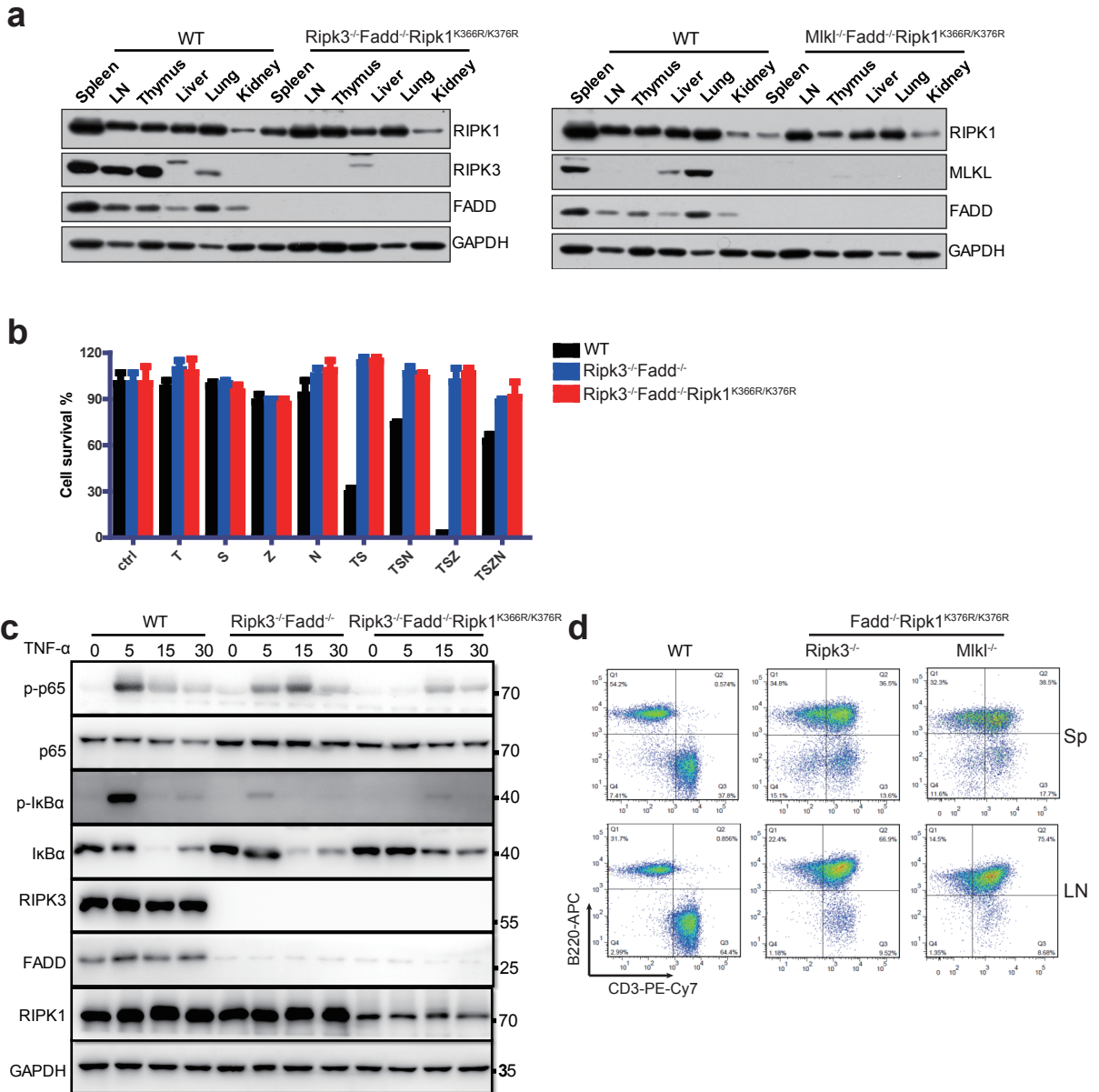
Stage	Ripk1 ^{+/+}	Ripk1 ^{K376R/+}	Ripk1 ^{K376R/K376R}	Total
E13.5	4	13	6	23
E14.5	6	10	12	28
E15.5	9	17	2 *	28
>P0	12	18	0	30

Mlkl^{-/-}Ripk1^{K376R/+} intercrossing
Mlkl^{-/-}

Stage	Ripk1 ^{+/+}	Ripk1 ^{K376R/+}	Ripk1 ^{K376R/K376R}	Total
E13.5	6	15	4	25
E14.5	7	15	8	30
E15.5	4	21	3 *	28
>P0	10	22	0	32

Supplementary Figure 3. Deletion of Ripk3 or Mlkl fails to prevent the embryonic lethality of Ripk1^{K376R/K376R} mice. Related to Fig. 7

a Embryos of the indicted genotypes at E14.5. Images are representative of embryos indicated each genotypes (n=4-15/genotype). **b** Expected and observed frequency of genotypes in offspring at weaning from crosses of Ripk3^{-/-}Ripk1^{K376R/+} (up) or Mlkl^{-/-}Ripk1^{K376R/+} (down).



Supplementary Figure 4. Analysis of $Ripk3^{-/-}Fadd^{-/-}Ripk1^{K376R/K376R}$ and $Mlkl^{-/-}Fadd^{-/-}Ripk1^{K376R/K376R}$ mice. Related to Fig. 7

a Expression levels of the indicated protein in tissues from $Ripk3^{-/-}Fadd^{-/-}Ripk1^{K376R/K376R}$ and $Mlkl^{-/-}Fadd^{-/-}Ripk1^{K376R/K376R}$ mice. **b** $Ripk1^{+/+}$, $Ripk3^{-/-}Fadd^{-/-}Ripk1^{K376R/K376R}$ and $Mlkl^{-/-}Fadd^{-/-}Ripk1^{K376R/K376R}$ MEFs were treated with TS, TSN, TSZ, TSZN for 24h. Cell viability was determined using the CellTiter-Glo kit. The data are represented as the mean $\pm$ SEM of three independent experiments. Abbreviations are as follows: Ctrl, untreated; T, TNF- α (20ng/ml); S, Smac mimetic (100nM); Z, zVAD (20 μ M); N, Necrostatin-1(20 μ M) **c** $Ripk1^{+/+}$, $Ripk3^{-/-}Fadd^{-/-}$ and $Ripk3^{-/-}Fadd^{-/-}Ripk1^{K376R/K376R}$ were treated with TNF- α (20ng/ml) for the indicated periods of time, NF- κ B signaling pathway was examined by western blotting. **d** Representative flow cytometric images of cells stained with anti-CD3 and anti-B220 taken from 12-week-old animal of the indicated genotypes. At least three mice each genotype were analyzed independently with similar results.

Genotype		E13.5	E14.5	P14	Adulthood
<i>WT</i>					
<i>Ripk1^{K376R/K376R}</i>	<i>WT</i>				
	<i>Ripk3^{-/-}</i>				
	<i>Mlkl^{-/-}</i>				
	<i>Tnfr1^{-/-}</i>				
	<i>Tnfr1^{-/-} Ripk3^{-/-}</i>				
	<i>Ripk3^{-/-} Fadd^{-/-}</i>				
	<i>Mlkl^{-/-} Fadd^{-/-}</i>				

Supplementary Figure. 5 Genetic evidence highlights the role of RIPK1 ubiquitination on K376 during development. Related to Fig. 6,7

Genetic crosses of *Ripk1^{K376R/K376R}* mice with different combinations of gene deletions were shown

Supplementary Figure 6. Source data of the indicated Figures

Fig.1f

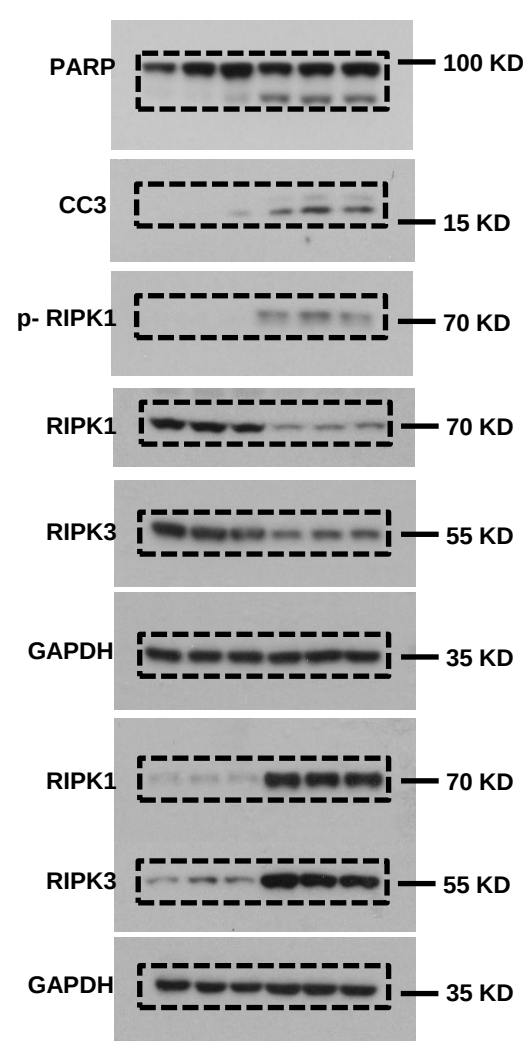


Fig.2a

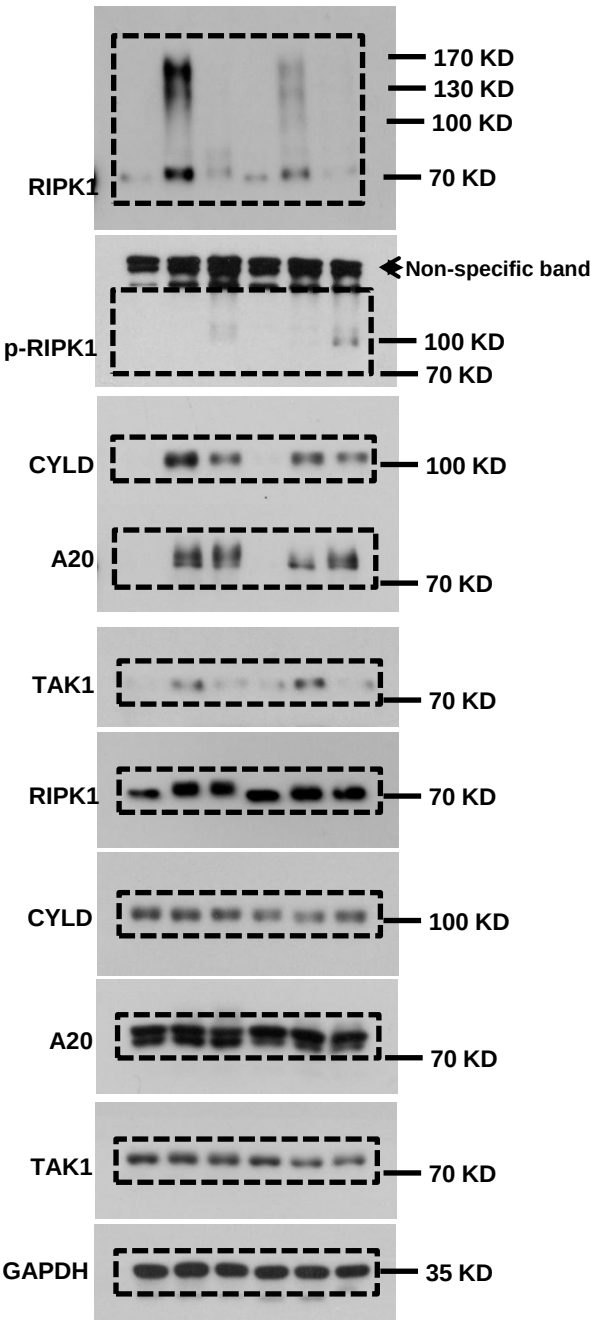


Fig.2b

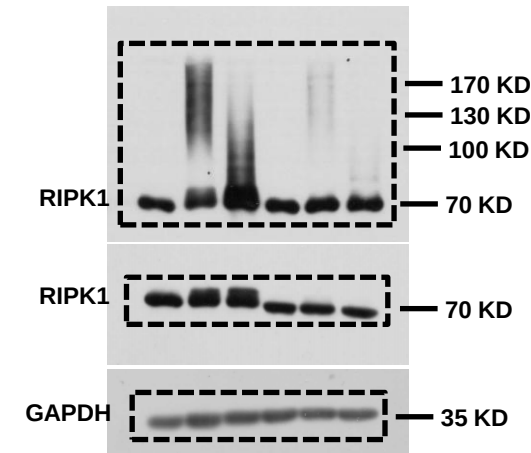


Fig.2c

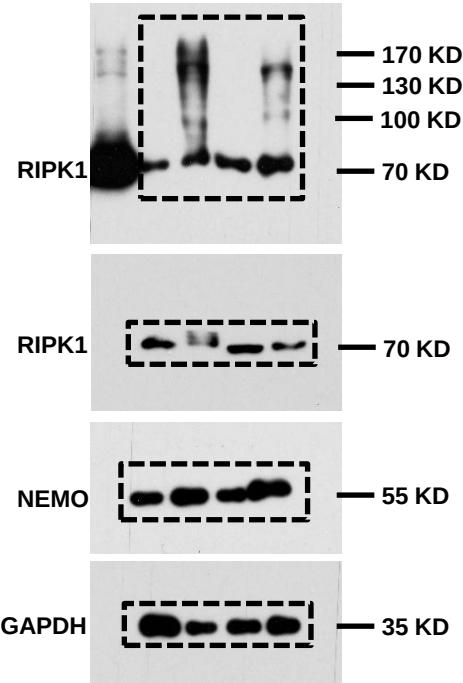


Fig.2d

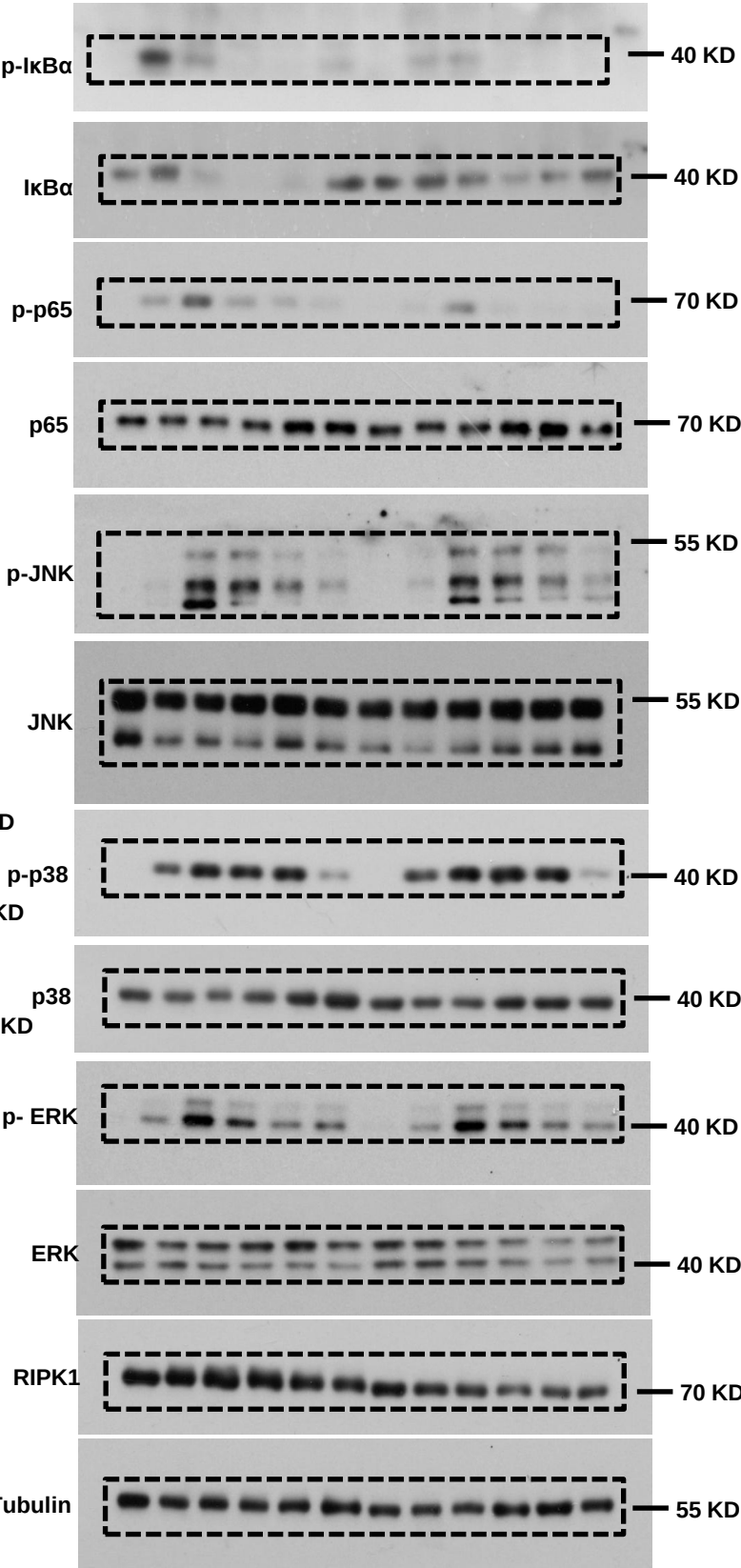


Fig.2e

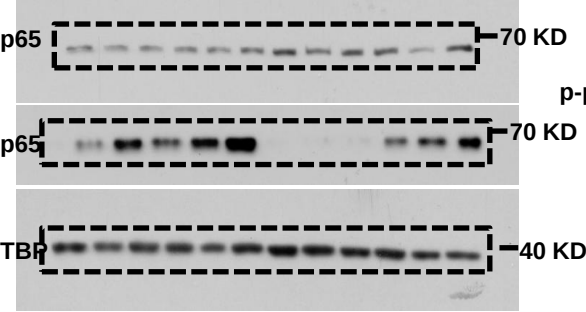


Fig.3b

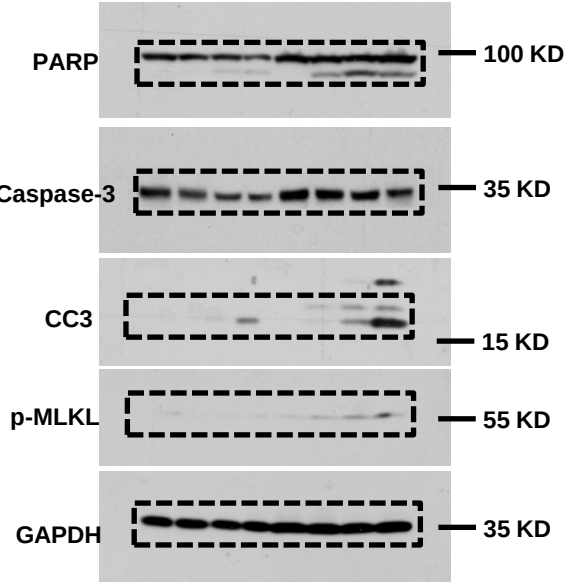


Fig.3c

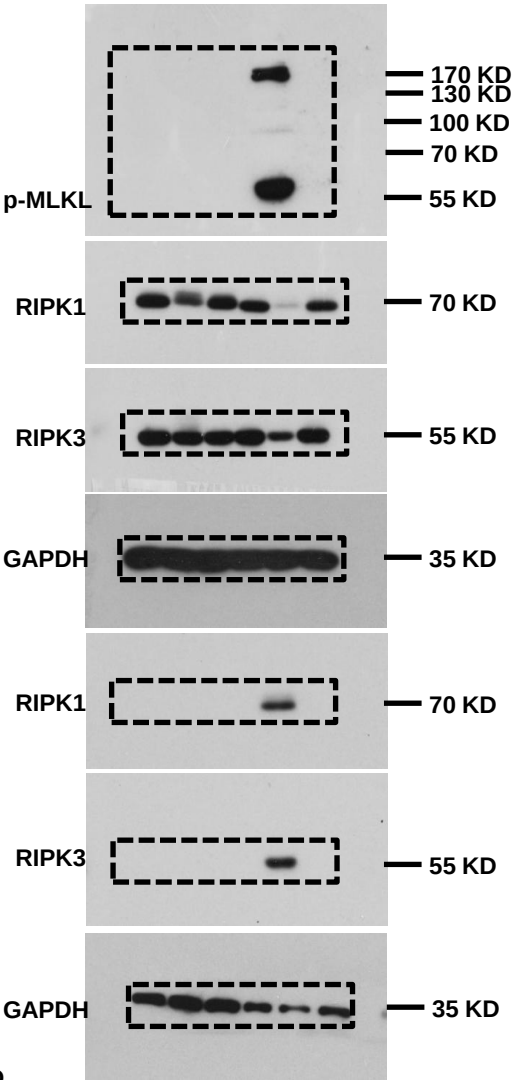


Fig.3d

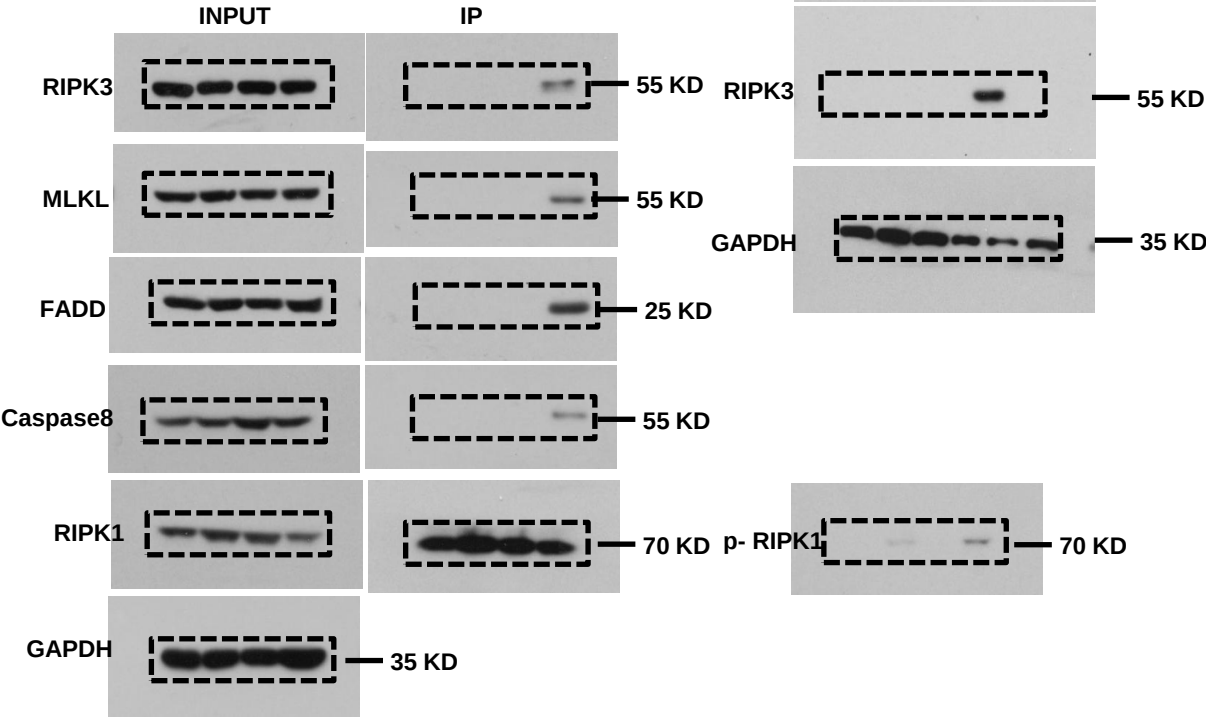


Fig.4f

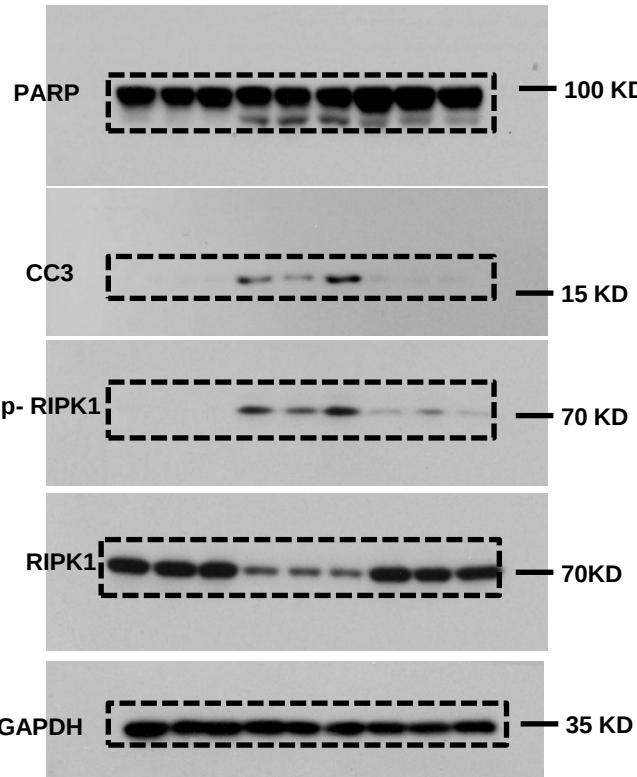


Fig.5c

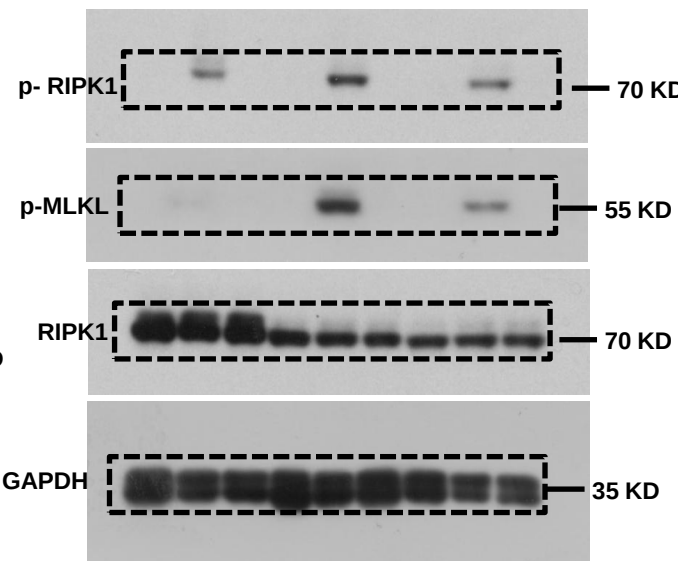
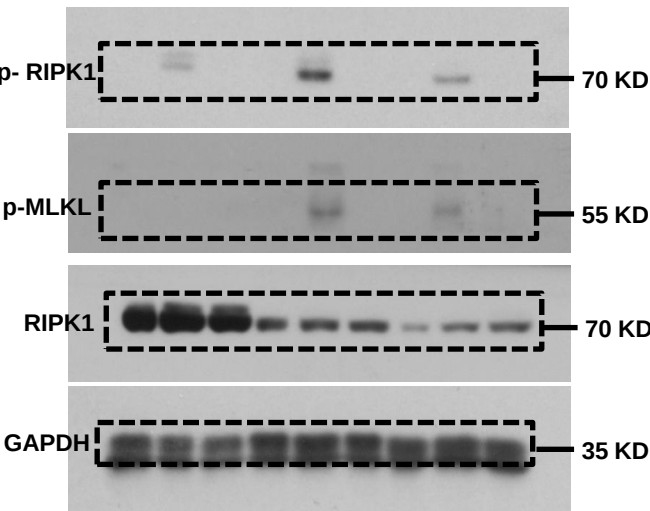
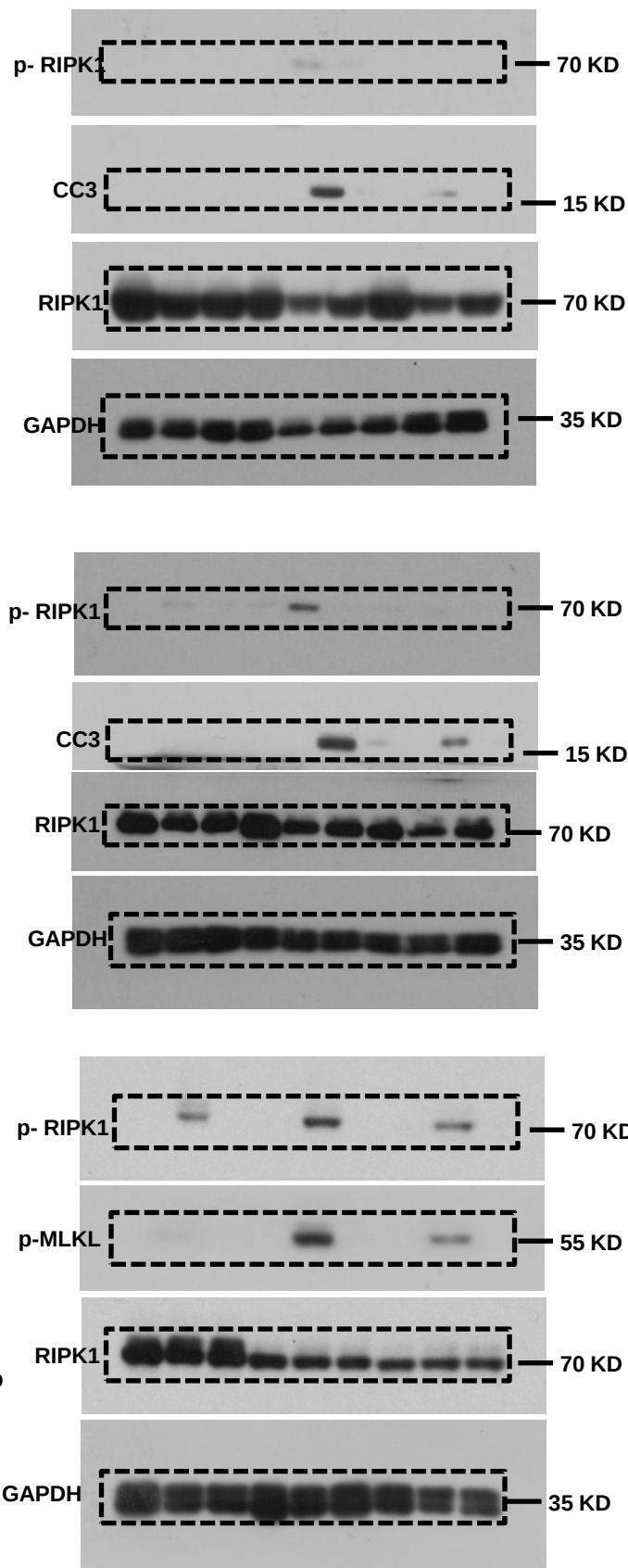


Fig.S1a

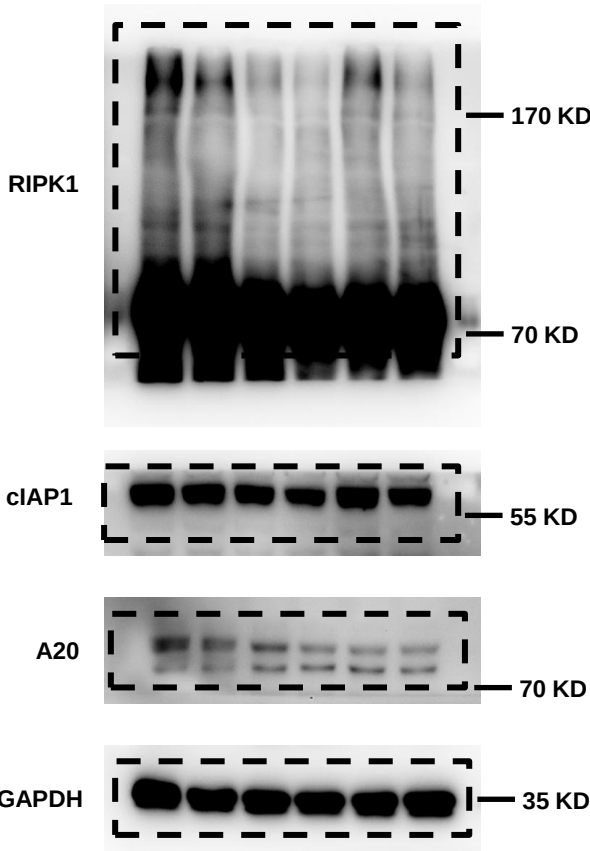


Fig.S2g

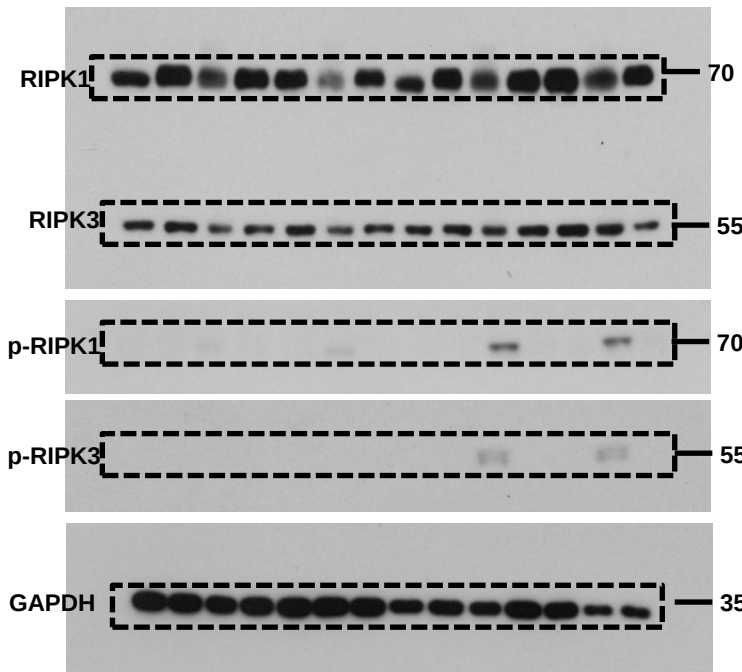


Fig.S4a

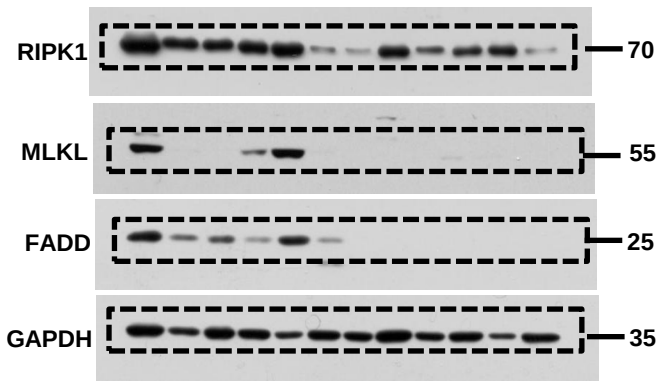
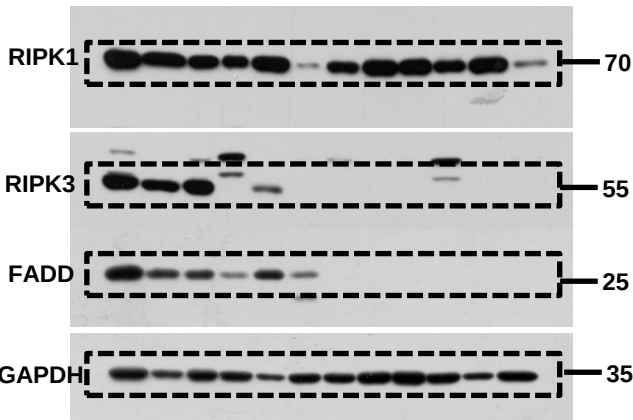


Fig.S4c

