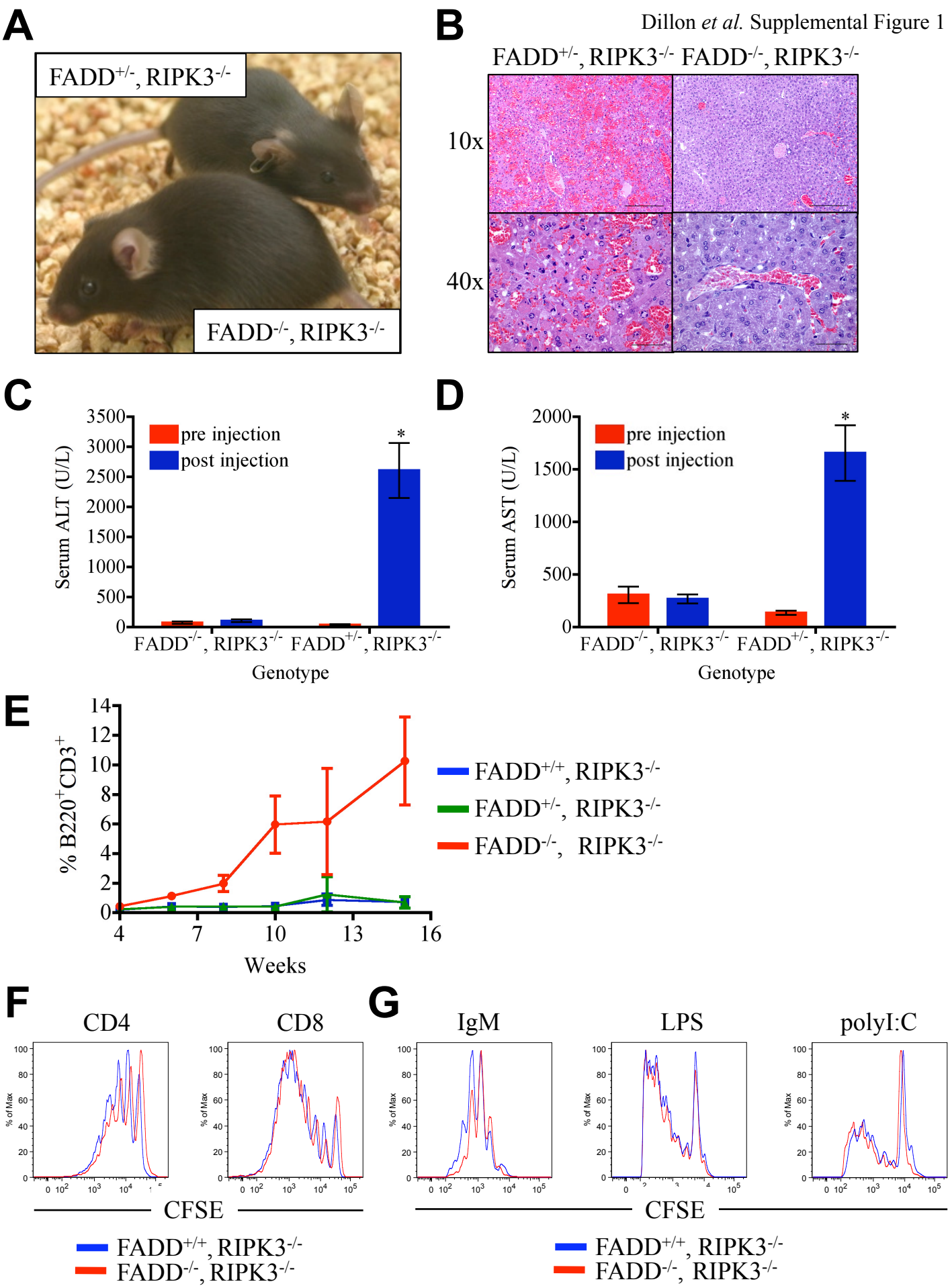
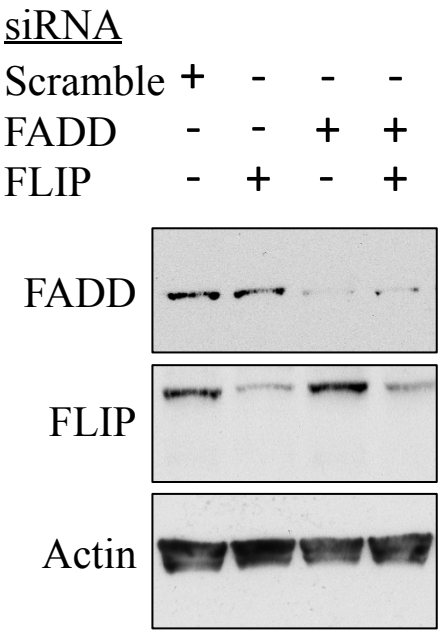
Dillon *et al.* Supplemental Figure 1



Dillon *et al.*

Supplemental Figure 2

A



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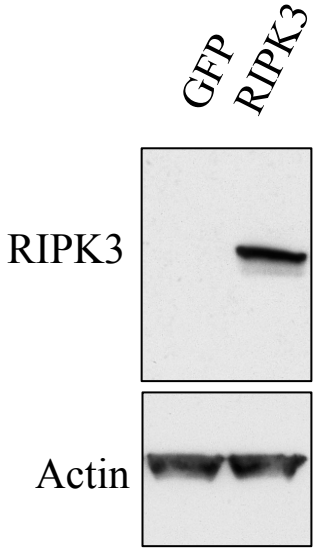
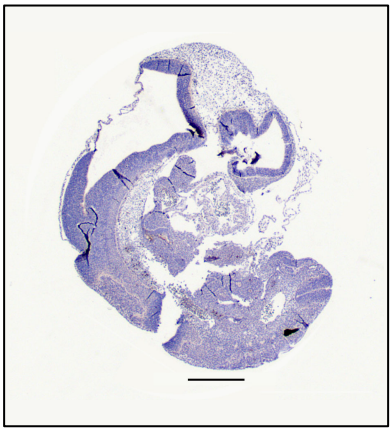
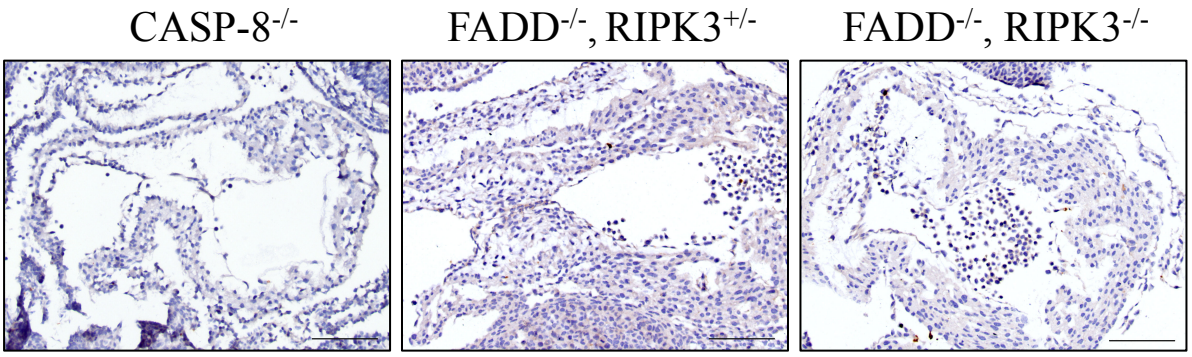


C

FADD$^{-/-}$, RIPK3$^{-/-}$

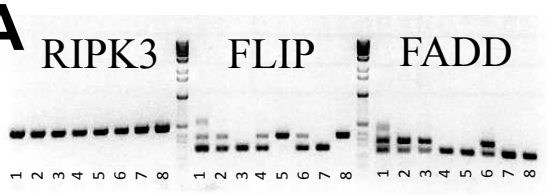


D



Dillon *et al.* Supplemental Figure 3

A



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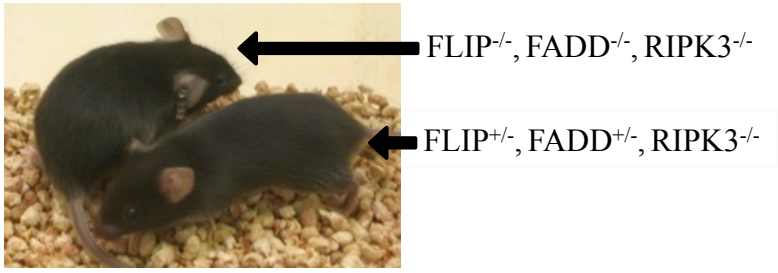

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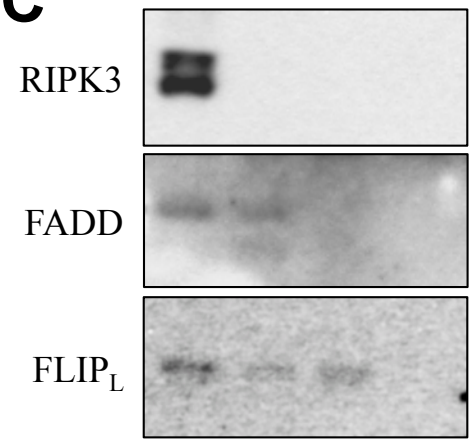


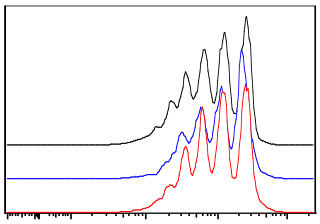
RIPK3	+/-	-/-	-/-	-/-
FADD	+/-	+/-	-/-	-/-
FLIP	+/+	+/+	+/+	-/-

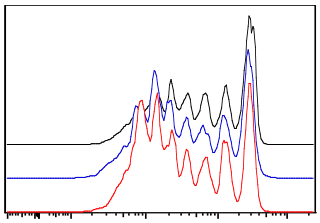
D

FLIP$^{+/-}$, FADD$^{+/-}$, RIPK3$^{-/-}$ x FLIP$^{+/-}$, FADD$^{-/-}$, RIPK3$^{-/-}$

	Expected	Observed
FLIP$^{+/+}$, FADD$^{+/-}$, RIPK3$^{-/-}$	9.875	12
FLIP$^{+/-}$, FADD$^{+/-}$, RIPK3$^{-/-}$	19.75	22
FLIP$^{-/-}$, FADD$^{+/-}$, RIPK3$^{-/-}$	9.875	0
FLIP$^{+/+}$, FADD$^{-/-}$, RIPK3$^{-/-}$	9.875	10
FLIP$^{+/-}$, FADD$^{-/-}$, RIPK3$^{-/-}$	19.75	22
FLIP$^{-/-}$, FADD$^{-/-}$, RIPK3$^{-/-}$	9.875	13
Total:	79	79

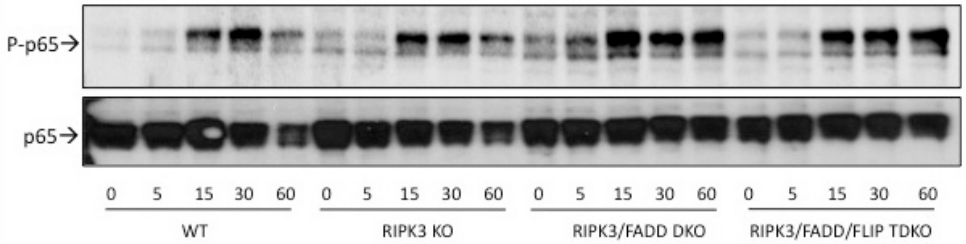
E



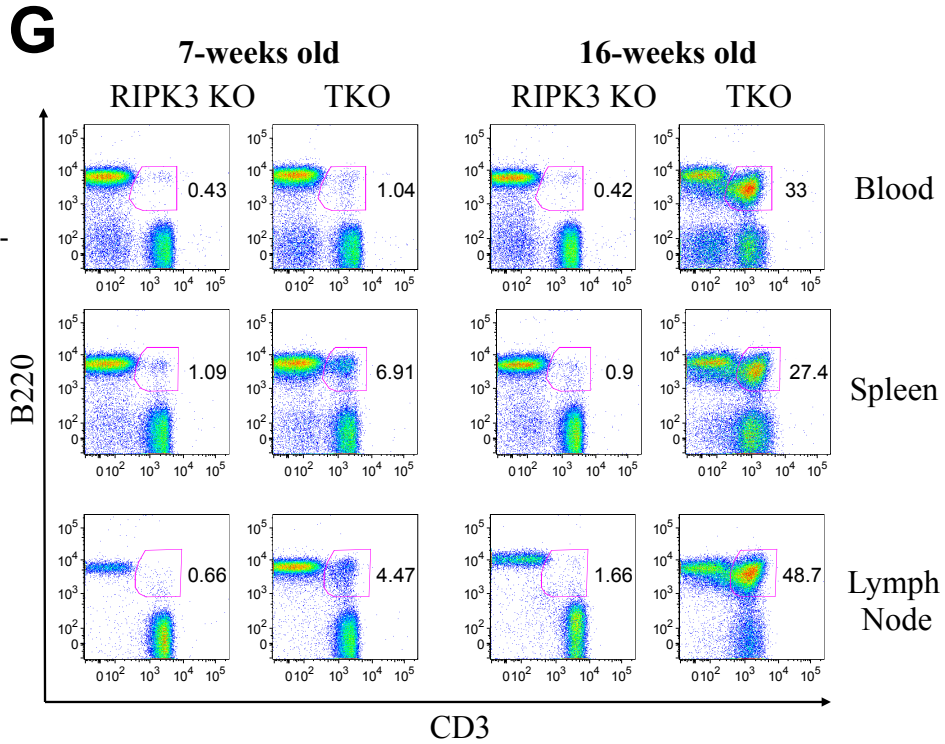


FLIP$^{+/+}$, FADD$^{+/+}$, RIPK3$^{-/-}$
FLIP$^{+/+}$, FADD$^{-/-}$, RIPK3$^{-/-}$
FLIP$^{-/-}$, FADD$^{-/-}$, RIPK3$^{-/-}$

F

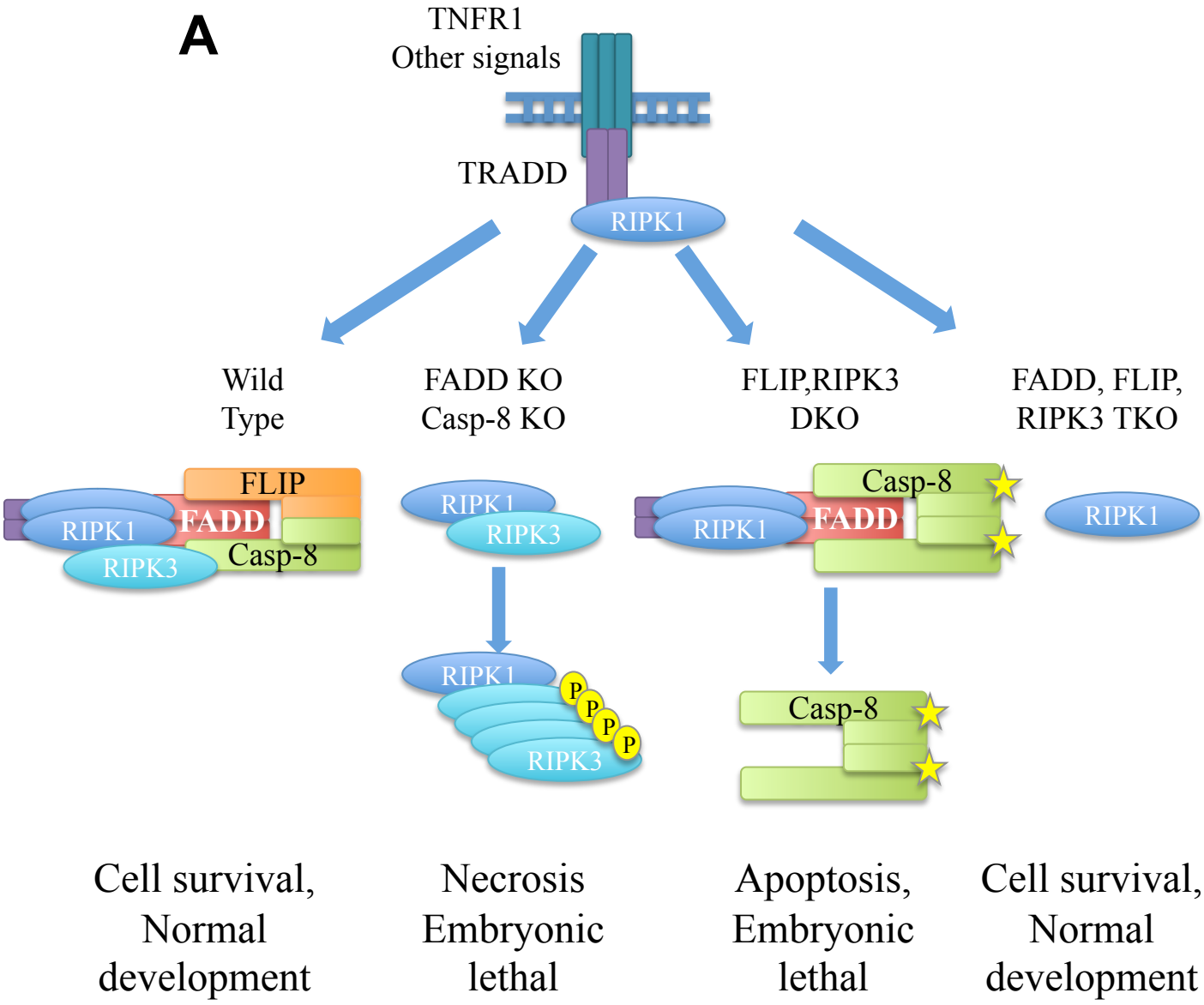


G



Dillon *et al.*

Supplemental Figure 4



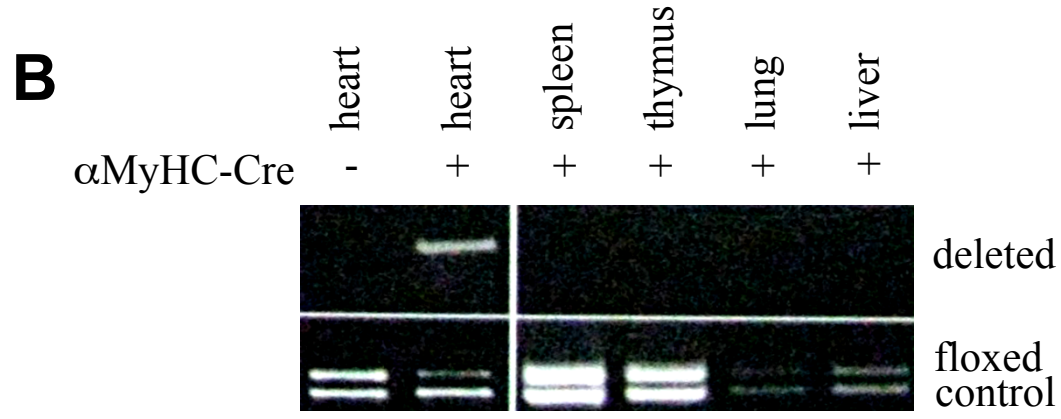


Figure S1. Characterization of FADD$^{-/-}$, RIPK3$^{-/-}$ mice. Related to Figure 1. (A) FADD$^{+/-}$, RIPK3$^{-/-}$ and FADD$^{-/-}$, RIPK3$^{-/-}$ littermates at 6 weeks. (B) Effects of agonist anti-CD95 injection in indicated genotypes 3 hr post-injection as shown by 10X and 40X magnified sections of livers stained with hematoxylin and eosin (H&E). Scale bars are 250 and 50 μm, respectively. Serum levels of alanine aminotransferase (ALT) (p=0.0059) (C) and aspartate aminotransferase (AST) (p=0.0045) (D) in indicated genotypes pre- and post-(3 hr)injection of anti-CD95. (E) Percentage of B220^{+}CD3^{+} cells among peripheral blood mononuclear cells (following red blood cell lysis) in mice of the indicated genotypes and ages. (F) Proliferation of anti-CD3 plus anti-CD28 activated splenic T cells of the indicated genotypes. Cells were stained with carboxyfluorescein succinimidyl ester (CFSE) and proliferation was assessed at 72 hr. (G) Proliferation of splenic B cells of the indicated genotypes following the indicated treatments. Cells were stained with CFSE and proliferation was assessed at 72 hr.

Figure S2. Lack of cleaved caspase-3 staining in embryos of selected genotypes. Related to Figure 3. (A-B) Western blot analysis of cells from Figure 3B and C. (C) Cleaved caspase-3 immunostaining of an E10 FADD$^{-/-}$, RIPK3$^{-/-}$ embryo. Scale bar is 500 μm. (D) Cleaved caspase-3 immunostaining in heart sections from E9.5-10 embryos of the indicated genotypes. Scale bars are 100 μm. For C and D, representative images are presented, $n \geq 3$ for each genotype.

Figure S3. Characterization of FLIP$^{-/-}$, FADD$^{-/-}$, RIPK3$^{-/-}$ mice. Related to Figure 4. (A) PCR-based genotyping analysis of two litters from crosses of FLIP$^{+/-}$, FADD$^{-/-}$, RIPK3$^{-/-}$ males and FLIP$^{+/-}$, FADD$^{+/-}$, RIPK3$^{-/-}$ females. For FLIP, lower band is wt, upper is knockout. For FADD, lower band is knockout, upper is wt. Note animals #5 and #8 are FLIP$^{-/-}$, FADD$^{-/-}$, RIPK3$^{-/-}$ TKO animals. (B) Photograph of FLIP$^{-/-}$, FADD$^{-/-}$, RIPK3$^{-/-}$ and FLIP$^{+/-}$, FADD$^{+/-}$, RIPK3$^{-/-}$ littermates at 6 weeks. (C) Western blot analysis of splenic T cells from animals of the indicated genotypes. (D) Expected and observed frequency of FLIP and FADD status in offspring from crosses of FLIP$^{+/-}$, FADD$^{-/-}$, RIPK3$^{-/-}$ males and FLIP$^{+/-}$, FADD$^{+/-}$, RIPK3$^{-/-}$ females. (E) Proliferation of anti-CD3 plus anti-CD28 activated splenic T cells of the indicated genotypes. Cells were stained with CFSE and proliferation was assessed at 72 hr. (F) Western blot analysis of T cells from animals of the indicated genotypes harvested at the indicated times post anti-CD3 plus anti-CD28 stimulation. (G) Percentage of B220^{+}CD3^{+} cells among peripheral blood mononuclear cells, spleen, or lymph node (following red blood cell lysis) in mice of the indicated genotypes and ages.

Figure S4. Model of the survival function of the FADD-Caspase-8-FLIP complex. Related to Figure 4. (A) TNF, or other poorly defined upstream signals, cause the formation of a complex containing RIPK1, RIPK3, FADD, FLIP, and caspase-8. In this context, there is no RIPK3 activation and no caspase-8 homodimer formation, so cells survive and development is normal. In the absence of FADD or caspase-8, RIPK3 becomes activated leading to necrosis and embryonic lethality. However, if both FLIP and RIPK3 are absent, caspase-8 homodimer can form, driving apoptosis

and causing embryonic lethality. Ablation of FADD together with FLIP and RIPK3 prevents both necrosis and apoptosis, so cells survive and development is normal. (B) Assessment of cre-mediated recombination in αMyHC-Cre^{+}, Casp8$^{flox/flox}$ mice via PCR-based genotyping.

Supplementary Experimental Procedures:

Knockdown experiments: NIH 3T3 cells with or without exogenous RIPK3 were maintained in D-MEM (Invitrogen) supplemented with 10% FCS, L-glutamine, and pen/strep. siGenome SMARTpool siRNAs against caspase-8(M-043044-01-0005), FLIP(M-041091-01-0005), and FADD(M-040488-01-0005) as well as scramble siRNA control (D-001206-13-05), were ordered from Dharmacon, and introduced into cells using Lipofectamine RNAiMAX (Invitrogen) according to manufacturer's guidelines.

Antibodies: Antibodies used for western blot were: Anti-RIPK1 from BD Biosciences (clone 38), anti-RIPK3 from Imgenex (IMG-5523-2), anti-caspase-8 (1G12) and anti-FLIP (Dave-2) both from Alexis, and anti-FADD (M19) from Santa Cruz. Purified anti-murine CD95 (clone Jo2) came from BD Biosciences. Fluorescent-conjugated anti-CD3-FITC (clone 145-2C11), anti-B220-APCeFluor780 (clone RA3-6B2), anti-CD4-eFluor450 (clone RM4-5), and anti-CD8-PECy7 (clone 53-6.7) came from eBioscience. Anti-cleaved caspase-3 (D175) came from Cell Signaling, and AlexaFluor647 donkey anti-rabbit was obtained from Invitrogen.

Proliferation Assays and Culture Conditions: For proliferation assays, splenic T cells were isolated from whole spleen using magnetic separation (Pan T cell isolation kit II, Miltenyi Biotec (130-095-130)). Cells were stained with CFSE and plated at 1×10^5 cells per well in 96-well plates with Dynabeads® Mouse T-Activator CD3/CD28 per manufacturer's instructions. Splenic B cells were from whole spleen isolated using magnetic separation (CD45R/B220 Microbeads, Miltenyi Biotec (130-049-501)). Cells were stained with CFSE and plated at 1×10^5 cells per well in 96-well plates with anti-IgM (Jackson Immunoresearch, 109-005-129), LPS (Sigma) or poly I:C (Invivogen) at concentrations previously described (Zhang et al., 2011). Splenocytes were cultured in RPMI 1640 (Invitrogen) supplemented with 55 μM β-mercaptoethanol, 1 mM sodium pyruvate and non-essential amino acids (Gibco).

Statistical analysis: To determine the statistical significance of embryonic crosses, a chi-squared test was used (Graph Pad). Analysis of serum ALT and AST was performed using a 2-way ANOVA with Prism software (Graph Pad).

References

Zhang, H., Zhou, X., McQuade, T., Li, J., Chan, F.K., and Zhang, J. (2011). Functional complementation between FADD and RIP1 in embryos and lymphocytes. Nature *471*, 373-376.