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Report

Survival Function of the FADD-CASPASE-8-cFLIP$_L$ Complex

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SUMMARY

Caspase-8, the initiator caspase of the death receptor pathway of apoptosis, its adapter molecule, FADD, required for caspase-8 activation, and cFLIP$_L$, a caspase-8-like protein that lacks a catalytic site and blocks caspase-8-mediated apoptosis, are each essential for embryonic development. Animals deficient in any of these genes present with E10.5 embryonic lethality. Recent studies have shown that development in *caspase-8*-deficient mice is rescued by ablation of *RIPK3*, a kinase that promotes a form of programmed, necrotic cell death. Here, we show that *FADD*, *RIPK3* double-knockout mice develop normally but that the lethal effects of *cFLIP* deletion are not rescued by *RIPK3* deficiency. Remarkably, in mice lacking *FADD*, *cFLIP*, and *RIPK3*, embryonic development is normal. This can be explained by the convergence of two cell processes: the enzymatic activity of the FADD-caspase-8-cFLIP$_L$ complex blocks RIPK3-dependent signaling (including necrosis), whereas cFLIP$_L$ blocks RIPK3-independent apoptosis promoted by the FADD-caspase-8 complex.

INTRODUCTION

Apoptosis, or programmed cell death, is an essential process for development and homeostasis of multicellular organisms. Insight into the important roles apoptosis plays in these processes has come from knockout animals ablated for genes in cell death pathways (Weinlich et al., 2011). Although knockout animals of proapoptotic genes such as *Apaf-1*, *caspase-9*, and *caspase-3* all demonstrate phenotypes consistent with failure to eliminate cells, animals ablated for key components of the death receptor pathway suffer early embryonic lethality associated with defective vascularization (Green et al., 2011; Weinlich et al., 2011). Embryos deficient in *caspase-8*, the adaptor molecule *FADD*, or the noncatalytically active caspase-8 homolog *cFLIP* die around E10.5 (Varfolomeev et al., 1998; Yeh et al., 1998, 2000; Zhang et al., 1998) with similar defects in vascularization of the yolk sac (Oberst and Green, 2011; Sakamaki et al., 2002), which suggests that these proteins perform significant nonapoptotic roles in development. Caspase-8, FADD, and cFLIP$_L$ (herein called "FLIP") have also been implicated in cell-cycle regulation and NF-kB activation (Budd et al., 2006; Oberst and Green, 2011; Tourneur and Chiocchia, 2010) in various tissues post-development. Recent work has shown that *caspase-8* deficiency can be rescued by concurrent deletion of *RIPK3* (Kaiser et al., 2011; Oberst et al., 2011), a kinase that promotes a form of programmed necrotic cell death (sometimes called "necroptosis"; Galluzzi et al., 2012), indicating a more specific and limited role for caspase-8 in development. Biochemical evidence demonstrates that a caspase-8-FLIP heterodimer acts to inhibit the function of RIPK3 in vitro, suggesting that this complex might play the same role in vivo (Oberst et al., 2011). In this work we address the survival functions of FADD and FLIP in the context of RIPK3-dependent necrosis and apoptosis during development.

RESULTS AND DISCUSSION

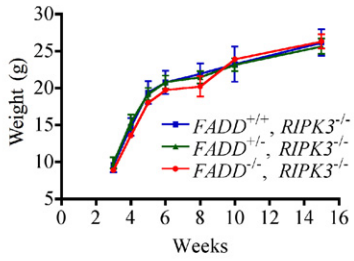
FADD$^{-/-}$ Embryonic Lethality Is Rescued by *RIPK3* Ablation

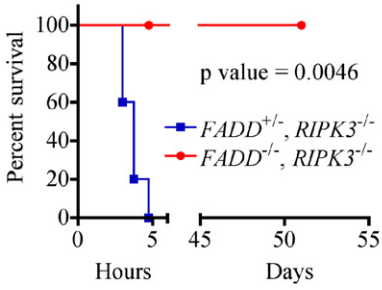
The activation of caspase-8 depends on the adaptor molecule FADD (Oberst and Green, 2011). We therefore hypothesized that deletion of RIP kinases might rescue the embryonic lethality of *FADD*$^{-/-}$ mice similar to *caspase-8*$^{-/-}$ animals (Kaiser et al., 2011; Oberst et al., 2011). However, in one study the lethal effects of *FADD* deletion were only partially rescued by ablation of *RIPK1* (Zhang et al., 2011) because these animals died perinatally, as is also seen in *RIPK1*$^{-/-}$ mice (Kelliher et al., 1998). Because RIPK1 can promote necrosis that depends on RIPK3 (Cho et al., 2009), we asked if deletion of *RIPK3* rescues development in *FADD*$^{-/-}$ mice. We found that *FADD*, *RIPK3* double-knockout (DKO) mice were weaned at expected frequencies (Figure 1A) and grew with kinetics identical to those of their *FADD*$^{+/+}$ and *FADD*$^{+/-}$ littermates (Figure 1B and Figure S1A). As

A

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

	Expected	Observed
FADD$^{+/+}$, *RIPK3*$^{-/-}$	40.25	44
FADD$^{+/-}$, *RIPK3*$^{-/-}$	80.5	73
FADD$^{-/-}$, *RIPK3*$^{-/-}$	40.25	44
Total:	161	161
p value:	0.4972	





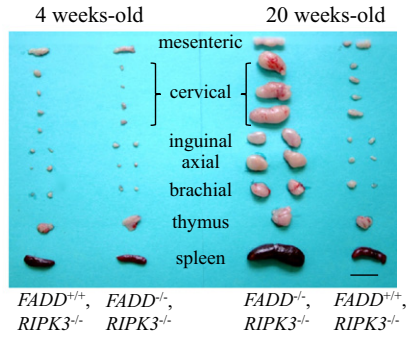


Figure 1. *FADD*$^{-/-}$, *RIPK3*$^{-/-}$ Mice Are Viable and Overtly Normal, Functionally Deficient for FADD, and Display Severe Progressive Lymphoaccumulation

(A) Expected and observed frequency of *FADD* status in offspring from crosses of *FADD*$^{+/-}$, *RIPK3*$^{-/-}$ animals. The resulting offspring were genotyped at weaning.

(B) Plot of weight of littermate *FADD*$^{+/+}$, *RIPK3*$^{-/-}$, *FADD*$^{+/-}$, *RIPK3*$^{-/-}$, and *FADD*$^{-/-}$, *RIPK3*$^{-/-}$ animals.

(C) Effect of anti-CD95 in vivo. A total of 15 μg of agonist anti-CD95 antibody Jo2 was injected intravenously into *FADD*$^{+/-}$, *RIPK3*$^{-/-}$ or *FADD*$^{-/-}$, *RIPK3*$^{-/-}$ animals. Animals were monitored and euthanized when moribund.

(D) Lymphoid organs removed from young (4 weeks) and old (20 weeks) littermate mice of the indicated genotypes. Scale bar represents 1 cm. See also Figure S1.

seen in *caspase-8*, *RIPK3* DKO mice (Kaiser et al., 2011; Oberst et al., 2011), *FADD*, *RIPK3* DKO mice were completely resistant to lethal hepatic injury induced by injection of agonistic anti-CD95 antibody (Ogasawara et al., 1993) (Figures 1C and S1B–S1D). Also, as seen in *caspase-8*, *RIPK3* DKO mice, *FADD*, *RIPK3* DKO animals accumulated over time a population of B220^{+}CD3^{+} T lymphocytes (Figure S1E), resulting in a lymphoaccumulative disease (Figure 1D), resembling that seen in mice or humans lacking CD95 or its ligand (Wilson et al., 2009). Therefore, like caspase-8 (Kaiser et al., 2011; Oberst et al., 2011), FADD is required for prevention of RIPK3-mediated embryonic lethality and for the function of CD95.

FADD, *RIPK1* DKO mice display defects in B cell activation-induced proliferation (Zhang et al., 2011). Because FADD had been previously suggested to play a role in cell-cycle progression (Tourneur and Chiocchia, 2010), we examined lymphocytes from young (5 weeks) *FADD*, *RIPK3* DKO mice, and observed no differences in activation-induced proliferation of T or B cells between these and *FADD*-sufficient cells (Figures S1F and S1G). Therefore, the defect observed in the *FADD*, *RIPK1* DKO (Zhang et al., 2011) is likely to be a consequence of *RIPK1* deficiency rather than due to the absence of *FADD*.

Elimination of *RIPK3* Does Not Rescue *FLIP*-Deficient Embryos

To investigate the role of RIPK3 in the embryonic lethality observed in *FLIP*-deficient mice, we crossed *FLIP*$^{+/-}$, *RIPK3*$^{-/-}$ animals (Figure 2A). No *FLIP*$^{-/-}$, *RIPK3*$^{-/-}$ mice were detected at weaning. Examination of embryos from timed matings showed developmental abnormalities beginning around E10–E10.5 (Figures 2A and 2B). As seen in animals lacking *caspase-8*, *FADD*, or *FLIP* (Sakamaki et al., 2002; Varfolomeev et al., 1998; Yeh et al., 1998, 2000; Zhang et al., 1998), the vasculature of the yolk sacs of *FLIP*, *RIPK3* DKO mice showed severe defects, readily observable in live embryos or by staining for the endothelial marker, PECAM-1 (Newman et al., 1990) (Figure 2C).

Formally, the ability of *RIPK3* ablation to rescue development of *caspase-8*$^{-/-}$ (Kaiser et al., 2011; Oberst et al., 2011) or *FADD*$^{-/-}$ mice (Figure 1), but not that of *FLIP*$^{-/-}$ mice (Figure 2), indicates that FLIP has a function distinct from those of FADD and caspase-8 in embryogenesis. To gain more insight into these interrelated functions, we turned to an established in vitro system in which ligation of TNF receptor 1 induces RIPK1-RIPK3-dependent necrosis that is inhibited by the activity of the caspase-8-FLIP heterodimer (Oberst et al., 2011). Cells were treated with TNF with or without the caspase inhibitor zVAD-fmk. In the presence of the inhibitor, TNF induced the formation of a complex, identified upon immunoprecipitation of FADD, containing FADD, caspase-8, FLIP, RIPK1, and RIPK3 (Figure 3A). In contrast, no components of the complex were found in the absence of caspase inhibition (Figure 3A). This is consistent with findings suggesting that caspase-8 (presumably in the form of the caspase-8-FLIP heterodimer) cleaves RIPK1 and RIPK3 (Feng et al., 2007; Rébé et al., 2007). Furthermore, additional findings suggest that caspase-8-FLIP is rapidly degraded in cells (Feoktistova et al., 2011), and therefore, this instability may extend to the entire complex to control both apoptosis and necrosis (Green et al., 2011).

Ablation of *RIPK3* in *FLIP*-Deficient Cells and Embryos Converts Death from Necrosis to Apoptosis

We then examined the interplay of FLIP and FADD in cells with or without RIPK3. In the presence of RIPK3, knockdown of FLIP, FADD, or both sensitized cells to TNF-induced cell death with characteristics of necrosis (i.e., rapid loss of plasma membrane integrity as detected by propidium iodide [PI] staining

A

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

	E9.0-9.5		E10-10.5		E11-11.5		E12-13.5		Weaned	
	Expected	Observed	Expected	Observed	Expected	Observed	Expected	Observed	Expected	Observed
FLIP$^{+/+}$, *RIPK3*$^{-/-}$	12.5	18	9.25	7	12.5	12	10. 5	14	42.25	59
FLIP$^{+/-}$, *RIPK3*$^{-/-}$	25	23	18.5	24	25	31	21	27	84.5	110
FLIP$^{-/-}$, *RIPK3*$^{-/-}$	12.5	9	9.25	6*	12.5	7*	10.5	1*	42.25	0
Total:	50	50	37	37	50	50	42	42	169	169
p value:	0.1686		0.1897		0.1437		0.0032		<0.0001	

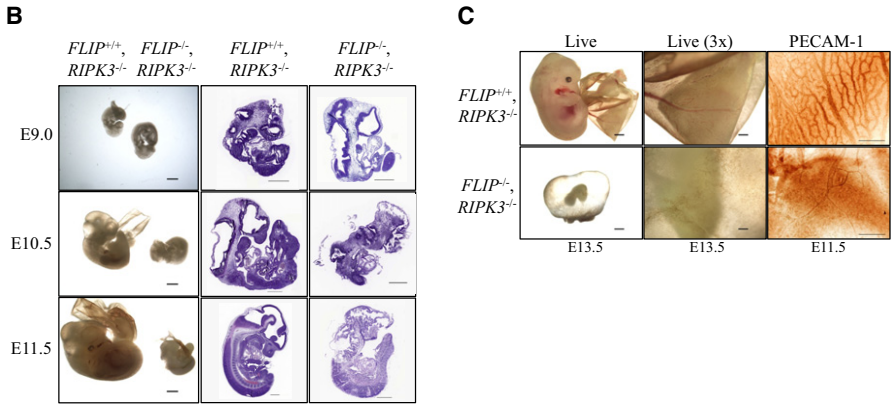


Figure 2. *FLIP*$^{-/-}$, *RIPK3*$^{-/-}$ Mice Are Embryonic Lethal

(A) Expected and observed frequency of *FLIP* status in offspring from crosses of *FLIP*$^{+/-}$, *RIPK3*$^{-/-}$ animals. The resulting offspring were genotyped at the indicated developmental time points or at weaning. Asterisks reflect malformed embryos.

(B) Embryos (left) and hematoxylin and eosin-stained sections of fixed embryos (right) of the indicated genotypes at the indicated time points. Representative images are presented ($n \geq 3$ for each genotype). Scale bars for images on left are 1 mm and for sections on right are 500 μm.

(C) Embryos of the indicated genotypes at E13.5 (left and middle) showing vascularization of the embryos and yolk sacs. Left scale bar is 1 mm, and middle scale bar is 333 μm. Right panels show PECAM-1 immunostaining on sections from E11.5 embryos of the indicated genotypes. Representative images are presented ($n \geq 3$ for each genotype). Scale bar represents 250 μm.

without downstream effector caspase activation) (Figures 3B, S2A, and S2B). However, in the absence of RIPK3, knockdown of FLIP sensitized cells to TNF-induced apoptosis (Annexin V$^+$, PI$^-$ and downstream effector caspase activation as detected by intracellular cleaved caspase-3 staining), whereas knockdown of FADD did not (Figures 3C, S2A, and S2B). Importantly, knockdown of both FADD and FLIP did not sensitize cells to apoptosis (Figures 3C, S2A, and S2B). These results are consistent with the interpretation, supported here by caspase-8 knockdown (Figure 3C) and extensively by other studies (Wilson et al., 2009), that FADD promotes caspase-8-mediated apoptosis that is inhibited by FLIP. However, in the presence of RIPK3, loss of function of the FADD-caspase-8-FLIP complex results in RIPK3-dependent necrosis (Figure 3B).

Based on these observations, we reasoned that in embryos, a lack of both FLIP and RIPK3 would result in uncontrolled activation of caspase-8, as we observed (Figure 3C) and as described in cell lines (Oberst et al., 2011). We therefore examined apoptosis in embryos at E9.5–E10, lacking *caspase-8*, *FADD*, or both *FLIP* and *RIPK3* (Figures 3D, 3E, S2C, and S2D). *Caspase-8*$^{-/-}$ embryos at this stage did not display obvious apoptotic cell death, consistent with previous observations (Sakamaki et al., 2002), and this was also the case for embryos lacking *FADD* (Yeh et al., 1998) (Figures 3D, S2C, and S2D). In contrast, *FLIP*, *RIPK3* DKO embryos showed apoptosis in the endothelium, the first branchial arch, and other regions (Figures 3D and 3E). Thus, whereas embryonic lethality in *caspase-8*$^{-/-}$ or *FADD*$^{-/-}$ animals may occur due to unregulated RIPK3 necrosis (supported by the survival of *caspase-8*, *RIPK3* DKO; [Kaiser et al., 2011; Oberst et al., 2011] and *FADD*, *RIPK3* DKO mice [Figure 1A]), apoptotic cell death may be responsible for lethality in *FLIP*, *RIPK3* DKO mice.

Mice Lacking *FADD*, *FLIP*, and *RIPK3* Are Developmentally Normal

Because knockdown of FADD protected cells lacking FLIP and RIPK3 from TNF-induced apoptosis (Figure 3C), we examined the consequences of the triple knockout (TKO) of *FADD*, *FLIP*, and *RIPK3*. Using two different breeding strategies, we found that animals lacking all three genes were born at expected frequencies (Figures 4A and S3A–S3D). Here, we have found a situation in which a combination of two lethal genotypes (*FADD* KO; *FLIP*, *RIPK3* DKO) yields a TKO with normal development. Young TKO animals were grossly normal (Figure S3B), and gained weight with age indistinguishably from littermates (Figure 4B). Furthermore, because FADD (Tourneur and Chiocchia, 2010) and FLIP (Budd et al., 2006) have been implicated in proliferation and activation of NF-kB in activated T cells, we examined proliferation and phosphorylation of p65^{RelA} in activated TKO T lymphocytes. No abnormalities in activation-induced proliferation (Figure S3E) or activation of NF-kB (Figure S3F) were observed. Finally, as in *caspase-8*, *RIPK3* DKO (Kaiser et al.,

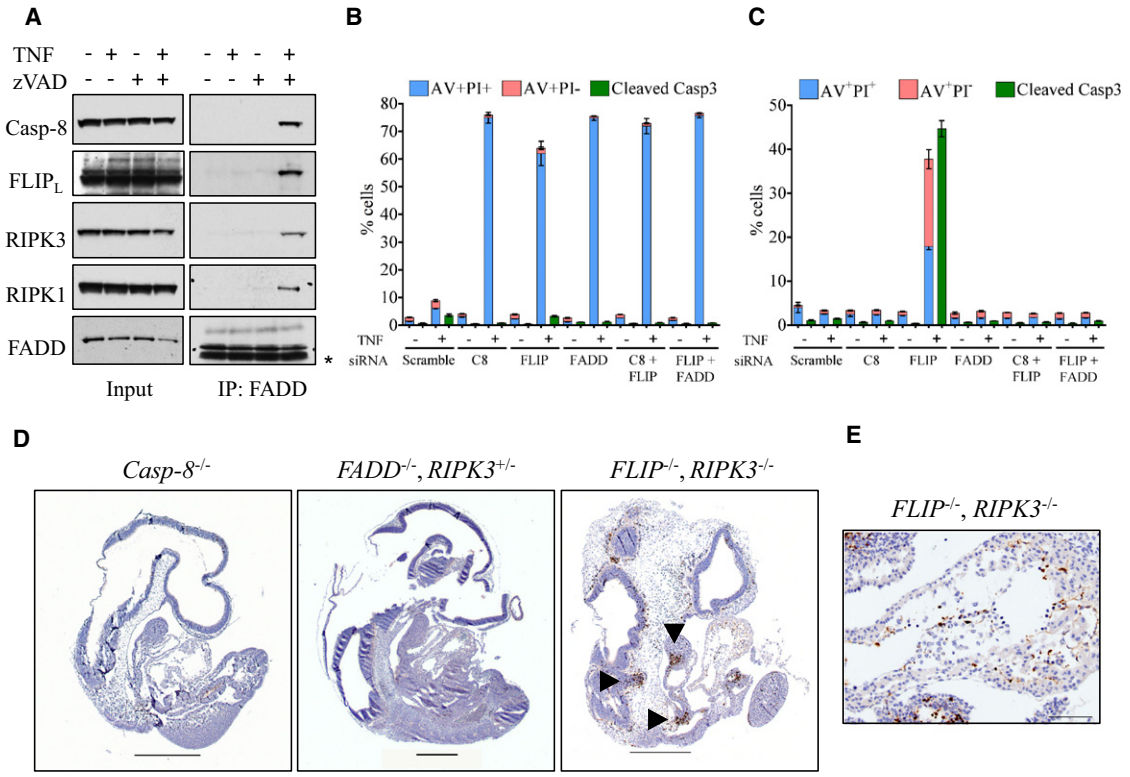


Figure 3. *FLIP*-Deficient Cells and Embryos Undergo Apoptosis in the Absence of RIPK3

(A) Immunoprecipitation of a FADD-containing complex from SVEC4-10 cells treated with or without 20 ng/ml TNF and 50 μM zVAD-fmk. NIH 3T3 cells, with (B) or without (C) stably expressed RIPK3, were transfected with the indicated siRNAs for 48 hr, followed by treatment with TNF for 9 hr. At harvest, cultures were split, and cell death was assessed by AnnexinV-APC and PI staining (with AnnexinV$^+$, PI$^-$ as apoptotic [pink] and AnnexinV$^+$, PI$^+$ as necrotic or late apoptotic [blue]). The presence of cleaved caspase-3 (green) was assessed by intracellular staining.

(D) Cleaved caspase-3 immunostaining in sections from E9.5–E10 embryos of the indicated genotypes. Arrowheads mark areas of focal cleaved caspase-3 staining. Scale bars are 500 μm.

(E) Cleaved caspase-3 immunostaining in heart section from an E9.5 *FLIP*$^{-/-}$, *RIPK3*$^{-/-}$ embryo. Scale bar represents 100 μm.

For (D) and (E), representative images are presented (n ≥ 3 for each genotype).

See also Figure S2.

2011; Oberst et al., 2011), and *FADD*, *RIPK3* DKO (Figure 1D) mice, these animals accumulated over time a population of B220$^+$CD3$^+$ T cells (Figure S3G), resulting in severe lymphoaccumulation (Figure 4C).

The normal development of *FADD*, *FLIP*, *RIPK3* TKO mice provides strong support for the emerging model (Figure S4A) that the FADD-caspase-8-FLIP complex inhibits RIPK3-mediated embryonic lethality while not promoting apoptosis in affected cells. In the absence of FLIP, the function of FADD promotes lethality, most likely via the action of caspase-8 (Oberst and Green, 2011). Unfortunately, due to the close linkage of *FLIP* and *caspase-8*, this could not be readily tested in vivo. However, this idea is strongly supported by the normal development of *caspase-8*, *RIPK3* DKO mice (Kaiser et al., 2011; Oberst et al., 2011), but not *FLIP*, *RIPK3* DKO mice (Figure 2). Furthermore, because *FADD*, *RIPK1* (Zhang et al., 2011) and *FADD*, *RIPK3* (Figure 1) DKO mice develop normally in utero, both RIPK1 and RIPK3 mediate embryonic lethality, most likely by the process of RIPK1-RIPK3-mediated programmed necrosis (Weinlich et al., 2011).

Mice lacking *FADD* (Yeh et al., 1998; Zhang et al., 1998), *FLIP* (Yeh et al., 2000), or *caspase-8* (Sakamaki et al., 2002; Varfolomeev et al., 1998) are embryonic lethal around E10.5 due to, at least partially, a failure in yolk sac vascularization, an effect we also observed in *FLIP*, *RIPK3* DKO mice (Figure 2C). According to the model (Figure S4A), this lethality in *FLIP*, *RIPK3* DKO mice is predicted to be caused by FADD-caspase-8-dependent apoptosis. Indeed, we observed early, focal apoptosis in endothelium and other structures in these embryos that was not observed at this stage in embryos lacking *caspase-8* or *FADD* (Figures 3D, 3E, S2C, and S2D). An early study, using mixed chimeras, suggested that embryonic lethality in *FADD*$^{-/-}$ mice correlates with the null allele associated with the region of the embryonic heart (Yeh et al., 1998). We found, however, that conditional deletion of *caspase-8* in the heart (*α myosin heavy-chain Cre*; Agah et al., 1997) did not cause embryonic lethality (Figures 4D and S4B), and weaned animals showed no differences in body weight (WT 22.6 ± 6.4 g versus KO 25.2 ± 4.3 g. n = 5) or heart weight (WT 0.14 ± 0.02 g versus KO 0.14 ± 0.03 g n = 5). Previous studies using conditional deletion

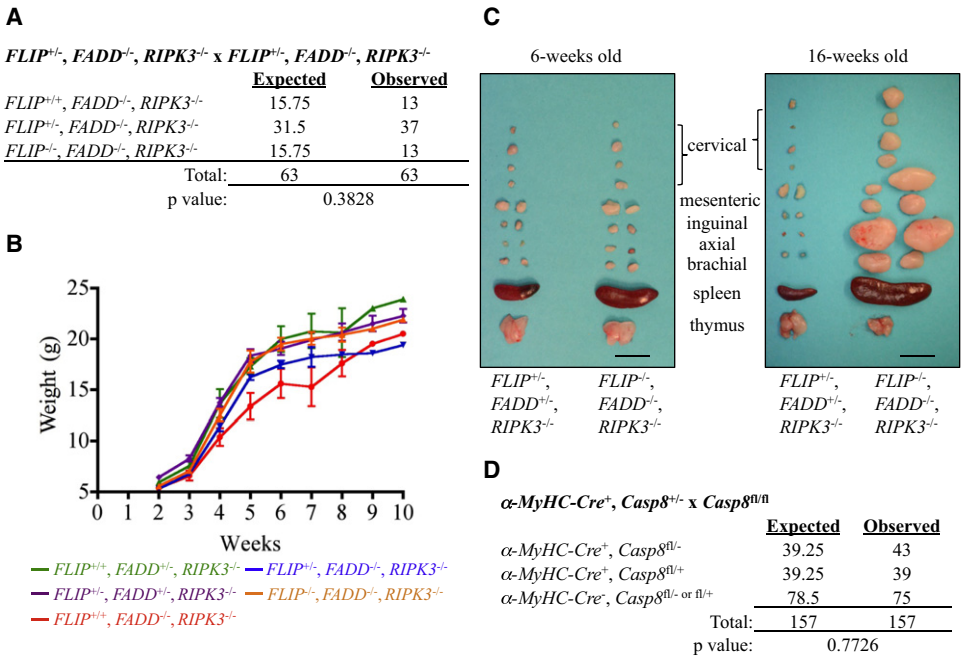


Figure 4. *FLIP*$^{-/-}$, *FADD*$^{-/-}$, *RIPK3*$^{-/-}$ Mice Are Viable with Overtly Normal Development but Display Severe Progressive Lymphoaccumulation

(A) Expected and observed frequency of *FLIP* and *FADD* status in offspring from crosses of *FLIP*$^{+/-}$, *FADD*$^{-/-}$, *RIPK3*$^{-/-}$ animals. The resulting offspring were genotyped for *FLIP* status at weaning.

(B) Plot of weight of littermate animals of the indicated genotypes at different ages.

(C) Lymphoid organs removed from young (6 weeks) and old (16 weeks) littermate mice of the indicated genotypes. Scale bars represent 1cm.

(D) Expected and observed frequency of *caspase-8* status in offspring from crossing of *αMyHC-Cre*$^{+}$, *Casp8*$^{+/-}$ with *Casp8*$^{flox/flox}$ animals.

See also Figures S3 and S4.

of *caspase-8*, however, have shown that E10.5 lethality is observed upon ablation of this gene in endothelium (*TIE1-Cre*) (Kang et al., 2004). *Caspase-8* ablation in liver (*Albumin-Cre*) (Kang et al., 2004), skin (*Keratin 5* or *14-Cre*) (Kovalenko et al., 2009; Lee et al., 2009), or intestinal epithelium (*Villin-Cre*) (Günther et al., 2011), while having effects, did not cause E10.5 lethality, nor did conditional deletion of *FADD* in the intestine or skin (Bonnet et al., 2011; Welz et al., 2011). It is possible that the precursors of definitive hematopoiesis, which arise in the endothelium of the aorta, move to the yolk sac, and are required for remodeling of the yolk sac vasculature (Dzierzak and Speck, 2008), are the targets of RIPK3-dependent necrosis in the absence of FADD or caspase-8, and of caspase-8-dependent apoptosis in the absence of FLIP and RIPK3.

Therefore, there is a close interplay between two cell death pathways: one leading to caspase-8-dependent apoptosis, and one leading to RIPK3-dependent necrosis. The presence of FLIP prevents the formation of FADD-dependent, active caspase-8 homodimers, required for apoptosis, whereas the resulting caspase-8-FLIP heterodimers prevent RIPK3 activation, required for programmed necrosis. In the absence of the FADD-caspase-8-FLIP complex, RIPK3 drives necrosis, resulting in embryonic lethality. In the presence of FADD, but in the absence of FLIP, caspase-8 drives apoptosis, also resulting in embryonic lethality at the same stage. Thus, either death pathway disrupts the proper development of the yolk sac vasculature, unless both are held in check through the interactions we have described (Figure S4A).

If so, why is the system "built" in this complex manner? One possibility relates to the dissemination of DNA viruses, which often carry endogenous inhibitors of caspase-8 (Weinlich et al., 2011). Such viruses may thereby trigger RIPK3-dependent necrosis, which would limit the infection. Indeed, cytomegalovirus (Weinlich et al., 2011) has been shown to cause such necrosis. Therefore, the developmental regulation of RIPK3-dependent necrosis by FADD-caspase-8-FLIP may represent a "failsafe" mechanism to prevent viral dissemination if the apoptotic pathway is compromised, resulting in the untoward consequences of deletion of any of the latter three components. A more direct role for these death processes in embryonic development may only be unveiled when the specific cell types and triggers that initiate both caspase-8-apoptosis and RIPK3-necrosis are elucidated.

EXPERIMENTAL PROCEDURES

Mice, Treatments, and Timed Matings

Mice with a deleted allele of caspase-8 were generated by germline deletion of a *caspase-8*^{flox} allele described previously by Salmena et al. (2003). A distinct set of *caspase-8*^{flox} animals was generated as previously described by Kang et al. (2004) and was crossed to *α myosin heavy-chain Cre* animals (Agah et al., 1997). *RIPK3*-deficient animals were obtained from V. Dixit (Newton et al., 2004). *FADD* and *FLIP*-deficient animals have been previously

described by Yeh et al. (1998, 2000). Genotypes were confirmed by tail snip PCR as described previously. For anti-CD95 injections, animals were injected via tail vein with 15 μg purified Jo2 antibodies in lipopolysaccharide-free PBS per animal. Liver enzymes were assayed using a Trilogy Multi-Purpose Analyzer System from Drew Scientific, and liver sections were created and stained with hematoxylin and eosin, in the St. Jude Veterinary Pathology Core facility. Timed matings were performed by mating animals and then verifying developmental age through palpation and ultrasound, with post-dissection staging performed by the St. Jude Veterinary Pathology Core facility. Dissections were performed using a Leica M844/F40 surgical microscope scope. Image capture of embryos was performed using a Nikon SMZ1500 Epi-fluorescence Stereoscopic Zoom Microscope with a DS-Fi1 Camera and Nikon Elements Imaging Software. Sections from embryos were generated and stained with hematoxylin and eosin, anti-cleaved caspase-3 (BioCare), or anti-CD31/PECAM-1 antibody (BD PharMingen) and biotinylated rabbit anti-rat antibody (Vector). The St. Jude Institutional Animal Care and Use Committee approved all procedures in accordance with the Guide for the Care and Use of Animals.

Knockdown Experiments

NIH 3T3 cells that do not express endogenous RIPK3 were stably transduced with a multicistronic pBabe-PURO vector containing full-length, untagged murine RIPK3 followed by a T2A ribosome-skipping sequence, followed by eGFP. Control cells received the same vector containing eGFP alone. Cells were treated with siRNAs as described in Extended Experimental Procedures.

Immunoprecipitation of FADD

SVEC 4-10 cells were treated with or without 20 ng ml^{-1} recombinant murine TNF-α (PeproTech) and 50 μM zVAD (SM Biochemicals) for 90 min. Immunoprecipitation of DISC-associated complexes was carried out using buffer and lysis conditions previously described by Geserick et al. (2009). FADD was immunoprecipitated using the M19 polyclonal anti-FADD antibody conjugated to Protein A/G-PLUS Agarose beads, both from Santa Cruz Biotechnology.

Immune Cell Staining, Cell Death, and Activation Assays

For immune cell staining the spleen, thymus, and lymph node were harvested from animals, and single-cell suspensions were generated. For immune cell staining from the blood, blood was harvested from the retrorbital sinus from animals anesthetized with 2%–2.5% isoflurane in 1 l oxygen. Red blood cells were lysed in hypotonic buffer, and samples were stained with the appropriate antibodies as described in Extended Experimental Procedures. Data were acquired using a FACSCalibur or LSRII using FlowJo Collectors or FACSDiva software, respectively. Data analysis was performed using FlowJo (Tree Star). For cell death assays, cells were harvested at 9 hr, stained with AnnexinV-APC (Invitrogen) and PI (Sigma-Aldrich), and assayed for viability using flow cytometry. For cleaved caspase-3 intracellular staining, cells were harvested, fixed, permeabilized, and stained per manufacturer's instructions (eBioscience). Proliferation assays and culture conditions were performed per standard protocols as detailed in Extended Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and four figures and can be found with this article online at doi:10.1016/j.celrep.2012.03.010.

LICENSING INFORMATION

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