

# A Function of Fas-Associated Death Domain Protein in Cell Cycle Progression Localized to a Single Amino Acid at Its C-Terminal Region

Zi Chun Hua,<sup>1,2</sup> Sue J. Sohn,<sup>1</sup> Chulho Kang,<sup>1</sup> Dragana Cado,<sup>1</sup> and Astar Winoto<sup>1\*</sup>

<sup>1</sup>Department of Molecular and Cell Biology  
Division of Immunology and  
Cancer Research Laboratory  
469 LSA

University of California  
Berkeley, California 94720

<sup>2</sup>The State Key Laboratory  
of Pharmaceutical Biotechnology and  
Institute of Molecular and Cell Biology  
Nanjing University  
Nanjing 210093  
People's Republic of China

## Summary

FADD is an adaptor known to transmit apoptotic signals from members of the tumor necrosis factor receptor family. We show here that FADD has a domain implicated in cell proliferation. Mice bearing the Asp mutation in the serine 191 phosphorylation site are runted and anemic and display splenomegaly. Apoptosis is unimpaired in these mice, but they exhibit many immune developmental problems indicative of proliferative defects. Mutant FADD T cells are defective in cell cycle progression, suggesting that regulation of phosphorylation at serine 191 is essential for growth/proliferation. Remarkably, serine 191 is conserved among mammalian FADD proteins, but this C-terminal region is absent in lower organisms, suggesting that FADD acquired a domain during evolution, rendering it a “proliferation-apoptosis coupler” that balances cell proliferation and apoptosis.

## Introduction

Members of the tumor necrosis factor receptor (TNF-R) superfamily have been shown to play diverse roles in apoptosis, growth, and differentiation. A subclass of these contains a death domain in each of their cytoplasmic tails that is crucial for transmission of apoptotic signals. These include Fas, TNF-R1, DR3, DR4/5, and DR6 (Krammer, 2000; Strasser et al., 2000). Fas is crucial for lymphocyte homeostasis, as its mutation leads to uncontrolled lymphocyte proliferation followed by autoimmunity, which is exacerbated in *Fas/TNF-R1* double mutants. Mice with *DR6* mutation exhibit enhanced T cell proliferation (Liu et al., 2001; Zhao et al., 2001), and mutation at either *DR3* or *TRAIL*, the ligand for DR4/5, results in only minor defects (Cretney et al., 2002; Wang et al., 2001). These data are consistent with the known role of death domain-containing receptors in apoptosis. FADD (Fas-associated death domain) is a protein containing a death effector- and a death domain. It mediates apoptotic signaling of all the death domain receptors

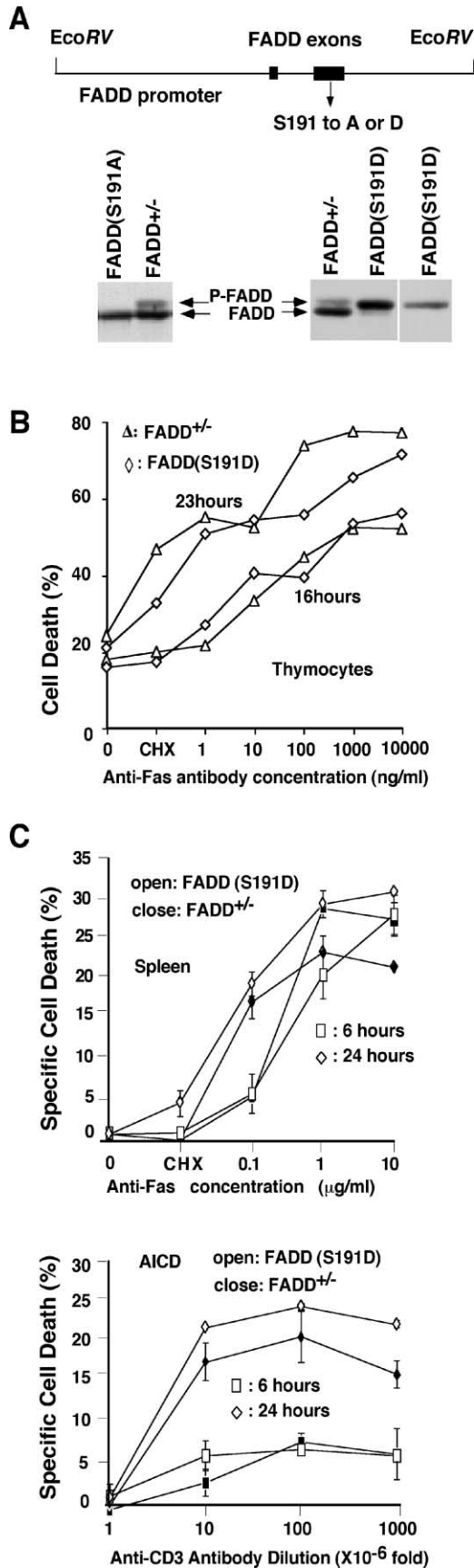
(Kuang et al., 2000; Yeh et al., 1998; Zhang et al., 1998). Its recruitment to the membrane leads to activation of caspase-8 and subsequent downstream proteases. Mutations in *FADD*, *caspase-8*, or *FLIP*, a caspase-8 activator/inhibitor that can bind FADD, lead to embryonic lethality (Varfolomeev et al., 1998; Yeh et al., 2000, 1998; Zhang et al., 1998), suggesting that some of the death receptors are additionally important for embryonic development. Analysis of T cell-specific FADD-knockout and *FADD*<sup>-/-</sup>→*RAG-1*<sup>-/-</sup> complementation mice showed that FADD deficiency in the immune system leads to inhibition of the proliferation stage in the CD4<sup>+</sup>CD8<sup>-</sup> (DN) compartment during thymocyte development. Furthermore, *FADD*<sup>-/-</sup> mature T cells fail to proliferate in response to anti-CD3/anti-CD28 stimulation (Kabra et al., 2001; Zhang et al., 1998). Similarly, expression of transgenic human FADD dominant-negative proteins in T cells engenders some of the above FADD deficiency phenotype (Newton et al., 1998; Walsh et al., 1998; Zornig et al., 1998), leading to a hypothesis that death domain receptors play crucial roles in T cell proliferation. Although this appears inconsistent with their role in initiating apoptosis, there are also reports that caspase activation is seen in anti-CD3/CD28-stimulated mature T cells in the absence of apoptosis and that FasL can costimulate proliferation and IL-2 production of primary T cells triggered with suboptimal dose of anti-CD3 antibodies (Alam et al., 1999; Elkon, 1999; Kennedy et al., 1999). In addition, FLIP<sub>L</sub> was reported to be capable of binding to Raf-1 and TRAF1/2 to activate ERK and NF-κB (Kataoka et al., 2000), leading to the suggestion that the proliferation defects of *FADD* null mutation are due to the inability of death domain receptors to trigger the FLIP<sub>L</sub> pathway. Inconsistent with the hypothesis that death domain receptors regulate proliferation are the observations that Fas deficiency leads to uncontrolled lymphoproliferation. Most investigators also find that addition of FasL to mature T cells leads to apoptosis and not proliferation (Krammer, 2000). *FADD*<sup>-/-</sup> T cells exhibit disarray of the cell cycle machinery at all phases of the cell cycle, and human FADD was reported to be phosphorylated specifically in G<sub>2</sub>/M phases and binds to a yet-to-be identified G<sub>2</sub>/M-specific kinase, suggesting that FADD might directly play a role in the cell cycle progression (Scaffidi et al., 2000; Zhang et al., 2001). However, the significance of FADD phosphorylation is not clear. Here, we show that one of the sites of FADD phosphorylation is the key to its role in the regulation of growth and proliferation.

## Results

### Generation of FADD(S191A) and FADD(S191D) Mutant Mice

Human FADD is serine phosphorylated at amino acid 194 (Scaffidi et al., 2000), while mouse FADD is phosphorylated at both serine and threonine residues (Zhang and Winoto, 1996). We confirmed that one of the phosphorylation sites of mouse FADD is serine 191, an amino

\*Correspondence: winoto@uclink4.berkeley.edu



acid that is equivalent to serine 194 of human FADD. This position is outside the FADD death effector- and death domains. In Western blot analysis, FADD can be seen both as a slower-migrating phosphorylated band and a faster nonphosphorylated species (Zhang and Winoto, 1996). Transfection of a construct expressing mutant FADD(S191A) into FADD-deficient fibroblasts leads to expression of only the faster-migrating FADD protein (data not shown), suggesting that serine 191 phosphorylation is responsible for the molecular weight shift detected in Western blot analysis. However, mouse FADD(S191A) still retains other sites of phosphorylation which we did not map (Zhang and Winoto, 1996). We introduced an alanine or an aspartic acid substitution at the corresponding serine in a FADD genomic construct (Figure 1A). Previous work has shown that a 12 kb EcoRV FADD genomic fragment, when introduced as a transgene, is sufficient to reconstitute FADD deficiency and that this region also contains a locus control region activity that recapitulates endogenous FADD transcription (Kabra et al., 2001). Every transgenic founder of this fragment expresses FADD in a copy number-dependent manner. Four transgenic founders were generated for FADD(S191A) transgene, and two founders were obtained for FADD(S191D). All of them were mated to FADD<sup>+/-</sup> mice for at least two generations to obtain mice that express only FADD(S191A) or FADD(S191D) (with FADD<sup>-/-</sup> alleles). These mice are henceforth termed FADD(S191A) or FADD(S191D) mice, respectively. All FADD(S191A) mice appear normal. A low percentage of FADD(S191D) mice are small and die within 4 weeks of age; the surviving FADD(S191D) mice look normal at first but become runted with age (see below). Western blot analysis shows that the majority of phosphorylated FADD has disappeared in FADD(S191A) mice and, conversely, only the slower FADD species are detected in FADD(S191D) mice (Figure 1A), suggesting that FADD(S191A) represents the underphosphorylated form of FADD while FADD(S191D) mimics constitutively serine-

Figure 1. Generation of Mutant Mice with Unphosphorylated and Constitutively Phosphorylated FADD

(A) Major phosphorylation site of mouse FADD was mapped to serine 191 and changed to either alanine (S191A) or aspartic acid (S191D). Transgenic mice (Tg) were generated and mated to FADD<sup>+/-</sup> mice for at least two generations to obtain FADD(S191A) or FADD(S191D) with FADD<sup>-/-</sup> alleles. Western blot analysis of total splenic lysates with FADD-specific antibodies was performed to confirm the loss of upper FADD phosphorylated band (P-FADD) in S191A mice and the loss of the lower FADD unphosphorylated band (FADD) in S191D mice.

(B) Fas-initiated apoptosis proceeds normally in FADD(S191D) thymocytes. With the exception of time 0, cycloheximide (CHX) was added to all samples containing anti-Fas antibody. Samples were harvested at two different time points (16 or 23 hr), and the percent apoptotic cells were measured by 7-AAD (7-amino-actinomycin-D) incorporation.

(C) Fas-initiated apoptosis and AICD proceed normally in FADD(S191D) spleen cells. Spleen cells are activated with ConA for 2 days, followed by addition of IL-2 and stimulation with either anti-Fas antibody (upper panel) or anti-CD3 antibody (lower panel). At two time points (6 or 24 hr later), samples were harvested and the percent apoptotic cells was measured using 7-AAD. Specific cell death is calculated by subtracting the percent apoptotic cells cultured in the absence of anti-Fas or anti-CD3 treatment.

phosphorylated FADD. Both the FADD(S191D) and two of the FADD(S191A) lines possess low copy numbers of the transgenes (lines D5 and D6 contain two and three copies, respectively; lines A12 and A9 contain one and three copies, respectively) and express mutant FADD at roughly equivalent levels to that in the wild-type mice (Figure 1A).

#### FADD(S191A) and FADD(S191D) Cells Exhibit Normal Receptor-Mediated Apoptosis

To test the ability of mutant FADD to transmit apoptotic signals, we treated thymocytes from FADD(S191D) or FADD(S191A) mice with increasing concentrations of anti-Fas antibody. The extent of apoptosis is indistinguishable from their FADD<sup>+/-</sup> littermate controls (Figure 1B and data not shown). Similar data were obtained when we tested Fas-mediated cell death of ConA-activated splenocytes (Figure 1C). Anti-CD3-mediated activation-induced cell death (AICD) of previously activated mature T cells in the presence of excess IL-2 (Lenardo, 1991) is also equivalent between mutant and wild-type T cells (Figure 1C). In addition, apoptosis mediated by S191D or S191A mutant FADD is similar to that of wild-type protein when they are used to reconstitute FADD-deficient fibroblast cells (data not shown). Thus, mutation at the FADD phosphorylation site does not affect its ability to transmit apoptotic signals in normal cells.

#### Defective Immune System in FADD(S191D) Mice

Thymocytes, splenocytes, lymph nodes, and bone marrow cells of 3- to 9-week-old FADD mutant mice were analyzed for hematopoietic and lymphocyte populations. Thymocyte numbers for FADD(S191D) mice are on the average 3-fold less than the wild-type counterparts, while FADD(S191A) thymi contain normal numbers of cells (Figure 2A and data not shown). Flow cytometric analysis of CD4 and CD8 expression showed an arrest at the DN to DP (CD4<sup>+</sup>CD8<sup>+</sup>) transition in FADD(S191D) but not in FADD(S191A) mice (Figure 2B). The defects in T cell development in FADD(S191D) mice are seen in both transgenic lines {FADD(S191D)-5 and FADD(S191D)-6}, although their severity varies. In the DN population, staining with CD44 and CD25 shows a decrease in the DN4 (CD25<sup>-</sup>CD44<sup>-</sup>) and a concomitant increase of DN3 (CD25<sup>+</sup>CD44<sup>-</sup>) subpopulations (data not shown). This is similar to the observation made in T cell-specific FADD knockout mice where loss of FADD leads to an arrest at the DN3 to DN4 transition (Kabra et al., 2001). CD25<sup>+</sup>CD4<sup>-</sup>CD8<sup>-</sup> thymocytes can be divided further into two subpopulations based on their cell sizes and cell cycle progression, the E subset cells are mostly in the G<sub>1</sub>/G<sub>0</sub> state whereas the L (larger size) cells are mostly in the S/G<sub>2</sub>/M phases (Hoffman et al., 1996). We show that FADD(S191D) thymocytes contain fewer L cells than the wild-type counterparts (Figure 2C), consistent with the notion that proliferation at the DN3 stage is defective in these mice. This early inhibition of T cell proliferation is further reflected in the fewer mature T cells that accumulate in FADD(S191D) peripheral lymphoid organs (Figure 2D, left panel). FADD(S191D) mice contain normal spleen cell numbers but slightly fewer lymph node cells (data not shown). Interestingly, staining for B cell and macrophage/granulocyte markers

also showed abnormalities in these mice. A decrease of CD19<sup>+</sup> B cells and an increase of Mac-1<sup>+</sup> cells can be seen (Figure 2D). A similar phenotype was previously seen in FADD<sup>-/-</sup>→RAG-1<sup>-/-</sup> chimeras (Zhang et al., 1998), although the significance of the B cell problem was not appreciated at the time due to the possibility that embryonic stem cells are intrinsically inefficient at populating the B lineage cells. The decrease in the number of CD19<sup>+</sup> cells in FADD(S191D) mice suggests FADD functional deficiency leads to defective B cell development.

#### Aged FADD(S191D) Mice Are Runted and Anemic and Exhibit Splenomegaly

When FADD(S191D) mice get older (4–12 months), they look runted in comparison to their FADD<sup>+/-</sup> littermates (Figure 3A, left panel). In addition to the immune system problems described above, these mice develop splenomegaly but not lymphadenopathy (Figure 3A, middle and right panels). We examined their B and T cell populations to look for B220<sup>+</sup>Thy1<sup>+</sup> T cells, an aberrant population of cells that arise in *lpr/lpr* mice correlating with T cell autoimmunity (Cohen and Eisenberg, 1991). In contrast to aged *lpr/lpr* strain of mice, no Thy1<sup>+</sup> B220<sup>+</sup> cells were found in 5- to 12-month-old FADD(S191D) mice (Figure 3B). In addition, the prevalence of CD4<sup>-</sup>CD8<sup>-</sup> T cells in *lpr/lpr* spleen and lymph nodes was not seen in these FADD mutant mice (data not shown). We conclude that FADD(S191D) mice do not suffer from the same autoimmunity as found in the *lpr/lpr* strain of mice, further confirming the notion that FADD(S191D) can mediate normal Fas function. Further analysis of the FADD(S191D) splenocytes shows an increased number of Gr-1<sup>+</sup> cells but not NK1.1<sup>+</sup> cells or cells bearing F4/80, a macrophage-specific marker (Figure 3C). Thus, the splenomegaly in these mice may simply reflect homeostatic expansion of Gr-1<sup>+</sup> granulocytes due to the low numbers of B and T cells.

Analysis of blood from 6- to 10-month-old FADD(S191D) mice showed decreased counts and percentages of their red blood cells (RBC) (the average values for FADD<sup>+/-</sup> and FADD(S191D), respectively, are RBC:  $7.39 \pm 0.87 \times 10^6/\text{mm}^3$  versus  $3.91 \pm 0.9 \times 10^6/\text{mm}^3$ ; hematocrit:  $42.7 \pm 3.76\%$  versus  $24.3 \pm 2.29\%$ ; hemoglobin:  $14.2 \pm 0.8$  g/dl versus  $7.97 \pm 1.61$  g/dl). To determine if erythropoiesis in these mice is abnormal, flow cytometric analysis of their bone marrow cells was performed. Antibody specific for TER119, a marker for erythrocyte lineage cells, shows a significant drop in the RBC population in FADD(S191D) mice (Figure 3D). As in the spleen, we found an increase of granulocytes (Gr-1<sup>+</sup>) in the FADD(S191D) mutant mice (Figure 3D and data not shown). This was also seen in FADD<sup>-/-</sup>→RAG-1<sup>-/-</sup> chimeras (our unpublished data). Similar to the young mice, older FADD(S191D) mice have decreased CD19<sup>+</sup> (or B220<sup>+</sup>) cell numbers. The block is at the transition from CD43<sup>+</sup> to CD43<sup>-</sup> stage, suggesting a defective transition between pro-B and pre-B cell stages (Figure 3D, right panel). Overall, these data suggest a general problem of hematopoiesis resulting from a mutation in the FADD phosphorylation site at serine 191. The defects in both T and B cell development correlate with the problems at their respective proliferative stages.

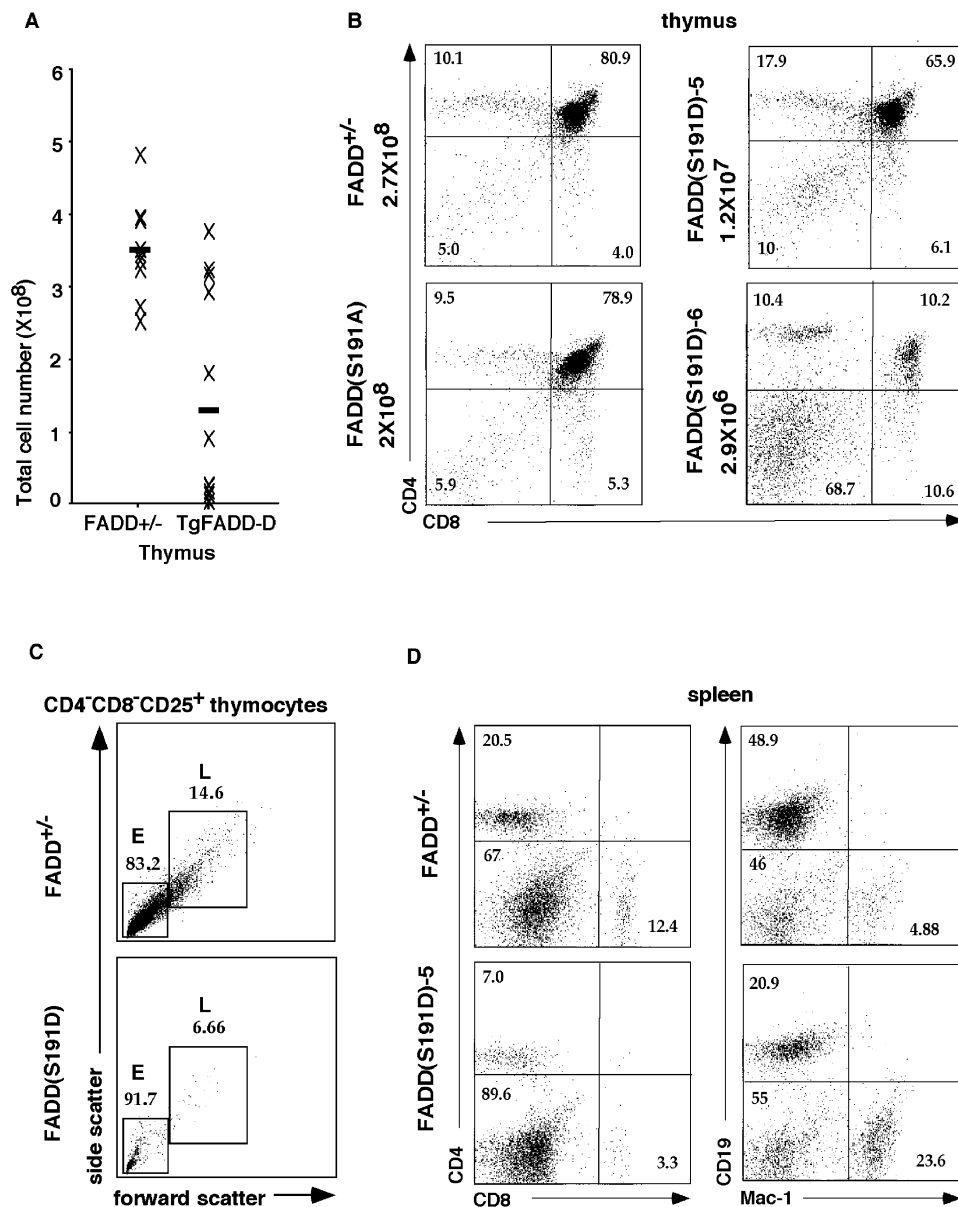


Figure 2. Analysis of FADD(S191D) Mice

Mice at 3–9 weeks of age were sacrificed, and their thymocytes, splenocytes, and lymph nodes were analyzed.

(A) Thymocyte number of transgenic Tg FADD(S191D) (both lines 5 and 6) and their FADD<sup>+/-</sup> heterozygous littermates. Bars indicate the average thymocyte cell number of each genotype (individual animal is denoted as a cross).

(B) Flow cytometric profiles of thymocytes from FADD<sup>+/-</sup> heterozygous littermate, and FADD(S191D) lines 5 and 6 as well as FADD(S191A) mice are shown. All the FADD Tg mutants are in FADD<sup>-/-</sup> alleles. The total cell number of each thymus is indicated.

(C) Thymocytes from FADD(S191D) were stained with antibodies specific for CD4, CD8, and CD25. Side and forward scatter profiles of DN thymocytes that express CD25 are shown.

(D) Representative flow cytometric profiles of splenocytes from FADD(S191D) mice and their littermate FADD<sup>+/-</sup> mice were obtained using anti-CD4 and anti-CD8 (left panel) or anti-CD19 and anti-Mac-1 (right panel) antibodies. Both FADD(S191D) lines display similar phenotypes.

### FADD(S191D) T Cells Are Defective in Cell Cycle Progression

To look at the effect of FADD mutation on cell cycle more directly, we used CFSE (Carboxy-Fluorescein-diacetate-Succinimidyl Ester) to label resting T cells. Activation of these cells with anti-CD3 and anti-CD28 antibodies leads to extensive cell proliferation, which can be visualized by decreases of CFSE fluorescence (labeled

as 0, 1, 2, 3, and 4 in Figure 4). In contrast to wild-type T cells, which undergo at least four cell divisions over the course of 4 day stimulation, FADD(S191D) T cells exhibit only one cell division (Figure 4, left and middle panels). Consistent with these findings, cell cycle analysis with propidium iodide shows significantly fewer cells in S and G<sub>2</sub>/M phases in the stimulated mutant FADD(S191D) T cells compared to the controls (Figure

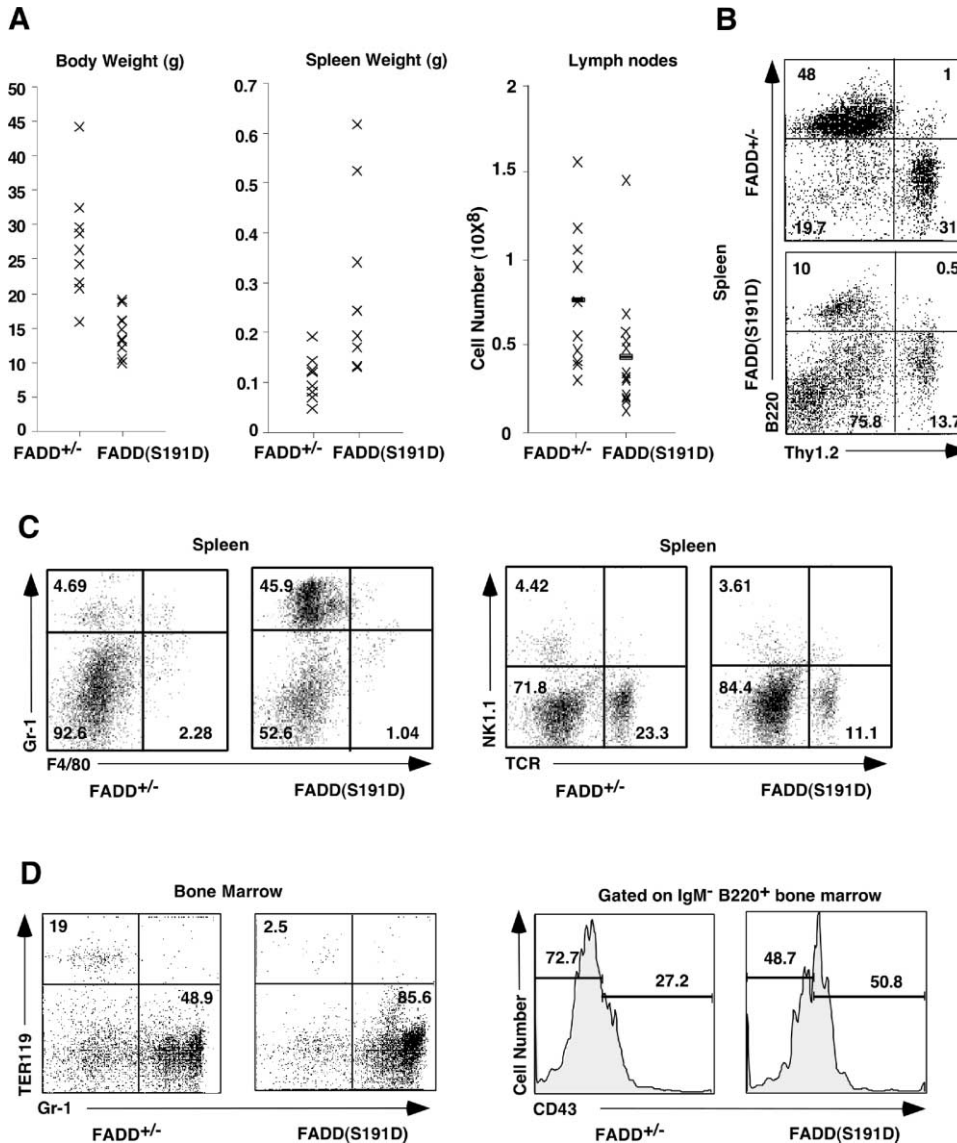


Figure 3. Characterization of Aged FADD(S191D) Mice

(A) Body weight, spleen weight, and lymph node cell numbers of 4- to 12-month-old FADD(S191D) mice were measured along with their wild-type littermates. Each cross denotes one animal.

(B) Anti-Thy-1 and B220 staining profiles of spleen cells from aged FADD(S191D) and the wild-type littermates are shown.

(C) Flow cytometric analysis of splenocytes from aged FADD(S191D) mice and their littermate controls.

(D) Flow cytometric analysis of bone marrow cells from FADD(S191D) mice and their littermate controls.

4, right panels). Similar data were also obtained when cells were stimulated with ConA or a combination of phorbol ester and ionomycin (data not shown).

## Discussion

Previous characterization of FADD-deficient T cells indicates that FADD deficiency results in not only apoptotic but also proliferative defects. IL-2 signaling is not affected, but there is a global cell cycle problem at both the G<sub>2</sub>/M and G<sub>1</sub> to S phases (Zhang et al., 1998, 2001). This correlates with an elevated level of p21 inhibitor and reduced levels of cyclin D2/D3 in stimulated FADD<sup>-/-</sup> T cells as well as constitutive expression of

several other cell cycle proteins in resting FADD<sup>-/-</sup> T cells (Zhang et al., 2001). Expression of a truncated human FADD protein lacking its death-effector domain (FADD-DN) in T cells was also reported to result in a cell proliferation problem (Newton et al., 1998; Walsh et al., 1998; Zornig et al., 1998), although there are several discernible phenotypic differences between the transgenic mice generated in different groups (possibly due to variable expression of FADD-DN). Because FADD-DN is a powerful dominant-negative protein in blocking the interaction of the endogenous FADD protein to Fas and TNF-R1 death receptors, it was thought that the role of FADD in proliferation is similarly dependent on its adaptor function. Contrary to this belief, our data show

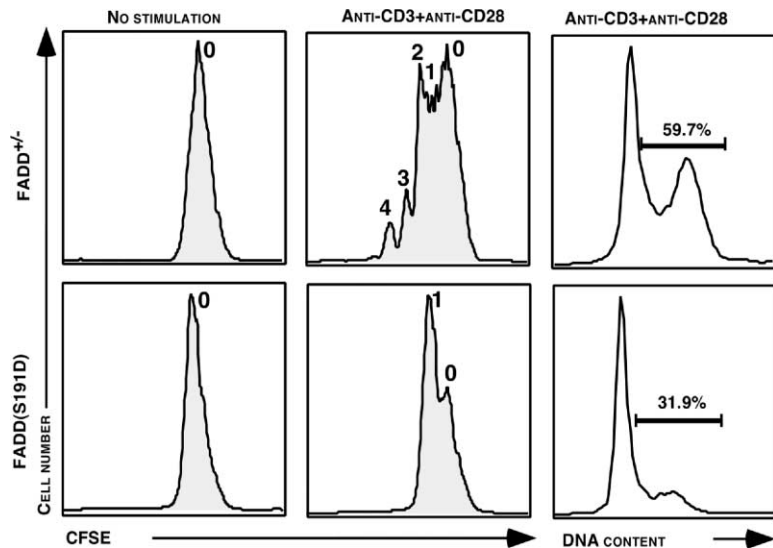


Figure 4. FADD(S191D) T Cells Are Defective in Their Proliferative Response to Anti-CD3/CD28 Stimulation

Cells are stained with CFSE and stimulated for 4 days with plate-bound anti-CD3/CD28 antibodies. Numbers in the left and middle panels represent CFSE peaks of undivided cells (0) and after 1, 2, 3, or 4 rounds of cell division. Each division results in a 50% decrease in the CFSE fluorescence from previous cell cycle. Right panel: instead of CFSE, cells are stained with propidium iodide for DNA content to quantify the percentages of cells in G<sub>2</sub>/M and S phases.

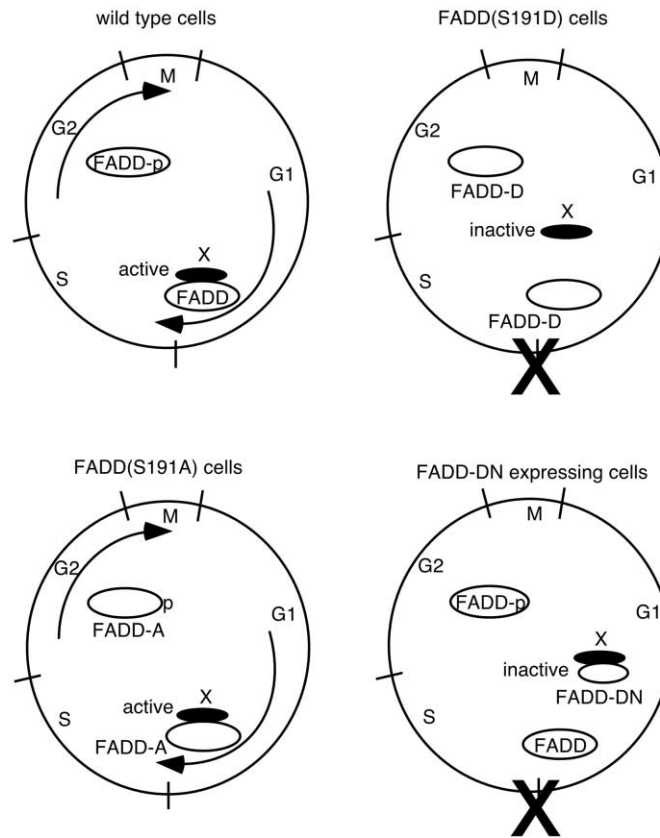
that the defects in proliferation can occur in the presence of intact FADD death effector- and death domains. FADD is phosphorylated at serine 194 in human during G<sub>2</sub>/M phases of the cell cycle (Scaffidi et al., 2000) and at serine 191 (this paper) as well as other unmapped residues in mouse (Zhang and Winoto, 1996). Serine 191 is located outside the domains directly implicated in the adaptor function of FADD in apoptosis and as expected, the Ser191→Asp or Ser191→Ala mutation does not affect its ability to transmit apoptotic signals in vivo and in vitro. However, mice expressing FADD(S191D) but not FADD(S191A) are small and anemic and exhibit many immunological defects that are remarkably similar to the phenotype associated with the complete loss of FADD protein. In addition, FADD(S191D) mature T cells are also defective in T cell receptor-induced cell cycle progression with problems at the G<sub>1</sub> to S transition. These results suggest that serine 191 at the FADD C-terminal region plays a regulatory role in the FADD proliferative function and that the roles of FADD in apoptosis and in proliferation represent two distinct functions utilizing separate domains. Consistent with this notion, the growth inhibition of human FADD-DN molecule was recently shown to depend on serine 194, an equivalent position to serine 191 of mouse FADD (M. Peter, personal communication).

Based on preliminary binding studies of the cellular proteins with various GST-FADD proteins (data not shown), we propose a hypothetical model to explain the striking similarities of the immune phenotype between FADD(S191D) and FADD<sup>-/-</sup>→RAG-1<sup>-/-</sup> mice, the comparatively normal phenotype of FADD(S191A) mice, and the ability of FADD-DN to block cell proliferation. In this model, the unphosphorylated FADD protein binds to an intracellular protein (called X for this discussion) and acquires an activity required for the G<sub>1</sub> to S cell cycle progression (Figure 5A). Phosphorylation of FADD leads to dissociation of the FADD/X complex. The mutant FADD(S191D) protein fails to bind protein X at all phases of the cell cycle, leading to an inactive X protein and subsequent failure of the cell cycle progression. In contrast, the FADD(S191A) mutant protein, at its unphosphorylated state, can still bind to protein X. As mouse

FADD is also phosphorylated on threonine residues (Zhang and Winoto, 1996), the residual phosphorylation in FADD(S191A) might be sufficient to allow its dissociation from X during the G<sub>2</sub>/M phases, leading to a somewhat normal regulation of the protein function. For FADD-DN, its intact C-terminal region would allow it to bind to protein X but its N-terminal truncation might result in an inactive FADD-DN/X complex. Furthermore, overexpression of FADD-DN leads to sequestration of protein X from the endogenous normal FADD protein, resulting in inhibition of cell growth. Further biochemical work to identify the putative interacting protein will be necessary to validate/refine the model and to see if FADD(S191D) mutation indeed mimics phosphorylation or simply represents a mutation that blocks the function of the FADD C-terminal region.

Our work also illustrates the importance of FADD in mouse physiology where a single amino acid change results in dramatic effects in growth and hematopoiesis. Alignment of FADD proteins from different mammalian species shows a remarkable conservation of serine 191 and its surrounding amino acids (Figure 5B). Interestingly, while the death effector- and death domains are highly conserved across all species examined, the region containing serine 191 is absent from puffer fish (*Fugu rubripes*), mosquito (*Anopheles gambiae*), and fly (*Drosophila melanogaster*) FADD proteins. Consistent with the lack of proliferation function for FADD in lower organisms, FADD null *Drosophila* has no apparent defects except for its inability to clear bacterial infection (Hu and Yang, 2000; Leulier et al., 2002; Naitza et al., 2002). These data suggest that the C-terminal region of the mammalian FADD may represent a domain distinct from its more conserved (and primitive) apoptotic region, and that this novel domain was acquired during evolution. Based on the phenomena of cell competition and compensation during development whereby “weaker” cells are actively eliminated through apoptosis, Martin Raff and, more recently, John Abrams have hypothesized for the existence of a class of molecules that links proliferation and apoptosis (Abrams, 2002; Raff, 1992). These “proliferation-apoptosis couplers” coordinate the balance between cell proliferation and cell death. Our

A



B

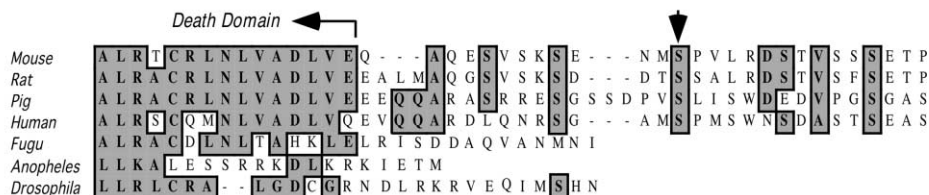


Figure 5. A Model and the C-Terminal Sequences of FADD in Cell Cycle Regulation

(A) A model of FADD-mediated cell cycle regulation.

(B) Alignment of FADD proteins from seven different species. Shaded boxes indicate conserved amino acids. Only the C-terminal regions are shown to highlight the conservation of serine 191 (arrow) in mammals.

study indicates that FADD may belong to this class of proteins. Deletion of the *FADD* gene leads to protection from cell death (Zhang et al., 1998) and also loss of proliferative function. We have now identified a domain in FADD that regulates cell proliferation independent of its role in receptor-mediated cell death. This further strengthens the notion that FADD directly controls both cell proliferation and cell death. Future work is necessary to dissect its exact molecular role in cell cycle progression and how FADD balances the process of apoptosis and cell proliferation.

#### Experimental Procedures

##### Plasmids

Mutagenesis was carried out by several rounds of PCR amplifications. First, using the *FADD* genomic clone as a template, two PCR

fragments were generated using primers 1 and 2 or 3 and 4. Primers 2 and 3 are complementary to each other and contain the corresponding mutation. For FADD(S191D) mutant, primer 2 is GAATCC CTTAGTACGGGGTCCATATTCTCACTC, while for FADD S191A, primer 2 is GAATCCCTTAGTACGGGGGCCATATTCTCACTC. The changes are TCC (serine) to GAC (Asp) or GCC (Ala) and a base pair silent mutation AGTACT to CGTACT to abolish the *Scal* site. The two PCR products were then mixed together and re-amplified using primers 1 (GATTTACCCATGGACCCATTTC) and 4 (AGACCACATGGAGGCAAGG) to yield a 2.5 kb product. After *Xho*I and *Nco*I digestion, the 1.6 kb fragment was cloned into *Morte*4.7 plasmid, which contains a 4.7 kb *Eco*RI *FADD* genomic fragment in pSP72 (Kabra et al., 2001). The resulting constructs were verified by sequencing for the lack of any other changes. For transgene constructs, the mutant clones were first digested with *Xho*I and *Eco*RI. The corresponding 3.3 kb insert was mixed with a 8.8 kb *Eco*RV/partial *Xho*I wild-type fragment and ligated to the *Eco*RI-*Eco*RV plasmid fragment of p318 in a three-way ligation reaction (note: p318 contains a 12.2kb *Eco*RV wild-type fragment of *FADD* gene in

pSP72 [Kabra et al., 2001]). For microinjection, the inserts were liberated with Sall and ClaI digestion and purified. The DNA was injected into fertilized (C57BL/6 X CBA) F<sub>2</sub> eggs, and the resulting transgenic mice were bred to C56Bl/6 for two generations before mating to *FADD*<sup>+/-</sup> mice (>2 generations) to generate *FADD*(S191A) and *FADD*(S191D) mice.

#### Mouse Genotyping

For genotyping transgenic *FADD* mutant mice, the primers used are: CACAGTTGAATCCCTTAGTACG and AGCGGTAAGGGAGAG TCTGAAAG. For the endogenous *FADD* alleles, the presence of *FADD* mutation with the neomycin-resistant gene was typed using two neomycin primers: CGCTCGGTGTTGAGGCCACACGC and ACTGTAGTGCCAGCAGACACCAGC. The following primers were then used to distinguish *FADD*<sup>+/-</sup> from *FADD*<sup>-/-</sup> GGACGGC GACCGCTGCGC and CACAGTTGAATCCCTTAGTACT. These primers are specific for the wild-type endogenous *FADD* and do not amplify the *FADD* mutant transgenes.

#### Flow Cytometry and Apoptosis Assay

Cell suspensions were prepared from lymphoid organs of littermates with the appropriate genotypes. After red blood cell lysis, they were stained with the indicated antibodies. Normal serum cocktail was added first to eliminate nonspecific staining. For CD25 and CD44 staining of DN thymocytes, staining with tricolor-conjugated CD4 and CD8 antibodies was done with FITC-CD25- and PE-CD44-specific antibodies. The CD25 versus CD44 profiles were analyzed on cells gated on CD4<sup>-</sup>CD8<sup>-</sup>. For AICD, spleen cells were activated according to published protocols (Holler et al., 2000; Zheng et al., 1995). Cell cycle analyses were performed by first staining with antibodies for CD25 (for purified CD4<sup>-</sup>CD8<sup>-</sup> thymocytes), or CD4 and CD8 (for splenocytes), and then fixed with 70% ethanol. They were then stained with propidium iodide and analyzed by flow cytometry.

#### CFSE

1 × 10<sup>7</sup> splenocytes/ml were labeled with 2.5 μM CFSE in 0.1% bovine serum albumin/phosphate buffer saline at 37°C for 10 min, washed twice with 10% fetal bovine serum/RPMI 1640, and then stimulated with plate-bound anti-CD3 (0.5 μg/ml) and anti-CD28 (0.5 μg/ml), or ConA (2 μg/ml), or PMA (10 ng/ml) and ionomycin (1 μM) for 4 days. The stimulated splenocytes were stained with anti-CD4-PE antibody, and CD4<sup>+</sup> gated cells were analyzed for CFSE fluorescence by flow cytometry. For cell cycle analysis, splenocytes were stimulated as above, stained with anti-CD4-FITC and CD8-FITC antibodies, fixed with ethanol, then stained with propidium iodide for flow cytometric analysis.

#### Bioinformatics

Most of the *FADD* protein sequences were obtained from <http://www.ensembl.org> or <http://www.ncbi.nlm.nih.gov>. For the Fugu sequence, the annotated *FADD* contains only the N-terminal part. To obtain the rest of the Fugu sequence, we performed the blast program with the C-terminal sequence of mouse *FADD* protein. The pig (*Sus Scrofa*) *FADD* sequence was assembled from the EST database.

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