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The E3 Ubiquitin Ligase Itch Couples JNK Activation to TNFα-induced Cell Death by Inducing c-FLIP$_L$ Turnover

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SUMMARY

The proinflammatory cytokine tumor necrosis factor (TNF) α signals both cell survival and death. The biological outcome of TNFα treatment is determined by the balance between NF-κB and Jun kinase (JNK) signaling; NF-κB promotes survival, whereas JNK enhances cell death. Critically, identity of a JNK substrate that promotes TNFα-induced apoptosis has been outstanding. Here we show that TNFα-mediated JNK activation accelerates turnover of the NF-κB-induced antiapoptotic protein c-FLIP, an inhibitor of caspase-8. This is not due to direct c-FLIP phosphorylation but depends on JNK-mediated phosphorylation and activation of the E3 ubiquitin ligase Itch, which specifically ubiquitinates c-FLIP and induces its proteasomal degradation. JNK1 or Itch deficiency or treatment with a JNK inhibitor renders mice resistant in three distinct models of TNFα-induced acute liver failure, and cells from these mice do not display inducible c-FLIP$_L$ ubiquitination and degradation. Thus, JNK antagonizes NF-κB during TNFα signaling by promoting the proteasomal elimination of c-FLIP$_L$.

INTRODUCTION

Tumor necrosis factor (TNF) α is a prototypical, proinflammatory cytokine that can signal, through its type 1 receptor (TNFR1), either cell survival, cell proliferation and activation, or cell death (Wajant et al., 2003). Binding of TNFα to TNFR1 results in sequential formation of two signaling complexes (Micheau and Tschopp, 2003; Muppidi et al., 2004). The rapidly forming complex I is assembled on the receptor's cytoplasmic tail and consists of the adaptor TRADD, the protein kinase RIP1, and the signal transducer TRAF2. This complex signals inflammation and cell survival through IκB kinase (IKK) dependent activation of transcription factor NF-κB (Liu et al., 1996). Subsequently, complex I dissociates from the receptor, and TRADD together with RIP1 associate with the adaptor protein FADD and pro-caspase-8 (also called FLICE) to form complex II. In cells that lack substantial NF-κB activity, formation of complex II results in caspase-8 activation and apoptotic cell death (Muppidi et al., 2004). However, if NF-κB has been activated during complex I formation, it prevents caspase−8 activation by complex II through induction of cellular FLICE-inhibitory protein (c-FLIP, also called CASH, Casper, CLARP, FLAME, I-FLICE, MRIT, or Usurpin), an adaptor protein with a pseudo-caspase domain, which specifically inhibits caspase-8 (Thome and Tschopp, 2001). Thus, NF-κB may negatively regulate TNFα-induced apoptosis through regulation of c-FLIP levels, and its activation serves as a checkpoint that determines whether complex II will signal cell death or not (Muppidi et al., 2004). NF-κB was also suggested to inhibit TNFα-induced cell death through additional mediators (Wang et al., 1998). Nonetheless, the c-FLIP-dependent mechanism has received physiological support through analysis of *Flip*$^{-/-}$ fibroblasts which undergo TNFα−induced apoptosis even without inhibition of NF-κB-dependent gene expression (Yeh et al., 2000).

Multiple *c-Flip* mRNA splice isoforms give rise to either a long FLIP (c−FLIP$_L$) polypeptide of 55 kDa, a short FLIP polypeptide (c−FLIP$_S$) of 26 kDa (Irmler et al., 1997a; Krueger et al., 2001a), or a third 23 kDa recently identified form, called FLIPR (Golks et al., 2005). Due to its structural similarity to caspase-8 and caspase-10, c-FLIP$_L$ undergoes caspase−mediated cleavage to generate a 43 kDa isoform that still contains death effector domains (DED) and the pseudo-caspase motif and can therefore remain bound to

FADD and inhibit complete caspase-8 processing and activation (Krueger et al., 2001b).

In addition to NF-κB, another important regulator of TNFα signaling is Jun kinase (JNK), a member of the MAP kinase (MAPK) subfamily (Varfolomeev and Ashkenazi, 2004). It is well established that the balance between NF-κB and JNK activities determines the outcome of TNFα signaling, such that NF-κB promotes cell survival, whereas prolonged JNK activation enhances TNFα-induced death (De Smaele et al., 2001; Karin and Lin, 2002; Papa et al., 2004; Tang et al., 2001). We have shown that NF-κB controls the duration of JNK activation through induction of genes encoding antioxidant proteins, such as superoxide dismutase (SOD) 2, that prevent oxidation and subsequent inhibition of MAPK phosphatases (MKPs) by reactive oxygen species (ROS) (Kamata et al., 2005). The molecular mechanism through which prolonged JNK activation promotes TNFα-induced death, however, remained elusive. It was recently proposed that JNK activation somehow promotes processing of the BH3-domain protein Bid to proapoptotic cleavage products, such as tBid (Luo et al., 1998) and jBid (Deng et al., 2003), that allow TNFα-induced apoptosis to proceed. Nonetheless, a JNK-dependent phosphorylation event that controls Bid processing or the activity of any defined component of the TNFR1-activated death or survival machinery has not been identified.

We now show that JNK activity controls TNFα-induced death through the proteasomal processing of c-FLIP$_L$, the very molecule that inhibits the apoptotic activity of complex II and whose expression is NF-κB dependent (Micheau and Tschopp, 2003). Instead of direct c-FLIP$_L$ phosphorylation, JNK promotes accelerated decay of c-FLIP$_L$ through activation of the ubiquitin ligase Itch, the first member of the HECT family of E3 ligases whose catalytic activity was shown to be phosphorylation (JNK1) dependent (Gao et al., 2004). Importantly, JNK1- and Itch-deficient mice, as well as mice given a specific JNK inhibitor, are resistant to three different models of fulminant hepatitis, a pathological response that depends on TNFR1 activation (Maeda et al., 2003). These mice fail to ubiquitinate and degrade c-FLIP$_L$ in response to hepatitis-inducing challenges, and their hepatocytes do not undergo apoptosis when challenged with TNFα and a transcriptional inhibitor.

RESULTS

JNK1 Is Specifically Required for TNFR1-Induced Apoptosis but Cannot Induce Apoptosis on Its Own

The midgestational lethality due to massive liver apoptosis of NF-κB-deficient mice, which is common to mice lacking RelA, IKKβ, or IKKγ, is completely suppressed by removal of either TNFα or TNFR1 (reviewed by Karin and Lin, 2002). To investigate the role of JNK1 in TNFR1 death signaling in mice lacking IKKβ, we crossed *Ikkβ*$^{+/-}$ and *Jnk1*$^{+/-}$ heterozygote mice to generate *Ikkβ*$^{-/-}$/*Jnk1*$^{-/-}$ double mutants. In these and subsequent experiments, we chose to use JNK1-deficient mice and cells because it has been noticed that JNK1 rather than JNK2 is required for TNFα-induced apoptosis (Liu et al., 2004; L.C., unpublished data). The absence of JNK1 delayed the lethality caused by removal of IKKβ by 4 days (Figure 1A). Histological analysis of livers from E14.5 fetuses revealed extensive hepatocyte apoptosis in *Ikkβ*$^{-/-}$ fetuses, which was absent in *Ikkβ*$^{-/-}$/*Jnk1*$^{-/-}$double mutants (Figure 1B). These results underscore the physiological importance of the previously described crosstalk between IKKβ-NF-κB and JNK1. Accordingly, primary hepatocytes from *Jnk1*$^{-/-}$ mice were resistant to apoptosis induced by incubation with TNFα plus cycloheximide (to inhibit expression of survival genes) and failed to cleave caspase-8 and -9 (Figure 1C). These cells also exhibited reduced levels of caspase-3 cleavage and cytochrome *c* release in comparison to similarly treated wild-type (wt) cells.

Despite the clear evidence, here and elsewhere (De Smaele et al., 2001; Kamata et al., 2005; Tang et al., 2001), that prolonged JNK activation is important for TNFα-induced death of NF-κB-deficient cells, it has been questioned whether prolonged JNK activation alone is sufficient to induce apoptosis in the absence of TNFR1 activation (Liu et al., 1996; Tang et al., 2001; Varfolomeev and Ashkenazi, 2004). This is an important issue, as its resolution should indicate whether JNK regulates a specific component of the TNFR1 death signaling pathway or a more general and downstream aspect of the apoptotic machinery. To answer this question, we transfected *Ikkβ*$^{-/-}$ fibroblasts, which undergo cell death upon incubation with TNFα alone, with a JNK1 expression vector alone or together with a dominant-negative MKP-7 mutant, MKP-7ΔC(C244S), that allows prolonged JNK activation in response to cell stimulation due to inhibition of endogenous MKPs (Kamata et al., 2005). As expected, prolonged JNK activation was observed in cells expressing MKP-7ΔC(C244S) that were stimulated with IL-1, but despite that and the absence of IKKβ, these cells did not die (Figure 1D). As before, substantial cell death was seen in TNFα-stimulated *Ikkβ*$^{-/-}$ fibroblasts, and it was not further enhanced by expression of the mutant MKP. These results indicate that prolonged JNK1 activation alone is not sufficient for activation of the apoptotic machinery even in IKKβ- (and NF-κB-) deficient cells. These findings therefore suggest that JNK1 targets a specific component of TNFR1 signaling to allow TNFα-induced apoptosis and does not act on a downstream target that is common to several distinct apoptosis signaling pathways.

JNK Controls c-FLIP$_L$ Stability

As mentioned above, TNFR1 death signaling depends on the progression from complex I to complex II, and the ability of caspase-8, a key component of complex II, to undergo activation (Micheau and Tschopp, 2003). Activation of caspase-8 is modulated by the levels of c-FLIP, its specific inhibitor (Krammer, 2000). Importantly, transcription of the *c-Flip* gene, which gives rise to c-FLIP$_L$ and c-FLIP$_S$ isoforms, is controlled by NF-κB (Micheau et al., 2001), and *Flip*$^{-/-}$ fibroblasts undergo apoptosis when incubated with TNFα alone (Yeh et al., 2000). To examine the effect of JNK1 on various components of the TNFR1 signaling machinery, including

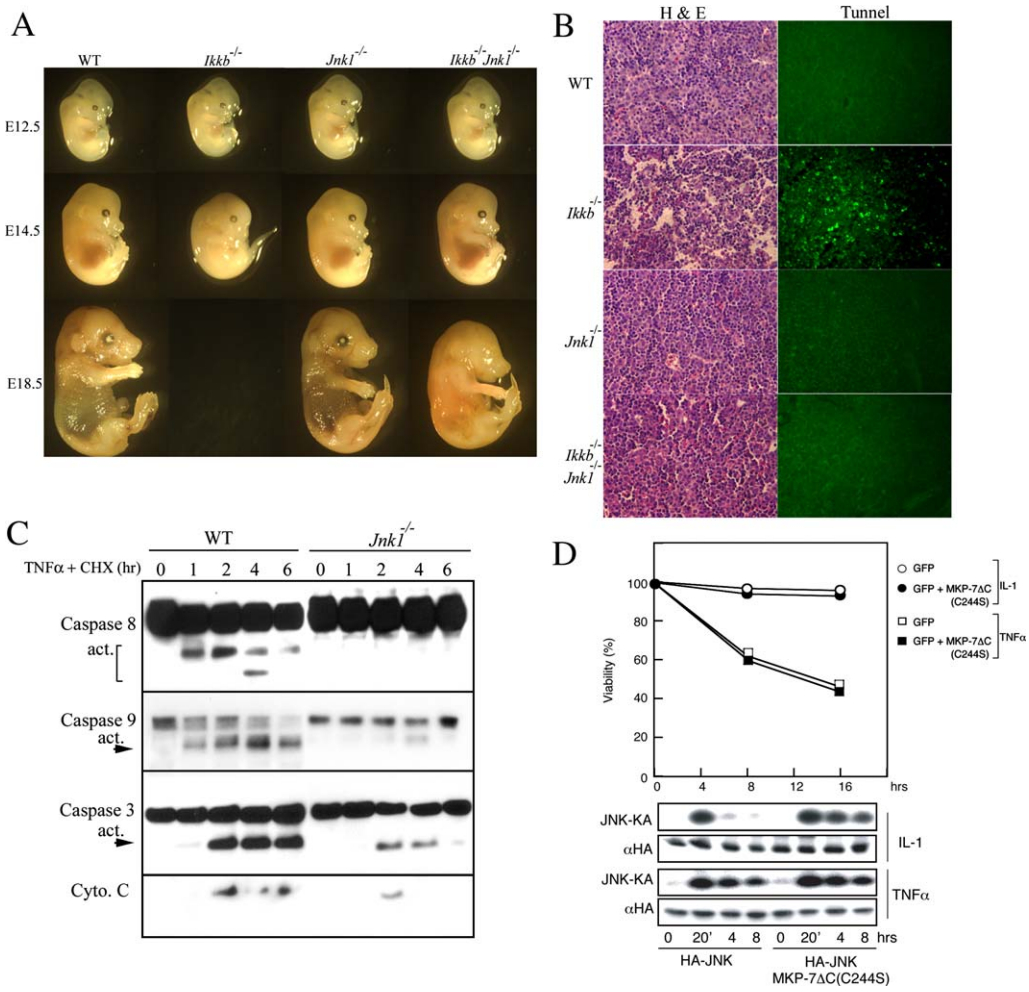

Figure 1. Prolonged JNK1 Activation Is Required for TNFα-Dependent Cell Death In Vivo but Does Not Kill Cells in the Absence of TNFα

(A) wt, *Ikkβ*$^{-/-}$, *Jnk1*$^{-/-}$, and *Ikkβ*$^{-/-}$/*Jnk1*$^{-/-}$ mouse fetuses were isolated and examined at the indicated embryonic days (E). *Ikkβ*$^{-/-}$ fetuses are dead at E14.5, whereas *Ikkβ*$^{-/-}$/*Jnk1*$^{-/-}$ fetuses survive until E17.5.

(B) Histological analysis of livers from wt, *Ikkβ*$^{-/-}$, *Jnk1*$^{-/-}$, and *Ikkβ*$^{-/-}$/*Jnk1*$^{-/-}$ E14.5 fetuses. Tissue sections were analyzed by H&E and TUNEL staining (original magnification 400×).

(C) JNK1 is required for TNFα-induced hepatocyte apoptosis. Primary hepatocytes from wt and *Jnk1*$^{-/-}$ E14.5 fetuses were treated with TNFα (10 ng/ml) plus cycloheximide (CHX, 100 μg/ml) for the indicated times. Caspase cleavage and cytochrome *c* release were monitored by immunoblotting of postmitochondrial supernatants with appropriate antibodies.

(D) Prolonged JNK activation without TNFα is not sufficient for killing of *Ikkβ*$^{-/-}$ fibroblasts. Upper panel: Viability of *Ikkβ*$^{-/-}$ fibroblasts after transfection with HA-tagged JNK1 or GFP expression vectors with or without a catalytically inactive MKP-7 expression plasmid [MKP-7ΔC(C244S)] was assessed by propidium iodide (PI) dye exclusion assay and flow cytometry (upper panel). The cells were incubated at time = 0 (36 hr after transfection) with TNFα or IL-1. Lower panels: The kinetics of JNK activation were determined by the solid state kinase assay (KA) with GST-c-Jun (1–79) as a substrate.

c-FLIP, in an in vivo setting, we injected mice with ConA, a potent activator of T cells that triggers TNFR1-dependent hepatocyte death, thereby causing fulminant and lethal hepatitis (Maeda et al., 2003). Immunoblot analysis revealed that mouse liver contained mostly the c-FLIP$_L$ isoform, even prior to ConA administration (Figure 2A). c-FLIP$_L$, unlike c-FLIP$_S$, contains a pseudo-caspase domain and is therefore a specific caspase-8 inhibitor (Irmler et al., 1997b; Krueger et al., 2001a, 2001b). ConA injection to wt mice caused degradation of c-FLIP$_L$ and appearance of an apoptotic DNA ladder (Figure 2A). ConA injection also caused cleavage of caspases-8 and -3 and cytochrome *c* release (Kamata et al., 2005). By contrast, very little c-FLIP$_L$ degradation or apoptosis were detected upon ConA injection to *Jnk1*$^{-/-}$ mice (Figure 2A). Such mice also showed little or no cleavage of caspase-3 and cytochrome *c* release (Kamata et al., 2005).

For further biochemical analysis of c-FLIP turnover, we switched to cultured hepatocytes. Again, treatment with TNFα plus cycloheximide reduced the levels of c-FLIP$_L$, the predominant form of c-FLIP in wt hepatocytes, and this

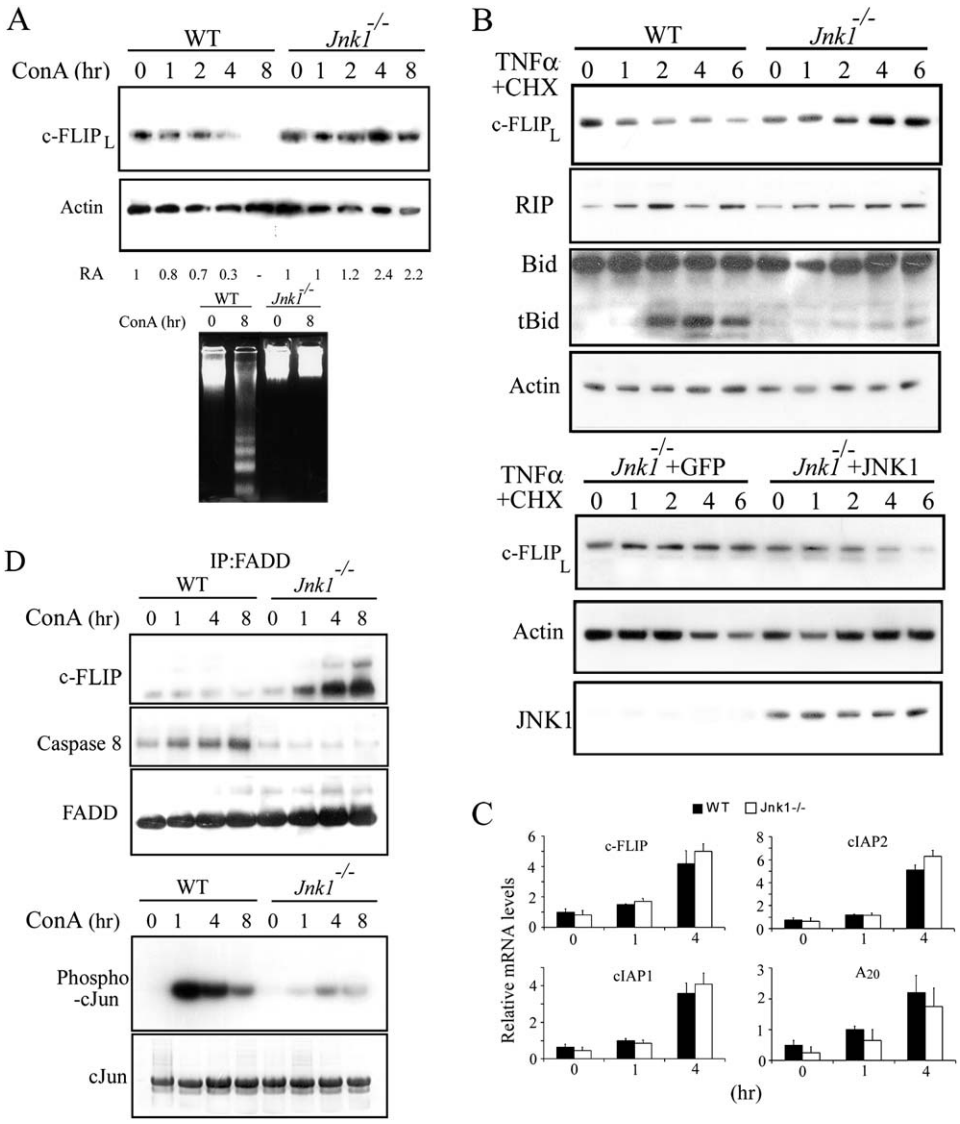

Figure 2. TNFα Induces JNK1-Dependent c-FLIP Degradation

(A) Livers were isolated at the indicated times after ConA injection (25 mg/kg) from wt and *Jnk1*$^{-/-}$ mice, lysed, gel separated, and immunoblotted with an anti-c-FLIP antibody. The same membrane was reprobed with anti-actin antibodies to monitor loading. Relative c-FLIP amounts (RA) determined by phosphoimaging are presented underneath the upper panels. Liver genomic DNA was isolated before and 8 hr after ConA injection and analyzed by agarose gel electrophoresis for a nucleosomal ladder indicative of apoptosis (lower panel).

(B) JNK1 is required for c-FLIP degradation. Upper panels: Primary wt and *Jnk1*$^{-/-}$ hepatocytes were treated with TNFα (10 ng/ml) and CHX (100 μg/ml) and analyzed at the indicated times (hr) by immunoblotting for levels of c-FLIP, RIP1, and Bid. Actin was used as a loading control. Lower panels: Primary *Jnk1*$^{-/-}$ hepatocytes were transduced with lentiviruses expressing GFP or HA-tagged JNK1. c-FLIP and JNK1 levels were examined by immunoblotting.

(C) Total RNA isolated at the indicated times (hr) from primary wt and *Jnk1*$^{-/-}$ hepatocytes after stimulation by TNFα (10 ng/ml) was subjected to Q-PCR analysis to determine mean relative mRNA levels of c-FLIP, cIAP1, cIAP2, and A20. The data shown are the means ± SD (n = 3).

(D) wt and *Jnk1*$^{-/-}$ mice were injected with ConA (25 mg/kg) and at the indicated times their livers were isolated and homogenized. Upper panels: Liver lysates were immunoprecipitated with anti-FADD antibodies and analyzed by immunoblotting with antibodies against c-FLIP, caspase-8, and FADD. Lower panels: Total JNK activity in liver lysates was determined by the solid state kinase assay.

was followed by cleavage of Bid and appearance of tBid (Figure 2B, upper panels). By contrast, the same treatment of *Jnk1*$^{-/-}$ hepatocytes did not decrease c-FLIP$_L$ expression and caused very little Bid cleavage. The TNFα-induced decrease in c-FLIP$_L$ was indeed JNK1 dependent because infection of *Jnk1*$^{-/-}$ hepatocytes with a JNK1-expressing lentivirus, but not a GFP lentivirus, restored TNFα-induced c-FLIP degradation (Figure 2B, bottom panels). Real-time quantitative PCR (Q-PCR) analysis revealed normal induction of *c-Flip* mRNA in *Jnk1*$^{-/-}$ hepatocytes and livers after

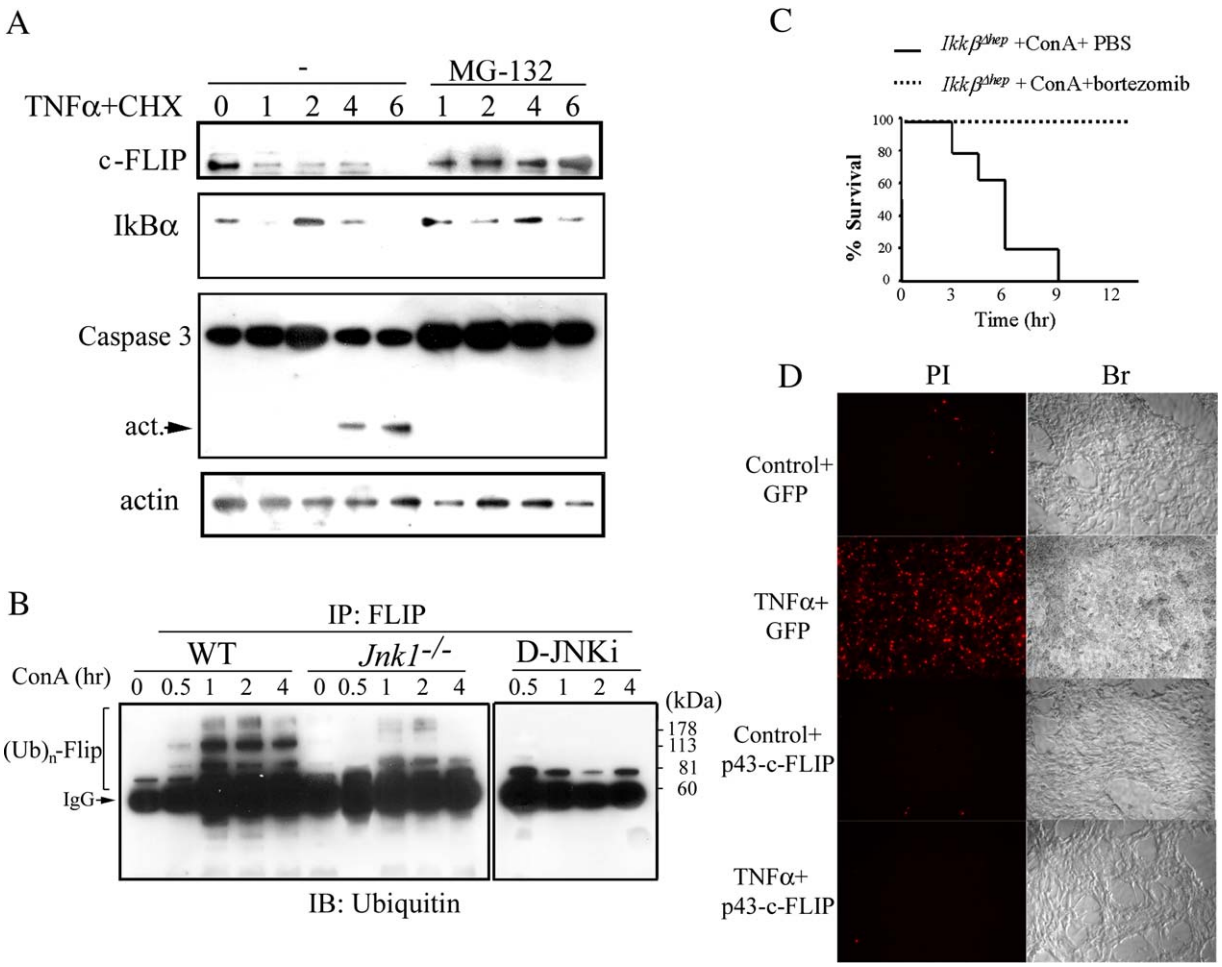

Figure 3. Proteasome Activity Is Required for TNFα-Induced c-FLIP Degradation and Cell Death

(A) Primary wt hepatocytes were incubated with or without the proteasome inhibitor MG-132 30 min prior to addition of TNFα (10 ng/ml) and CHX (100 μg/ml). At the indicated times (hr), cell lysates were prepared and analyzed by immunoblotting with antibodies for c-FLIP$_L$, IκBα, caspase-3, and actin.

(B) shows c-FLIP ubiquitination in livers of ConA-injected mice. wt and *Jnk1*$^{-/-}$ mice were given either vehicle (PBS) or 15.7 ng of a D-JNKi peptide in PBS i.c.v. 30 min before injection of ConA (25 mg/kg). Livers were isolated at the indicated times (hr), lysed, and immunoprecipitated with anti-c-FLIP antibodies, gel separated, and immunoblotted with anti-ubiquitin antibodies.

(C) shows Kaplan-Meier survival plot for *Ikkβ*$^{\Delta hep}$ mice given PBS or the proteasome inhibitor bortezomib (1 mg/kg) 1 day prior to injection of ConA (25 mg/kg).

(D) *Ikkβ*$^{-/-}$ fibroblasts were infected with either GFP- or p43-c-FLIP$_L$-expressing lentiviruses and incubated with TNFα (10 ng/ml). After 8 hr, the cells were examined by either PI staining and fluorescent microscopy or bright-field (Br) microscopy.

stimulation with either TNFα plus cycloheximide or ConA (Figure 2C and data not shown). Thus, JNK1 is likely to control c-FLIP$_L$ abundance at a posttranscriptional level. Consistent with the results of the RNA and immunoblot analyses, very little c-FLIP$_L$ was associated with FADD in livers of wt mice either before or after ConA injection, whereas the levels of FADD-associated c-FLIP$_L$ were increased in livers of *Jnk1*$^{-/-}$ mice after ConA injection (Figure 2D). A reciprocal relationship was found for caspase-8 association with FADD: very little pro-caspase-8 was brought down by the anti-FADD antibody in *Jnk1*$^{-/-}$ livers, whereas pro-caspase-8 was readily detected in FADD immunoprecipitates from wt liver—especially after ConA administration.

A major posttranscriptional mechanism that controls protein levels in eukaryotic cells is ubiquitin-dependent proteasomal turnover (Ciechanover and Schwartz, 2004). To examine proteasome involvement in TNFα-induced c-FLIP decay, we treated cultured hepatocytes with the specific proteasome inhibitor MG132 (Rock et al., 1994). Pretreatment with MG132 completely prevented TNFα-induced c-FLIP$_L$ degradation in wt hepatocytes (Figure 3A). MG132 pretreatment also inhibited TNFα-induced IκBα degradation (seen at the 1 hr time point) and prevented caspase-3 cleavage. These results suggest that TNFα induces JNK1-dependent proteasomal degradation of c-FLIP.

Proteasomal degradation depends on protein polyubiquitination (Ciechanover and Schwartz, 2004). Indeed, in wt mice ConA administration resulted in appearance of polyubiquitinated forms of c-FLIP$_L$ as early as 30 min postinjection (Figure 3B). Much less ConA-induced c-FLIP$_L$ ubiquitination was seen in ConA-injected *Jnk1*$^{-/-}$ mice or wt mice pretreated with a specific JNK-inhibitory peptide, D-JNKi (Borsello et al., 2003). This peptide also conferred resistance to ConA-induced fulminant hepatitis (Figure S1). Protection against ConA-induced fulminant hepatitis and death was also seen in *Ikkβ*$^{\Delta hep}$ mice injected with another specific proteasome inhibitor—bortezomib (Valcade, formerly PS-341) (Adams, 2004) (Figure 3C). Thus, despite the ability of proteasome inhibitors to inhibit NF-κB activation, when added prior to TNFα or ConA, they can prevent TNFα-induced cell death in hepatocytes or liver of animals that are compromised in NF-κB activation. This protective effect is most likely due to stabilization of c-FLIP$_L$ (Figure 3A) because infection of *Ikkβ*$^{-/-}$ cells with a lentivirus encoding the p43 form of c-FLIP$_L$, but not with a GFP lentivirus, prevented TNFα-induced apoptosis (Figure 3D).

Itch Activity Is Required for TNFα-Induced c-FLIP$_L$ Degradation

The results shown above indicate that TNFα induces c-FLIP$_L$ degradation in a JNK-dependent manner by inducing its polyubiquitination. As phosphorylation can tag specific proteins for polyubiquitination and proteasomal degradation, e.g., the IκBs (Karin and Ben-Neriah, 2000), we investigated whether JNK1 could directly phosphorylate c-FLIP$_L$. The results of these experiments were negative, and examination of the c-FLIP$_L$ sequence did not reveal obvious JNK docking or phosphoacceptor sites (data not shown). Recently we found that JNK1 can signal specific protein ubiquitination and degradation via phosphorylation-dependent activation of the ubiquitin ligase Itch (Gao et al., 2004). We therefore examined the susceptibility of fibroblasts from *Itchy* mutant mice, which do not express Itch (Fang et al., 2002) to TNFα-induced apoptosis. Strikingly, incubation of such cells with TNFα plus cycloheximide resulted in very little apoptosis within 8 hr, a time point at which many wt fibroblasts displayed an apoptotic phenotype (Figure 4A). Accordingly, incubation of *Itch*$^{-/-}$ fibroblasts with TNFα plus cycloheximide resulted in very little and extensively delayed caspase-8 cleavage, delayed caspase-3 activation, and no c-FLIP$_L$ degradation (Figure 4B). Similar results were obtained when instead of cycloheximide the different fibroblast cultures were transduced with an adenovirus encoding IκBα superrepressor (IκBα-SR) to inhibit NF-κB. Whereas TNFα treatment of wt cells transduced with this virus resulted in apoptosis and c-FLIP$_L$ degradation, very little apoptosis or c-FLIP$_L$ degradation were seen in *Itch*$^{-/-}$ or *Jnk1*$^{-/-}$ fibroblasts (Figure 4C). Reconstitution of *Itch*$^{-/-}$ fibroblasts with a lentivirus encoding wt Itch restored TNFα-induced c-FLIP$_L$ degradation and apoptosis, while very little apoptosis or c-FLIP$_L$ degradation was seen in *Itch*$^{-/-}$ cells transduced with a lentivirus encoding a mutant form of Itch, AA-Itch, which is refractory to JNK1-induced phosphorylation (Figure 4D; Figure S2).

In transient transfection experiments, we found that coexpression of Itch with c-FLIP$_L$ resulted in polyubiquitination of the latter that was seen only in cells treated with the proteasome inhibitor MG132 (Figure 4E). Itch-induced ubiquitination of c-FLIP$_L$ was augmented by coexpression of activated JNK1, a JNK1-JNKK2 fusion protein (Zheng et al., 1999). Coexpression of the antiapoptotic proteins cIAP1 or cIAP2, which contain a RING-finger domain that possesses E3 ligase activity (Yang et al., 2000), instead of Itch did not stimulate c-FLIP$_L$ ubiquitination (Figure 4E). Coexpression of normal JNK1 had only a small stimulatory effect on basal Itch-induced c-FLIP$_L$ ubiquitination, but the ubiquitination response seen in the presence of JNK1 was strongly stimulated by incubating the transfected cells with TNFα (Figure 4F). TNFα weakly enhanced Itch-induced c-FLIP$_L$ ubiquitination in the absence of cotransfected JNK1.

Itch Selectively Interacts with c-FLIP$_L$

To further characterize the functional interaction between Itch and c-FLIP, we examined whether the two proteins directly interact. In addition to full-length c-FLIP$_L$ and c-FLIP$_S$, we also used two different subfragments of c-FLIP$_L$ corresponding to its N-terminal DEDs and its C-terminal caspase-8-like domain (CASP). In GST-pulldown experiments, full-length c-FLIP$_L$ or its CASP domain, but neither c-FLIP$_S$ nor the DED portion, were found to bind GST-Itch (Figure 5A). These interactions were of functional relevance because in cotransfection experiments similar to those described in Figure 4E, coexpression of Itch and activated JNK1 promoted the ubiquitination and degradation of c-FLIP$_L$ but not c-FLIP$_S$ (Figure 5B). Note that no TNFα-enhanced c-FLIP$_L$ ubiquitination is seen in this experiment because the JNK1 construct is a constitutively active JNKK2-JNK1 fusion protein. The time-dependent c-FLIP$_L$ degradation in this figure is due to the inhibition of new protein synthesis by cycloheximide. Even after TNFα plus cycloheximide treatment, c-FLIP$_S$, unlike c-FLIP$_L$, remained stable. Similar results were obtained in pulse-chase experiments (Figure 5C). Whereas Itch together with activated JNK1 induced rapid decay of both full-length (p55) and p43 c-FLIP$_L$, it failed to alter the amount of radiolabeled c-FLIP$_S$. Thus, Itch selectively interacts with the CASP domain that is present in c-FLIP$_L$ and this interaction is of functional relevance.

Itchy Mutant Mice Are Resistant to TNFα-Dependent Fulminant Hepatitis

If Itch is required for TNFα-induced c-FLIP$_L$ degradation in vivo and this is required for TNFα-induced PCD, the absence of Itch activity should protect mice from TNFα-induced lethality. To examine this prediction, we injected wt, *Jnk1*$^{-/-}$, and *Itchy* mice (all in the C57BL6 background) with ConA. Histological analysis revealed extensive liver damage in wt mice within 8 hr of ConA administration, but very little damage was observed and tissue integrity was retained in *Jnk1*$^{-/-}$ and *Itchy* mice (Figure 6A). Similar observations were made by measuring the circulating levels of

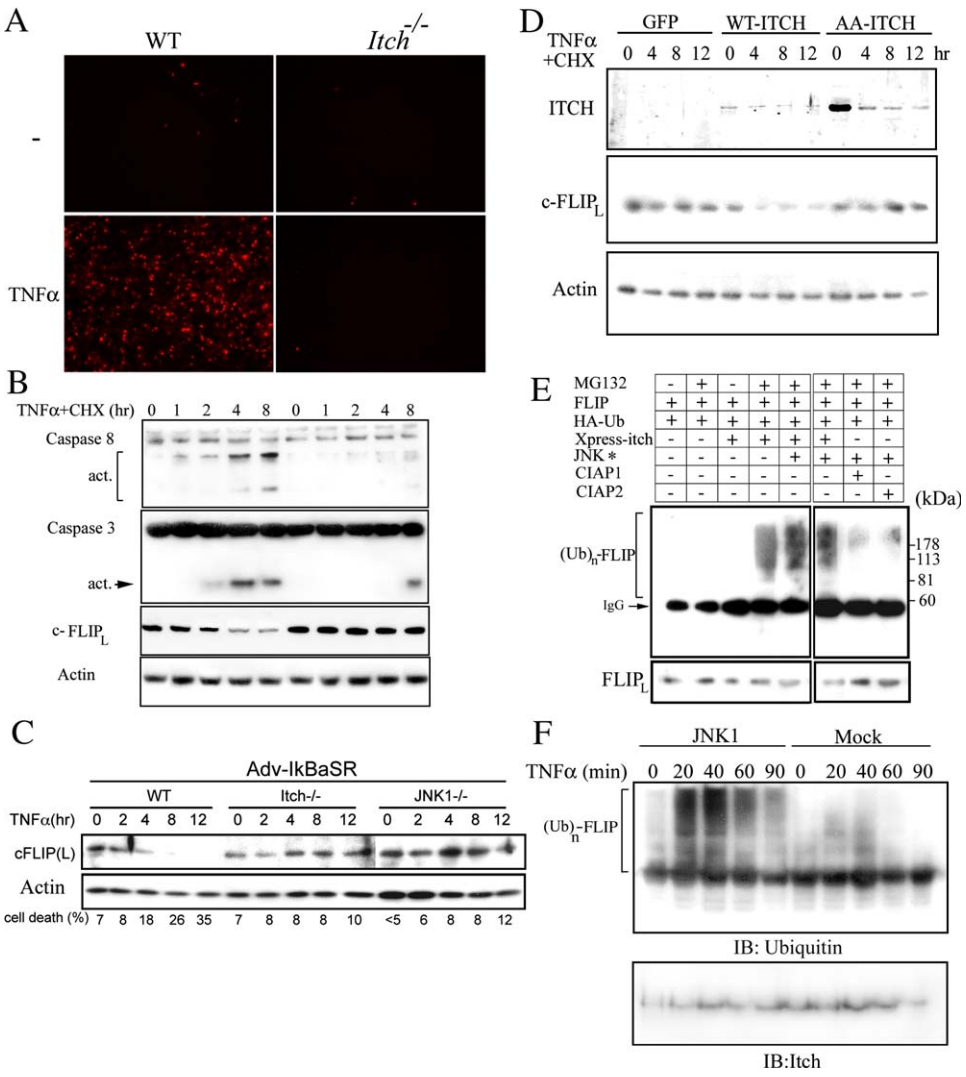

Figure 4. Itch Is Required for c-FLIP Degradation and TNFα-Induced Apoptosis

(A) wt and *Itch*$^{-/-}$ fibroblasts were incubated with TNFα (10 ng/ml) and CHX (100 μg/ml). After 8 hr, apoptotic cells were visualized by PI staining.

(B) c-FLIP protein levels and caspase-8 and -3 cleavage were analyzed in wt and *Itch*$^{-/-}$ fibroblasts after TNFα plus CHX treatment.

(C) wt, *Itchy* (Itch$^{-/-}$), and *Jnk1*$^{-/-}$ fibroblasts were transduced with an adenovirus encoding IκBα superrepressor (IκBαSR) to inhibit NF-κB. TNFα (10 ng/ml) was added and at the indicated times cell lysates were prepared and c-FLIP$_L$ degradation was examined as above. The extent of apoptosis in parallel treated cultures was examined by propidium iodide staining, and relative rates of apoptotic cell death are indicated below.

(D) *Itchy* fibroblasts were transduced with lentiviruses encoding GFP, wt Itch, or AA-Itch, a mutant in which the D domain required for JNK1 binding and Itch phosphorylation was inactivated (Gao et al., 2004). After 24 hr, the cells were incubated with TNFα plus CHX and Itch expression and c-FLIP degradation was assessed by immunoblotting. Note that the titre of the AA-Itch virus was somewhat higher than that of the wt-Itch virus, and the cells at the 0 time point received more virus.

(E) Itch induces c-FLIP$_L$ ubiquitination. 293T cells were transfected with Myc-tagged-c-FLIP$_L$, Xpress-tagged Itch, HA-ubiquitin with or without JNKK2-JNK1 (JNK*) expression plasmids. cIAP1 and cIAP2 expression vectors were used as controls for the Itch expression vector. After 36 hr, cells were incubated with TNFα plus CHX for 8 hr and c-FLIP$_L$ ubiquitination was examined by immunoprecipitation with anti-Myc antibodies and immunoblotting with anti-HA antibodies. Expression of Itch (not shown) was detected with anti-Xpress antibodies.

(F) TNFα stimulates c-FLIP$_L$ ubiquitination. 293T cells were transiently transfected with plasmids encoding Myc-tagged c-FLIP$_L$, ubiquitin, and Itch with or without (Mock) a JNK1 vector. The cells were preincubated with MG-132 for 30 min and stimulated with TNFα for the indicated duration (min). After immunoprecipitation with anti-Myc, ubiquitination of c-FLIP$_L$ was examined by immunoblotting as described above. Itch levels were examined by immunoblotting.

alanine aminotransferase (ALT), a liver enzyme whose presence in the circulation is an indicator of liver damage: circulating ALT levels were strongly elevated in wt mice 8 hr after ConA administration, while only a small increase in circulating ALT was seen in similarly treated *Itchy* mice (Figure 6B). Accordingly, ConA administration led to c-FLIP$_L$ degradation in wt but not in *Itchy* mice (Figure 6C). ConA-induced hepatitis depends on production of several cytokines, including

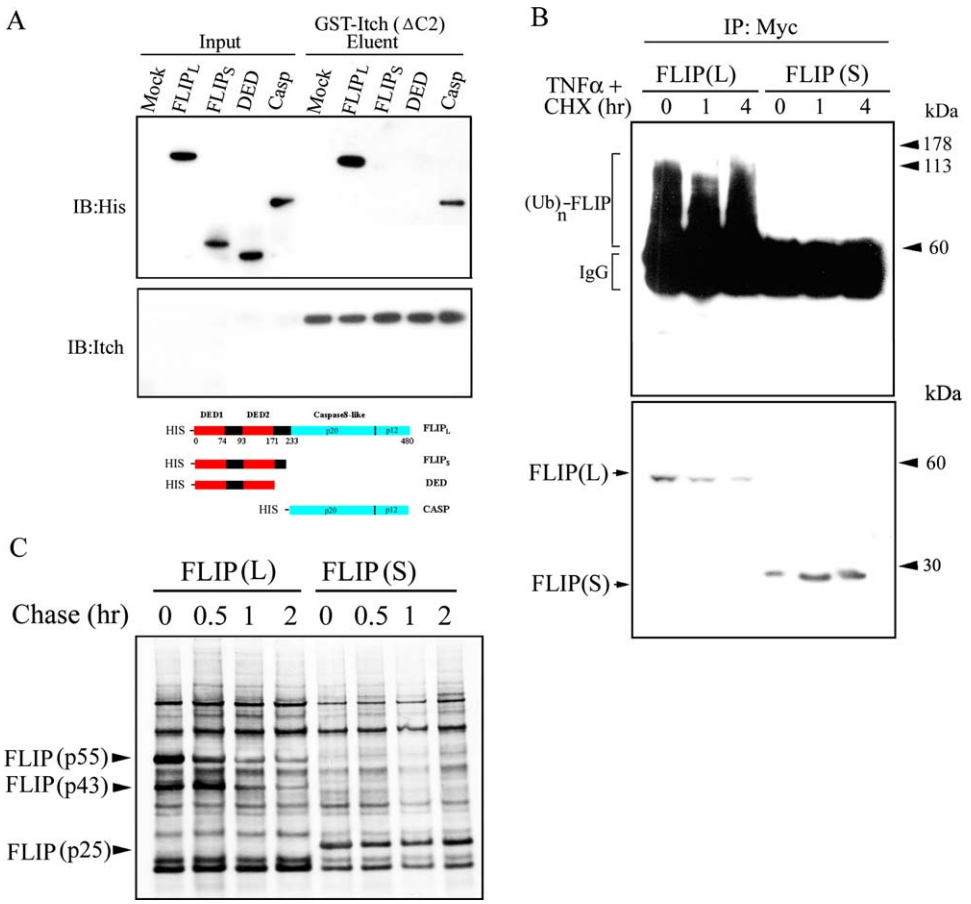

Figure 5. Itch Selectively Interacts with c-FLIP$_L$ and Induces Its Degradation but Does Not Affect c-FLIP$_S$

(A) Reticulocyte lysates were programmed with His-tagged expression vectors for c-FLIP$_L$, c-FLIP$_S$, and the DED or CASP regions of c-FLIP$_L$. A portion of each lysate was saved, whereas the rest was subjected to pulldown with recombinant GST-Itch. The saved lysates and eluates from the GST-Itch beads were analyzed by immunoblotting with anti-His antibodies. An anti-Itch antibody was used to examine the amount of GST-Itch in each precipitate. Shown below is a schematic representation of the FLIP expression constructs.

(B) shows comparison of c-FLIP$_L$ and c-FLIP$_S$ ubiquitination. 293T cells were transfected with either Myc-tagged c-—FLIP$_L$ or c-FLIP$_S$ vectors together with Itch, ubiquitin, and JNKK2-JNK1 expression plasmids. After 36 hr, the cells were incubated with TNFα (10 ng/ml) and CHX (100 μg/ml) for the indicated times. Ubiquitination of c-FLIP isoforms was assessed as described in Figure 4 after immunoprecipitation with anti-Myc antibody. The total levels of c-FLIP isoforms were examined by immunoblotting.

(C) Itch induces selective degradation of c-FLIP$_L$. 293T cells were transfected with Myc-tagged c-FLIP$_L$ or c-FLIP$_S$ expression vectors along with Itch, ubiquitin, and JNKK2-JNK1 expression plasmids. After 24 hr, the cells were pulse labeled with a [^{35}S]*trans*-label for 1 hr and chased with nonradioactive cysteine and methionine containing medium for the indicated times. Cell lysates were immunoprecipitated with anti-Myc antibodies, separated on 12% NuPAGE gels, and fluorographed.

TNFα, interferon (IFN) -γ, IL-4, and Fas ligand (FasL), by NKT cells (Tiegs et al., 1992). The *Itchy* mutation increases the expression of Th$_2$ cytokines, such as IL-4, while having no effect on Th$_1$ cytokines, such as IFN-γ (Fang et al., 2002). As expected, we found no change in expression of TNFα, IFN-γ, and FasL mRNAs after ConA injection between wt and *Itchy* mice, while the levels of IL-4 mRNA were elevated in the mutant (Figure 6D). Thus the protection against ConA-induced fulminant hepatitis in *Itchy* mice is unlikely to be due to defective cytokine gene expression.

We also examined JNK-dependent c-FLIP$_L$ degradation in a different model of acute liver failure, this time induced by coadministration of bacterial lipopolysaccharide (LPS) and D-galactosamine (GalN). While LPS leads to rapid activation of macrophages that produce copious amounts of TNFα, GalN is a specific inhibitor of gene transcription in hepatocytes (Decker and Keppler, 1974), and it therefore prevents the induction of antiapoptotic genes only in this cell type. While administration of LPS + GalN to wt mice resulted in massive liver damage and lethality, treatment of these mice with the JNK inhibitor D-JNKi prevented hepatocyte apoptosis and no liver damage could be detected (Figure S3). Importantly, administration of the JNK inhibitor inhibited the ubiquitination of c-FLIP$_L$ and prevented its degradation (Figure 6E). The effect was not due to reduced TNFα production (Figure S3). Nonetheless, to completely rule out

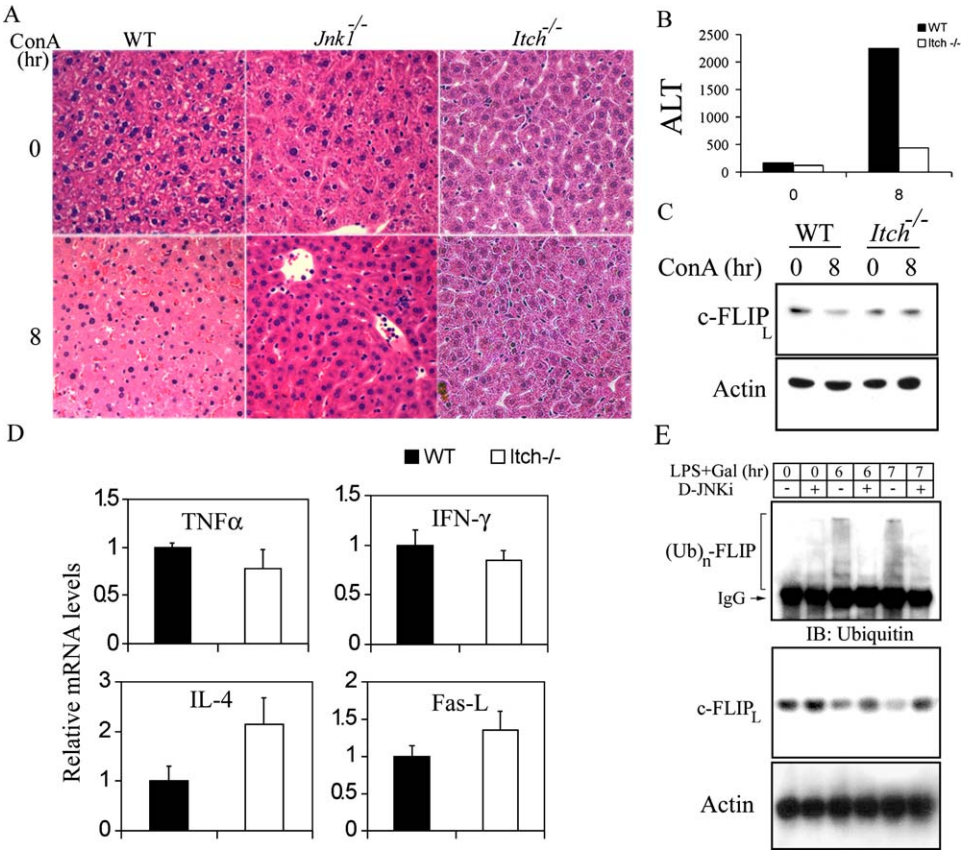

Figure 6. Itch Is Required for ConA-Induced Fulminant Hepatitis and c-FLIP$_L$ Degradation

(A) Histological analysis of livers from wt, *Jnk1*$^{-/-}$, and *Itch*$^{-/-}$ mice before and 8 hr after ConA (25 mg/kg) challenge. Tissue sections were subjected to H&E staining and examined by microscopy (original magnification 400×). Similar results were obtained by analysis of three additional mutant mice on two separate occasions.

(B) wt and *Itch*$^{-/-}$ mice were injected with ConA, and serum samples were removed at 0 and 8 hr for measurement of ALT levels (units/ml). The results are average values for two mice per genotype.

(C) Livers of wt and *Itc* $^{-/-}$ mice were isolated 0 and 8 hr after ConA injection, and c-FLIP$_L$ levels were determined by immunoblotting. Actin was used as loading control.

(D) Livers from wt and *Itch*$^{-/-}$ mice were collected 0 and 8 hr after ConA injection and total RNA was isolated for Q-PCR analysis of the indicated cytokine mRNAs. The data shown are the means ± SD (n = 3 or 4).

(E) wt mice were injected i.p. with LPS and Gal-N. Half of the treatment group was also injected with a JNK inhibitor (D-JNKi, 30 ng/mouse) or PBS. At the indicated points, the mice were sacrificed and their livers removed for biochemical and histological analyses. c-FLIP ubiquitination was analyzed as described above by immunoprecipitation with anti-FLIP antibody and immunoblotting with anti-ubiquitin antibody. c-FLIP amounts were determined by immunoblotting.

a contribution of altered TNFα synthesis, we administered TNFα + GalN to wt and *Itch*$^{-/-}$ mice. Whereas this treatment led to severe liver damage within 7 hr in wt mice, *Itch*$^{-/-}$ mice did not display any obvious histological liver damage and were resistant to TNFα-induced morbidity (Figure S4). In addition, we reconstituted lethally irradiated wt mice with bone marrow of either wt of *Jnk1*$^{-/-}$ mice, whereas lethally irradiated *Jnk1*$^{-/-}$ mice were reconstituted with wt bone marrow. After 8–10 weeks, these mice were challenged with TNFα + GalN. Whereas mice with wt stroma (i.e., the radiation-resistant compartment) were highly sensitive to TNFα + GalN and exhibited severe liver damage with characteristic necrotic and hemorrhagic lesions, mice with a *Jnk1*$^{-/-}$ stroma were resistant to TNFα + GalN and their livers retained normal morphology at 8 hr post-challenge (Figure S5). These results confirm that the sensitivity to TNFα-induced death is determined by the genotype of the hepatocyte and not bone marrow-derived cells and that the resistance displayed by Itch- or JNK1-deficient mice is not due to reduced TNFα production, processing, or secretion.

DISCUSSION

The ability of NF-κB to inhibit TNFα-induced cell death is well established and was seen on many different occasions both in vitro and in vivo (reviewed by Karin and Lin, 2002). Furthermore, rapid activation of IKK and NF-κB by TNFα explains, in part, its poor ability to trigger cell death in comparison to

other family members that are weak NF-κB activators, such as FasL (Micheau and Tschopp, 2003; Muppidi et al., 2004). By contrast, the role of JNK in the control of TNFα-induced cell death has been controversial, and its mechanism of activation remained enigmatic. In recent years, it was shown, initially in cultured cells (De Smaele et al., 2001; Tang et al., 2001) and now in vivo (Figure 1) that one of the antiapoptotic mechanisms through which NF-κB acts depends on termination of JNK activation. This was shown to be due to the ability of NF-κB to induce genes encoding antioxidants (Pham et al., 2004), which prevent oxidative inhibition of JNK phosphatases (Kamata et al., 2005). Yet, the exact mechanism through which JNK activation promotes TNFα-induced apoptosis remained unknown. Recently, it was suggested that JNK is likely to act at a step upstream to caspase-8 perhaps through generation of a novel Bid cleavage product, jBid (Deng et al., 2003). But even that work failed to identify a JNK-dependent phosphorylation event or a JNK substrate that promotes TNFα-induced apoptosis. The experiments described above address this deficiency in our understanding of TNFα signaling by identifying a novel regulatory step and a direct JNK substrate needed for progression from TNFR1-assembled signaling complexes to caspase-8 activation. Our results demonstrate, both in vitro and in vivo, that TNFα-induced apoptosis requires the JNK1-dependent degradation of the caspase-8 inhibitor, c-FLIP$_L$. The induced degradation of c-FLIP$_L$, which is mediated by the 26S proteasome, requires activation of the JNK1-responsive ubiquitin ligase Itch, which specifically interacts with the long isoform of c-FLIP.

These results are satisfying because they are consistent with many previous observations to which they provide new and important mechanistic insights. First and foremost, JNK1 promotes apoptosis by directly antagonizing a critical NF-κB-dependent antiapoptotic step. It was established that the primary mechanism by which NF-κB activation, during early stages of TNFR1 signal transduction, prevents activation of caspase-8 through complex II depends on induction of c-FLIP (Micheau and Tschopp, 2003). Furthermore, ablation of the *c-Flip* gene renders cells sensitive to TNFα-induced apoptosis even without inhibition of NF-κB or macromolecular synthesis (Yeh et al., 2000). In addition, c-FLIP but not another NF-κB-dependent antiapoptotic regulator, cIAP1, blocks TNFα-induced cell death (Micheau et al., 2001). We now show that JNK1 antagonizes the very same process that blocks TNFα-induced death in response to NF-κB activation, by promoting c-FLIP$_L$ degradation. Second, the progression from TNFR1 ligation to caspase-8 activation in cells with compromised NF-κB activity depends on formation at later time points of the soluble complex II, whose assembly depends on binding of RIP1, TRAF2, and TRADD to the caspase-8 activator FADD (Micheau and Tschopp, 2003). Yet the ability of complex II to activate caspase-8 is inhibited by c-FLIP, whose synthesis is induced by NF-κB. We now show in three different pathophysiologically relevant in vivo models that injection of ConA, LPS plus GalN, or TNFα plus GalN into mice results in degradation of c-FLIP$_L$ (Figures 2A and 6E) with very similar kinetics to those of pro-caspase-8 recruitment to FADD (Figure 2D). These biochemical changes correlate with induction of fulminant liver failure associated with hepatocyte apoptosis. By contrast, in *Jnk1*$^{-/-}$ mice, which are resistant to ConA-induced liver failure (Maeda et al., 2003), ConA injection does not lead to c-FLIP$_L$ degradation (Figure 2A), and therefore FADD activation results in recruitment of c-FLIP$_L$ at the expense of caspase-8 (Figure 2D). Injection of a specific JNK-inhibitory peptide into wt mice treated with either ConA (Figure 3B) or LPS plus GalN (Figure 6E) also blocked c-FLIP$_L$ ubiquitination and degradation and caspase-8 cleavage.

We also observed that JNK1-induced c-FLIP degradation is specific to the c-FLIP$_L$ isoform and does not apply to c-FLIP$_S$ (Figures 5B and 5C). Specificity is due to the selective interaction of c-FLIP$_L$ with Itch, mediated by its CASP domain, which is absent from c-FLIP$_S$ or FLIPR. This result may explain why activated T cells mainly upregulate c-FLIP$_S$ and thereby resist activation-induced death (Kirchhoff et al., 2000a, 2000b). We previously found that potent T cell activation results in the JNK1-dependent activation of Itch (Gao et al., 2004). This is expected to prevent the accumulation of c-FLIP$_L$ by inducing its degradation but have no effect on c-FLIP$_S$, the very isoform found to accumulate in strongly activated T cells. Conversely, c-FLIP$_L$ was found to be the relevant inhibitor of caspase-8 activation by TNFα (Micheau and Tschopp, 2003). Furthermore, c-FLIP$_L$ is the predominant form of FLIP in the liver, present even prior to TNFα challenge, an observation that explains why TNFα-induced apoptosis in hepatocytes is prevented by proteasomal inhibitors, which may enhance apoptosis in other cell types. Thus, cell type-specific modulation of c-FLIP isoform expression levels may strongly affect the ability of JNK1-dependent Itch activation to promote apoptosis in response to engagement of TNFR1 or other receptors.

Interestingly, Itch is the second HECT domain E3 ubiquitin ligase found to control programmed cell death through ubiquitin-induced degradation of antiapoptotic proteins. Recently, another HECT domain ubiquitin ligase, Mule/ARF-BP1, was shown to catalyze the ubiquitin-dependent elimination of Mcl-1 during DNA-damage-induced apoptosis (Zhong et al., 2005). Thus, there may be a newly emerging but general role for HECT domain ubiquitin ligases as positive regulators of apoptosis through their ability to catalyze polyubiquitination of antiapoptotic proteins. It would be of interest to examine whether like Itch (Gao et al., 2004), Mule activity is also controlled by its phosphorylation.

The present results lead to a further refinement of the two-step model of TNFR1 signal transduction (Micheau and Tschopp, 2003; Muppidi et al., 2004) and provide a detailed and rather definitive explanation of the mechanism by which the intricate interplay between NF-κB and JNK determines the outcome of TNFα signaling (Figure 7). By inducing c-FLIP$_L$ synthesis, NF-κB activation prevents caspase-8 activation at later time points, during which the TNFR1-associated complex I dissociates and the FADD-containing complex II appears. The amount of c-FLIP$_L$ in the cell determines the extent of pro-caspase-8 recruitment to FADD and its rate of activation. At the same time, NF-κB activation

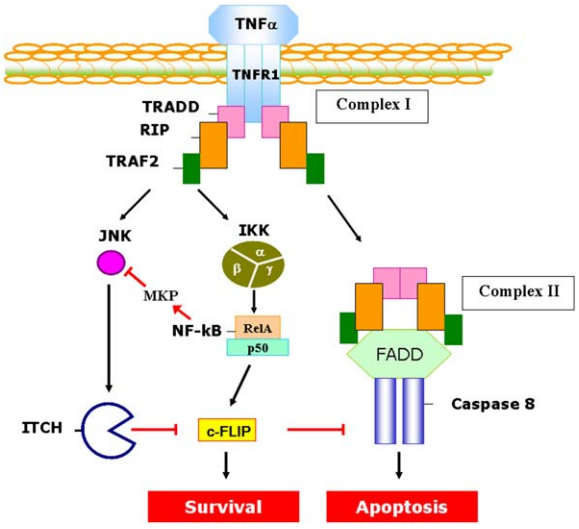

Figure 7. TNFα-Induced Apoptosis Depends on Itch Activation by JNK1 and c-FLIP$_L$ Degradation

Engagement of TNFR1 results in formation of complex I at the cytoplasmic tail of the receptor leading to JNK1 and IKK activation. IKK activates NF-κB, which induces c-FLIP expression, whereas JNK1 activates Itch, which induces c-FLIP$_L$ ubiquitination and proteasomal degradation. After awhile, complex I dissociates from the receptor and complex II, which includes FADD, is formed. If c-FLIP$_L$ levels are high (high NF-κB, low JNK1), caspase-8 recruitment to FADD and its subsequent activation are inhibited and the cells survive. If c-FLIP$_L$ levels are low (low NF-κB, high JNK1), caspase-8 is recruited to FADD and activated, leading to apoptotic cell death.

induces expression of antioxidant enzymes that keep JNK activation in check by preserving MKP activity (Kamata et al., 2005). However, under conditions where NF-κB activity is reduced or attenuated, the amount of newly synthesized c-FLIP$_L$ decreases, and MKPs are inhibited due to accumulation of ROS. This promotes strong and sustained JNK1 activation, which in turn activates Itch to promote c-FLIP$_L$ degradation. As Itch phosphorylation is reversible, prolonged JNK1 activation is required for maintaining Itch in an activated state needed for inducing the decay of the entire c-FLIP$_L$ pool. This facilitates the recruitment of procaspase-8 to FADD and its subsequent activation. Thus, even subtle changes in the relative activities of NF-κB and JNK1 can have rather dramatic effects on the ability of TNFα to induce cell survival and proliferation versus cell death. In addition to explaining why prolonged, rather than transient, JNK activation is required for progression of TNFα-induced apoptosis, our results also explain the recently observed differences in the abilities of JNK1 and JNK2 to promote TNFα-induced apoptosis (Liu et al., 2004), as only JNK1 and not JNK2 can lead to Itch phosphorylation and activation (Gao et al., 2004).

EXPERIMENTAL PROCEDURES

Animals

Mice deficient in either IKKβ in either all cells or only in hepatocytes (Maeda et al., 2003), JNK1 (Chang et al., 2003), or Itch (Fang et al., 2002) were previously described and were backcrossed to C57BL/6 mice for more than six generations.

Primary Hepatocyte Culture

Hepatocytes were isolated and plated as described (Maeda et al., 2003). Cell viability exceeded 90% as determined by Trypan-blue exclusion. Hepatocytes were cultured for 24 hr in arginine-free medium containing 10% dialyzed serum to eliminate other cell types.

Gene Expression Analysis

Total cellular RNA was extracted using TRIZOL (Invitrogen, Carlsbad, CA). cDNA was generated using SuperScript II (Invitrogen). The relative levels of c-FLIP, cIAP1, cIAP2, TNFα, IL-4, IFN-γ, and FasL mRNAs were determined by real-time quantitative PCR (Q-PCR) using cyclophilin mRNA for normalization (Park et al., 2002). Primer sequences are available upon request.

Immunohistochemistry

Livers were fixed in 4% formaldehyde, dehydrated, embedded in paraffin, and sectioned (5 μm). Sections were stained with Harris hematoxylin and eosin (H&E). TUNEL staining was as described (Maeda et al., 2003).

Liver Injury Models

ConA was injected i.v. at 25 mg/kg in PBS. Alternatively, mice were injected i.p. with 35 μg/kg LPS (Sigma) and 700 mg/kg GalN (Sigma). Animals were sacrificed, blood was collected by cardiac puncture, and livers were surgically removed. Serum was separated and analyzed for transaminase and TNFα levels and liver histology was analyzed by H&E staining (Maeda et al., 2003).

Immunochemistry and Kinase Assays

For immunoprecipitations, lysates were mixed with antibodies (1 μg) for 2 hr, followed by addition of 30 μl of proteinA/G Plus-Sepharose beads (Santa Cruz Biotechnology, Santa Cruz, CA) for an additional hr at 4ºC. Immunoprecipitates were washed three times with RIPA buffer and boiled in Laemmli buffer. Samples were separated on 12% NuPAGE gels (Invitrogen) or by standard SDS-PAGE and electrotransferred to PVDF membranes. Membranes were probed with primary antibodies (1 μg/ml), followed by horseradish peroxidase-conjugated secondary antibodies, washed and visualized with chemiluminescence detection system (Pierce). We used polyclonal antibodies against caspase-8 (Cell Signaling), c-FLIP (Stressgene), RIP1 (Cell Signaling), p21 (Santa Cruz Biotech), and Bid (Gift from Dr. J. Reed) and monoclonal antibodies against HA (Roche), Myc, His (Invitrogen), c-FLIP (Alexis), actin (Sigma), FADD (Stressgene), ubiquitin, IκBα (Imgenex) JNK1, caspase-3, and cytochrome *c* (BD biosciences).

Glutathione *S*-transferase (GST)-Itch fusion proteins were expressed and purified as described (Fang et al., 2002). Proteins were in vitro transcribed and translated in reticulocyte lysates (Promega). For pull-down assays, GST fusion proteins (5 μg) were mixed with in vitro translates and incubated at 4ºC for 4 hr, followed by addition of 30 μl of GSH-Sepharose beads (Amersham Pharmacia) for 30 min. Precipitates were washed four times with lysis buffer, eluted in Laemmli buffer, and separated on 12% NuPAGE gels and immunoblotted as above. JNK immunocomplex kinase assays were as described (Maeda et al., 2003).

Metabolic Labeling and Pulse-Chase Experiment

Transfected 293T cells were grown to confluence, washed with PBS, and incubated at 37ºC for 1 hr in serum-free DMEM without methionine and cysteine. Cells were pulse-labeled for 2 hr with 200 μCi ProMix L-[^{35}S]methionine and cysteine (Amersham Biosciences), washed extensively with unlabeled DMEM, and chased at 37ºC in DMEM containing serum and supplemented with unlabeled methionine and cysteine (Sigma). At the indicated times cells were washed with PBS and lysed.

Plasmids and Cell Transfection

Fibroblasts from wt, *Ikkβ*$^{-/-}$, and *Itch*$^{-/-}$ (Bai et al., 2004) mouse embryos and 293T cells were cultured in DMEM plus 10% fetal calf serum (FCS). c-FLIP expression plasmids encoding HA-tagged full-length ($FLIP_L$), short form ($FLIP_S$), caspase-8 like domain (CASP) and the DEDs were transfected into cells using Lipofectamine 2000 (Invitrogen). Human recombinant TNFα and IL-1 were from R&D Systems (Minneapolis, MN). The protease inhibitor MG132 and JNK inhibitor D-JNKi were from Calbiochem. The proteasome inhibitor bortezomib (PS-341) was a gift from Millennium Pharmaceuticals (Boston).

Lentivirus Constructs and Infection

Lentivirus expressing GFP under control of CMV promoter was kindly provided by Dr. I. Verma (Salk Institute). Myc-tagged p43-c-FLIP, HA-JNK1, Itch, or Itch (AA) cDNAs were PCR amplified and subcloned into lentivirus vector pLL3.7 (Dull et al., 1998). Titers were determined by infecting wt fibroblasts with serial dilution of concentrated lentivirus. For scoring p43-c-$FLIP_L$-positive cells, infected cells were immunostained with anti-Myc antibody and visualized with Rhodamine. For GFP expression, infected cells were examined and scored under an inverted microscope (Zeiss). The titers were approximately 1–10 × 10^8 infectious units (IFU) per ml.

Supplemental Data

Supplemental data include five figures and can be found with this article online at http://www.cell.com/cgi/content/full/124/3/601/DC1/.

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