Tweak-FN14 signaling induces lysosomal degradation of a cIAP1/TRAF2 complex to sensitize tumour cells to TNF

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Abstract: Synthetic inhibitor of apoptosis (IAP) antagonists induce degradation of IAP proteins such as cellular IAP1 (cIAP1), activate nuclear factor kappaB (NF-kappaB) signaling, and sensitize cells to tumor necrosis factor alpha (TNFalpha). The physiological relevance of these discoveries to cIAP1 function remains undetermined. We show that upon ligand binding, the TNF superfamily receptor FN14 recruits a cIAP1-TNF receptor-associated factor 2 (TRAF2) complex. Unlike IAP antagonists that cause rapid proteasomal degradation of cIAP1, signaling by FN14 promotes the lysosomal degradation of cIAP1-TRAF2 in a cIAP1-dependent manner. TNF-like weak inducer of apoptosis (TWEAK)/FN14 signaling nevertheless promotes the same noncanonical NF-kappaB signaling elicited by IAP antagonists and, in sensitive cells, the same autocrine TNFalpha-induced death occurs. TWEAK-induced loss of the cIAP1-TRAF2 complex sensitizes immortalized and minimally passaged tumor cells to TNFalpha-induced death, whereas primary cells remain resistant. Conversely, cIAP1-TRAF2 complex overexpression limits FN14 signaling and protects tumor cells from TWEAK-induced TNFalpha sensitization. Lysosomal degradation of cIAP1-TRAF2 by TWEAK/FN14 therefore critically alters the balance of life/death signals emanating from TNF-R1 in immortalized cells.

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TWEAK-FN14 signaling induces lysosomal degradation of a cIAP1–TRAF2 complex to sensitize tumor cells to TNFα

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SYNTHETIC INHIBITOR OF APOPTOSIS (IAP) antagonists induce degradation of IAP proteins such as cellular IAP1 (cIAP1), activate nuclear factorκB (NF-κB) signaling, and sensitize cells to tumor necrosis factorα (TNFα). The physiological relevance of these discoveries to cIAP1 function remains undetermined. We show that upon ligand binding, the TNF superfamily receptor FN14 recruits a cIAP1–Tnf receptor-associated factor 2 (TRAF2) complex. Unlike IAP antagonists that cause rapid proteosomal degradation of cIAP1, signaling by FN14 promotes the lysosomal degradation of cIAP1–TRAF2 in a cIAP1-dependent manner. TNF-like weak inducer of apoptosis (TWEAK)/FN14 signaling nevertheless promotes the same noncanonical NF-κB signaling elicited by IAP antagonists and, in sensitive cells, the same autocrine TNFα-induced death occurs. TWEAK-induced loss of the cIAP1–TRAF2 complex sensitizes immortalized and minimally passaged tumor cells to TNFα-induced death, whereas primary cells remain resistant. Conversely, cIAP1–TRAF2 complex overexpression limits FN14 signaling and protects tumor cells from TWEAK-induced TNFα sensitization. Lysosomal degradation of cIAP1–TRAF2 by TWEAK/FN14 therefore critically alters the balance of life/death signals emanating from TNF-R1 in immortalized cells.

Introduction

Upon binding of their cognate ligand, TNF receptor superfamily (TNFRSF) members transmit signals via their cytoplasmic domains. Several TNF receptors bear death domains (DD) that allow them to directly promote apoptotic cell death. Activation of the TNFRSF receptors, such as Fas or TNF-related apoptosis-inducing ligand (TRAIL)–R2 (Tartaglia et al., 1993), allows the binding of FADD in a DD–DD interaction, which initiates apoptotic signaling by the recruitment and activation of caspase 8 or 10 by oligomerization. TNF-R1–induced activation of caspase 8 or 10 is less direct, involving recruitment of the DD-containing adaptor TRADD, followed by the formation of an internalized secondary complex which can bind FADD and caspase 8 to initiate the apoptotic program (Micheau and Tschopp, 2003).

Despite its name, most tumor cells do not die when exposed to TNFα but must also be treated with inhibitors of translation or transcription, such as actinomycin D or cycloheximide. These agents are thought to sensitize cells to TNFα by preventing production of survival proteins induced via NF-κB. Many of the TNFRSF members, including FN14, contain a consensus Tnf receptor-associated factor (TRAF) binding motif (Park et al., 1999; Ye et al., 1999) that recruits TRAFs to activate transcription factors including NF-κB and AP1 (Lee et al., 1997; Yeh et al., 1997).

TRAF1 and TRAF2 were initially identified in protein complexes that bound to the cytoplasmic domain of TNF-R2 (Rothe et al., 1994), together with cellular inhibitor of apoptosis...
FN14 is expressed by most tumor cell lines

To understand how endogenous cIAPs regulate TNFRSF signaling, we used TNFSF ligands to screen for cell lines containing detectable levels of endogenous TNFSF receptors. To facilitate the screen, we generated TNFSF ligands as recombinant proteins tagged with the Fc portion of human IgG (Fig. S1A, available at http://www.jcb.org/cgi/content/full/jcb.200801010/DC1; Bossen et al., 2006). These molecules are cross-linked via the Fc portion that promotes higher order aggregation of the corresponding receptors, closely mimicking engagement by membrane-bound ligands (Holler et al., 2003). The Fc portion also facilitates reliable detection of these proteins by Western blot (Fig. S1 B) and allows for their simple purification with protein A (Fig. S1 B, right) and for immunoprecipitation of interacting protein complexes (see results below).

We tested the purified Fc ligands for specific binding to their cognate receptor using FpIn stable cell lines inducibly expressing different TNFSF receptors and only observed binding when the ligand was added to cells in which expression of the corresponding cognate receptor was induced, i.e., CD27/CD70 and FN14/TWEAK (Fig. S1 C). Satisfied with the specificity of the ligands, we used them to screen a panel of tumor cell lines (including those from kidney, brain, colon, melanoma, breast, and ovarian cancers). Only one of the ligands, TWEAK, bound to a high proportion of the tumor cell lines examined (Fig. 1 A, Fig. S1 D, and not depicted), suggesting that in culture, many tumor cells constitutively express the TWEAK receptor FN14.

Some studies have suggested that TWEAK binds other receptors in addition to FN14 (Polek et al., 2003; Bover et al., 2007). To confirm that the signal caused by binding of TWEAK correlated with expression of FN14, we used a commercial antibody against FN14. The specificity of this FN14 antibody was demonstrated by flow cytometry using cells inducible for FN14 expression (Fig. 1 B). Importantly, cell lines that bound TWEAK also stained strongly with the antibody to FN14 (Fig. 1 C). These results demonstrate that a large number of transformed cell lines of both human and mouse origin constitutively express the TWEAK receptor FN14.

TWEAK binding to FN14 recruits TRAF2 and cIAP1

Because yeast two-hybrid screens suggested a potential interaction between TRAF2 and FN14 (Brown et al., 2003), we tested whether TRAF2 could interact with FN14 in vivo. Recombinant Fc-TWEAK successfully immunoprecipitated endogenous
TNFα signaling sensitizes tumor cells to TNFα via its TRAF2 binding domain. (A) Cells were harvested and incubated with Fc-CD70 or Fc-TWEAK, Tricolor-labeled anti-Fc, and analyzed by flow cytometry. (B) FN14 antibody specifically detects FN14. Stable Flp-In T-REx 293 cells inducible for FN14 were induced with or without doxycycline overnight, stained with anti-FN14, and analyzed as in A. Controls were stained with secondary antibody alone. (C) Cell lines that bind TWEAK also stain with antibodies to FN14. Cells were harvested and stained with the FN14 antibody as in B. (D) D645 cells were harvested and then treated for 15 min at 37°C with or without 2 μg Fc-TWEAK. Cells were lysed and Fc-TWEAK protein complexes precipitated with protein A beads and analyzed by Western blot. *: carryover signal from cIAP1 blot. (E) Binding of cIAP1 to FN14 is mediated by its association with TRAF2. D645 glioma cells were transfected with the indicated FLAG-cIAP1 constructs, harvested, and then treated with Fc-TWEAK for 20 min at 37°C. Cells were lysed and Fc-TWEAK complexes precipitated and analyzed as in D. Molecular mass is indicated in kDa on the left of the autoradiograph.

Figure 1. TWEAK specifically binds to endogenous FN14 in many tumor cell lines and cIAP1 binds to FN14 via its TRAF2 binding domain. (A) Cells were harvested and incubated with Fc-CD70 or Fc-TWEAK, Tricolor-labeled anti-Fc, and analyzed by flow cytometry. (B) FN14 antibody specifically detects FN14. Stable Flp-In T-REx 293 cells inducible for FN14 were induced with or without doxycycline overnight, stained with anti-FN14, and analyzed as in A. Controls were stained with secondary antibody alone. (C) Cell lines that bind TWEAK also stain with antibodies to FN14. Cells were harvested and stained with the FN14 antibody as in B. (D) D645 cells were harvested and then treated for 15 min at 37°C with or without 2 μg Fc-TWEAK. Cells were lysed and Fc-TWEAK protein complexes precipitated with protein A beads and analyzed by Western blot *: carryover signal from cIAP1 blot. (E) Binding of cIAP1 to FN14 is mediated by its association with TRAF2. D645 glioma cells were transfected with the indicated FLAG-cIAP1 constructs, harvested, and then treated with Fc-TWEAK for 20 min at 37°C. Cells were lysed and Fc-TWEAK complexes precipitated and analyzed as in D. Molecular mass is indicated in kDa on the left of the autoradiograph.
Because TWEAK did not induce proteasomal degradation of the cIAP1–TRAF2 complex, we tested other protease inhibitors. Cells preincubated with a protease inhibitor cocktail showed reduced TWEAK-mediated degradation of TRAF2 and a modest protection of cIAP1 when serum was removed from the medium before addition of the inhibitor (Fig. 2D). We therefore tested whether TWEAK-mediated TRAF2 and cIAP1 depletion was dependent upon lysosomal function. Consistent with this hypothesis, inhibitors of lysosomal function, such as chloroquine and ammonium chloride, prevented TWEAK-mediated TRAF2 degradation, whereas ammonium chloride also substantially blocked TWEAK-mediated cIAP1 degradation, although not to the same extent as it blocked TRAF2 depletion (Fig. 2, D and F).

To further test a role for lysosomal proteases, we used specific protease inhibitors. The serine protease inhibitor AEBSF failed to block TWEAK-mediated cIAP1–TRAF2 degradation, whereas TLCK, which can inhibit both serine and cysteine proteases, partially blocked TWEAK-mediated TRAF2 loss (Fig. 2E). The cathepsin B inhibitor CA-074Me (Fig. 2, E and F) also provided protection against loss of both cIAP1 and TRAF2, implying that lysosomal cathepsins may be important for the degradation of this complex. Importantly, neither CA-074Me nor the inhibitors of lysosomal function perturbed the proteasomal degradation pathway because they did not prevent the loss of cIAP1 induced by IAP antagonist (compound A) treatment (Fig. 2F), which we have previously shown is proteasomal dependent (Vince et al., 2007).

Although endogenous TRAF2 and cIAP1 was difficult to detect by confocal microscopy, analysis of D645 cells transiently transfected with FLAG-TRAF2 revealed that in unstim-
ulated cells, TRAF2 was exclusively cytosolic and did not overlap with the acidotropic lysosome marker lysotracker (Fig. S2, C and D). However upon stimulation with TWEAK ligand for 3–6 h, TRAF2 showed a significant redistribution to punctate vesicles (Fig. S2, C and E). TRAF2-containing vesicles were juxtaposed with lysotracker-stained compartments and often directly overlapped (Fig. S2 E), suggesting that TRAF2 degradation occurs in the lysosome or in compartments that are in close association. It is probable that the Triton X-100–insoluble fraction contains MVB/lysosomal membranes because the inhibitors NH4Cl and CA-074Me significantly blocked degradation of TRAF2 and cIAP1 in the Triton X-100–insoluble fraction (unpublished data).

TWEAK activates noncanonical NF-κB by depleting cIAP1 and TRAF2

TWEAK/FN14 signaling has previously been shown to activate both canonical and noncanonical NF-κB (Saitoh et al., 2003). Because TRAF2 knockout B cells and either immortalized cIAP1 or TRAF2 knockout MEFs show constitutive activation of noncanonical NF-κB (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200801010/DC1; Grech et al., 2004; Vince et al., 2007), we hypothesized that TWEAK-mediated NF-κB signaling may be a direct result of the depletion of cIAP1 and TRAF2 and noncanonical in nature, despite the fact that degradation of cIAP1 after TWEAK signaling is never complete.

To measure TWEAK-induced NF-κB activation, we created stable cell lines containing an NF-κB reporter, where expression of EGFP is driven by a promoter containing four NF-κB binding elements. As expected, NIH 3T3 cells bearing the NF-κB reporter showed strong NF-κB induction when stimulated with TNF (Fig. 3 A). TWEAK also induced a significant NF-κB response, although this was slower and not as large as the TNF response (Fig. 3 A). TWEAK-induced NF-κB was not dependent upon autocrine-produced TNFα because induction of NF-κB could not be blocked by anti-TNFα (unpublished data). To investigate whether NF-κB was noncanonical, we examined processing of the NF-κB2 subunit from the p100 form to the active, processed p52 form. In both OVCAR4 and KYM1 cell lines, processing of p100 to p52 became visible after 24 h. FN14 was induced overnight, the cells were treated with 100 ng/ml Fc-TWEAK for a further 24 h, and NF-κB activity was measured by flow cytometry. Histograms are representative of three independent experiments.

(E) NH4Cl blocks TWEAK-induced noncanonical NF-κB. Cells were pretreated with NH4Cl, CA-074Me, or MG132 for 2 h and then treated with 100 ng/ml TWEAK for the indicated times, lysed, and analyzed by Western blot. (F) Wild-type and knockout MEFs were treated with TWEAK for the indicated times, and lysates were analyzed as in E. Molecular mass is indicated in kD on the left of the autoradiograph.

Figure 3. TWEAK-induced cIAP1–TRAF2 loss activates noncanonical NF-κB. (A) An NIH 3T3 cell clone stably transformed with a lentiviral NF-κB reporter vector was stimulated with Fc-TNF or Fc-TWEAK for the indicated times. (B) KYM1 or OVCAR4 cells were stimulated with 100 ng/ml Fc-TWEAK for the indicated times and analyzed by Western blot for p100 processing to p52. (C) KYM1 or OVCAR4 cells were treated with or without Fc-TWEAK for 6 h and lysates were analyzed by Western blot. (D) FN14-inducible FlpIn 293 cells infected with the lentiviral NF-κB reporter were transiently transfected with the indicated constructs for 24 h. FN14 was induced overnight, the cells were treated with 100 ng/ml Fc-TWEAK for a further 24 h, and NF-κB activity was measured by flow cytometry. Histograms are representative of three independent experiments. (E) NH4Cl blocks TWEAK-induced noncanonical NF-κB. Cells were pretreated with NH4Cl, CA-074Me, or MG132 for 2 h and then treated with 100 ng/ml TWEAK for the indicated times, lysed, and analyzed by Western blot. (F) Wild-type and knockout MEFs were treated with TWEAK for the indicated times, and lysates were analyzed as in E. Molecular mass is indicated in kD on the left of the autoradiograph.
vation of NF-κB, we observed that TWEAK treatment caused a remarkable stabilization of NIK (Fig. 3, C and E), which correlated with processing of p100 to p52, but observed no change in NF-κB1 p105 processing (Fig. 3, C and E).

If TWEAK-induced loss of the cIAP1–TRAF2 complex is required to activate the noncanonical pathway, then genetic loss of either cIAP1 or TRAF2 might also result in constitutive activation of this pathway. Consistent with this model and our previous observations (Vince et al., 2007), MEFs deleted for either cIAP1 or TRAF2 showed elevated p52 levels and an increase in p52 localization to a nucleus-containing fraction (Fig. S3 A). In contrast, p50 localization was unaffected by loss of these genes and was predominantly present in the unprocessed p105 form in the cytoplasm (Fig. S3 A).

If depletion of the cIAP1–TRAF2 complex is sufficient to activate NF-κB, then overexpression of these two proteins should inhibit TWEAK/FN14-induced NF-κB activity. To test this hypothesis, we used FN14-inducible NF-κB EGF reporter cells in which maximal NF-κB activity was detected in cells that were simultaneously induced for FN14 expression and stimulated with TWEAK ligand and tested the effect of transiently transfecting cIAP1, TRAF2, or both (Fig. 3 D) in this system. Individual expression of either TRAF2 or cIAP1 failed to block TWEAK/FN14-induced NF-κB activation (Fig. 3 D). However, the overexpression of both proteins together significantly reduced the amount of NF-κB activation (Fig. 3 D). Importantly, this was dependent on cIAP1 binding to TRAF2, because coexpression of TRAF2 with the ΔBIR1 cIAP1 mutant that is unable to bind TRAF2 (Fig. S1 E), was unable to inhibit FN14/TWEAK-induced activation of NF-κB (Fig. 3 D). NIK stabilization and p100 processing to p52 could be blocked by pretreatment of cells with NH4Cl but not by pretreatment with CA-074Me (Fig. 3 E). This suggests that relocation to the lysosomal compartment is sufficient to trigger stabilization of NIK and subsequent processing of p100 rather than degradation in the lysosome per se.

Because TWEAK has been reported to activate the canonical pathway, we also examined the effects of TWEAK and cIAP1 or TRAF2 loss on canonical signaling markers. Consistent with previous observations (Saitoh et al., 2003), we observed TWEAK-induced rapid phosphorylation of IκB and p65. Loss of either cIAP1 or TRAF2 resulted in almost identical responses, with higher basal phosphorylation of IκB and p65 and a significantly delayed TWEAK-induced increase (Fig. 3 F). This highlights that the cIAP1–TRAF2 complex plays an important role in both NF-κB pathways induced by TWEAK.

**TWEAK induces cell death through NF-κB-dependent induction of TNFα.**

Tumor cell lines sensitive to synthetic IAP antagonists are killed through NF-κB–dependent autocrine production of TNFα (Vince et al., 2007). Moreover, it has been described that TWEAK can kill Kym1 cells in a TNFα-dependent manner (Schneider et al., 1999), although how TWEAK stimulated TNFα in Kym1 cells remains unknown. We therefore asked whether TWEAK acted in a similar manner to synthetic IAP antagonists by causing an increase in the abundance of TNFα driven through the activation of NF-κB.

![Figure 4](image-url)  
**Figure 4.** TWEAK-mediated NF-κB activation induces TNFα in TWEAK-sensitive lines. Kym1 (A) or SKOV3 and OVCAR4 (B) cells were treated with Fc-TWEAK for 8 or 24 h, respectively and the amount of TNFα in cell lysates was measured by ELISA. (C) Supernatant from TWEAK-treated SKOV3 and OVCAR4 cells was collected and filtered and TNFα was measured by ELISA. (D and E) SKOV3 and OVCAR4 cells containing inducible IκBα were induced or not before Fc-TWEAK treatment for 24 h. The levels of TNFα in the cell lysate (D) or cell supernatant (E) were measured by ELISA. Error bars represent SEM from three to five independent experiments.

We observed that the levels of TNFα in the cell lysate of TWEAK-treated cell lines increased significantly in all three cell types that are killed by TWEAK treatment alone (Fig. 4, A and B; and Fig 5 A) with a concomitant increase of TNFα released into the media supernatant (Fig. 4 C). In contrast, cell lines that are not killed by TWEAK treatment alone did not produce TNFα when TWEAK was added (unpublished data), suggesting that induction of TNFα is necessary for TWEAK to cause apoptosis.

To test whether activation of NF-κB by TWEAK/FN14 was required for the enhanced TNFα production observed in TWEAK-sensitive cell lines, we created stable inducible nondegradable IκBα (IκB superrepressor) SKOV3 and OVCAR4 cell lines. Induction of IκBα inhibited TWEAK-induced NF-κB activity (Fig. S3 A) and significantly reduced the TWEAK-dependent increase in levels of cellular and secreted TNFα in both SKOV3 and OVCAR4 cells (Fig. 4, D and E).

**Inhibition of TNFα signaling or caspase 8 blocks TWEAK/FN14 cell death.**

Previous work with synthetic IAP antagonists (Gaither et al., 2007; Varfolomeev et al., 2007; Vince et al., 2007) and the data presented here with TWEAK demonstrate that either treatment...
results in an increase in TNFα, which is driven by NF-κB. Remarkably, tumor cell lines that are killed by treatment with a synthetic IAP antagonist alone, such as OVCAR4, SKOV3, and Kym1 cells, (Vince et al., 2007) are also killed by TWEAK.

TWEAK killing of sensitive cell lines was prevented by TNFα-blocking antibodies but not by TRAIL- or Fas ligand–neutralizing antibodies in both short-term (Fig. 5 A), and long-term clonogenic survival assays (Fig. S4A), which is consistent with a conserved mechanism of cell death between synthetic IAP antagonist compounds and TWEAK. In addition, expression of the extracellular domain of TNF-R2 fused to a GPI-anchor (dnTNFR2), which is able to sequester and hence neutralize TNFα (Vince et al., 2007), significantly inhibited cell death caused by TWEAK (Fig. 5 B). In contrast, neither dnCD27 nor dnTRAIL-R2 had any protective effect (Fig. 5 B).

Caspase 8 activity was necessary for TWEAK to induce apoptosis because Kym1 and SKOV3 cell lines inducibly expressing the caspase 8 inhibitor crmA were significantly resistant to TWEAK killing in both short-term (Fig. 5 C) and long-term clonogenic survival assays with Kym1 cells (Fig. S4 B).

To provide a nongenetic test that TWEAK-driven NF-κB was sufficient to kill cells, we used Geldanamycin because it completely blocked TWEAK-induced NF-κB (Fig. S4 C). As has been shown before (Wang et al., 2006), inhibiting the IKK1/2 complex with Geldanamycin is sufficient to sensitize OVCAR4 and wild-type MEFs to TNFα (Fig. S4 E), presumably by blocking NF-κB–induced transcription of prosurvival genes (Wang et al., 2006). Remarkably, however, Geldanamycin was able to block TWEAK-induced NF-κB (Fig. S4, C and D) and TWEAK-induced cell death of Kym1 and OVCAR4 cells (Fig. S4 E). Moreover, although Geldanamycin-treated Kym1 cells showed reduced survival in long-term clonogenic growth assays, cells treated with TWEAK and Geldanamycin still showed clonogenic protection when compared with TWEAK treatment alone (Fig. S4 A).

Figure 5. TWEAK-induced cell death is mediated by TNFα. (A) TWEAK-induced death is blocked by neutralizing TNFα antibodies. Kym1, SKOV3, and OVCAR4 cells were incubated with Fc-TWEAK or Fc-CD27 (control) for 24 (Kym1) or 48 (SKOV3 and OVCAR4) h in the absence or presence of 10 μg/ml of neutralizing antibodies against TNFα, FasL, or TRAIL. Cell death was measured by propidium iodide staining and flow cytometry. (B) Dominant-negative (dn) TNF receptor blocks TWEAK-induced cell death. Cells containing inducible dominant-negative GPI-anchored TNF-R2, CD27, or TRAIL-R2 receptors were induced for 24 h before Fc-TWEAK treatment. Cell death was measured as in A. (C) TWEAK-induced death is blocked by crmA. CrmA-inducible Kym1 or SKOV3 cells were induced for 24 h before Fc-TWEAK or Fc-TNFα treatment for 24 (Kym1) or 48 (SKOV3) h. Cell death was measured as in A. All errors bars represent SEM of at least three independent experiments.
treatment reduced cIAP1 and TRAF2 levels, whereas TNF treatment alone had no effect (Fig. 6 C). Consistent with the lack of cell death (Fig. 6 A), the individual treatments of TWEAK or TNF did not alter caspase 8 cleavage (Fig. 6 C). In contrast, upon cotreatment of TWEAK with TNF, processing of caspase 8 into the p43/p41 forms and the active p18 subunit was observed within 3 h (Fig. 6 C) and correlated with the loss of cIAP1–TRAF2 and the rapid death of these cells (Fig. 6 A). To allow a direct comparison with our synthetic IAP antagonist, we also incubated D645 cells with compound A alone or compound A and TNF. Treatment with compound A alone resulted in the rapid loss of cIAP1 but did not affect either TRAF2 or caspase 8 levels (Fig. 6 C). Significantly, cIAP1 loss alone was sufficient to sensitize D645 cells to TNF to a similar level as that of TWEAK-induced depletion of the cIAP1–TRAF2 complex (Fig. 6 C).

To test this hypothesis, exogenous TNFα was applied to TWEAK-sensitive (OVCAR4) and -resistant (D645 and MEF) cell lines alone or in combination with TWEAK for 24 h. Consistent with an additional sensitizing role for TWEAK, OVCAR4 cells were killed by TWEAK/TNF treatment far more efficiently and rapidly than with TWEAK alone. Even more significantly, D645 and MEF cells (among many other cell types; not depicted) were resistant to treatment with TWEAK or TNF alone but were extremely sensitive to combined TWEAK/TNF treatment (Fig. 6 A and B). Even a subset (2/12) of primary human tumor lines was significantly sensitized to TNF treatment (Fig. 6 A). TWEAK sensitization to TNFα killing was examined further by Western blot on the TWEAK (and TNFα)-resistant D645 glioma cell line. As in TWEAK-sensitive cell lines, TWEAK treatment reduced cIAP1 and TRAF2 levels, whereas TNF treatment alone had no effect (Fig. 6 C). Consistent with the lack of cell death (Fig. 6 A), the individual treatments of TWEAK or TNFα did not alter caspase 8 cleavage (Fig. 6 C). In contrast, upon cotreatment of TWEAK with TNFα, processing of caspase 8 into the p43/p41 forms and the active p18 subunit was observed within 3 h (Fig. 6 C) and correlated with the loss of cIAP1–TRAF2 and the rapid death of these cells (Fig. 6 A). To allow a direct comparison with our synthetic IAP antagonist, we also incubated D645 cells with compound A alone or compound A and TNF. Treatment with compound A alone resulted in the rapid loss of cIAP1 but did not affect either TRAF2 or caspase 8 levels (Fig. 6 C). Significantly, cIAP1 loss alone was sufficient to sensitize D645 cells to TNF to a similar level as that of TWEAK-induced depletion of the cIAP1–TRAF2 complex (Fig. 6 C).
Further evidence supporting the observation that TWEAK/TNFα kill in a death receptor–dependent pathway was obtained using FADD−/− MEFs, as these were completely resistant to TWEAK/TNFα-induced death (Fig. 6 D). In contrast, TWEAK/TNFα killing was independent of the Bax/Bak-dependent apoptotic pathway, as Bax/Bak double knockout MEFs showed a similar TWEAK/TNFα sensitivity to wild-type MEFs (Fig. 6 D).

As expected, cIAP1−/− (Vince et al., 2007), TRAF2−/−, and TRAF2/TRAF5−/− double knockout or compound A–treated MEFs were all extremely sensitive to killing by TNFα alone (Fig. 6 E; Tada et al., 2001), supporting the hypothesis that TWEAK-induced loss of the cIAP1–TRAF2 complex is sufficient to sensitize MEFs to TNFα killing. Surprisingly, cIAP2−/− MEFs were not sensitive to TNFα-mediated cell death (Fig. 6 E), making it unlikely that cIAP2 has a role in TWEAK-mediated sensitization to TNFα.

Pretreating wild-type MEFs with TWEAK for 8 h before addition of TNFα caused a reduction in the total canonical response. However simultaneous treatment with TWEAK/TNFα resulted in an augmented canonical response (Fig. S5 A, available at http://www.jcb.org/cgi/content/full/jcb.200801010/DC1), making it unlikely that a reduction in prosurvival NF-κB signal from TNFα is the reason for TWEAK-induced sensitization to TNFα when the two cytokines are added simultaneously. Consistent with this data, pretreating wild-type MEFs with either TNFα or TWEAK alone for 24 h before cotreatment with TWEAK/TNFα or compound A/TNFα did not change the amount of cell death observed when cells were cotreated for the same time period (Fig. S5 B). This suggests that the prosurvival signals elicited by TNFα, such as NF-κB–induced gene transcription, are not sufficient to counteract TWEAK/TNFα killing.

TWEAK/TNFα treatment distinguishes between normal and transformed cells

Genetic knockout cIAP1 mice display no obvious phenotypic defects in apoptotic signaling (Conze et al., 2005; unpublished data), raising the possibility that primary cells may be less sensitive to TWEAK/TNFα–induced death. Consistent with this possibility, primary MEFs showed only a twofold increase in death after TWEAK/TNFα stimulation, whereas a 14-fold increase was observed in SV40T immortalized MEFs (Fig. 7 A). TWEAK-induced loss of cIAP1–TRAF2 was observed in both primary MEFs and transformed MEFs (Fig. 7 B), as was activation of noncanonical NF-κB (Fig. 7 B). Although similar levels of FN14 were initially present in both MEF lines, these increased dramatically after TWEAK stimulation (Fig. 7 B), implying that FN14 expression is regulated by TWEAK.

Figure 7. TWEAK/TNFα treatment distinguishes between normal and transformed cells, and TWEAK/TNFα killing is suppressed by elevated cIAP1–TRAF2 levels. (A) Primary MEFs are resistant to TWEAK/TNFα–induced death. Three primary MEF cell lines derived from three separate embryos were treated with 100 ng/ml Fc-TWEAK and/or 100 ng/ml Fc-TNFα for 24 h, and the amount of cell death was measured by propidium iodide staining and flow cytometry. Error bars are SEM of three to five independent experiments. (B) TWEAK increases FN14 expression and induces cIAP1–TRAF2 degradation in primary and transformed MEFs. Primary MEFs or SV40T transformed MEFs were treated with 100 ng/ml Fc-TWEAK for the indicated times, and DISC lysates were analyzed by Western blot. (C) PIL2 cells or PIL4 cells were treated with 100 ng/ml Fc-TWEAK and/or 100 ng/ml Fc-TNFα for 24 h and cell death was analyzed as in A. Error bars are SEM of three independent experiments. (D) Fluorescence microscopy. Bars, 20 μm. (E) Enhanced cIAP1 and TRAF2 levels in PIL2 cells are resistant to TWEAK-induced degradation. PIL2 and PIL4 cells were treated with 100 ng/ml Fc-TWEAK for the indicated times and lysates were analyzed by Western blot. Molecular mass is indicated in kD on the left of the autoradiograph.
complex to lysosomes and subsequent degradation. In cell lines that can be killed by TWEAK, NF-κB induces production of TNFα and simultaneously sensitizes tumor cells to TNF-R1–induced death. Transformation of MEFs renders these cells significantly more sensitive to killing by TWEAK/TNFα than their nontransformed progenitors, but tumor cells that express high levels of cIAP1–TRAF2 are comparatively resistant to TWEAK/TNFα killing (Fig. 8). Our data demonstrate that tumor cell sensitivity to TWEAK correlates with their sensitivity to synthetic IAP antagonists and that the mechanism of tumor cell killing between this class of chemical compounds and naturally occurring ligand is remarkably similar. Synthetic IAP antagonists target cIAP1 and cIAP2 for complete proteasomal degradation (Gaither et al., 2007; Varfolomeev et al., 2007; Vince et al., 2007), whereas TWEAK targets a proportion of the cIAP1–TRAF2 complex (presumably the proportion that can be recruited to FN14) for degradation in a lysosomal cathepsin-dependent manner. The end result is, however, the same, with activation of NF-κB. In sensitive tumor cell lines, activated NF-κB drives autocrine TNFα production (Fig. 8).

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Because TWEAK-mediated loss of the cIAP1–TRAF2 complex is sufficient to sensitize tumor cells to TNFα-induced death, we tested whether a liver progenitor tumor cell line, PIL2, which expresses high levels of the cIAP1–TRAF2 complex (Fig. 7E), was resistant to TWEAK/TNFα killing relative to a liver progenitor cell line, PIL4, with lower levels (Fig. 7E). Treatment of these cells with TWEAK/TNFα killed >90% of PIL4 cells, whereas only 35% of PIL2 (cIAP1 high) cells were killed (Fig. 7, C and D). Western blot analysis showed that PIL2 and PIL4 cells expressed equal levels of FN14 (Fig. 7E). Significantly, TWEAK-induced degradation of cIAP1–TRAF2, and increased FN14 levels, were attenuated in the PIL2 cells, implying that enhanced expression of cIAP1–TRAF2 inhibits FN14 signaling and counters TWEAK-induced sensitivity to TNFα-induced death.

**Discussion**

Recent work using synthetic IAP antagonists has shown that cIAPs play a pivotal role in regulating NF-κB signaling from TNF-R1 (Gaither et al., 2007; Varfolomeev et al., 2007; Vince et al., 2007). However, the binding of cIAP1 to TNF receptors other than TNF-R1 or TNF-R2, or the physiological regulation of cIAP1 by TNF receptor signaling, remains poorly characterized. In this paper, we show that when ligated with TWEAK, endogenous FN14 recruits a cIAP1–TRAF2 complex that is subsequently degraded by a cathepsin-mediated lysosomal pathway. TWEAK/FN14 signaling results in both canonical and noncanonical NF-κB activity. Noncanonical NF-κB activity is most probably the result of relocation of the cIAP1–TRAF2 complex to lysosomes and subsequent degradation. In cell lines that can be killed by TWEAK, NF-κB induces production of TNFα and simultaneously sensitizes tumor cells to TNF-R1–induced death. Transformation of MEFs renders these cells significantly more sensitive to killing by TWEAK/TNFα than their nontransformed progenitors, but tumor cells that express high levels of cIAP1–TRAF2 are comparatively resistant to TWEAK/TNFα killing (Fig. 8).

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pletely unexpected that inhibition of the proteasome did not prevent TWEAK/FN14-mediated loss of cIAP1–TRAF2, although TRAF2 depletion required cIAP1 function. However, TWEAK/FN14-induced cIAP1–TRAF2 degradation was prevented by several different classes of inhibitors of lysosome proteases or function and could be specifically blocked by an inhibitor of the lysosome cysteine protease cathepsin B. Consistent with this observation, TWEAK mediated relocalization of TRAF2 to punctate vesicles that often overlapped with, and were in contact with, lysotracker-stained vesicles.

Given previous reports implicating the proteasome in TRAF2 degradation, it remains unclear whether other TNFSF ligands can also stimulate cIAP1–TRAF2 loss through lysosomal mechanisms. However, it has been demonstrated that CD30-induced TRAF2 degradation was blocked by nonproteosomal inhibitors (Duckett and Thompson, 1997). It is likely that lysosomal degradation of the cIAP1–TRAF2 complex occurs by the well documented multivesicular body (MVB) pathway, whereby endocyted cell surface material is further internalized to form endosomal MVBs, which subsequently fuse with lysosomes (Fig. 8; Williams and Urbé, 2007). The finding that cIAP1 was required for TRAF2 degradation raises the possibility that cIAP1-mediated ubiquitination of TRAF2/FN14 targets the FN14 complex to the MVB pathway, as has previously been demonstrated for ubiquitin-dependent MVB targeting of several cell surface receptors. Depletion of cIAP1–TRAF2 by TWEAK/FN14 is the most likely cause of noncanonical NF-κB activation. Both cIAP1 and TRAF2 are required to inhibit noncanonical NF-κB activation from TNF receptors, and genetic knockout of either component alone results in spontaneous noncanonical NF-κB activity. Underlining the requirement for their concerted action, overexpression of either cIAP1 or TRAF2 alone does not affect FN14 signaling, but overexpression of both components is able to block TWEAK-induced NF-κB.

The NF-κB activity observed after FN14 signaling resulted in an increase in production of TNFα in TWEAK-sensitive cells but not in TWEAK-resistant cells. TWEAK-resistant cell lines were nevertheless sensitized to exogenously added TNFα, emphasizing that it is the production of autocrine TNFα that determines sensitivity of cells to TWEAK. This finding is consistent with previous observations that activation of TNF-R2, CD30, or CD40 can also induce TNFα and enhance TNF-R1 apoptotic signaling (Grell et al., 1999).

We demonstrated TWEAK binding and FN14 expression on both primary and tumor cells in culture. Surprisingly, however, primary nontransformed MEFs were insensitive to TWEAK/TNFα-induced death, whereas the same MEFs transformed by SV40 Large T were efficiently killed by this treatment. It is not clear why tumor cells show heightened sensitivity to TWEAK/TNFα-induced apoptosis, as TWEAK stimulation of primary MEFs still resulted in the loss of cIAP1–TRAF2 and NF-κB activation.

Not all tumor cell lines were sensitive to TWEAK/TNFα treatment. In particular, PIL2 liver progenitor oval cells that express much higher levels of cIAP1 and TRAF2 than PIL4 cells were resistant to TWEAK/TNFα-induced death. TWEAK stimulation of PIL4 cells resulted in the loss of cIAP1–TRAF2, and these cells were highly sensitive to TWEAK/TNFα killing. In contrast, the increased levels of cIAP1–TRAF2 in the tumorigenic PIL2 cells appeared to block FN14 signaling, as the cIAP1–TRAF2 complex was resistant to TWEAK-induced degradation and the cells were more resistant to TWEAK/TNFα-induced death. These results are also consistent with our findings that the overexpression of cIAP1 and TRAF2 is sufficient to block FN14-induced NF-κB activity.

The fact that the cIAP1-cIAP2 locus is amplified in some human tumors and a mouse model of liver cancer (Zender et al., 2006) and that high levels of expression of cIAP1 have been observed in several cancers argues that increased cIAP1 can contribute to oncogenesis. Our results suggest that one possible mechanism by which it does is by regulating the balance between life and death signaling from TNFSF receptors. Our findings provide further support for using IAP antagonists as tumor therapy and extend options by highlighting the fact that TWEAK is a physiological regulator of TNFSF signaling that targets the cIAP1–TRAF2 complex rather than the IAPs alone.

Materials and methods

Cell lines, transient transfections, antibodies, and protease inhibitor

SW480, K562, MCF7, BJAB, G401, ColoF, and NIH 3T3 cell lines were a gift from L. O’Reilly (The Walter and Eliza Hall Institute, Melbourne, Australia; O’Reilly et al., 2000, 2002), RM, WW, and A2058 were a gift from P. Hersey (Calvary Mater Newcastle Hospital, Newcastle, Australia; Zhang et al., 1999), and HT29, SKOV3, OVCAR4, and MDAMB231 were purchased from American Type Culture Collection. Kym1 cells were a gift from M. Grell (ICBI, Stuttgart, Germany; Grell et al., 1999). D2234 and D2247 early passage lines were derived from specimens obtained from patients who had undergone tumor resection at Duke University Hospital (Durham, NC; Ashley et al., 2008). Transient transfections (typically using 1 μg of plasmid DNA per 10 cm plate of cells) were performed with effectene as described by the manufacturer (QIAGEN). Antibodies used in this study for flow cytometry were anti-FN14 (Abcam), Goat anti–mouse RPE (Millipore), and Goat anti–human RPE (SouthernBiotech). Antibodies used for Western blots were Goat anti–human IgG HRP (Jackson Immunoresearch Laboratories), anti-cIAP1 (in house), anti-TRAF2 (Santa Cruz Biotechnology, Inc.), anti-FN14 (Cell Signaling Technology), anti-NIK (Cell Signaling Technology), anti–phospho–NF-κB (Ser536) p65 (Cell Signaling Technology), anti-κB, (Cell Signaling Technology), anti–phospho-κB (Ser32/36; Cell Signaling Technology), anti–NF-κB p65 (Santa Cruz), anti–NF-κB2 (Cell Signaling Technology), anti–NF-κB p50 (Santa Cruz), anti-NIK (Sigma-Aldrich), anti–ubiquitin (Cell Signaling Technology), anti–TRAF3 (BD Biosciences), anti–FLAG M2 (Sigma-Aldrich), anti–IgG bio- tin (Jackson Immunoresearch Laboratories), and anti–GS–VSV (MBL International). Protease inhibitor cocktail final concentrations were the following: AEBSF, 1.3 μM; aprotinin 1.1 μM; benaztin, 66 μM; E-64, 20 μM; leupeptin, 27 μM; and pepstatin A, 13 μM.

Cell culture and lentivirus production

All cell lines were maintained in DME supplemented with 10% FCS, 2 mM l-glutamine, and penicillin/streptomycin and grown at 37°C in 5% CO2. PIL2, PIL4, and BMOL liver progenitor cells were maintained in Williams Media E supplemented with 2 mM l-glutamine, penicillin/streptomycin, 20 ng/ml mouse EGF, 30 ng/ml human IGF II, and 0.25 U/ml human insulin and grown at 37°C in 5% CO2.

To generate lentiviral particles, 293T cells were transfected with packaging constructs pCMV ΔR8.2, VSVg, and the relevant lentiviral plasmid in the ratio of 1:0.4:0.6. After 24–48 h, 0.45 μM of the virus-containing supernatants were harvested and filtered. 12 μg/ml Polybrene was added and target cells were infected with virus supernatant for 24–48 h. The media was subsequently changed and successful infection selected for with 2–5 μg/ml puromycin (pf 5xUAS selection) or 100–500 μg/ml hygromycin B (GEV16 selection) or by screening for GFP fluorescence (pTRH). pf 5xUAS-inducible constructs were induced with 100 nM A2OQ2.
ting or before death assays. Flp In T-Rex 293 cells (Invitrogen) containing doxycycline-inducible VSV-tagged FN14 were generated according to the manufacturer’s instructions.

Con structs
The NF-κB lentiviral reporter vector pRH1 mCMV NF-κB dscGFP was pur- chased from System Biosciences. Cre recombinase and SV40 Large T anti gen were cloned into the lentiviral vector pFU. In the inducible lentiviral system, the inducible transcriptional activator Gal4 1–147 ERZ2 VP16 (GEV16) was cloned into pFU PGK Hygro, and the genes dnTNF-R2, dn-TRAIL-R2 or dnCd72 (Bossen et al., 2006), IκBα super repressor (Van Ant werp et al., 1996), and N-Flag cma, were cloned into a pF5xUAS SV40 Puro vector. D. Baltimore provided us with pFU and lentiviral packaging components, Theodor Mantamadiotis (Monash University, Melbourne, Australia) provided the ER2 construct. Complete sequence of all constructs can be obtained upon request.

Generation of MEFs
Knockout MEFS were generated from embryonic day–15 embryos from wild-type and XAP–/–; mice using standard procedures and infected with SV40 Large T antigen-expressing lentivirus. cIAP1, cIAP2, or TRAF2 condi tional knockout MEFS were similarly generated from cIAP1 LoxP/LoxP or TRAF2 LoxP/LoxP embryonic day–15 embryos. To delete cIAP1 or TRAF2, the transformed MEFS were infected with a cre-expressing lentivirus (pFU cre SV40 pur), and deletion was confirmed by PCR and Western blotting. To delete cIAP2, the transformed MEFS were infected with a Flp-express ing lentivirus (pFU FlpE PGK Hygro). W.-C. Yeh (University of Toronto, Toronto, Canada) provided FADD knockout MEFS, H. Nakano (Juntendo University, Tokyo, Japan) provided TRAF2/TRAF5 double knockout MEFS, and D. Huang (The Walter and Eliza Hall Institute, Melbourne, Australia) provided Box/Bak double knockout MEFS.

Immunofl ow cytometry
Approximately 3 × 10^5 cells were harvested, washed in PBS, and resus pended in buffer (KDS/BSS containing 3% FCS) on ice. 500 ng Fc-TWEAK or 1 µg FN14 antibody was incubated with cells on ice for 20 min, and then the cells were washed and incubated with anti–human IgG biotin for 20 min on ice, followed by Streptavidin Tri-color (for Fc-TWEAK staining) or anti–mouse IgG-RPE (for anti-FN14 staining). Cells were washed and then analyzed by flow cytometry.

Death assays
Cells were seeded on 12-well tissue culture plates at ∼40% confluency and were allowed to adhere for 16–20 h. Compound A (5 nM Kym1 cells or 500 nM of all other cell types), 70 ng/ml of human Fc-TNFα, or 500 nM of human Fc-TWEAK were added to cells for 24 or 48 h, and cell death was measured by propidium iodide staining and flow cytometry. In each sample, 10,000 events were measured and the cell death (percentage of propidium iodide-positive cells) was quantified.

Western blotting and immunoprecipitations
For immunoprecipitation of endogenous FN14, cells were grown on 15-cm tissue culture plates and, when approaching confluency, were harvested and resuspended in 800 µl of ice-cold DME. 1.6 µg Fc-TWEAK was added for 30 min on ice (or indicated times) at 37°C. Cells were subsequently washed in ice-cold PBS and lysed in DISC buffer (1% Triton X-100, 10% glycerol, 150 mM NaCl, 20 mM Tris, pH 7.5, 2 mM EDTA, and complete protease inhibitor cocktail [Roche] on ice. Cell lysate was spun at 14,000 g for 10 min, and the soluble material was preclarified with Sepharose 6B beads at 4°C for 1 h. Fc-TWEAK–bound material was immunoprecipitated by adding the preclarified lysate to EZI View Protein A–agarose (Sigma-Ald rich) for 1–2 h at 4°C. Samples were washed four times with DISC buffer and then eluted with 1% SDS and β-mercaptoethanol at 95°C for 3 min. Samples were separated on 4–20% Bio-Rad Laboratories or 4–12% Invit rogen) polyacrylamide gels and transferred to nitrocellulose membranes for antibody detection. All membrane-blocking steps and antibody dilu tions were performed with 5% skim milk in PTBS (PBS containing 0.1% Tween 20), and washing steps were performed with PTBS. Proteins on Western blots were visualized by ECL (GE Healthcare) after incubation of membranes with HRP-coupled secondary antibodies.

Immunoprecipitation of FLAG-tagged cIAP1 constructs or VSV tagged FN14 were performed similarly, except one 10-cm plate of cells was used per immunoprecipitation, and anti–FLAG M2–conjugated agar ose (Sigma-Aldrich) or anti-VSV-conjugated agaro se (Sigma-Aldrich) were used for immunoprecipitation of the relevant proteins.

Nuclear fractionation
Cells were harvested, washed in PBS, and resuspended in lysis buffer (10 mM hepes-KOH, pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.5 mM DTT, and protease inhibitor cocktail) and incubated on ice for 15 min. The lysis buffer was adjusted to 0.6% NP-40 and immediately vortexed for 10 s, and the pellet (membrane) and supernatant (cytosol) fractions were sepa rated by centrifugation at 14,500 g for 5 min. Equal amounts of mem bran (nuclear) and cytosolic fractions were analyzed by SDS-PAGE and Western blotting.

EUSA assays
Cells were grown on 10-cm plates, harvested, washed thoroughly with ice-cold PBS, and lysed in 300 µl DISC buffer for 20 min on ice. Cell lys ate was spun for 10 min at 14,000 g, and the soluble material was col lected. Alternatively, the cell supernatant from the same plates was collected and filtered to remove cellular debris. Soluble cell lysate or the filtered cell supernatant was used for human or mouse TNFα ELISA as says (mouse; HSTA00; R&D Systems) according to the manufacturer’s protocol. Protein from the cell lysate was quantified using the BCA assay (Thermo Fisher Scientific).

Immunofluorescence, image acquisition, and processing
D645 cells grown on glass coverslips were fixed with 3.2% PFA for 20 min, washed in PBS, and permeabilized with 0.5% Triton X-100 for 5 min. Cells were blocked, incubated with primary antibody, washed four times with PBS, and then incubated with anti–rat or anti–mouse Alexa Fluor 488–conjugated secondary antibody (Invitrogen) and washed four times again. All blocking steps and antibody incubations were performed with PBS containing 1% BSA for 30 min. The primary antibodies used were anti-FLAG (Amrad) or anti-TRAF2 (BD Biosciences). Cells were viewed on an inverted confocal microscope (TCS-S2P2; Leica) using a 63x 1.4 NA oil immersion objective at room temperature. Images were collected and analyzed with SP2 imaging software (Leica) and Image software (National Institutes of Health; http://rsb.info.nih.gov/ij). All images were in TIF format and imported into Freehand MX (Macro media) for the compilation of figures.

Online supplemental material
Fig. S1 shows that Fc-TWEAK binds to a large selection of adherent trans formed cell lines. Fig. S2 shows analysis of TWEAK-induced cIAP1–TRAF2 degradation. Fig. S3 shows that C3a induces proinflammatory gene expres sion in BEAS-2B cells. We thank Jurg Tschopp for generous provision of materials and advice, Wen Chien Yeh for FADD knockout MEFS, Hiroko Nakano for TRAF2/TRAF5 double knockout MEFS, David Vaux for pFU and lentiviral packaging components, Theo Mantamadiotis for the ERT2 construct, Robert Geri for early work with the GAL4 ERT2 VP16 system, David Huang for Box/Bak double knockout MEFS, and an anonymous reviewer for their suggestions. J. Silke is supported by NHMRC grants (433013 and 356256). D.L. Vaux is an Australian fellow, funded by the Leukemia and Lymphoma Society and an NHMRC grant (AJ1221). M. McKinlay, C.A. Benetatos, S.M. Condon, and SK. Chunduru are employees of TetraLogic Pharmaceuticals. M. McKinlay, C.A. Benetatos, S.M. Condon, and SK. Chunduru are employees of TetraLogic Pharmaceuticals. D.L. Vaux is on the scientific advisory board. We thank Jurg Tschopp for generous provision of materials and advice, Wen Chen Yeh for FADD knockout MEFS, Hiroko Nakano for TRAF2/TRAF5 dou ble knockout MEFS, David Baltimore for pFU and lentiviral packaging con structs, Theo Mantamadiotis for the ERT2 construct, Robert Geri for early work with the GAL4 ERT2 VP16 system, David Huang for Box/Bak double knockout MEFS, and an anonymous reviewer for their suggestions.

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