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The KSHV oncoprotein vFLIP contains a TRAF-interacting motif and requires TRAF2 and TRAF3 for signalling

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Primary effusion lymphomas (PELs) characterized by infection with the Kaposi's sarcoma herpesvirus (KSHV; also called human herpesvirus 8) depend on the expression of the viral FADD-like interleukin-1-β-converting enzyme (FLICE)/caspase-8-inhibitory protein (vFLIP) for their survival. This effect is achieved by activation of the transcription factor nuclear factor-κB (NF-κB). Tumour necrosis factor (TNF) receptor-associated factors (TRAFs) are direct mediators of NF-κB signalling by TNF family receptors and the Epstein–Barr virus oncoprotein latent membrane protein 1 and so we assessed the role of TRAFs in signalling by vFLIP. Here, we report the identification of a TRAF-interacting motif (PYQLT) in vFLIP, which is not present in other FLIP molecules. We show that vFLIP directly binds to TRAF2 *in vitro* and in PEL cells. TRAF2 and TRAF3 are required for induction of NF-κB and associated cell survival, as well as Jun amino-terminal kinase phosphorylation by vFLIP, whereas TRAF1, TRAF5 and TRAF6 are dispensable. Mutations in the P93 or Q95 amino acids within the TRAF-interacting motif of vFLIP abolish its ability to bind to TRAF2 and to signal to NF-κB. TRAF2, but not TRAF3, mediates the association of vFLIP with the IκB kinase complex. These data indicate that vFLIP uses TRAF2 and TRAF3 for signalling to NF-κB, which is crucial for KSHV-associated lymphomagenesis.

Keywords: Kaposi's sarcoma-associated herpesvirus; human herpesvirus 8; tumour necrosis factor receptor-associated factor; viral FADD-like interleukin-1-β-converting enzyme (FLICE)/caspase-8-inhibitory protein; nuclear factor-κB

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INTRODUCTION

Kaposi's sarcoma herpesvirus (KSHV), also called human herpesvirus 8, is causally associated with Kaposi's sarcoma (Chang *et al*, 1994) and several lymphoproliferative diseases that include primary effusion lymphoma (PEL; Cesarman *et al*, 1995a) and multicentric Castleman's disease (Soulier *et al*, 1995). The mechanisms by which KSHV causes these diseases are complex, and are probably distinct among the different diseases associated with infection by this virus. Studies to understand the molecular pathogenesis of KSHV-associated lymphomas have shown that the transcription factor nuclear factor-κB (NF-κB) is constitutively active and is essential for the survival of PEL cells (Keller *et al*, 2000), and that the viral gene responsible for this activity is vFLIP (viral FADD-like interleukin-1-β-converting enzyme (FLICE)/caspase-8-inhibitory protein; Chaudhary *et al*, 1999; An *et al*, 2003; Guasparri *et al*, 2004). We have recently shown that vFLIP is essential for the survival of PEL cells, and therefore functions as a viral oncogene in KSHV lymphomagenesis (Guasparri *et al*, 2004). The importance of vFLIP in PEL cell survival *in vivo* has also been demonstrated (Godfrey *et al*, 2005).

vFLIP is part of a group of viral and cellular proteins that have recently received significant attention as inhibitors of death receptor-induced apoptosis, called FLIP (Liu *et al*, 2002). FLIP proteins contain two death effector domains (DEDs) and were shown to inhibit DED–DED interactions between FADD and procaspases 8 and 10. Two forms of cellular FLIP have been identified and characterized—$cFLIP_L$ (long form) and $cFLIP_S$ (short form)—which correspond to splice variants. Several viruses contain proteins with homology to cFLIP. One of these is ORF71/K13 from KSHV (Thome *et al*, 1997; Chaudhary *et al*, 1999). KSHV vFLIP has two DEDs, and structurally most closely resembles $cFLIP_S$. It has been shown to bind to other DED-containing proteins FADD and caspases 8 and 10, and to inhibit activation of these caspases by preventing their association with FADD, therefore inhibiting apoptosis when transfected into a T-cell lymphoma cell line (Djerbi *et al*, 1999). Chaudhary *et al* (1999) showed that vFLIP can activate NF-κB when ectopically expressed in 293T cells, but that other viral FLIP homologues tested did not. Although $cFLIP_L$ was also shown to be able to

activate NF-κB, vFLIP is a much more potent inducer of this transcription factor in several cell lines, including those of B-cell origin (Chaudhary *et al*, 1999; Guasparri *et al*, 2004). vFLIP is present in a large macromolecular IκB kinase (IKK) complex, which has also been reported to contain IKKα, IKKβ, NEMO/IKKγ, RIP and the chaperone protein Hsp90 (Liu *et al*, 2002; Field *et al*, 2003). KSHV vFLIP has also been shown to affect proliferation of PEL cells by activating the NF-κB alternative pathway (Matta & Chaudhary, 2004). Even though all these studies have shed light on some of the mediators of vFLIP signalling, this process is still not well understood, and it is unclear how a relatively simple adaptor molecule, containing only two DEDs, can lead to activation of the IKK complex.

One important set of adaptor molecules, clearly involved in signalling from tumour necrosis factor (TNF) receptors to the IKK complex, is the TNF receptor-associated factor (TRAF) family. All TRAFs with the exception of TRAF4 have been implicated in signalling in B cells (Bishop, 2004). Several TRAFs have also been shown to bind to and colocalize with Epstein–Barr virus (EBV) latent membrane protein 1 (LMP1), but only TRAF6 (Luftig *et al*, 2003) and TRAF3 (Xie *et al*, 2004) have been shown to be essential for NF-κB activation by LMP1. As KSHV vFLIP seems to resemble EBV LMP1 in its ability to activate NF-κB, as well as Jun amino-terminal kinase (JNK) phosphorylation (An *et al*, 2003), it is possible that TRAFs mediate this function. One study showed that vFLIP can bind to TRAFs, in a yeast two-hybrid assay and in transfected 293T cells (Chaudhary *et al*, 1999), but another study failed to confirm this association (Field *et al*, 2003). Here, we report the identification of a TRAF-binding motif in vFLIP, and we show that both TRAF2 and TRAF3 are essential for vFLIP induction of NF-κB, but through different mechanisms.

RESULTS

vFLIP contains a TRAF-binding motif

A sequence consistent with a TRAF-interacting motif, PYQLT, was identified in the second DED of vFLIP. This is imperfect according to the major structure-based consensus sequence (P/S/A/T)x(Q/E)E, but it is in agreement with the reported binding site for CD40, PXQX(S/T), and the consensus sequence found in LMP1, PxQxT (Franken *et al*, 1996). Alignment with other FLIP homologues showed that this motif is unique to KSHV vFLIP (supplementary Fig 1 online). To test whether this motif is involved in NF-κB activation, we made three conservative single-base-pair mutations in the first, third and fifth consensus amino acids of this motif, as indicated in Fig 1. Specific substitutions were chosen to replace the wild-type sequence to represent conservative changes or amino acids that are present in the same position in other DEDs, not to disrupt this adaptor function. Plasmids encoding these mutations were transfected into Namalwa cells, together with an NF-κB luciferase reporter plasmid. Changes in the P93 and Q95 residues, structurally predicted to be more important for TRAF binding than T97, severely impaired the ability of vFLIP to activate NF-κB (Fig 1A). Impairment of function and equal expression of the mutant constructs was confirmed using vFLIP–Flag fusion proteins (supplementary Fig 2 online). Computer modelling of the second DED of vFLIP showed that both P93 and Q95 are exposed on the surface and available for interaction with TRAFs (Fig 1B). This result suggests that P93 and Q95 are essential for vFLIP signalling to NF-κB, whereas T97 is dispensable for this effect.

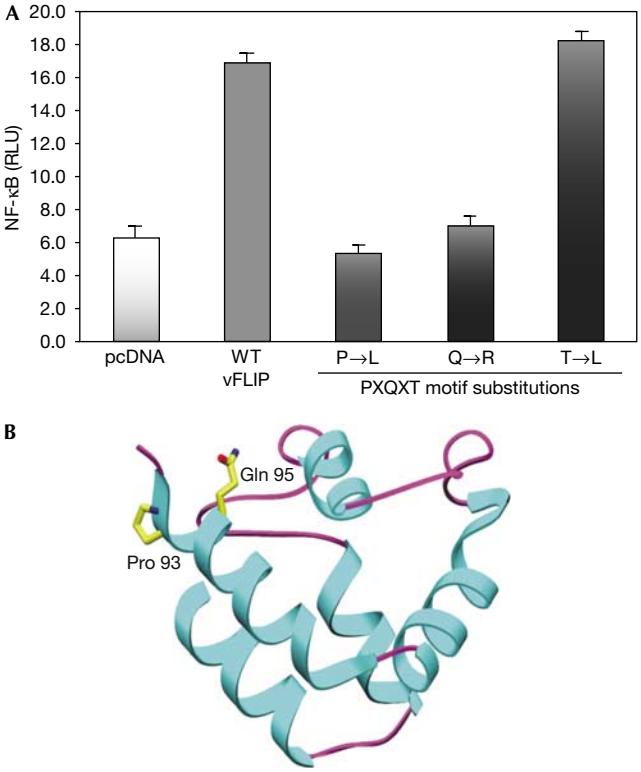

Fig 1 | Mutations in the TRAF-interacting motif abolish nuclear factor-κB signalling by vFLIP. (**A**) Namalwa cells were transfected with wild-type (WT) vFLIP or the indicated vFLIP mutants, and an nuclear factor-κB (NF-κB) reporter plasmid. Luciferase assays were carried out 48 h after transfection. Values are averages (±s.e.m.) of one representative experiment in which each transfection was carried out in triplicate, normalized with transfected *Renilla* luciferase (pRSV-RL), and reported as relative luciferase units (RLUs). Mutations of P93 or Q95 amino acids in the PxQxT motif of vFLIP abolish its NF-κB-inducing ability. (**B**) A molecular model of the second death effector domain of vFLIP (residues F91–M171). The potential TRAF2-binding motif PxQ is located at the amino-terminal end of the first helix. It is possible that the PxQ sequence unwinds on binding to TRAF2 and/or a related TRAF. The model was produced with 3D-JIGSAW (Bates *et al*, 2001). TRAF, tumour necrosis factor receptor-associated factor; vFLIP, viral FADD-like interleukin-1-β-converting enzyme/caspase-8-inhibitory protein.

vFLIP associates with TRAF2 *in vitro* and in PEL cells

To determine whether vFLIP interacts with TRAFs in PEL cells, we carried out co-immunoprecipitation analysis in BC3 cells. We chose BC3 cells because they are relatively easy to transfect and also because it is a KSHV-positive cell line with high endogenous expression of vFLIP and TRAF2. Extracts from BC3 cells transfected with vFLIP–Flag were precipitated with antibodies to TRAF2, and the presence of transfected vFLIP could be detected with antibodies to the Flag tag (Fig 2A). Mutations in P93 and Q95 of vFLIP abolished binding to TRAF2, showing that this is a bona fide TRAF-interacting motif. Mutation of this domain also affected localization of vFLIP to the IKK complex (Fig 2A), implicating TRAF in this process. When extracts were precipitated with an antibody to TRAF3, no Flag–vFLIP could be detected in the

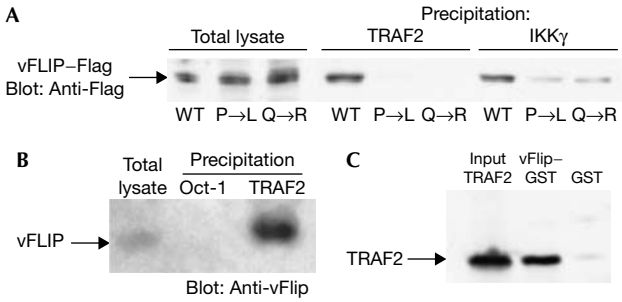

Fig 2 | vFLIP binds to TRAF2 in primary effusion lymphoma cells and *in vitro*. (**A**) BC3 cells were transfected with a plasmid encoding vFLIP–Flag, of the indicated vFLIP–Flag mutants, and 48 h later, total cell extracts were immunoprecipitated with anti-TRAF2 or anti-IκB kinaseγ (IKKγ) antibodies and then analysed by immunoblotting with antibodies to Flag. Transfected wild-type (WT) vFLIP–Flag could be detected in the TRAF2 and IKKγ precipitates but the mutated plasmids had decreased binding. (**B**) BC-3 cell extracts were immunoprecipitated with anti-TRAF2, as well as with anti-Oct-1 (as a negative control), and then analysed by immunoblotting with an antibody to vFLIP, showing the presence of endogenous vFLIP in complexes containing TRAF2. Total BC3 lysate (40 μg) was also loaded as a positive control. (**C**) The TRAF domain of TRAF2 was labelled with [^{35}S]methionine by *in vitro* translation and tested for binding to glutathione *S*-transferase (GST)–vFLIP. A 10% of input of TRAF2 is shown in the first lane. GST pull-down showed the presence of TRAF2 when incubated with vFLIP–GST but not with GST alone. TRAF, tumour necrosis factor receptor-associated factor; vFLIP, viral FADD-like interleukin-1-β-converting enzyme/caspase-8-inhibitory protein.

precipitates (not shown). This may reflect a real lack of association, or a technical problem, such as disruption of the complex by the antibody used. We also carried out immunoprecipitation of cell extracts from untransfected BC3 cells with antibodies to TRAF2, and endogenous vFLIP could be detected in the precipitates with an antibody to vFLIP (Fig 2B). These results confirm that vFLIP is in the same complexes as TRAF2 in PEL cells. The interaction between TRAF2 and vFLIP is direct, because a purified *Escherichia coli*-derived fusion protein of glutathione *S*-transferase (GST) and vFLIP, but not GST alone, efficiently binds to purified recombinant TRAF domain from TRAF2 (Fig 2C).

TRAF2 and TRAF3 are essential for vFLIP signalling

A TRAF2 dominant-negative (TRAF2.DN) mutant was previously reported to inhibit vFLIP signalling in transfected 293T cells (Chaudhary *et al*, 1999), and we found that the same is true in human lymphoma B cells and PEL cells (supplementary Fig 4 online). We used RNA interference to confirm the involvement of endogenous TRAF2 in vFLIP signalling, because TRAF2.DN is not likely to be entirely specific owing to similar binding motifs in other TRAFs. In addition, we tested the effect of suppression of all the TRAF molecules that have been implicated in TNF family receptor signalling in B cells, which include TRAF1, TRAF2, TRAF3, TRAF5 and TRAF6. Short interfering RNA (siRNA) to these TRAFs was transfected into a PEL cell line (BC3) and a significant inhibition of TRAF1, TRAF2, TRAF3, TRAF5 and TRAF6 proteins was obtained, as assessed by immunoblot analysis (Fig 3A). This inhibition was specific to the TRAF targeted; that is, siRNA to TRAF2 reduced the levels of TRAF2 but the levels of the other TRAF proteins remained unchanged and the same was true for the other TRAFs (not shown). siRNA to TRAF2 or TRAF3 was able to inhibit endogenous NF-κB activity by more than 80%, but suppression of the other TRAFs did not have any effect (Fig 3B). These results confirm the need for TRAF2 in NF-κB activation by vFLIP and indicate that TRAF3 also is involved in the activation of this pathway. Elimination of both TRAF2 and TRAF3 resulted in a marked decrease of nuclear accumulation of P65, and also in decreased nuclear accumulation of P50 (supplementary Fig 5A online). No effect was seen in the amount of nuclear P52 or RelB. Therefore, TRAF2 and TRAF3 mediate activation of the classical, but not the alternative, pathway in PEL cells. In addition, suppression of both TRAF2 and TRAF3, but not TRAF1, TRAF5 or TRAF6, resulted in markedly decreased levels of JNK phosphorylation. Therefore, JNK activation by vFLIP seems to share some of the same upstream adaptors with the NF-κB pathway (supplementary Fig 5B online).

TRAFs depletion leads to apoptosis of PEL cells

As inhibition of both NF-κB and vFLIP results in apoptosis of PEL cells (Keller *et al*, 2000; Guasparri *et al*, 2004), it is likely that both TRAF2 and TRAF3 are essential for the survival of PEL cells. To test this, we measured apoptosis by flow cytometry for annexin V on inhibition of the different TRAFs by RNA interference. siRNA to TRAF2 and TRAF3 induced apoptosis by 85% and 78%, respectively, but suppression of the other TRAFs did not have any effect (Fig 3C). These results confirm the need for TRAF2 and TRAF3 in NF-κB activation by vFLIP and in associated cellular survival.

TRAF2 mediates localization of vFLIP to the IKK complex

To evaluate the mechanism by which TRAF2 and TRAF3 mediate signalling by vFLIP, we determined whether these TRAFs serve as adaptors to bring vFLIP to the IKK complex. It has been shown that vFLIP binds to IKKγ, and is present in complexes with IKKα, IKKβ and IKKγ, as well as in complexes with the chaperone protein Hsp90 in PEL cells (Field *et al*, 2003). We therefore determined whether vFLIP localizes to the IKK complex in the absence of TRAF2 and TRAF3. Immunoprecipitation of IKKγ in PEL cells results in efficient co-precipitation of endogenous vFLIP, but on suppression of TRAF2 by siRNA, no vFLIP could be identified in the precipitates (Fig 3D). In contrast, vFLIP still co-precipitated with IKKγ in the absence of TRAF3. These data indicate that although both TRAF2 and TRAF3 are essential for vFLIP signalling, they do so by different mechanisms, whereas only TRAF2 is necessary for bringing vFLIP to the IKK complex.

DISCUSSION

These experiments show that vFLIP requires TRAF2 and TRAF3 for signalling and cellular survival, but that TRAF1, TRAF5 and TRAF6 are dispensable. The role of specific TRAFs in EBV and CD40 signalling has been carefully evaluated, most recently through elegant studies evaluating the effects of expression in cell lines in which individual TRAFs have been knocked out. These studies have shown that TRAF3 and TRAF6 are involved in LMP1 signalling—although they both have not yet been shown to be essential in the same experimental system—and that LMP1 can

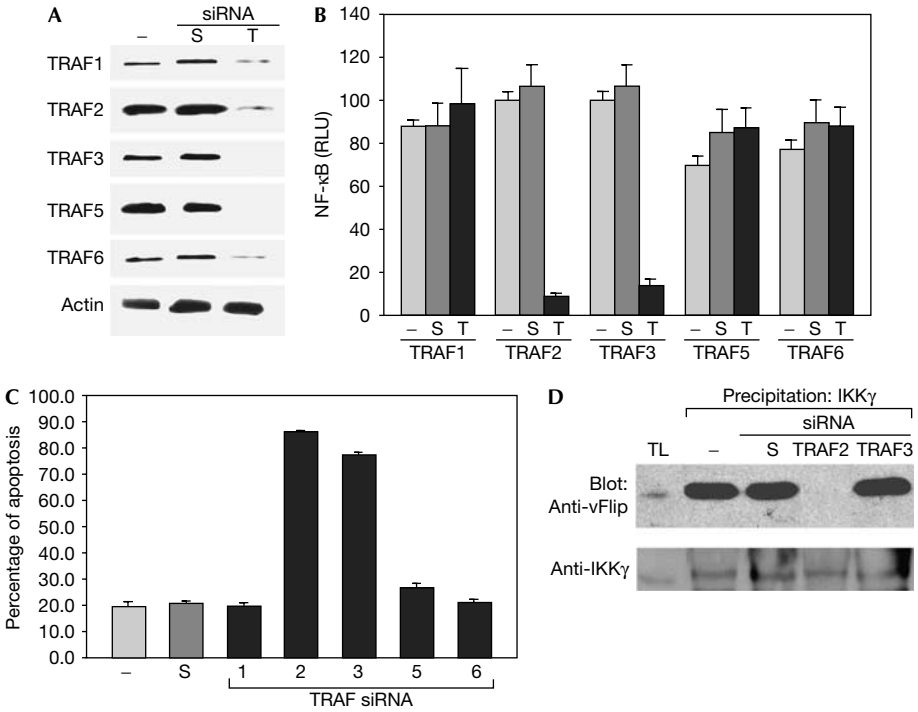

Fig 3 | Inhibition of endogenous TRAF2 and TRAF3 by short interfering RNA abolishes activation of nuclear factor-κB. (**A**) BC3 cells were transfected with either short interfering RNA (siRNA) to TRAF1, TRAF2, TRAF3, TRAF5 or TRAF6 (T) or scramble siRNA or were mock transfected (−), and protein extracts were prepared 48 hours later. Immunoblot analysis was carried out using antibodies to the indicated TRAFs and showed effective suppression of the corresponding TRAF proteins. Actin reprobing was carried out for all the blots to confirm even protein loading, and a representative example corresponding to the TRAF2 blot is shown. (**B**) BC3 cells were transfected with a nuclear factor-κB (NF-κB) luciferase reporter plasmid and either scramble siRNA (S) or siRNA to TRAF, as indicated. Luciferase assays were carried out 48 h after transfection. Values shown in normalized relative luciferase units (RLUs) are averages (±s.e.m.) of one representative experiment in which each transfection was carried out in triplicate. siRNA to TRAF2 and TRAF3 resulted in >80% reduction of NF-κB activity, but no effect was seen on suppression of TRAF1, TRAF5 or TRAF6. (**C**) BC3 cells were transfected with either scramble siRNA (S) or siRNA to TRAF, as indicated, or were mock transfected (−), and flow cytometry using antibodies to annexin V was carried out 8 days later. Bars represent the average number of annexin V-positive cells (+s.e.m.) of a representative experiment in which each transfection and analysis was carried out in triplicate. (**D**) BC3 cells were transfected with either siRNA to TRAF2 or TRAF3 or scramble siRNA (S) or were mock transfected (−). Extracts were made 48 h later and immunoprecipitated with rabbit polyclonal antiserum to IκB kinaseγ (IKKγ). Precipitates were analysed by immunoblotting with a monoclonal antibody to viral FADD-like interleukin-1-β-converting enzyme/caspase-8-inhibitory protein (vFLIP; top panel), or a mouse monoclonal antibody to IKKγ, to confirm equal immunoprecipitation. Total BC3 lysate (TL) was also loaded as a positive control. Suppression of TRAF2 resulted in the inability of vFLIP to co-precipitate with IKKγ.

still signal in TRAF1-, TRAF2- and TRAF5-negative cells (Luftig *et al*, 2003; Xie *et al*, 2004). These requirements are different from those of CD40, which uses TRAF2, TRAF5 and TRAF6 (Rothe *et al*, 1995; Ishida *et al*, 1996a,b), and in which TRAF3 acts as a negative regulator (He *et al*, 2004; Xie *et al*, 2004; Hauer *et al*, 2005). Here, we used RNA interference as an approach to eliminate expression of the different TRAFs and test for their requirement in vFLIP signalling. This method has the advantage over knockout cells in that the endogenous TRAFs can be eliminated in naturally infected KSHV-positive lymphoma cells; therefore, all the relevant proteins are present in physiological concentrations. We found that vFLIP requires TRAF2 and TRAF3; hence, the specific signalling requirements of vFLIP are different from those of CD40 and LMP1.

vFLIP is essential for the survival of KSHV-infected lymphoma cells, and this activity is mediated by NF-κB. A full understanding of the mechanisms by which vFLIP induces signalling to NF-κB is essential for rational drug design. Our findings identify the presence of a TRAF-interacting motif in vFLIP, and TRAF2 and TRAF3 as crucial mediators of vFLIP survival signals. Molecules that disrupt TRAF function, or more specifically the interaction between vFLIP and relevant TRAFs, are likely to be effective for the treatment of PEL and perhaps other KSHV-associated diseases.

METHODS

Cell lines and culture conditions. PEL cell lines used were BC1, BC2 (KSHV+/EBV+) and BC3 (KSHV+/EBV−), which were established in our laboratory from lymphomatous effusions, as described previously (Cesarman *et al*, 1995b; Arvanitakis *et al*, 1996). Namalwa, a Burkitt lymphoma cell line, was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were grown in RPMI-1640 (Gibco, BRL, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum

(Atlanta Biologicals, Lawrenceville, GA, USA) and 50 mg/ml gentamicin (Sigma-Aldrich, St Louis, MO, USA).

Cloning and expression vectors. The KSHV vFLIP and vFLIP–Flag expression vectors have been described previously (Guasparri *et al*, 2004). Comparable expression of transfected plasmids was confirmed by reverse transcription–PCR and immunoblot with an anti-Flag monoclonal antibody. TRAF2.DN (aa 241–501, haemagglutinin-tagged) was a kind gift from Yongwon Choi. For mutations in the PxQxT motif in the second DED of vFLIP, three single-base-pair mutations were made in vFLIP and vFLIP–Flag expression vectors using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA), according to the manufacturer's protocols. Mutations were made to result in the following amino-acid substitutions: P93 to L, Q95 to R, or T97 to L. These were chosen to be conservative substitutions or to be present in the corresponding position of other DEDs.

Transient transfection and luciferase assay. Transient transfection of Namalwa and BC3 cells was carried out using FuGene 6 Transfection Reagent (Roche, Nutley, NJ, USA) or TransFectin Lipid Reagent (Bio-Rad, Hercules, CA, USA), according to the manufacturer's protocol and as described (Guasparri *et al*, 2004). The amount of plasmid DNA transfected was equalized by addition of pcDNA3.1 control empty vector. The NF-κB firefly luciferase reporter derived from a κ-immunoglobulin promoter was kindly provided by Hsiou-Chi Liou, and 2 μg was used for each transfection. All the transfections were carried out with the addition of 0.5 μg pRSV-RL vector (Promega, Madison, WI, USA), encoding *Renilla* luciferase, to normalize the results for the transfection efficiencies. After 48 h, lysates were prepared using 1 × cell culture lysis reagent, as specified by the manufacturer (Promega). Luciferase assays were carried out with a luminometer (Dynex Technologies, Chantilly, VA, USA).

RNA interference. RNA duplex siRNA for TRAF1, TRAF2, TRAF3, TRAF5 and TRAF6 was obtained from Santa Cruz Biotechnology Inc. A control scramble siRNA duplex was obtained from Dharmacon (Scramble II Duplex; Lafayette, CO, USA). We carried out transfections of siRNA using oligofectamine reagent (Invitrogen, Carlsbad, CA, USA), as described previously (Guasparri *et al*, 2004) and assayed for silencing 48 h after transfection.

Immunoblot analysis. Untransfected, mock-transfected or cells transfected with siRNA or the relevant expression plasmids were collected 48 h after transfection. Lysates were prepared and blotted using standard methodology (see the supplementary information online). The following primary antibodies were used: anti-TRAF1 (Santa Cruz H-3; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-TRAF2 (Santa Cruz C-20), anti-TRAF3 (Santa Cruz H-122), anti-TRAF5 (Santa Cruz H-257), anti-TRAF6 (Santa Cruz D-10), anti-RelB (Santa Cruz C-19), anti-P65 (Santa Cruz F-6), anti-P50 (Santa Cruz N-19), anti-Flag and anti-β-actin (Sigma-Aldrich), anti-phospho-SAPK/JNK (Thr 183/Tyr 185; Cell Signaling Technology, Beverly, MA, USA) and anti-JNK1 (Santa Cruz C-17) and anti-NF-κB2(p52) (Upstate, Waltham, MA, USA).

Immunoprecipitation and *in vitro* binding. Untransfected and transfected BC-3 cells were lysed with Triton buffer (50 mM Tris pH 7.5, 250 mM NaCl, 0.1% Triton, 1 mM EDTA and 50 mM NaF) at a concentration of 4×10^7 cells/ml at 4 °C for 20 min. The lysate was cleared by centrifugation. The proteins were quantified by the Bradford method and 8 mg of total cell lysate was precipitated with 5 μg of rabbit anti-TRAF2 (Santa Cruz c-20), rabbit anti-TRAF3 (Santa Cruz H-122), rabbit anti-IKKγ (Santa Cruz FL-419), mouse anti-Oct1 (Upstate) or rabbit anti-Flag (Sigma-Aldrich) for 3 h. The precipitate was washed four times with Triton buffer, resuspended in 5 × loading buffer, separated by SDS–polyacrylamide gel electrophoresis and probed with mouse monoclonal anti-Flag (Sigma-Aldrich), rat monoclonal anti-vFLIP (kindly provided by Mary Collins) or mouse anti-IKKγ (Santa Cruz B-3).

A plasmid encoding the TRAF domain of TRAF2 was used to produce [^{35}S]methionine-labelled recombinant protein by *in vitro* transcription/translation (TNT Quick Coupled System, Promega). vFLIP was cloned in-frame with a carboxy-terminal GST tag, and recombinant vFLIP–GST protein was prepared in *E. coli* and purified using the MagneGST protein purification system (Promega). GST protein was similarly prepared, as a control. The vFLIP–GST fusion and GST proteins were incubated with radiolabelled recombinant TRAF2, and protein complexes were collected by GST pull-down, washed thoroughly and subjected to SDS–polyacrylamide gel electrophoresis. *In vitro*-translated proteins were visualized by autoradiography.

Flow cytometry analysis. BC3 cells were placed in culture at a density of 7.5×10^5/ml. Annexin V analysis was carried out as described by the manufacturer (Clontech, Mountain View, CA, USA). Briefly, 2×10^5 cells were washed once with 1 × binding buffer and resuspended in 200 μl of 1 × binding buffer. A 5 μl portion of FITC-Annexin V was added and the cells were incubated for 15 min in the dark at 22 °C and analysed by flow cytometry using a FACSCalibur (Becton Dickinson, San Jose, CA, USA).

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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