Supporting Online Material

Materials and Methods

Mice, cell lines, antibodies and reagents

Traf2$^{+/-}$, *Traf6*$^{+/-}$ and *Traf3*$^{+/-}$ mice were obtained from Tak W. Mak (University of Toronto, Canada) and Hitoshi Kikutani (Osaka University, Japan), and used to generate the respective homozygote null mice which were used as a source of fetal liver stem cells. *Mekk1*$^{\Delta KD}$ (aka *Map3k1*$^{\Delta KD}$) mice were generated and maintained as described (*1*). Mice were housed under conventional barrier protection according to UCSD and NIH guidelines, and mouse protocols were approved by the UCSD Institutional Animal Care Committee.

1.3E2 (*Ikk*γ^{-}) and 70Z3 (*Ikk*γ^{+}) cells (*2*) were kindly provided by Gilles Courtois, INSERM U697. KMS-28BM (*WT*) and KMS-28PE (*c-IAP*$^{-/-}$) multiple myeloma cells, were a generous gift from Rafael Fonseca, Mayo Clinic (*3*). HEK293 cells stably transfected with murine CD40 (HEK293-CD40) were generated as described (*4*). Cells were maintained at 37°C, 5% CO_2 in RPMI medium supplemented with fetal calf serum, glutamine, and antibiotics (Gibco BRL, Invitrogen, USA).

Phospho-Thr1381 specific MEKK1 antibody and antibodies to MEKK1 were generated as described and affinity purified (*5*). A monoclonal antibody that specifically recognizes K63-linked polyubiquitin was generated as described elsewhere (H. Wang *et al.*, submitted for publication). The following commercial antibodies were used: HRP-

conjugated anti-mouse IgG and anti-rabbit IgG (Amersham; NA931 and NA 941), HRP-conjugated anti-goat IgG and anti-rat IgG (Santa Cruz; sc-2020 and sc-2006), anti-MEKK1 (Santa Cruz; sc-252), anti-TAK1 (Santa Cruz; sc-7162), anti-phosphorylated TAK1 (Cell Signaling; 4531), anti-JNK1/2 (Pharmingen; 554285), anti-JNK1 (Pharmingen; 554286), anti-phosphorylated JNK (Cell Signaling; 9261), anti-p38 (Santa Cruz; sc-535), anti-phosphorylated p38 (Cell Signaling; 9211), anti-ERK (Cell Signaling; 9102), anti-phosphorylated ERK (Cell Signaling; 9101), anti-MKK4 (Santa Cruz; sc-964), anti-MKK7 (Santa Cruz; sc-7103), anti-MKK3 (Santa Cruz; sc-959), anti-phosphorylated MKK4 (Cell Signaling; 9156), anti-phosphorylated MKK3/6 (Cell Signaling; 9231), anti-IκBα (Santa Cruz; sc-371), anti-phosphorylated IKKα/IKKβ (Cell Signaling; 2681), anti-IKKγ (Santa Cruz; sc-8330 or Pharmingen; 559675), anti-IKKα/IKKβ (Santa Cruz; sc-7607), anti-IKKα (Santa Cruz; sc-7183), anti-IKKβ (Cell Signaling; 2684), anti-CD40 (Santa Cruz; sc-9096 or Pharmingen; 553787), anti-TRAF2 (Santa Cruz; sc-7346 or Imgenex; IMG-162), anti-TRAF3 (Santa Cruz; sc-949 or sc-6933), anti-TRAF6 (Santa Cruz; sc-8409 or sc-7221), anti-c-IAP1 (Santa Cruz; sc-7943), anti-c-IAP2 (R&D Systems; AF8171), anti-Ubc13 (Santa Cruz; sc-19542), anti-ubiquitin (Zymed; 13-1600 or Santa Cruz; sc-8017), anti-HSP70 (Santa Cruz; sc-24), anti-γ-tubulin (Sigma; T9026), anti-β-actin (Sigma; A4700), anti-Flag (Sigma; F3165), and anti-GST (Santa Cruz; sc-33613). MG132 was purchased from EMD. The c-IAP inhibitor Smac mimic was kindly provided by Xiaodong Wang, University of Texas Southwestern (*6*). Ubiquitination assay reagents were purchased

from Boston Biochem.

Splenic B cell isolation

Splenic B cells were isolated as described (*7*). Splenocyte suspensions were made by grinding spleens between two cover slips. $CD43^-$ B cells were isolated with magnetic beads (MACS; Miltenyi) according to manufacturer's protocol. B cells or B cell lines (1×10^6 cells/ml) were stimulated with anti-CD40 (5 μg/ml; clone 3/23; Pharmingen), or BAFF (200 ng/ml; PeproTech).

Generation of chimeric mice

Single-cell suspensions of fetal liver from WT, *Traf2*$^{-/-}$, *Traf3*$^{-/-}$, or *Traf6*$^{-/-}$ mice were prepared, and 5×10^5 cells were injected into the tail vein of 6-week-old C57BL/6J host mice that were exposed to 900 rad of γ radiation. Host mice were maintained under sterile conditions, using autoclaved cages, food, and water containing 25 mg/L neomycin sulfate and 13 mg/L polymyxin B sulfate (*8*).

Immunoblot analysis

Cells were lysed in ice-cold lysis buffer containing 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 1 mM phenylmethyl sulfonyl fluoride and 20 μg/ml of aprotinin. After centrifugation, cell extracts were resolved by

SDS-PAGE and were analyzed by immunoblotting. The membranes were probed with the indicated antibodies. Blots were developed with ECL reagent (Amersham Pharmacia Biotech).

Short hairpin RNA (shRNA) constructs, lentiviral packaging and transduction

Oligonucleotides directed against indicated mRNAs were cloned into pLSLPw lentiviral plasmid, kindly provided by Peter M. Chumakov, Cleveland Clinic (*9*). The oligonucleotide sequences used for the shRNA constructs are as below: m/h-Ubc13: 5'-AATCCAGATGATCCATTAGCA (*10*); m-TRAF2: 5'-CTAGACCAGGACAAGATTG; m-TRAF3: 5'-GCAAGAGAGAGATTCTGGC; m-TRAF6: 5'-CGTCCTTTCCAGAAGTGCC. Lentiviral packaging was as described (*11*). 293T cells were transfected with the pLSLPw constructs along with packaging plasmids, pVSVG (Clontech) and pLV-CMV-delta 8.2 (provided by Inder Verma, Salk Institute) using Lipofectamine 2000 (Invitrogen). Virus-containing supernatants were collected at 48, 72 and 96 hrs post-transfection, pooled and concentrated 20-fold by centrifugation at 20,000 rpm for 2 hrs. Cells were transduced with lentivirus in the presence of 5 mg/ml polybrene (Sigma). After 24 hrs, the virus-containing medium was replaced with selection medium containing 5 mg/ml puromycin (EMD). After cell growth was stable, the cells were used in the described experiments.

Subcellular fractionation and immunoprecipitation assay

Subcellular fractionation was as described (*12*). Cells (1×10^6) were stimulated or not with CD40 or BAFF, washed twice in ice cold PBS, and resuspended in 1 ml homogenization buffer (250 mM sucrose, 20 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$, plus 1 mM phenylmethyl sulfonyl fluoride (PMSF) and 20 μg/ml of aprotinin) for 20 min on ice. Cells were disrupted with 15 strokes of a tight-fitting pestle in a dounce homogenizer. Nuclei and unbroken cells were removed by low-speed centrifugation (1000 × g for 10 min at 4°C) and homogenates were centrifuged at 100,000 × g for 1 hr at 4°C. Supernatants containing soluble cytosolic proteins were separated, and the pellets containing cellular membranes and other insoluble materials were resuspended in 10 mM Tris-HCl pH 7.4, 150 mM NaCl, and 0.2% Nonidet P-40 to dissolve membranes. Both fractions were used for immunoprecipitation.

Membrane or cytosol fractions were immunoprecipitated with anti-CD40 (Santa Cruz; sc-9096) or anti-MEKK1 (Santa Cruz; sc-252) antibodies, respectively, overnight at 4°C. Total cell lysates were prepared by ice-cold lysis buffer containing 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, 1 mM PMSF and 20 μg/ml of aprotinin, and immunoprecipitated with the indicated antibodies overnight at 4°C. Whenever protein ubiquitination was analyzed, 20 mM N-ethylmaleimide (NEM, Sigma) was added to the lysis buffer. All samples were precleared with Sepharose-6B (Sigma-Aldrich) for 1 hr at 4°C, and incubated with protein G–Sepharose (Amersham) for 1 hr to

overnight at 4°C. The beads were washed, eluted, separated by SDS-PAGE and analyzed by immunoblotting with indicated antibodies. Blots were developed with ECL reagent.

Plasmid constructs

pCMV5-Myc-MEKK1 was constructed as described (*13*). pcDNA-Flag-c-IAP2, pcDNA-HA-TRAF2, and pcDNA-Flag-TRAF2 were kindly provided by John Reed and Ze'ev Ronai (Burham), and pcDNA-HA-TRAF3 was previously described (*14*). MEKK1 and c-IAP2 mutations were introduced by a QuikChange mutagenesis kit (Stratagene).

***In vitro* ubiquitination assay**

In vitro ubiquitination was performed as described (*7*). c-IAP-deficient multiple myeloma cells were transfected with vector, flag-c-IAP2 (WT) and flag-c-IAP2 (RM) (c-IAP2 with RING finger mutations that render the E3 ligase inactive). After G418 selection for 1 week, the cells were stimulated with anti-CD40. Cells were lysed and immunoprecipitated with anti-Flag antibody. Washed immunecomplexes were incubated with or without GST-TRAF3 produced in *E. coli* in a reaction mixture including ubiquitin, E1 and a mixture of E2 ubiquitin-conjugating enzymes: UbcH2, UbcH3, Ubc5a, Ubc5b, Ubc5c, UbcH6, UbcH7, and UbcH10. Reactions were carried out at 30°C for 30 min in 50 mM HEPES pH7.8 containing 4 mM ATP and 10 mM $MgCl_2$. GST-TRAF3 was immunoprecipitated, gel-separated, and analyzed by immunoblotting with anti-ubiquitin

and anti-TRAF3 antibodies.

Fig. S1

TRAF2 requirement for MEKK1 signaling in CD40-stimulated B cells.

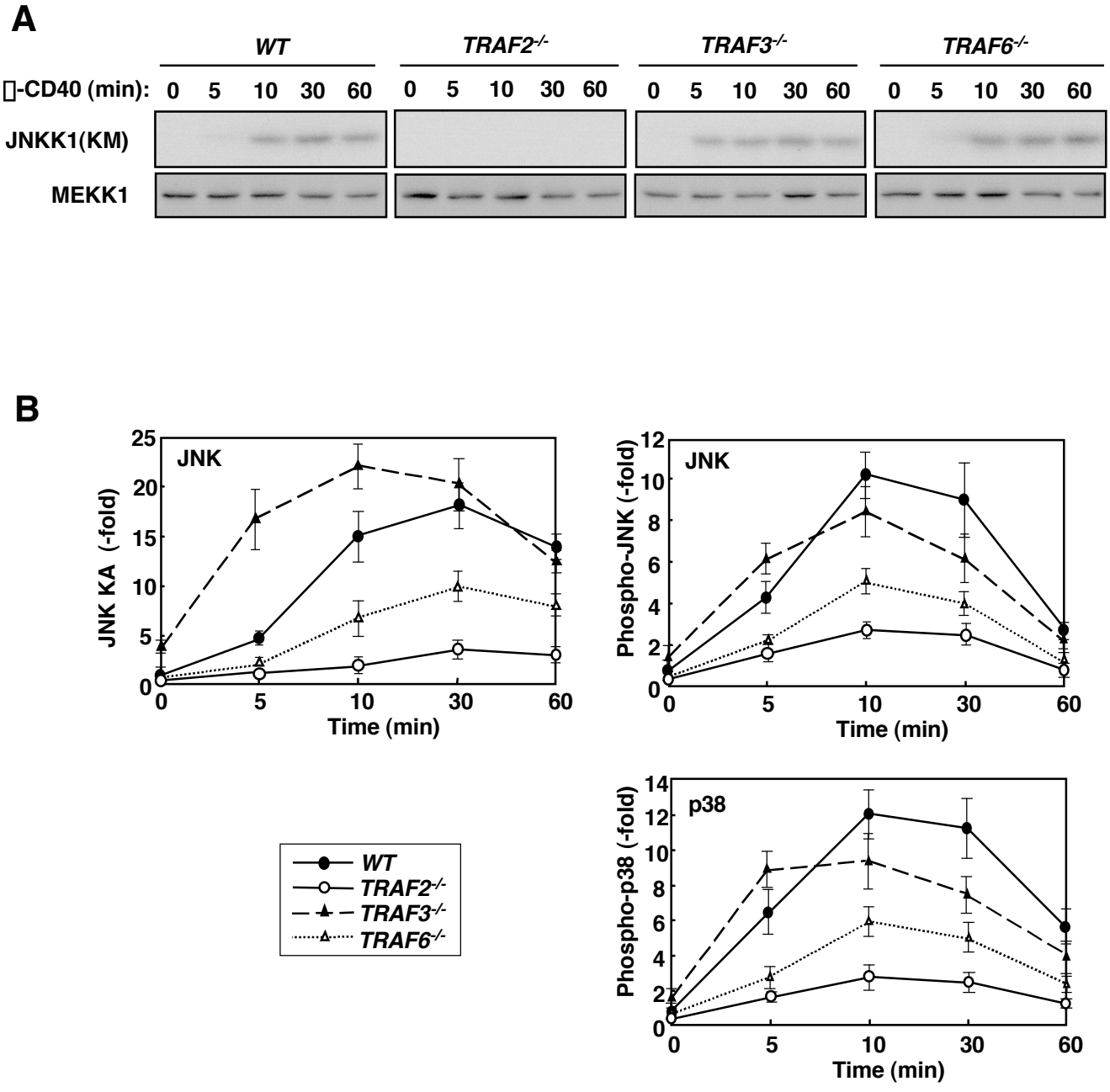


Fig. S2

TRAF2 requirement for MEKK1 ubiquitination.

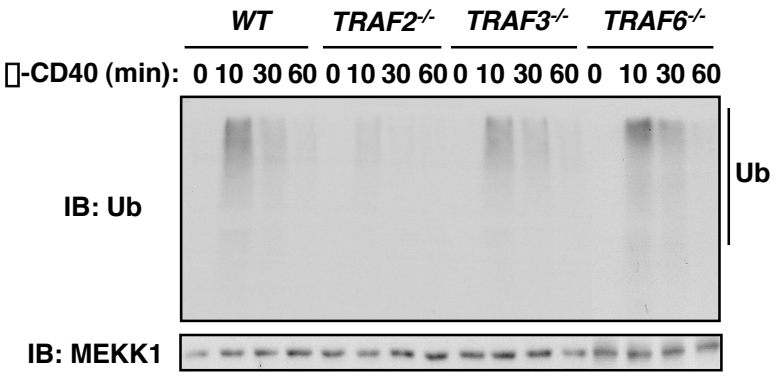


Fig. S3

Ubc13 requirement for MEKK1 ubiquitination.

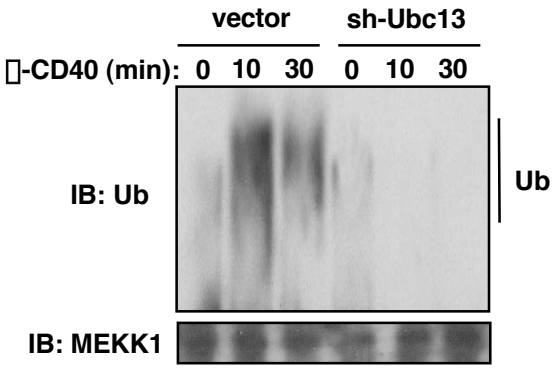
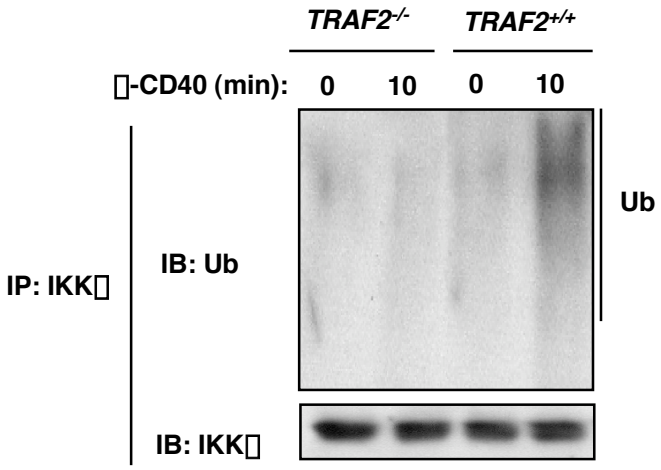


Fig. S4

TRAF2 and Ubc13 requirement for ubiquitination of IKK

A



B

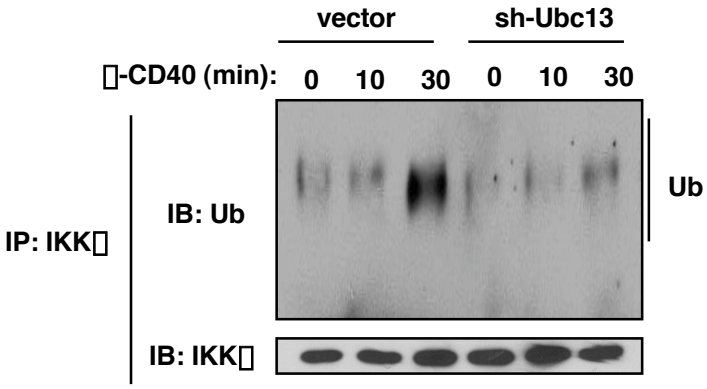


Fig. S5

MEKK1 requirement for ubiquitination of IKK-free IKKγ

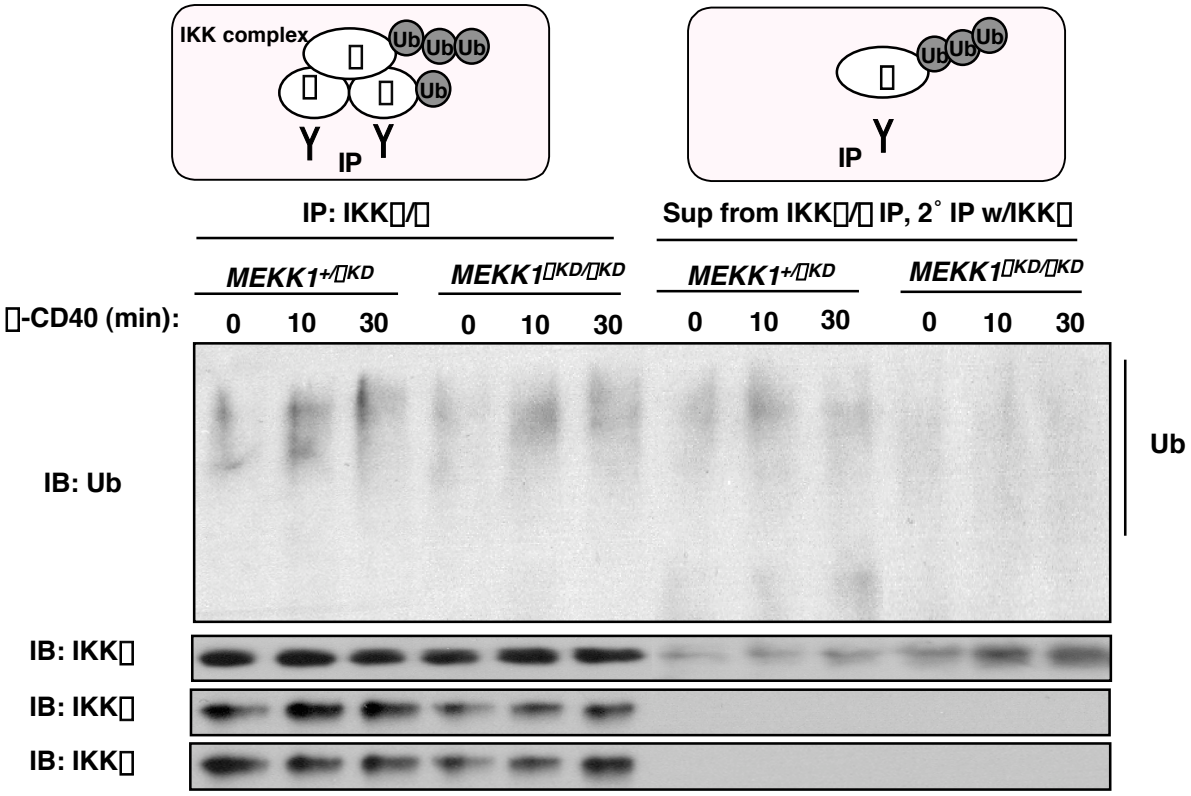


Fig. S6

Amounts of TRAF2, MEKK1, IKK□, Ubc13 and CD40 in total splenic B cell lysates and subcellular fractions.

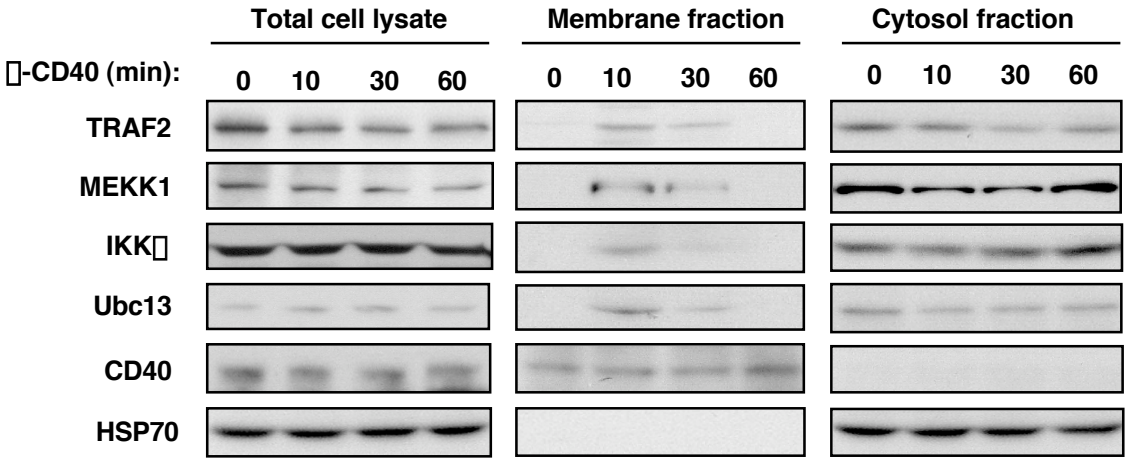


Fig. S7

TRAF proteins in the cytosolic MEKK1 signaling complex.

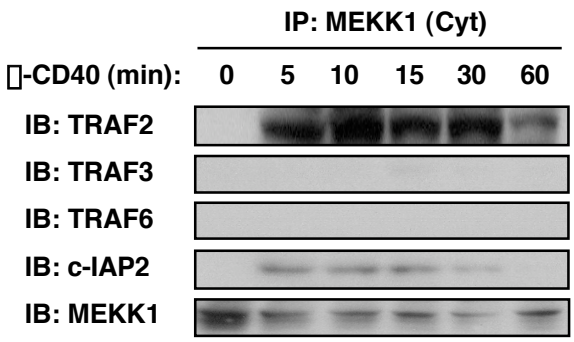


Fig. S8

c-IAP requirement for activation of MEKK1 signaling.

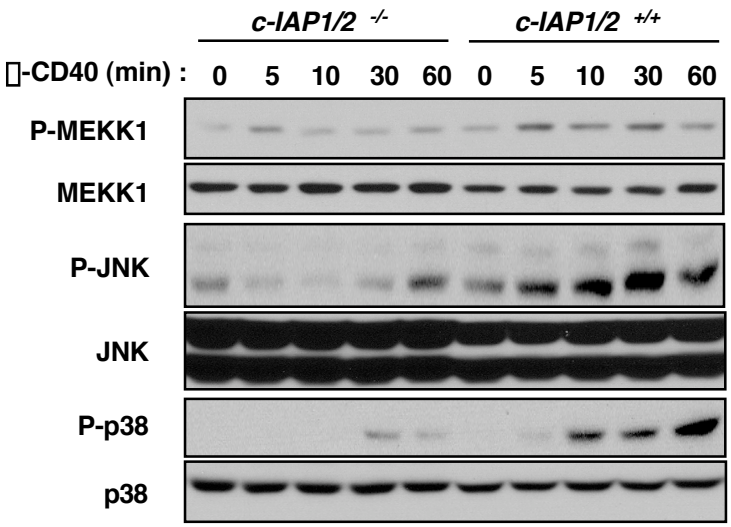


Fig. S9

Kinetics of MEKK1 and JNK activation.

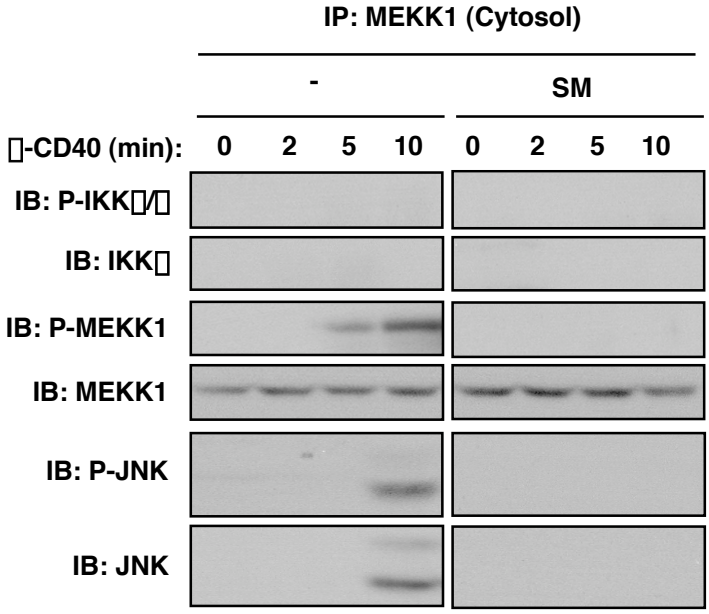


Fig. S10

MEKK1 ubiquitination occurs in the cytosol and requires c-IAP and proteasome activity.

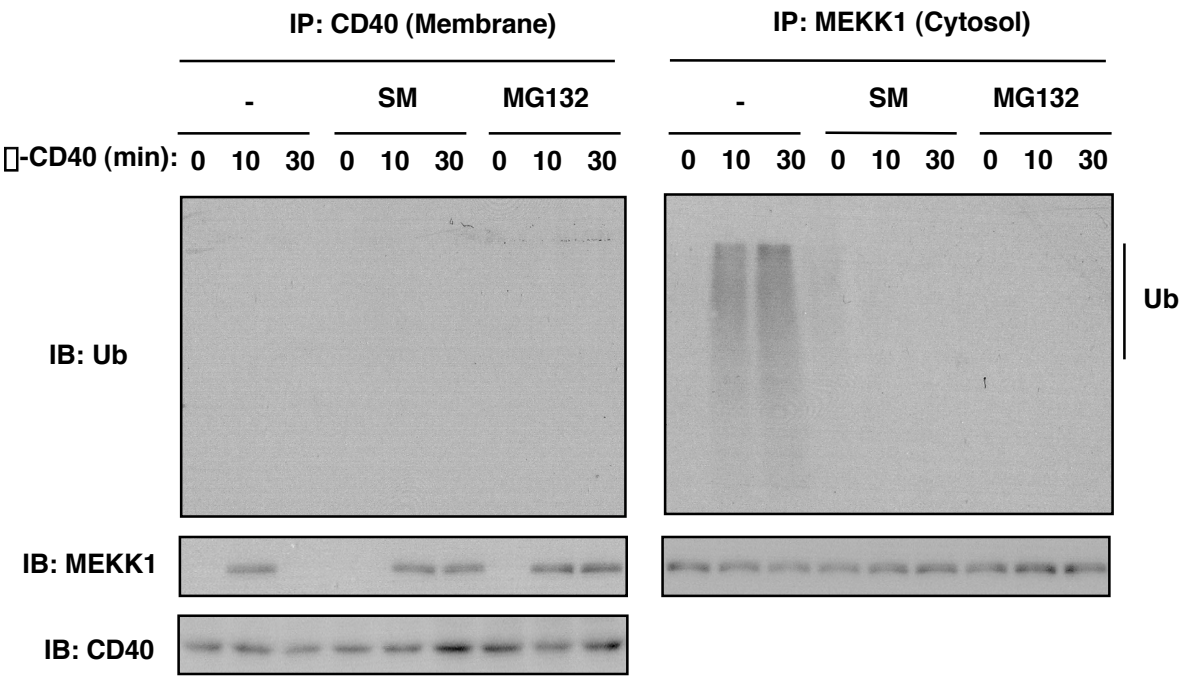


Fig. S11

Activation of MEKK1,TAK1 and JNK, but not IKK, by TNF-α is c-IAP dependent.

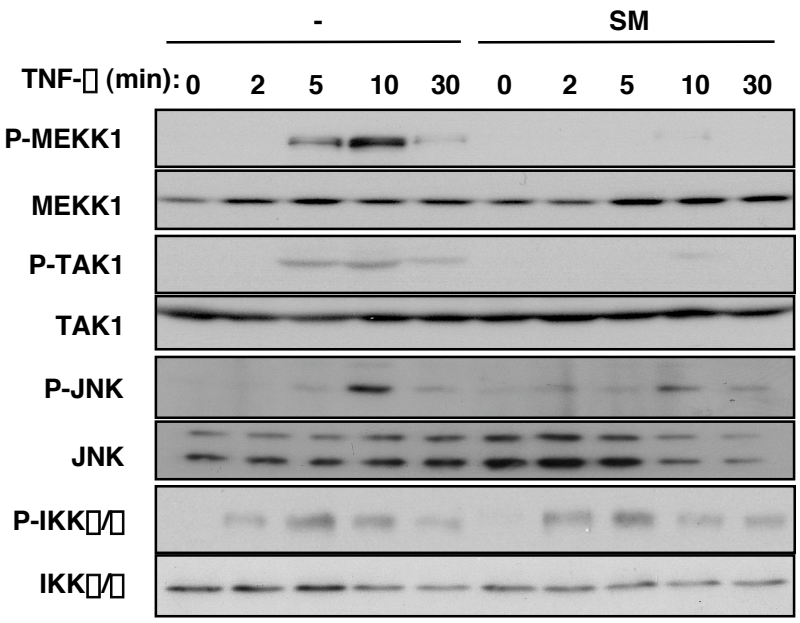
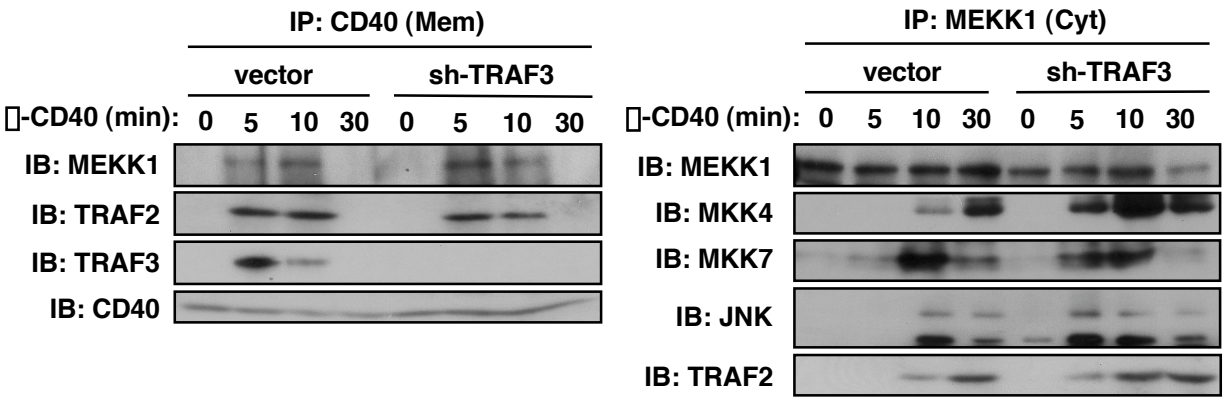


Fig. S12

Absence of TRAF3 accelerates and enhances the interaction of MEKK1 with MKK4/7 and JNK, but has no effect on recruitment of TRAF2 to CD40.



Supplemental Figures and Legends

Fig. S1 TRAF2 requirement for MEKK1 signaling in CD40-stimulated B cells. (**A**) Splenic B cells from mice reconstituted with WT, *Traf2*$^{-/-}$, *Traf3*$^{-/-}$, and *Traf6*$^{-/-}$ fetal liver cells were stimulated with anti-CD40 agonistic antibody and cell lysates were prepared at indicated times. The kinase activity of immunoprecipitated MEKK1 was measured by immunecomplex kinase assays with GST-JNKK1(KM) (JNKK1/MKK4 with alteration of the ATP-binding pocket that renders the kinase inactive) as a substrate (*7*). Immunoblotting with anti-MEKK1 served as the loading control. (**B**) Quantitation of JNK and p38 activation. JNK kinase activity (KA) was quantitated by densitometric analysis of two separate experiments examining GST-c-Jun(1-79) phosphorylation similar to the one shown in Fig. 1A. The extent of JNK and p38 phosphorylation in WT and TRAF-deficient B cells was determined by densitometric analysis of two separate experiments similar to the one in Fig. 1A. The figure shows average values.

Fig. S2 TRAF2 requirement for MEKK1 ubiquitination. Splenic B cells from mice reconstituted with WT, *Traf2*$^{-/-}$, *Traf3*$^{-/-}$, and *Traf6*$^{-/-}$ fetal liver were stimulated with anti-CD40. At indicated times, MEKK1 was immunoprecipitated, extensively washed and its ubiquitination was analyzed by immunoblotting.

Fig. S3 Ubc13 requirement for MEKK1 ubiquitination. Cell lysates were prepared from B

cells transduced with lentiviruses with no insert or shRNA to Ubc13 that were stimulated with anti-CD40. MEKK1 immunoprecipitated from cell lysates was analyzed by an *in vitro* ubiquitination assay. Washed MEKK1 immunecomplexes were incubated with ubiquitin, ATP, E1 and a mixture of E2 ubiquitin-conjugating enzymes. After 30 min, reaction mixtures were gel-separated and analyzed by immunoblotting with anti-ubiquitin and anti-MEKK1 antibodies.

Fig. S4 TRAF2 and Ubc13 requirement for IKK□ ubiquitination. (**A**) IKK□ ubiquitination in *Traf2*$^{+/+}$ or *Traf2*$^{-/-}$ CD43^{-} splenic B cells stimulated with anti-CD40. IKK□ was immunoprecipitated from non-stimulated and stimulated cells, and after extensive washing was gel separated and blotted with anti-ubiquitin and anti-IKK□ antibodies. (**B**) Ubiquitination of IKK□ in A20 B cells transduced with lentiviruses containing no insert or shRNA to Ubc13. IKK□ was immunoprecipitated and analyzed as above.

Fig. S5 MEKK1 requirement for ubiquitination of IKK-free IKK□. (**A**) *Mekk1*$^{+/\Delta KD}$ and *Mekk1*$^{\Delta KD/\Delta KD}$ splenic B cells were stimulated with anti-CD40. At the indicated times, the IKK complex was immunoprecipitated with anti-IKK□/□ antibodies, and the supernatant from this immunoprecipitation was re-precipitated with anti-IKK□ antibody. After extensive washing, both immunecomplexes were analyzed by immunoblotting with anti-ubiquitin (top panel), or anti-IKK□, anti-IKK□, and anti-IKK□ (bottom three panels) antibodies.

Fig. S6 Amounts of TRAF2, MEKK1, IKKγ, Ubc13 and CD40 in total B cell lysates and subcellular fractions. Splenic B cells from Fig. 3 were stimulated with anti-CD40 for the indicated times. Total cell lysates, membrane and cytosol fractions were gel-separated and immunoblotted with the indicated antibodies.

Fig. S7 TRAF proteins in the cytosolic MEKK1 signaling complex. Splenic B cells were treated and fractionated as above. Presence of the indicated proteins in the cytosolic MEKK1 immunecomplexes of SM-nontreated cells was examined.

Fig. S8 c-IAP1/2 requirement for activation of MEKK1 signaling. Control and c-IAP-deficient multiple myeloma cells were stimulated with anti-CD40. At the indicated time points, MEKK1, JNK and p38 phosphorylations were monitored by immunoblotting.

Fig. S9 Kinetics of MEKK1 and JNK activation and c-IAP1/2-dependence. The cytosolic fractions of the cells from Fig. 6E that were preincubated without or with SM were immunoprecipitated with anti-MEKK1. The gel-separated immunecomplexes were blotted with the indicated antibodies.

Fig. S10 MEKK1 ubiquitination occurs in the cytosol and requires c-IAP1/2 and

proteasome activity. Splenic B cells were stimulated with anti-CD40 in the absence or presence of SM. Cells were separated into membrane and cytosolic fractions that were immunoprecipitated with anti-CD40 and anti-MEKK1, respectively. The CD40-immunoprecipitated fraction was resuspended in cell lysis buffer and then diluted with immunoprecipitation buffer. This sample was re-precipitated with anti-MEKK1 antibody, and the gel-separated immunecomplexes were blotted with anti-ubiquitin and anti-MEKK1 antibodies. Aliquots of the CD40 immunecomplexs were also gel-separated and blotted with anti-CD40 antibody.

Fig. S11 TNF-□-induced activation of MEKK1, TAK1 and JNK, but not IKK, is c-IAP1/2 dependent. Mouse embryonic fibroblasts were stimulated with TNF-□ in the absence or presence of SM. Total cell lysates were gel-separated and immunoblotted with the indicated antibodies.

Fig. S12 Absence of TRAF3 accelerates and enhances the interaction of MEKK1 with MKK4/7 and JNK, but has no effect on recruitment of TRAF2 to CD40. A20 B cells transduced with lentiviruses containing no insert or shRNA to TRAF3 were stimulated with anti-CD40. Cells were separated into membrane and cytosolic fractions that were immunoprecipitated with anti-CD40 and anti-MEKK1, respectively. The immunocompelexs were gel-separated and immunoblotted with the indicated antibodies.

Supplemental References

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