Supplementary Data

Supplementary Materials and Methods

Reagents and cell culture

Recombinant murine and human TNFα, and murine IL-1 were purchased from BD Biosciences (San Jose, CA). Anisomycin, butylated hydroxyanisole (BHA), cycloheximide (CHX), Ac-DEVD-AMC, z-VAD-fmk, 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA), and MG132 were purchased from Wako, Peptide Institute, Molecular Probes, and Biomol. Anti-c-FLIP (Alexis), anti-tubulin and anti-Flag (Sigma-Aldrich), anti-HA (Roche), anti-Myc and IκBα (Santa Cruz Biotechnology) antibodies were purchased from the indicated sources. Antibodies specific for phospho-JNK, phospho-ERK, phospho-p38, phospho-MEK1, phospho-MKK3/6, phospho-MKK4, phospho-MKK7, total JNK, total ERK, total p38, total MEK1, and total MKK7 were purchased from Cell Signaling Technology. WT, *RelA*$^{-/-}$, *traf2*$^{-/-}$*traf5*$^{-/-}$, *c-Flip*$^{-/-}$ MEFs, HEK293, and Phoenix-Eco cells were cultured in high-glucose DMEM containing 10% fetal calf serum (FCS).

Western blotting

MEFs (5 ~ 8 x 10^5 cells) were lysed in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM PMSF, 1 μg/mL aprotinin,

and 1 μg/mL leupeptin). After centrifugation, cell lysates were subjected to SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were immunoblotted with the indicated antibodies. The membranes were developed with Enhanced Chemiluminescence (ECL) Western Blotting Detection System Plus (GE Healthcare Life Sciences). The intensities of the signals were quantified by Image Gauge Software (LAS 1000, Fuji Film)

Generation of stable transfectants

Retroviral expression vectors for c-FLIP$_L$ and c-FLIPs were generated by cloning PCR-amplified products from pcDNA3-CASHα and pcDNA3-CASHβ (provided by D. Wallach) into pMX-Flag-puro vector, respectively. Generation of stable transfectants of *RelA*$^{-/-}$, *traf2*$^{-/-}$ *traf5*$^{-/-}$, and *c-Flip*$^{-/-}$ MEFs by using retroviral vectors was performed as previously described (Sasazuki et al., 2002). After infection, puromycin-resistant pools were used for the experiments.

Cell viability assay

MEFs (5 x 10^3 cells) were plated onto 96-well plates and cultured for 12 hr in DMEM containing 10% FCS. Then the cells were stimulated with TNFα for the indicated times. Cell viability was determined by WST (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)[^{2}H]tetrazolium monosodium salt) assay using a Cell Counting kit (Dojindo).

Statistical analysis

Statistical analysis was performed by Student *t* test. *P* value < 0.05 was considered to be significant.

Measurement of ROS accumulation

MEFs (2 ~ 4 x 10^5 cells) were plated onto 6-well plates and stimulated with TNFα for the indicated times. After stimulation, the cells were washed with Opti-MEM (Invitrogen), and then incubated with CM-H_2DCFDA (1 μM) in the dark for 30 min at 37℃. Then, the cells were harvested and analyzed on a flow cytometer (FACSCalibur, BD Biosciences). Data were processed by using the CellQuest program (BD Biosciences).

Caspase activity

Caspase 3 activity was measured by the fluorometric assay as previously described (Nakayama et al., 2002). MEFs (5 ~ 8 x 10^5 cells) were stimulated with TNFα for the indicated times, then the cells were lysed in RIPA buffer. The cell lysates were incubated with Ac-DEVD-AMC (20 μM), and the release of fluorescent 7-amino-4-methylcoumarin was measured on a fluorometer (Labsystems).

Real-time PCR

Total RNAs were extracted by using RNA STAT60 (TEL-TEST). First-strand cDNAs were synthesized by using SuperScript II (Invitrogen). Real-time PCR was performed in

triplicates in the 7500 Real-Time PCR detection system using TaqMan Universal PCR Master Mix and Assays-on-Demand gene expression products of the mouse target genes including the *fhc* (Mm 00850707_g1) or *Mnsod* (Mm 00449726_m1) along with an endogenous control (*gapdh*; Mm 99999915_g1) (Applied Biosystems). The expression levels of these genes were expressed relative to those of *gapdh* using a 7500 SDS software (Applied Biosystems).

Phosphorylation of MAPKs and MAPKKs

HEK293 cells (0.5 x 10^6 cells) were transfected with appropriate amounts of the indicated expression vectors by Lipofectamine (Invitrogen). After 18 hr transfection, the cells were lysed in Buffer A (50 mM Tris-HCl, pH 8.0, 250 mM NaCl, 0.5% Nonidet P-40, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM PMSF, 1 μg/mL aprotinin, and 1 μg/mL leupeptin). After immunoprecipitation with anti-HA antibody, the immunoprecipitates were subjected to SDS-PAGE, and phosphorylated proteins were detected by immunoblotting with phospho-specific antibodies. Expression levels of transfected proteins in the lysates were analyzed by immunoblotting.

Co-immunoprecipitation

HEK293 cells (1.5 x 10^6 cells) were transfected with appropriate amounts of the indicated expression vectors. After 18 hr transfection, the cells were lysed in Buffer A. To detect the endogenous interactions of c-$FLIP_L$ with MKK7 and MEK1,

c-FLIP$_L$-*RelA*$^{-/-}$ MEFs (2 x 10^7 cells) or HEK293 cells (1 x 10^8 cells) were unstimulated or stimulated TNFα for the indicated times. After immunoprecipitation with the indicated antibodies, co-immunoprecipitated proteins were subjected to SDS-PAGE, and then detected by immunoblotting with the indicated antibodies. Expression levels of transfected or endogenous proteins in the lysates were analyzed as described above.

Supplementary References

Nakayama, M., Ishidoh, K., Kayagaki, N., Kojima, Y., Yamaguchi, N., Nakano, H., Kominami, E., Okumura, K. and Yagita, H. (2002) Multiple pathways of TWEAK-induced cell death. *J Immunol*, 168, 734-743.

Sasazuki, T., Sawada, T., Sakon, S., Kitamura, T., Kishi, T., Okazaki, T., Katano, M., Tanaka, M., Watanabe, M., Yagita, H., Okumura, K. and Nakano, H. (2002) Identification of a novel transcriptional activator, BSAC, by a functional cloning to inhibit tumor necrosis factor-induced cell death. *J Biol Chem*, 277, 28853-28860.