Supporting Information

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SI Methods

Reagents. Antibodies against JNK (333 for immunoprecipitation and 666 for immunoblotting) were from Pharmingen. Antibodies against phospho-JNK, p38, phospho-p38, phospho-JNKK1, and phospho-c-Jun(Ser63) were from Cell Signaling. Antibody against Miz1 (Myc-interacting zinc-finger protein 1) was previously described (1). Antibodies against TNF-receptor associated factor 2 (TRAF2), Xpress, ubiquitin conjugating enzyme (Ubc13), and HA were from Santa Cruz. All siRNA were purchased from Dharmacon. Creatine phosphate, creatine kinase, E1, Ub, and antibodies against M2-tag, ubiquitin, and β-actin were from Sigma. Ubc13/Uev1A was from Boston Biochem. Mouse inflammation cytometric bead array kit was from BD Biosciences. Murine TNF-α was from R&D Systems. [γ-^{32}P]ATP (3,000 mCi/nmol) and [^{35}S]methionine were from Dupont New England Nuclear.

Plasmids and siRNA. HA-JNK1, M2-JNK1, and GST-c-Jun were described previously (2). M2-TRAF2, M2-TRAF6, and GST-TRAF2 were kindly provided by Zhenggang Liu (National Cancer Institute, Bethesda, MD); HA-Ub (WT, K63O, and K48O) and HA-Ubc13 were kindly provided by James Z. Chen (University of Texas Southwestern, Dallas, TX); HA-TRAF2 (WT and C49/51A mutant) were kindly provided by Shao-Cong Sun (MD Anderson Cancer Center, Houston, TX); and M2-TRAF2 RING domain mutant (RM) (C49A, H51A, C54A and C57A) were kindly provided by Ze'ev Ronai (Sanford–Burnham Medical Research Institute, La Jolla, CA). K388/472R mutation was introduced into Miz1 using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). Xpress-Miz1, Xpress-Miz1 truncation mutants (Δ410–459, Δ466–514, Δ522–570, Δ576–635, Δ640–680, Δ680–710 and Δ710–795), Xpress-Ubc13, and GST-Miz1 were generated by PCR and subcloned into pcDNA3.1 vector (Invitrogen). All constructs were verified by DNA sequencing. The sequence of siMiz1 is UGCUGAACCUGCAUAGAA dTdT.

Mice. WT C57BL/6J mice were purchased from Charles River Breeding Laboratories. Animal care and experiments were performed in accordance with the Institution and National Institutes of Health guidelines and approved by the animal use committee at the University of Chicago.

Cell Culture and Transfection. Cell culture conditions and transfections have been described previously (2). WT and immortalized Miz1$^{-/-}$ mouse embryonic fibroblasts were described previously (1, 3). siRNAs were tranfected using Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen).

Protein Kinase Assays and Apoptosis Assays. Immune complex kinase assays and apoptosis assays were performed as previously described (4, 5).

Ex Vivo Ubiquitin Assembly Assay. Ex vivo ubiquitin assembly assay for TRAF2 E3 ligase activity was performed as described (6). Briefly, TRAF2 was immunoprecipitated from TNF-α–treated WT or Miz1-null mouse embryonic fibroblasts (MEFs) and used as E3 ligase to synthesize polyubiquitin chains from purified recombinant K63O-ubiquitin proteins in the presence of purified E1, Ubc13-Uev1a in an ATP regeneration system at 30 °C for 2 h (6). After the removal of Protein-A Sepharose beads-bound TRAF2 proteins or proteins that may be coimmunoprecipitated with TRAF2, the clarified supernatant, which only contained polyubiquitin chains, was subjected to SDS/PAGE, followed by analysis with anti-Ub antibody.

Mass Spectrometry. To map ubiquitination sites of Miz1, protein in-gel digestion of Miz1 and nano-HPLC/LTQ mass spectrometric analysis of the resulting tryptic peptides were carried out, as previously described (7). The acquired MS/MS spectra were searched against National Center for Biotechnology Information-nr protein sequence database with the specification of the possible ubiquitination modification at lysine residues using MASCOT database search engine. The candidate peptides were examined with the manual verification rules, as previously described (8), and the ubiquitination sites have been exclusively identified by consecutive *b* or *y* ions.

Preparation of Primary Macrophages, Retroviral infection, and Measurement of Proinflammatory Cytokine Synthesis. Bone marrow-derived macrophages were prepared as described (9). Briefly, bone marrow from 8- to 10-wk-old female mice was flushed from both mouse tibia and femur bones and was maintained in DMEM media supplemented with 20% FBS and macrophage colony-stimulating factor (20 ng/mL). Primary macrophages were isolated in 1 mg/mL dispase and replated for experiments. Retrovirus were produced using pSEB-3F (for expression of Xpress-Miz1) or pSEB-HUS (for expression of siRNAs) vector systems. On the third day of macrophages culture, cells were infected with the retroviruses two to three consecutive times (with 6-h intervals). After 24 h, the infection procedure was repeated. Forty-eight hours after the second round of infection, cells were split into 96-well plate (1×10^4 cells/plate) and then treated with TNF-α (10 ng/mL, 8 h).

Mouse Inflammation Cytometric Bead Array Assay. Cytokines (TNF, IL-6, and moncyte chemoattractant protein-1) production was measured with mouse inflammation cytometric bead array kit (BD Biosciences), according to the manufacturer's instruction, using a FACSCalibur flow cytometer at the Flow Cytometry Core Facility, University of Chicago. The sample results were generated in graphical and tabular format using cytometric bead array analysis software (BD Biosciences).

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4. Lin A, et al. (1995) Identification of a dual specificity kinase that activates the Jun kinases and p38-Mpk2. *Science* 268:286–290.
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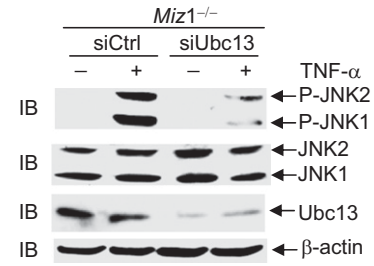

Fig. S1. Knockdown of Ubc13 also abrogates TNF-α–induced JNK activation induced by the loss of Miz1. Miz1-null MEFs were transfected with the control siRNA (siCtrl) or siUbc13 (100 nM each) and treated without or with TNF-α (5 ng/mL, 15 min). Phospho-JNK and expression levels of JNK, Ubc13, and β-actin were analyzed by immunoblotting with corresponding antibodies.

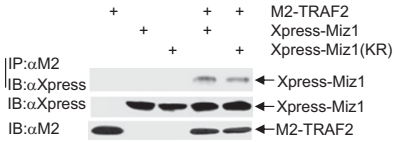

Fig. S2. Miz1(K/R) mutant binds to TRAF2 similarly to its wild-type counterpart. HEK293 cells were transfected with expression vector encoding M2-TRAF2 (5 μg), Xpress-Miz1, or Xpress-Miz1(K/R) mutant, in which lysines 388 and 472 has been replaced by arginines (5 μg each). M2-TRAF2 was immunoprecipitated by anti-M2 antibody and its associated Xpress-Miz1 or the K/R mutant was detected by immunoblotting with anti-Xpress antibody.