Supporting Information

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SI Materials and Methods

DNA Constructs and Chemicals. cDNAs encoding mouse mitogen-activated protein kinase kinase 1 (MKK1), human MKK6, and its cysteine mutants were cloned into pET32M for the production of thioredoxin(Trx)-6xHis fusion proteins (1). In addition, eukaryotic expression vectors encoding the wild-type HA-MKK6, HA-MKK6-4C, and HA-MKK6-6C were generated by subcloning cDNA inserts into a pcDNA3-based vector. Prokaryotic expression vectors expressing glutathione S-transferase (GST)-p38α(KM), GST-Jun N-terminal kinase 1 (JNK1), and GST-JNK kinase 1 (JNKK1), were described previously (2). GST-extracellular signal-regulated kinase (ERK) 2 was a gift from Michael Karin (University of California, San Diego, La Jolla, CA). N-(biotinoyl)-N′-(iodoacetyl)ethylenediamine (BIAM), and 2′-(or-3′)-O-(BODIPY FL)-β:γ-imidoadenosine 5′-triphosphate (BODIPY FL AMPPNP) were purchased from Invitrogen; Iodoacetic acid (IAA), thrombin, sorbitol, and 5′,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Sigma-Aldrich; H_2O_2 was from Merck.

Cell Culture. Mouse embryonic fibroblasts derived from MKK3/6 double-knockout embryos, HeLa, and RAW264.7 cells were maintained in DMEM supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin in a 37 °C incubator with 5% CO_2.

Protein Expression and Purification. Various protein-expressing constructs were transformed into BL21(DE3). Bacterial cells were induced at OD_{600} of 0.6 with 0.1 mM isopropyl-β-D-thiogalactopyranoside, and the induction was carried out for 4 h at room temperature or at 16 °C overnight. GST and Trx-6xHis fusion proteins were purified on glutathione-Sepharose beads (GE Healthcare) and Ni-nitrilotriacetate (Ni-NTA) His•Bind Resins (Merck), respectively. To remove the Trx-His tag, purified fusion proteins were first digested with thrombin followed by removal of Trx-His with a Ni-NTA His•Bind Resins column. To obtain air-induced oxidized kinases, we used reducing agent-free buffers throughout purification. Furthermore, we dialyzed purified kinases in a reducing agent-free buffer (25 mM Tris, PH 8.0, 100 mM KCl, 5 mM $MgCl_2$, and 10% glycerol) at 4 °C for three times (first time, 4 ∼ 6 h; second and third times, 12 h each).

Transfection and Cell Lysis. DNA transfection was performed with Lipofectamine/PLUS reagents from Invitrogen following manufacturer's instruction. Cells were harvested and resuspended in the lysis buffer (2) for 10 min on ice. Whole cell extracts (WCE) were prepared by centrifugation to remove cell debris.

In Vitro Kinase Assays. The in vitro protein kinase assays were performed as described previously (2). The phosphorylated p38 mitogen-activated protein kinase (MAPK) was revealed after SDS-PAGE by either autoradiography or Western blot analysis using an antibody specifically recognizing the dually phosphorylated (i.e., active) p38 MAPK (9211, Cell Signaling Technology). To generate reduced kinases, the purified MKK6, JNKK1, and MKK1 were preincubated with 1 mM DTT for 5 min on ice before the reaction.

Phorbol 12-Myristate 13-Acetate (PMA)-Induced Oxidative Burst in RAW264.7 Cells. RAW264.7 cells were cultured in DMEM containing 10% FBS under adherent conditions in 6-cm plates (80% confluent). Before PMA treatment, cells were cultured overnight in the serum-free medium. Cells were washed twice with PBS and subsequently resuspended in PBS containing 1 μM PMA for 30 min.

GST Pull-Down Assays. Fifteen micrograms of GST or GST-p38 immobilized on glutathione-Sepharose beads were incubated with 10 μg of purified MKK6, with or without 1 mM DTT, in 400 μL of the binding buffer (PBS with 0.25% Triton X-100) for 1 h at 4 °C. After brief spin-down, the beads were washed three times with the binding buffer, and boiled in 1× SDS sample buffer for 5 min. The supernatant was then subjected to Western blot analysis with an anti-MKK6 antibody.

Antibodies and Western Blot Analysis. Anti-phospho-MKK3/MKK6 (Ser189/207) and anti-phospho-p38 MAPK (Thr180/Tyr182) were from Cell Signaling; anti-HA(Y-11) was from Santa Cruz Biotechnology, Inc.; anti-β-tubulin was from Sigma-Aldrich; anti-MKK6/MKK3 was from Millipore; HRP-conjugated streptavidin was from Thermo Fisher Scientific. Western blot analysis was conducted as described previously (3).

Labeling of Reduced Cysteines by BIAM. BIAM-mediated alkylation of reduced cysteines was carried out as described previously with minor modification (4). HeLa or RAW264.7 cells were treated with 200 μM H_2O_2 for 10 min. After medium removal, the cells were frozen in liquid nitrogen and the frozen cells were then resuspended in 400 μL of oxygen-free (by bubbling with nitrogen gas at a low rate for 20 min) lysis buffer containing 100 μM BIAM for 15 min at room temperature. Soluble cell extracts were then prepared by centrifugation and subjected to immunoprecipitation with an anti-HA or anti-MKK6 antibody. For double-labeling with IAA and BIAM, HeLa cells were first resuspended in the lysis buffer containing 30 mM IAA at 37 °C for 15 min. HA-MKK6 was immunoprecipitated, then denatured with the addition of 200 μL of lysis buffer containing 8 M urea and 30 mM IAA for another 30 min at 37 °C to ensure the labeling of those reduced cysteines that were not exposed in the native protein. Proteins were then precipitated with cold acetone/HCl/H_2O (92:2:10) to remove IAA. After centrifugation, the pellets were resuspended in 50 μL lysis buffer containing 8 M urea, 3.5 mM DTT, and 10 mM BIAM for 15 min at 37 °C. The biotin-labeled MKK6 was detected by Western blot analysis using an HRP-conjugated streptavidin antibody.

Quantification of Free Thiols Using Ellman's reagent (DTNB). Ten microliters of protein samples, 50 μL of DTNB solution (50 mM sodium acetate, 2 mM DTNB in H_2O), and 100 μL of 1 M Tris solution (pH 8.0) were added into 840 μL 6 M guanidinium chloride, pH8.0 solution. The solution was mixed well and the optical absorbance was measured at 412 nm. The −SH content in the samples was calculated as follows: sample dilution factor (i.e., 1000 μL/10 μL = 100) × $OD_{412\,nm}$ ÷ 13600 $M^{-1}\,cm^{-1}$ (the extinction coefficient of the reagent). Immediately before the DTNB assays, the excess DTT in protein samples was removed by passing the protein samples through disposable PD-10 columns (GE Healthcare).

Circular Dichroism Measurement. CD spectra of both the reduced and oxidized MKK6 proteins were measured in a JASCO J-815 CD Spectropolarimeter (Jasco) at room temperature using a

cuvette of 1-mm path length. Each spectrum was collected with 10 scans spanning a spectral window of 200–250 nm. The proteins were dissolved in 25 mM Tris buffer (pH 8.0) with or without 1 mM DTT. The protein concentration used in the CD experiment was 10 μM.

Equilibrium Fluorescence Anisotropy. Fluorescence anisotropy binding assays were performed on a PerkinElmer LS-55 fluorimeter equipped with an automated polarizer at 25 °C. The fluorescence anisotropy was measured using a constant concentration of BODIPY FL AMPPNP (30 nM) and increasing amount of MKK6 proteins in the binding buffer (25 mM Tris, pH 8.0, 100 mM KCl, 5 mM $MgCl_2$, and 10% glycerol), with or without 1 mM DTT. The titration curves were fitted with the MicroCal Origin software package. BODIPY FL AMPPNP was excited at 490 nm and fluorescence emission was determined at 514 nm.

Sample Preparation, Tandem Mass Spectrometry, and Database Searching. Protein pellets dissolved in 8 M urea were alkylated with 10 mM iodoacetamide (IAM) in 100 mM ammonium bicarbonate for 30 min. The excess IAM was removed by precipitating proteins with trichloroacetic acid followed with acetone wash. Proteins were further reduced with 5 mM Tris(2-carboxyethyl)phosphine hydrochloride for 20 min, followed by treatment with 10 mM N-ethylmaleimide for 20 min. Sequencing grade soluble trypsin was used for digestion. The resulting peptides were subjected to multidimensional liquid chromatography analysis. The data-dependent tandem mass spectrometry analysis was performed in a LTQ-Orbitrap mass spectrometer (Thermo Electron).

1. Feng W, Long JF, Zhang M (2005) A unified assembly mode revealed by the structures of tetrameric L27 domain complexes formed by mLin-2/mLin-7 and Patj/Pals1 scaffold proteins. *Proc Natl Acad Sci USA* 102:6861–6866.
2. Wu Z, Wu J, Jacinto E, Karin M (1997) Molecular cloning and characterization of human JNKK2, a novel Jun NH2-terminal kinase-specific kinase. *Mol Cell Biol* 17:7407–7416.
3. Sun L, et al. (2007) JAK1-STAT1-STAT3, a key pathway promoting proliferation and preventing premature differentiation of myoblasts. *J Cell Biol* 179:129–138.
4. Giannoni E, Buricchi F, Raugei G, Ramponi G, Chiarugi P (2005) Intracellular reactive oxygen species activate Src tyrosine kinase during cell adhesion and anchorage-dependent cell growth. *Mol Cell Biol* 25:6391–6403.

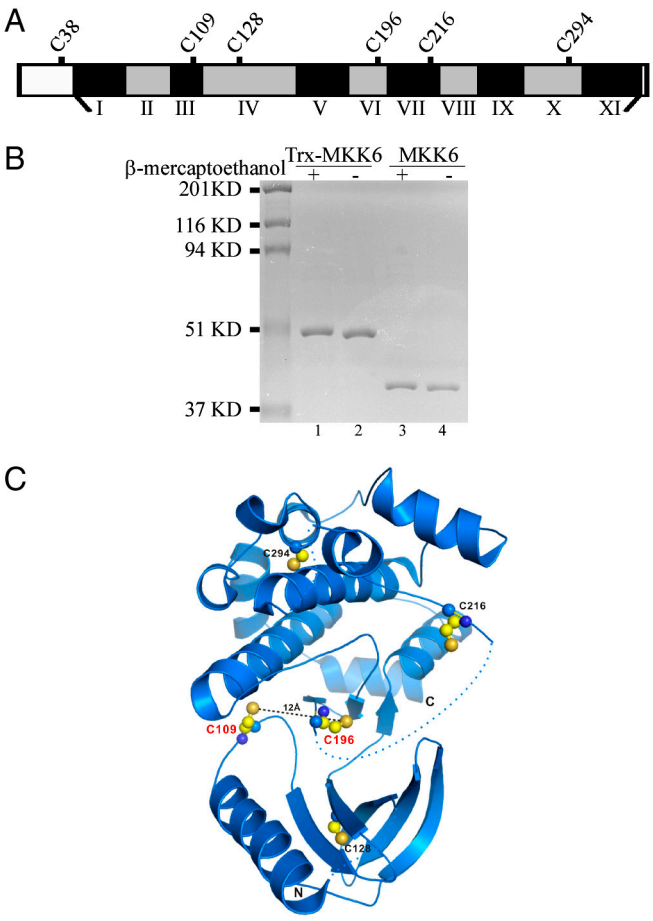

Fig. S1. (*A*) A schematic diagram showing the positions of six cysteine residues in human MKK6. Roman numerals I to XI denote 11 conserved kinase subdomains in MKK6. (*B*) The oxidized recombinant MKK6, with or without preincubation with 5% (vol/vol) β-mercaptoethanol, was electrophoresed on a nonreducing SDS-polyacrylamide gel and visualized with Coomassie blue. (*C*) The ribbon diagram of the structure of a constitutively active MKK6 mutant was shown. The positions of all five cysteines were indicated with the sulfur atoms indicated by brown balls.

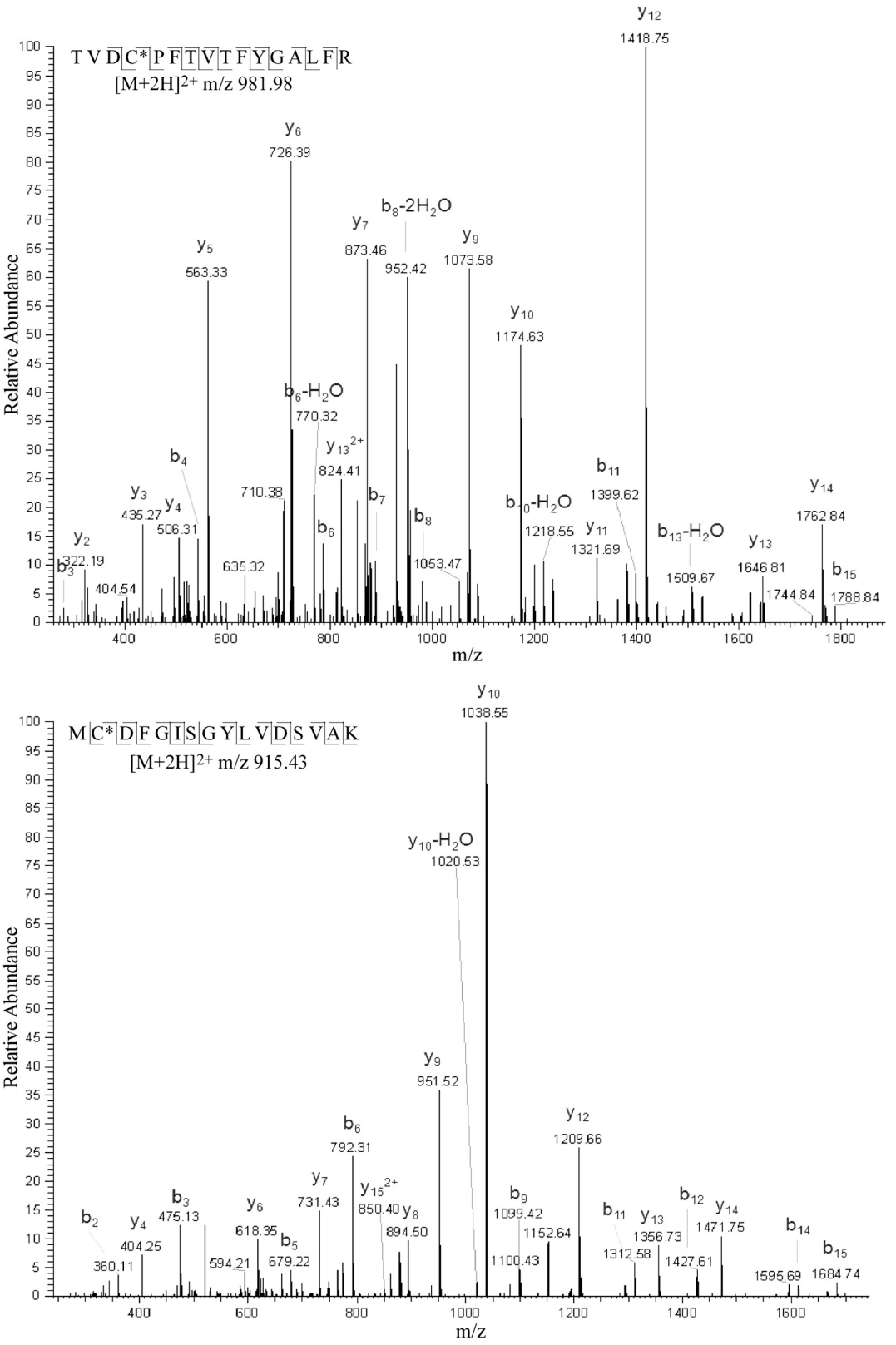

Fig. S2. The collision-induced dissociation mass spectra of the $[M + 2H]^{2+}$ ions at *m/z* 981.98 (*Upper*) and 915.43 (*Lower*). The fragment *b* and *y* ions were assigned on top of the peaks.