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Supplementary Information for

Effects of ALS-associated TBK1 mutations on protein-protein interactions and kinase activity

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Supplementary Text

Materials and Methods

Cell culture and the generation of TBK1 knockout cells
293T cells and primary fibroblasts derived from G217R TBK1 mutation carriers skins (a gift from Dr. Frank Baas of Leiden University Medical Center in the Netherlands) were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% Fetal Bovine Serum. To generate TBK1 knockout cells, cells were transfected with two modified px459 plasmids, each encodes Cas9 and a distinct gRNA sequence targeting the *Tbk1* gene (5'- gaagtgctctgcatcttggc-3 and 5'- gctactgcaaatgtctttcg-3'). Puromycin selection was initiated 24hrs after transfection, and lasted for two days. About 20 single clones were picked and expanded from surviving cells and tested for the loss of TBK1 by Western blot. The deletion of the *Tbk1* alleles from the positive clones was further confirmed by PCR.

Transfection and Luciferase reporter assays
All transfection experiments were carried out with Lipofectamine 2000 transfection reagent (Invitrogen) according to manufacturer's manual. For reporter assays, TBK1 knockout 293T cells (seeded in 96-well plates, with ~45,000 cells/well) were transfected with TBK1 expression constructs together with IFNβ promoter or PDRIIx3-driven firefly reporter and HSV TK promoter-driven Renilla luciferase constructs. Luciferase activity was measure 24 hrs after transfection with Dual-Glo luciferase assay system (Promega) by a BioTek synergy HT plate reader.

Co-immunoprecipitation, Western blots and antibodies
For Co-IP, cell lysates were prepared in a lysis buffer (20 mM Tris·HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 30 mM NaF, 1 mM glycerophosphate, 1X proteinase inhibitor (Roche), and 1 mM Na_3VO_4), and the magnetic anti-Flag M2 beads (Sigma) were added to lysates and rotated at 4ºC for 2 hrs. Beads were collected with a DynaMag™-2 magnet and washed for 4 times with the same lysis buffer. IP samples were denatured in sampling buffer and subjected to SDS/PAGE for Western blot analysis. Anti-pS172 TBK1, IRF3, p42/p44 MAPK, phospho-S403 p62 and GAPDH antibodies were purchased from Cell Signaling Technology; Anti-TBK1, phospho-S386 IRF3, anti-p62 and β-Actin antibodies were purchased from Abcam. Anti-Flag (M2) and anti-HA antibodies were from Sigma and Bethyl Laboratories, respectively. Anti-GFP antibody was from Santa Cruz Biotechnology. Anti-pS177 OPTN antibody was a gift from Ivan Dikic (Goethe University, Germany).

In vitro kinase assays
Kinase assays were conducted in a buffer system of 20 mM HEPES, pH 7.5, 20 mM β-Glycerophosphate, 0.1 mM Na_3VO_4, 10mM $MgCl_2$ and 50 μM ATP, with recombinant TBK1 (directly from immunoprecipitation beads) with either Flag-IRF3, GST-p62 or HA-OPTN as substrates at 30ºC for specified time in figure legends. Phosphorylation of target proteins was analyzed by Western blots probing with phospho-specific antibodies.

Analytical ultracentrifugation and SEC-MALS analysis
Sedimentation equilibrium measurements were performed in a Beckman XL-A/I analytical ultracentrifuge (Beckman-Coulter, Palo Alto CA, USA), utilizing six-cell centerpieces. Protein samples were diluted in the Dilution buffer (20 mM HEPES, 300 mM NaCl, and 1 mM Tris (2-carboxyethyl) phosphine (TCEP), pH 7.4) to 8.2 μM, 5.4 μM

and 2.9 μM in channels A, B and C, respectively. Samples were run at four different speeds (9000 rpm for 20 hrs, 11000 rpm, 14000 rpm and 17,000 rpm for 10hrs each, with four UV scans at 280 nm of 1 hr interval between different speeds). Solvent density and protein v-bar were determined using the program SednTerp. (Alliance Protein Laboratories, Corte Cancion, Thousand Oaks, CA, USA). K_d and molecular weight were calculated through global fit model in the program HeteroAnalysis (www.biotech.uconn.edu/auf). SEC-MALS analyses were performed on an Akta chromatograpy system (GE Healtcare), equipped with a Superdex 200 Increase 10/300 GL column (GE Healtcare). Samples (diluted to different concentrations in the Dilution buffer) were run at a flow of 0.5 mL/min at room temperature. Molecular weights were calculated from light scattering and refractive index data using Astra 6.1 software (Wyatt Technologies).

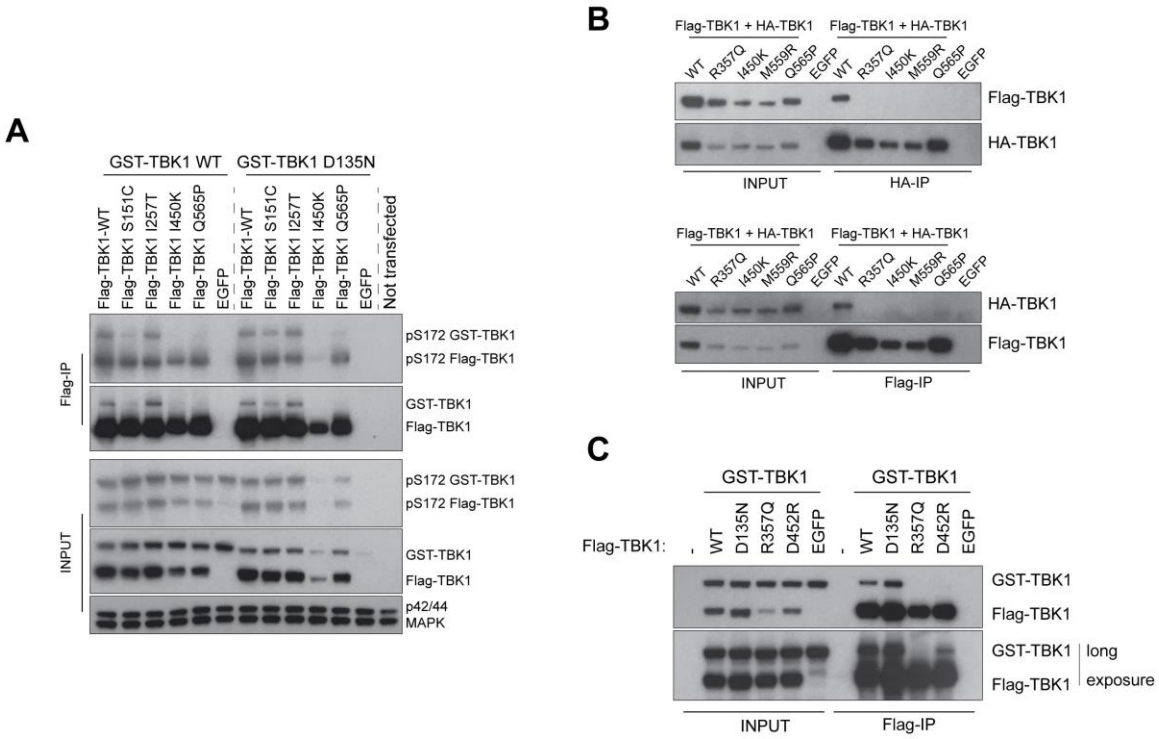

Figure S1. TBK1 I450K, Q565P and D452R mutants display reduced dimer formation. (A)TBK1 I450K and Q565P mutants display diminished dimerization. Expression constructs of Flag-tagged TBK1 WT, S151C, I257T, I450K and Q565P mutants were cotransfected with GST-tagged WT or D135N TBK1 constructs into TBK1 knockout (KO) 293T cells. Protein lysates were prepared 24hrs later and subjected to anti-Flag IP. The levels of TBK1 and phospho-S172 TBK1 in the lysates and IP samples were analyzed by Western blots with specific antibodies. (B) TBK1 R357Q, I450K, M559R and Q565P are defective in homodimer formation. Flag-tagged TBK1 WT, R357Q, I450K, M559R and Q565P mutants were co-expressed with corresponding HA-tagged mutants in TBK1 KO 293T cells for 24 hrs. Immunoprecipitations were conducted with either anti-Flag or anti-HA beads and the association of another partner was analyzed by Western blots with anti-HA or Flag antibodies. (C) TBK1 D452R mutation also affects dimerization. Flag-tagged TBK1 WT, D135N, R357Q and D452R mutants were co-expressed with GST-tagged WT TBK1 in TBK1 KO 293T cells. 24hrs after transfection, protein lysates were prepared and subjected to IP with anti-Flag beads. The levels of TBK1 in the lysates and IP samples were analyzed by Western blot with anti-TBK1 antibodies.

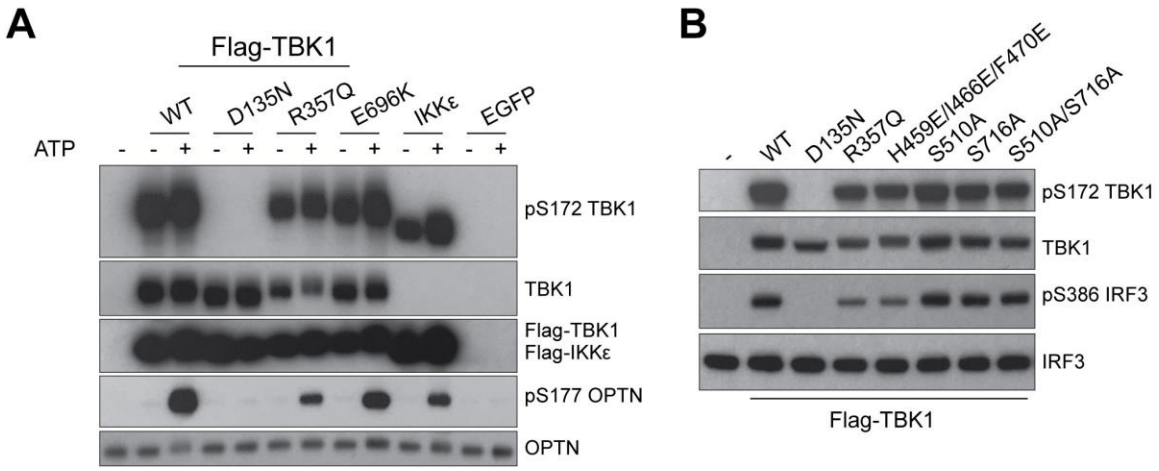

Figure S2. TBK1 R357Q is a functional kinase. (A) TBK1 R357Q is capable of phosphorylating OPTN. Expression constructs for Flag-tagged TBK1 WT, D135N, R357Q, E696K mutants, IKKε and EGFP were transfected into TBK1 knockout 293T cells. 24hrs later, protein lysates were prepared and subjected to anti-Flag IP. In vitro kinase assays were conducted directly with IP samples with recombinant OPTN as a substrate at 30ºC for 40 min. The levels of pS172 TBK1, total TBK1, Flag-tagged TBK1 or IKKε, pS177 OPTN and total OPTN were analyzed by Western blots with specific antibodies. (B) TBK1 R357Q behaves similarly to the monomeric TBK1 H459E/I466E/F470E (HIF) mutant. Expression constructs for WT TBK1, TBK1 D135N, R357Q, HIF, S510A, S716A and S510A/S716A mutants were transfected into TBK1 KO 293T cells, total cell lysates were prepared 24hrs later and the levels of phospho-S172 TBK1, phospho-S386 IRF3, total TBK1 and IRF3 were determined by Western blots with specific antibodies.

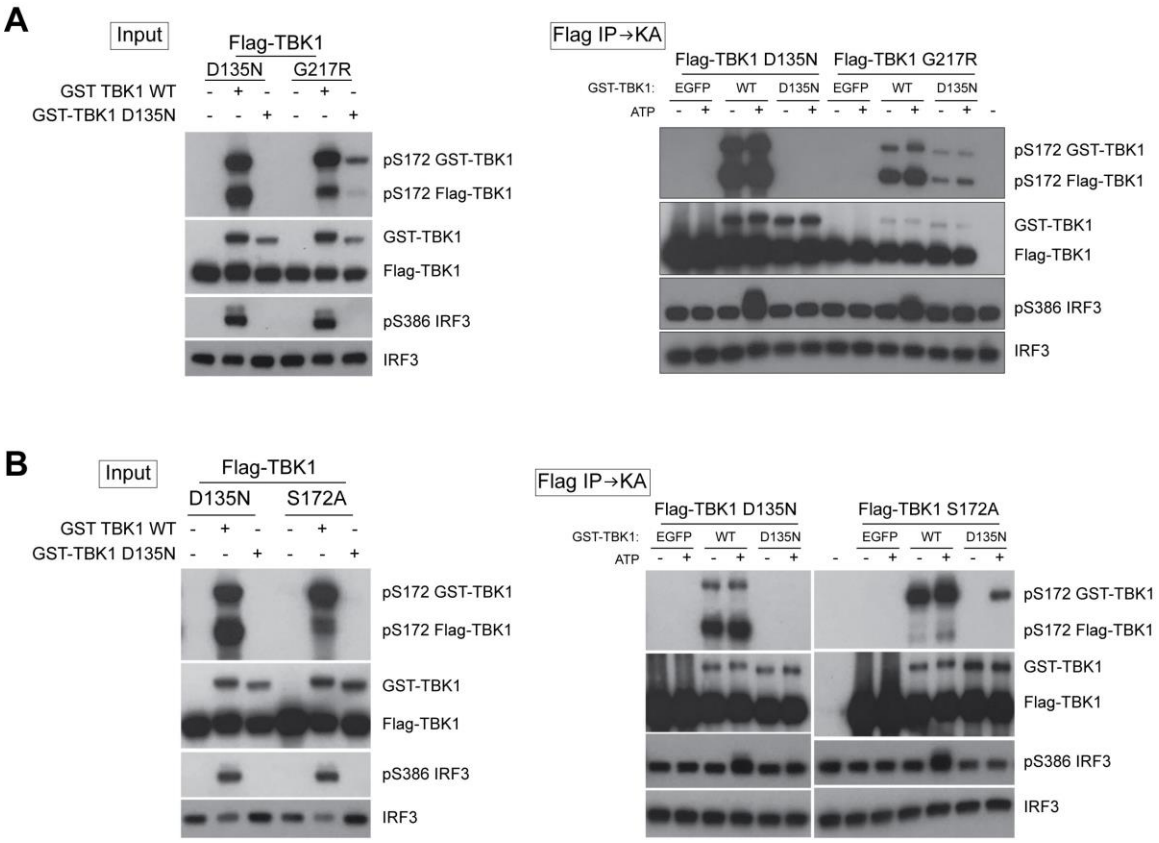

Figure S3. Complementation between mutant TBK1 subunits within a dimer. GST-tagged WT TBK1 or D135N TBK1 were co-expressed with Flag-tagged D135N or G217R (A), or S172A TBK1 (B) or EGFP as a control in TBK1 KO 293T cells. Cell lysates were prepared 24hrs after transfection, immunoprecipitations were conducted with anti-Flag beads, followed by kinase assays with recombinant IRF3 as a substrate at 30ºC for 40 min. The levels of pS172 TBK1, total TBK1, pS386 IRF3 and total IRF3 were determined by Western blots with specific antibodies. Note the exposure time to acquire the image of the top right panel in IP→KA assays in B was longer than that for the top left panel.

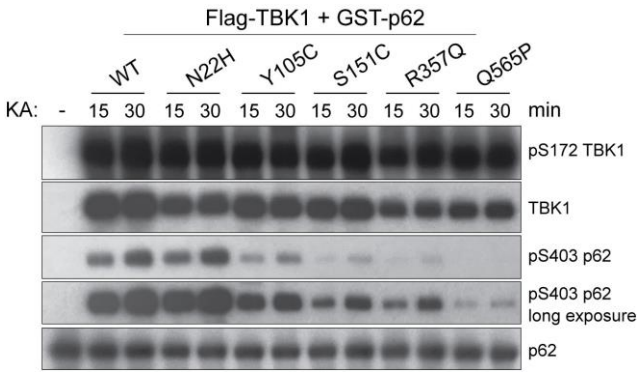

Figure S4. Reduced kinase activity of selected TBK1 ALS mutations towards p62. Flag-tagged WT TBK1, or TBK1 N22H, Y105C, S151C, R357Q or Q565P mutant proteins were expressed in TBK1 KO 293T cells and purified by anti-Flag beads IP. Kinase assays with GST-p62 as a substrate were conducted on beads at 30ºC for increasing time (15 and 30 min) and stopped by the addition of equal amount of 2X SDS-PAGE loading dye. Samples were resolved by SDS-PAGE and the levels of pS172 TBK1, total TBK1, pS430 p62 and total p62 from the reactions were determined by Western blots with specific antibodies.