SUPPLEMENTARY ONLINE DATA

TfR1 interacts with the IKK complex and is involved in IKK–NF-κB signalling

Niall S. KENNETH1,2, Sharon MUDIE1, Sanne NARON and Sonia ROCHA3

Wellcome Trust Centre for Gene Regulation and Expression, College of Life Sciences, MSI/WTB/JBC Complex, Dow Street, University of Dundee, Dundee DD1 5EH, Scotland, U.K.

Cells
IKKβ−/− and wild-type MEFs were a gift from Professor Inder Verma (Salk Institute, San Diego, CA, U.S.A.) and grown in DMEM supplemented with 10% (v/v) fetal bovine serum, 50 units/ml penicillin and 50 μg/ml streptomycin for no more than 30 passages. MDA-MB-231 breast carcinoma cell lines were grown in DMEM supplemented with 10% (v/v) fetal bovine serum, 50 units/ml penicillin and 50 μg/ml streptomycin for no more than 30 passages. IKKβ−/− TAP and TAP–IKKβ cells were maintained in DMEM supplemented with 10% (v/v) fetal bovine serum, 50 units/ml penicillin and 50 μg/ml streptomycin with 2.5 μg/ml puromycin (Sigma).

Creation of stable reconstituted IKKβ−/− TAP and TAP–IKKβ MEFs
Reconstitution of MEFs was generated by retroviral infection. Viral stocks were obtained from supernatant of HEK-293 cells, which were co-transfected with viral envelope plasmid, packaging plasmid and expression vector plasmid for 48 h. Cells infected with virus were selected in DMEM supplemented with 10% (v/v) fetal bovine serum, 50 units/ml penicillin and 50 μg/ml streptomycin with 2.5 μg/ml puromycin. Individual clones were screened by Western blotting for IKKβ expression levels.

TAP-tag and SILAC procedures
Cells were grown for at least five cell divisions in either light (i.e. non-labelled L-lysine and L-arginine) or heavy (L-[13C6]lysine hydrochloride and L-[13C6]arginine hydrochloride) labelled media before TAP-tag purification procedures. SILAC medium was obtained from Dundee Cell Products. TAP-tag purification protocol was followed as described in [12]. Liquid chromatography–MS/MS (tandem MS) was performed using an Ultimate U3000 nanoflow system (Dionex) and a linear ion-trap–orbitrap hybrid mass spectrometer (LTQ-Orbitrap XL, Thermo Fisher Scientific) via a nanoelectrospray ion source (Proxeon Biosystems) as described previously [13]. Data were acquired using Xcalibur software, and quantification was performed using Max-Quant (http://msquant.alwaysdata.net/) and Mascot search engine (Matrix Science; http://www.matrixscience.com/) for peptide identification against the International Protein Index (IPI) human protein database. The initial mass tolerance was set to 7 p.p.m., and MS/MS mass tolerance was 0.5 kDa. Enzyme was set to trypsin/P (trypsin with no proline restriction) with three missed cleavages. Carbamidomethylation of cysteine was searched as fixed modification, whereas N-acetyl-protein and oxidation of methionine were searched as variable modification. A minimum of two peptides was quantified for each protein.

DNA constructs
VSV-G (vesicular stomatitis virus glycoprotein; viral envelope protein), CMVR8.91 (cytomegalovirus viral packaging plasmid) and PIRESpuro-deNotI (viral vector plasmid) were a gift from Professor Ron Hay. PIRESpuro-deNotI-TAP and PIRESpuro-deNotI-TAP-IKKβ were cloned from pCNA3-IKKβ-Flag (a gift from Professor Ron Hay) and RSV-TAP (a gift from Professor Neil Perkins) by Elizabeth Farrell (CLS cloning service). GST (glutathione transferase)–TFR1-(1–64) were created by PCR subcloning into pGEX4T, using the primers 5′-GATATAGGATCCATGGATGATCAAGCT-3′ and 5′-GGCCAAGCTTAACTTCCACTACACCT-3′.

1 These authors contributed equally to this study
2 Present address: Department of Pathology, University of Michigan, Ann Arbor, MI 48109, U.S.A.
3 To whom correspondence should be addressed (email s.rocha@dundee.ac.uk).
Figure S1 Identification of TFR1 as an IKKβ-interacting protein

(A) Characterization of reconstituted IKKβ−/− MEFs. Western blot analysis for IKKβ and IKKα in cells reconstituted with TAP or TAP–IKKβ. β-Actin was used as loading control. (B) IKKβ reconstitution into IKKβ−/− MEFs rescues NF-κB DNA-binding activity. IKK wild-type (WT), IKKβ−/− and IKKβ−/− TAP–IKKβ MEFs were treated with 10 ng/ml TNFα for 30 min before nuclear extracts were prepared. NF-κB DNA binding was analysed by EMSA using a NF-κB consensus binding probe based on the HIV κB site. (C) TAP-tag purification using reconstituted IKKβ−/− MEFs followed by Western blot analysis for IKKβ. Lane 1, TAP lysate; lane 2, TAP–IKKβ lysate; lane 3, TAP lysate post-IgG beads; lane 4, TAP–IKKβ lysate post-IgG beads; lane 5, TAP bound to IgG beads; lane 6, TAP–IKKβ bound to IgG beads; lane 7, TAP–IκκβTEV eluate; lane 8, TAP–IKKβTEV eluate; lane 9, TAP–IKKβTEV eluate post-TEV protease; lane 10, TAP–IKKβTEV eluate post-calmodulin beads; lane 11, TAP–IKKβTEV eluate post-calmodulin beads; lane 12, TAP–IKKβTEV eluate post-calmodulin beads; lane 13, TAP calmodulin beads; lane 14, TAP–IKKβ calmodulin beads. (D) HEK-293 cells were transfected with the plasmids indicated and whole-cell lysates were prepared. Anti-GFP antibody-bound beads were used to immunoprecipitate (IP) GFP–TFR1, precipitates were resolved by SDS/PAGE and then analysed by Western blotting using the antibodies indicated. (E) Purified GST or GST–TFR1-(1–64), expressed in Escherichia coli and bound to glutathione–agarose, was used in a pull-down assay with reticulocyte lysate-translated IKKβ.

Figure S2 Characterization of U2OS-NF-κB reporter cells

U2OS-NF-κB luciferase reporter cells were treated with 10 ng/ml TNFα for the times indicated before luciferase activity was measured. Results are means ± S.E.M. for at least three independent experiments expressed as fold activation/repression relative to untreated cells.
Figure S3  TFR1 depletion impairs NF-κB activity

(A) Quantitative RT–PCR analysis of TFR1 mRNA prepared from U2OS cells depleted of TFR1 and subsequently stimulated with TNFα for the times indicated. (B) MDA-MB-231 cells were depleted of TFR1 and subsequently stimulated with TNFα for the times indicated. Whole-cell lysates were subjected to immunoblot analysis to determine the levels of the proteins indicated.

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