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

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The IKK [inhibitor of NF-κB (nuclear factor κB) kinase] complex has an essential role in the activation of the family of NF-κB transcription factors in response to a variety of stimuli. To identify novel IKK-interacting proteins, we performed an unbiased proteomics screen where we identified TfR1 (transferrin receptor 1). TfR1 is required for transferrin binding and internalization and ultimately for iron homoeostasis. TfR1 depletion does not lead to changes in IKK subunit protein levels; however, it does reduce the formation of the IKK complex, and inhibits TNFα (tumour necrosis factor α)-induced NF-κB-dependent transcription. We find that, in the absence of TfR1, NF-κB does not translocate to the nucleus efficiently, and there is a reduction in the binding to target gene promoters and consequentially less target gene activation. Significantly, depletion of TfR1 results in an increase in apoptosis in response to TNFα treatment, which is rescued by elevating the levels of RelA/NF-κB. Taken together, these results indicate a new function for TfR1 in the control of IKK and NF-κB. Our data indicate that IKK–NF-κB responds to changes in iron within the cell.

Key words: inhibitor of nuclear factor κB kinase (IKK), iron, nuclear factor κB (NF-κB), transferrin, transferrin receptor 1 (TfR1), tumour necrosis factor α (TNFα).

INTRODUCTION

NF-κB (nuclear factor κB) is the collective name of a family of transcription factors consisting of seven proteins, encoded by five genes: RelA, RelB, c-Rel, p105/p50 and p100/p52 [1]. All of these proteins are related via a highly conserved DNA-binding/dimerization domain, termed the Rel homology domain. Through this domain, NF-κB family members can form a variety of homo- and hetero-dimers to directly alter gene expression [1]. NF-κB-activation pathways can be classified as canonical, non-canonical and atypical regarding the mode and involvement of the IKK [IκB (inhibitor of NF-κB) kinase] complex [2].

IKK is a multimeric protein complex known to consist of at least three distinct subunits: two catalytic kinase subunits IKKα and IKKβ and a regulatory subunit IKKγ, also called NEMO (NF-κB essential modulator). IKKα and IKKβ are structurally related, but are functionally distinct, having different substrate specificities [3,4]. IKKγ, in contrast, has no catalytic activity, but instead is required for oligomerization and activation of the complex [3,4]. Despite recent findings of NF-κB-independent functions for IKK [5–8], the best understood role for these kinases is phosphorylation of IκB proteins and consequentially NF-κB activation [1].

IKK-mediated phosphorylation of IκBα generally triggers β-TrCP (β-transducin repeat-containing protein) binding, polyubiquitination and subsequently proteasomal degradation. The destruction of IκBα leads to the release of NF-κB dimers, translocation into the nucleus and binding to target gene promoters to either activate or repress their expression [1,4].

Early biochemical studies characterizing the IKKs revealed that they are part of a large molecular complex, of a size of approximately 900 kDa on size-exclusion chromatography analysis [9]. This suggests that other binding partners exist that could modulate the IKK complex activity and function. Indeed, proteins such as HSP90 (heat-shock protein 90) [10] and ELKS (glutamate-, leucine-, lysine- and serine-rich protein) [11] have been shown to interact with IKK and alter its function.

Given the importance of the IKK complex, we conducted an unbiased analysis for IKK-interacting proteins using quantitative MS. We have identified TfR1 (transferrin receptor 1), as a novel IKK-binding partner. Functionally, TfR1 is required for IKK complex activity, without altering IKK subunit levels. Importantly, depletion of TfR1 results in reduced NF-κB activation and increased apoptosis in response to TNFα (tumour necrosis factor α).

EXPERIMENTAL

Cells

U2OS osteosarcoma and HEK (human embryonic kidney)-293 cells were grown in DMEM (Dulbecco's modified Eagle's medium) (Lonza) supplemented with 10% (v/v) fetal bovine serum (Gibco), 50 units/ml penicillin (Lonza) and 50 μg/ml streptomycin (Lonza) for no more than 30 passages. U2OS-NF-κB luciferase reporter cells were grown in DMEM supplemented with 10% (v/v) fetal bovine serum, 50 units/ml penicillin and 50 μg/ml streptomycin with 150 μg/ml Hygromycin B (Roche).

Creation of stable U2OS-NF-κB luciferase reporter cells

U2OS cells were transfected with 2 μg of NF-κB-reporter construct pGL4.32[luc2P/NF-κB-RE/Hygro] (Promega) using GeneJuice® (Merck Biosciences). After 48 h, cells were split and cultivated under 300 μg/ml Hygromycin B. Individual clones were picked and selected on the basis of their response to

Abbreviations used: ChIP, chromatin immunoprecipitation; DFX, desferoxamine; DMEM, Dulbecco's modified Eagle's medium; EMSA, electrophoretic mobility-shift assay; GFP, green fluorescent protein; HEK, human embryonic kidney; HIF-1, hypoxia-inducible factor-1; HSP90, heat-shock protein 90; IAP2, inhibitor of apoptosis protein 2; c-IAP2, cellular IAP2; IκB, inhibitor of nuclear factor κB; IKK, IκB kinase; MEF, mouse embryonic fibroblast; NF-κB, nuclear factor κB; PARP, poly(ADP-ribose) polymerase; PCNA, proliferating-cell nuclear antigen; RT, reverse transcription; siRNA, small interfering RNA; TAP, tandem affinity purification; TfR1, transferrin receptor 1; TNFα, tumour necrosis factor α.

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TNFα. Selected cell lines were grown in DMEM supplemented with 10 % (v/v) fetal bovine serum, 50 units/ml penicillin and 50 μg/ml streptomycin with 150 μg/ml Hygromycin B.

DNA constructs

The GFP (green fluorescent protein)–TfR1 expression construct was a gift from Professor Wolfhard Almers (Oregon Health and Sciences University, Portland, OR, U.S.A.). pCNA3-IKKβ-Flag and pCNA3-IKKα were gifts from Professor Ron Hay (Dundee University, Dundee, U.K.) pGL4.32[luc2P/NF-κB-RE/Hygro] was obtained from Promega. RSV (respiratory syncytial virus)-RelA was a gift from Professor Neil Perkins (Newcastle University, Newcastle upon Tyne, U.K.). pCMVTAG-IKKγ-Flag was a gift from Professor Jon Ashwell (National Cancer Institute, Bethesda, MD, U.S.A.) (Addgene plasmid 11970) [12].

siRNA (small interfering RNA) transfection

siRNA duplex oligonucleotides were synthesized by MWG and transfected using Interferin (Polyplus) as per the manufacturer's instructions.

The siRNA sequences used were: Control, CAGUCGCGUUUGCGACUGG [13]; RelA, GCUGAUGUGCACCGACAAG [13]; TfR1_A, CCAAUACAGAGCAGACAUA; TfR1_B, ACUUGCUGUAGAUGAAGAA; TfR1_C, CUUCCAGACUAACAACAGA; and IKKγ, ACAGGAGGUGAUCGAUAAG [14].

RT (reverse transcription)–PCR and PCR sequences

Semi-quantitative RT–PCR and PCR was performed as described previously [14–16]. PCR products were resolved on 2 % agarose gels and scanned using a PhosphoImager (FujiFilm FLA-5100) into TIFF format. Quantitative RT–PCR was performed using cDNA templates [cDNA synthesis was performed using Quantitect Reverse Transcription kit (Qiagen)] amplified using specific primer sets and the Stratagene Brilliant II SYBR® Green qPCR mix according to the manufacturer's instructions. Amplification and detection were performed using a Stratagene Mx3005P detection system. Sample values obtained with specific primer sets were normalized to β-actin primer set values.

The PCR primer sequences used were: actin, 5′-CTGGGAGTGGGTGGAGGC-3′ (forward) and 5′-TCAACTGGTCTCAAGTCAGTG-3′ (reverse); IAP2 (inhibitor of apoptosis protein 2), 5′-GTCAAATGTTGAAAAAGTGCCA-3′ (forward) and 5′-GGGAAGAGGAGAGAGAAAGAGC-3′ (reverse); p100, 5′-AGCCTGGTAGACACGTACCG-3′ (forward) and 5′-CCGTACGCACTGTCTTCCTT-3′ (reverse); and TfR1-Quantitect primer assay (Qiagen). ChIP (chromatin immunoprecipitation) primers used were: p100 κB, 5′-CACTCCGAGGAGGAGACACT-3′ (forward) and 5′-GGAGCAACCTTGGGATTTTC-3′ (reverse); and p100 control, 5′-TATGGGGGATTGAAGCAGAG-3′ (forward) and 5′-TGGGCCAAATGGAATACTGT-3′ (reverse).

ChIP

Proteins were cross-linked with formaldehyde for 10 min. Glycine (0.125 mM) was added, and cells were washed with PBS. Cells were lysed with lysis buffer (1 % SDS, 10 mM EDTA, 50 mM Tris/HCl, pH 8.1, 1 mM PMSF, 1 mg/ml leupeptin and 1 mg/ml aprotonin), followed by sonication and centrifugation. The supernatant was pre-cleared with sheared salmon sperm DNA and Protein G–Sepharose beads (Sigma). The supernatant was incubated with specific antibodies overnight, and then with Protein G–Sepharose beads for 1 h. After an extensive wash step, the complexes were eluted with buffer (100 mM $NaHCO_3$ and 1 % SDS) and incubated with proteinase K. DNA was purified using QIAquick® PCR purification kit (Qiagen). PCR was performed on the purified immunoprecipitated DNA using specific primers.

Antibodies

Antibodies used were: anti-TfR1 (sc-51829, Santa Cruz Biotechnology), anti-phospho-RelA (Ser^{536}) (3031, Cell Signaling Technology), anti-RelA (sc-372, Santa Cruz Biotechnology), anti-PCNA (proliferating-cell nuclear antigen) (P8825, Sigma), anti-β-actin (A5441, Sigma), anti-IκBα (sc-371, Santa Cruz Biotechnology; 4812, Cell Signaling Technology), anti-phospho-IκBα (Ser^{32}/Ser^{36}) (9246, Cell Signaling Technology), anti-phospho-IKKα/β (2681, Cell Signaling Technology), anti-IKKγ (sc-8330, Santa Cruz Biotechnology), anti-IKKβ (2678, Cell Signaling Technology), anti-IKKα (2682, Cell Signaling Technology; sc-7182, Santa Cruz Biotechnology), anti-GFP (Roche), anti-FLAG (Sigma), anti-IAP2 (3130, Cell Signaling Technology), anti-p100/p52 (05-361, Millipore) and anti-(cleaved PARP) [poly(ADP-ribose) polymerase] (9541, Cell Signaling Technology).

Iron and DFX (desferoxamine) treatment

DFX mesylate (Sigma) was added for 2 h at a final concentration of 200 μM, ammonium iron(III) sulfate (Sigma) and ascorbate (Sigma) were also added for 2 h at final concentrations of 300 and 2 μM respectively.

Western blot quantification

Nuclear RelA blots were quantified using ImageJ (NIH), normalized with PCNA loading control and untreated control levels set to 1. All other conditions are compared with untreated non-targeting siRNA control levels.

Other experimental procedures

Whole-cell protein extracts, nuclear extracts, EMSAs (electrophoretic mobility-shift assays) and luciferase assays were performed as described previously ([15] and references therein).

RESULTS

TfR1 is an IKK-interacting protein

The IKK complex is central in the activation pathway of the transcription factor NF-κB [2]. As such, identification of a novel control mechanism would be of great interest for the understanding of the pathway, as well as for future therapeutic intervention. To unveil novel interacting proteins and potential novel regulators we employed the TAP (tandem affinity purification) technique followed by quantitative MS, utilizing SILAC (stable isotope labelling of amino acids in culture). We reconstituted IKK$\beta^{-/-}$ MEFs (mouse embryonic fibroblasts) with TAP–IKKβ or TAP alone (Supplementary Figure S1A at http://www.biochemj.org/bj/449/bj4490275add.htm). The TAP-tag consists of Protein A and a calmodulin-binding peptide separated by the recognition motif for the TEV (tobacco etch virus) protease. The functionality of the TAP–IKKβ-reconstituted MEFs was tested using NF-κB DNA binding following TNFα treatment. It was possible to see a rescue of IKK function in these cells, as measured by EMSA using an NF-κB consensus probe, to

Table 1 Protein significantly enriched in TAP–IKKβ purification

Following TAP purification, MS analysis and Mascot search, proteins identified were quantified using MSQuant software and enrichment levels of TAP–IKKβ were compared with TAP alone.

Protein ID	Fold enrichment in TAP–IKKβ cells
IKKβ	15.979
IKKα	15.299
IKKγ	6.258
TfR1	4.419
HSP90	4.157

a level comparable with that of wild-type MEFs (Supplementary Figure S1B).

Having established the cellular system, the TAP–IKKβ- or TAP-reconstituted IKK$\beta^{-/-}$ MEFs were cultured in either 'light' (i.e. non-labelled) L-lysine and L-arginine or heavy-isotope-labelled amino acids L-[$^{13}C_6$]lysine hydrochloride and L-[$^{13}C_6$]arginine hydrochloride medium. Lysates were obtained from TAP–IKKβ cells (heavy) and TAP cells (light) mixed at an equal ratio, before being subjected to a sequential purification procedure as outlined in Supplementary Figure S1(C). The purified material was separated by SDS/PAGE (4–12 % gels), and gel fragments were excised. A proteomic analysis was performed to identify and quantify the proteins that interact with TAP–IKKβ. Peptide ratios were quantified using MSQuant software to identify proteins that interact more with IKKβ. Comparison of the obtained peptide sequences with protein databases identified both IKKα and IKKγ, confirming that our purification technique pulls down known IKKβ-interacting proteins (Table 1). In addition, we also identified HSP90 as one of the IKKβ-associated proteins (Table 1). However, we did find a novel interaction partner for IKKβ: TfR1 (Table 1). We confirmed this interaction by Western blotting, using a specific antibody against TfR1 (Figure 1A).

TfR1 interacts with the endogenous IKK complex in human cells

To confirm that the interaction between TfR1 and IKK proteins is conserved in human cells, co-immunoprecipitation experiments between exogenous IKKα, IKKβ, IKKγ and TfR1 were performed. Our results demonstrate that TfR1 interacts with IKKα, IKKβ or IKKγ, but not with the negative controls (Figure 1B and Supplementary Figure S1D). To validate further our analysis, we performed co-immunoprecipitation experiments analysing endogenous proteins. This analysis confirmed that endogenous TfR1 interacts with all members of the IKK complex (Figure 1C). Finally, we tested whether under conditions of activation of the IKK complex, such as treatment with TNFα, TfR1 was still present in the complex. Our analysis revealed that TfR1 association with IKKβ or IKKγ does not change significantly with treatment with TNFα (Figure 1D).

To analyse the extent of association between TfR1 and the IKK complex, we analysed size-exclusion chromatography fractions for the presence of these proteins (Figure 2A). We found that endogenous TfR1 co-fractionates with the IKK complex subunits over several fractions, ranging from high-molecular-mass (1 MDa) to smaller complexes (Figure 2A). Since the bulk of TfR1 seems to co-fractionate mostly with IKKγ, we determined whether TfR1 was still able to associate with IKKα or IKKβ in the absence of IKKγ. Our analysis revealed that TfR1 remained associated with the catalytic IKKs, when IKKγ was depleted using siRNA (Figure 2B). These results indicate that TfR1 binds to the IKK complex in cells.

TfR1 is a single-pass type II membrane protein [17–19]. However, it does have a small intracellular portion, and it is internalized when bound to transferrin and subsequently recycled to the membrane again [17–19]. To test whether the intracellular portion of TfR1 was binding directly to IKK, we cloned the intracellular 64 residues of TfR1 into a bacterial expression construct. Protein fragments expressed in bacteria were purified and used in an *in vitro* binding assay with *in vitro* transcribed and translated IKKβ. Interestingly, we could not detect any specific binding of IKKβ to the small intracellular portion of TfR1 (Supplementary Figure S1E). These results suggest that the interaction is mediated by post-translational modifications on either of the proteins, requiring full-length TfR1, or it is indirect, via additional protein partners.

Depletion of TfR1 alters the stability of the IKK complex without changing IKK subunit levels

To address whether TfR1 influences IKK activity, we first sought to address whether IKK levels were altered by depleting TfR1 using RNAi (RNA interference). Relative to a non-targeting control, levels of the TfR1 protein were specifically decreased when U2OS cells were transfected with siRNAs against the TfR1 mRNA (Figure 3A). In contrast, TfR1 siRNA did not diminish the levels of the two IKK catalytic subunits IKKα and IKKβ or the regulatory subunit IKKγ.

The association between the regulatory subunit IKKγ, and the kinase subunits IKKα or IKKβ, is absolutely critical for the activity of the IKK complex [9]. To assess whether TfR1 was involved in IKK complex assembly, co-immunoprecipitation assays were used to investigate whether the integrity of the endogenous IKK complex is altered in the absence of TfR1. To this end, we depleted TfR1 using two different siRNAs, and immunoprecipitated IKKγ or IKKβ. Levels of associated IKKs were analysed by Western blotting (Figure 3B). Although expression of the IKK subunits is not altered by TfR1 depletion, association between the regulatory subunit IKKγ and the kinase subunit IKKβ is diminished in TfR1-compromised cells (Figure 3B). This is seen when either IKKβ or IKKγ are used to immunoprecitate the complex. Interestingly, IKKα interaction with IKKγ is still preserved, suggesting that TfR1 facilitates the IKKγ–IKKβ interaction, but has minimal effect on IKKγ–IKKα binding. These data indicate that TfR1 is required for the integrity of the full IKK complex.

Depletion of TfR1 impairs IKK and NF-κB activity in response to TNFα

The IKK complex responds to a number of stress stimuli, including cytokines such as TNFα [3]. Activation of the IKK complex can be assessed by phosphorylation in the T-loop, and by analysis of the phosphorylation status and levels of its substrate IκBα. To determine whether TfR1 is important for IKK activation following TNFα, TfR1 was depleted by siRNA and cells were treated with TNFα for different periods of time (Figure 4A). TNFα-induced IKK phosphorylation and activity were severely delayed and impaired when TfR1 was depleted (Figure 4A). As the IKK complex is the upstream kinase for the NF-κB family of transcription factors, we tested whether NF-κB transcriptional activity is altered in cells lacking TfR1. We created a U2OS cell line stably transfected with a NF-κB luciferase reporter construct. We initially tested NF-κB transcriptional activity in response to TNFα in a time-dependent manner (Supplementary Figure S2 at http://www.biochemj.org/bj/449/bj4490275add.htm). Luciferase

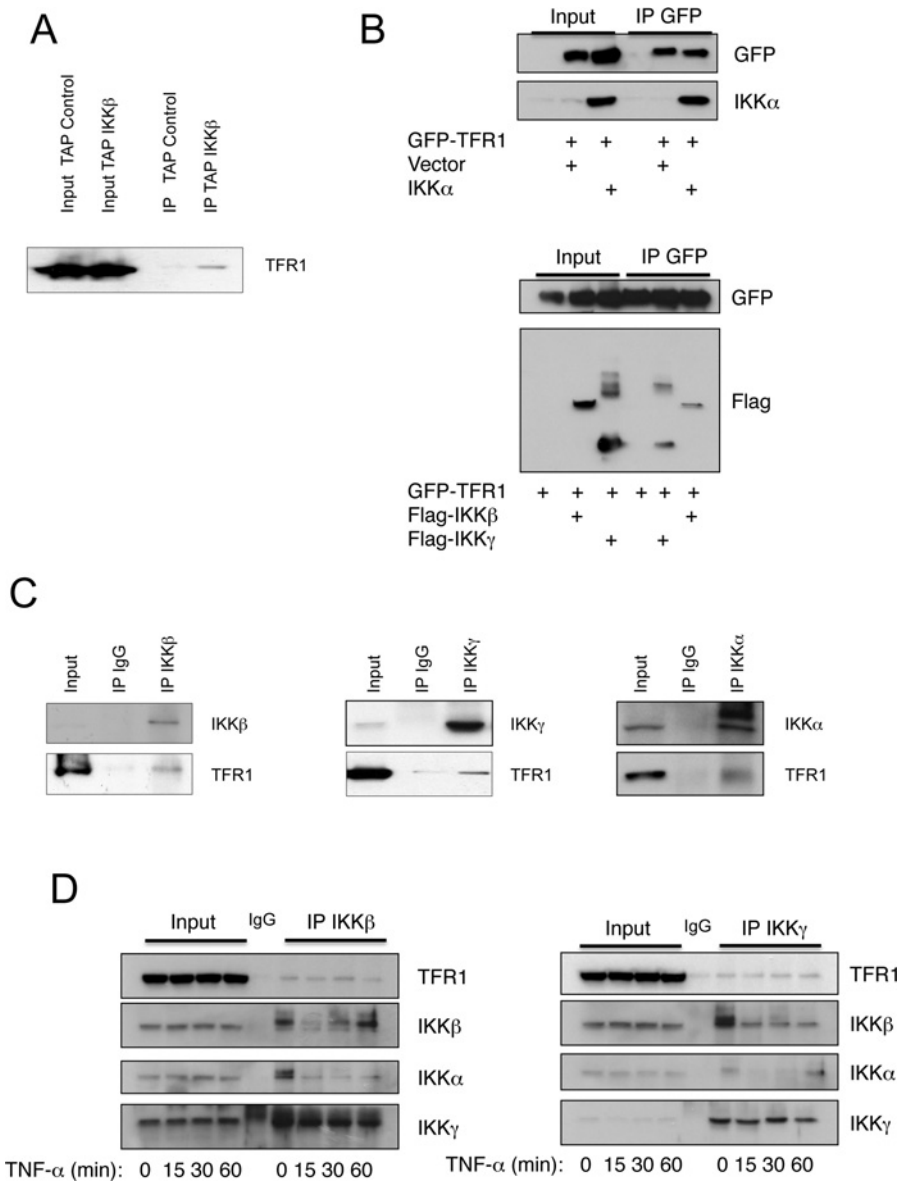

Figure 1 TfR1 interacts with IKK in cells

(**A**) TfR1 interacts with TAP–IKKβ. TAP-tag-purified material was analysed by Western blotting for the presence of TfR1. (**B**) HEK-293 cells were transfected with the plasmids indicated and whole-cell lysates were prepared. Anti-GFP antibody-bound beads were used to immunoprecitate GFP–TfR1. Precipitates were resolved by SDS/PAGE and then analysed by Western blotting using the antibodies indicated. (**C**) Whole-cell lysates were prepared from U2OS cells and immunoprecipitated with normal rabbit IgG or anti-IKK antibodies. Precipitates were resolved by SDS/PAGE and then analysed by Western blotting using the antibodies indicated. (**D**) U2OS cells were treated with 10 ng/ml TNFα for the times indicated before lysis. Whole-cell lysates were immunoprecipitated with normal rabbit IgG or anti-IKK antibodies. Precipitates were processed and analysed as in (**C**). IP, immunoprecipitation.

activity was increased following 2 h of TNFα treatment and reached maximal levels following 6 h of treatment (Supplementary Figure S2). These results are in agreement with the NF-κB-activation pattern in most cells, which is cyclical in nature [20,21]. Using the NF-κB reporter cells, we depleted TfR1 using siRNA, and stimulated for 5 h with TNFα before harvest and luciferase assay. In cells depleted of TfR1, both basal and TNFα-induced NF-κB activity were reduced compared with control cells (Figure 4B). Indeed, the fold decrease in NF-κB activity observed in cells lacking TfR1 is comparable with that observed with depletion of RelA, the NF-κB family member that is most responsive to TNFα (Figure 4B).

Our results based on siRNA-mediated depletion indicate that TfR1 is required for IKK complex formation and activation in response to TNFα. To assess whether increased TfR1 levels increase IKK activity, we overexpressed TfR1 and assessed the NF-κB transcriptional response to TNFα stimulation (Figure 4C). Our analysis revealed that increased TfR1 levels result in a slight increase in NF-κB transcriptional activity, supporting further the hypothesis that TfR1 modulates IKK–NF-κB activity. In addition, we tested whether NF-κB activity could be modulated by iron levels. As such, we treated cells with either an iron chelator or increased iron, and analysed NF-κB luciferase activity following TNFα treatment. We observed that depletion of iron resulted in lower luciferase activity, whereas addition of extra iron to the cells potentiated NF-κB activity (Figure 4D). This could also be observed at the level of two endogenous NF-κB targets: IAP2 and p100 (Figure 4E).

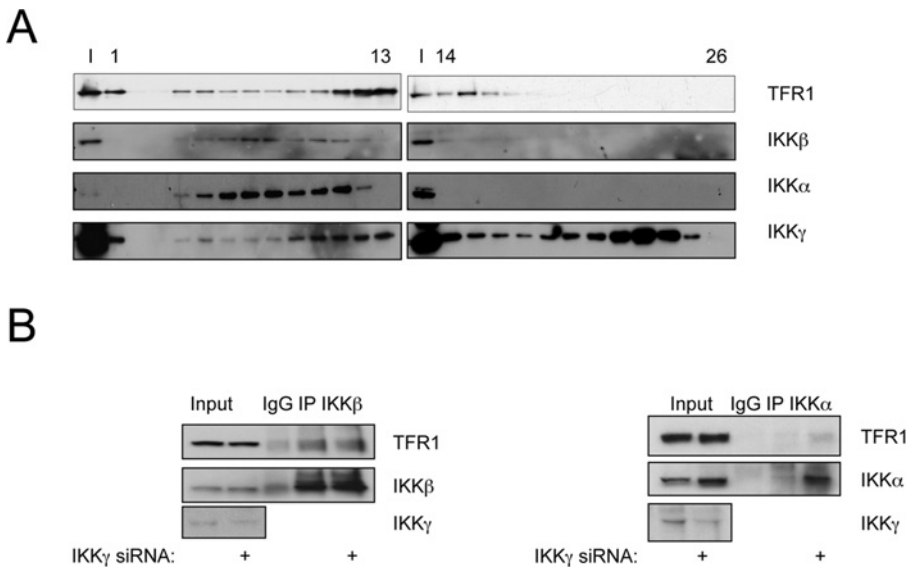

Figure 2 TfR1 forms several complexes with the IKK proteins

(**A**) Whole-cell lysates prepared from U2OS cells were run on a size-exclusion chromatography column, and gradient-eluted fractions (1–26) were immunoblotted with the antibodies indicated. (**B**) U2OS cells were transfected with control and IKKγ siRNA oligonucleotides before lysis. Immunoprecipitations were performed for IKKβ and IKKα, with IgG as a control. Precipitates were resolved by SDS/PAGE and then analysed by Western blotting using the antibodies indicated. IP, immunoprecipitation.

Depletion of TfR1 results in impaired NF-κB target gene activation

Activation of the canonical NF-κB signalling pathway by TNFα requires the translocation of the RelA subunit from the cytoplasm to the nucleus where it binds target gene promoters and enhancers. To determine the role of TfR1 in this process, we transfected U2OS cells with a control or TfR1 siRNA oligonucleotides, treated with TNFα for different periods of time and isolated nuclear fractions. In control cells, we observed robust translocation of RelA from the cytoplasm to the nuclear compartment within 30 min of TNFα stimulation, whereas in cells depleted of TfR1, the levels of nuclear RelA were significantly decreased (Figure 5A). No change in total levels of RelA was observed; however, when TfR1 was depleted, there was a reduction in the levels of phosphorylated Ser^{536} on RelA (Figure 5A). These results are consistent with impairment of IKK function as this residue can be phosphorylated by IKK [22].

We investigated further the functional significance of this reduced RelA translocation/activation by analysing the effects of TfR1 depletion on the levels of mRNA from endogenous target genes by quantitative RT–PCR (Figure 5B). Induction of NF-κB target genes c-IAP2 (cellular IAP2) and p100 in response to stimulation with TNFα was compromised in cells depleted of TfR1 (Figure 5B). We also noted an induction of TfR1 mRNA by TNFα treatment at 4 h (Supplementary Figure S3A at http://www.biochemj.org/bj/449/bj4490275add.htm). This is in agreement with previous studies that have found that HIF-1 (hypoxia-inducible factor-1) and NF-κB can control TfR1 gene expression in response to inflammatory signals [23].

We further validated our results with Western blot analysis, where we observed that TNFα induction of IAP2 and p100 protein is impaired in the absence of TfR1 in U2OS cells (Figure 5C) and MDA-MB-231 cells (Supplementary Figure S3B). Importantly, in the absence of TfR1, RelA recruitment to the p100 promoter following TNFα treatment is visibly decreased (Figure 5D). Taken together, these results demonstrate that TfR1 is required for TNFα-mediated activation of endogenous IKK and NF-κB in the cell.

Depletion of TfR1 impairs the anti-apoptotic function of NF-κB signalling

NF-κB signalling pathway in response to TNFα is to transactivate anti-apoptotic genes, in order to protect the cell against programmed cell death [1]. As we have demonstrated that depletion of TfR1 interferes with the IKK–NF-κB signalling pathway, we sought to determine whether loss of TfR1 could interfere with the anti-apoptotic functions associated with the IKK–NF-κB axis. We depleted U2OS cells of TfR1 and then treated them with increasing concentrations of TNFα for 24 h before lysis. Western blot analysis was performed to determine the levels of the known apoptosis marker cleaved PARP. In control cells, TNFα does not lead to significant levels of cell death owing to NF-κB anti-apoptotic function (Figure 6A). However, in cells lacking TfR1, TNFα-induced apoptosis is readily observed even with low concentrations of TNFα, as these cells display much higher levels of cleaved PARP when compared with control cells (Figure 6A).

To rule out the possibility that the effects of TfR1 depletion on TNFα-induced apoptosis are not dependent on modulation of IKK–NF-κB, we performed rescue experiments. Cells were depleted of TfR1, but, in addition, we expressed control or a plasmid for the NF-κB subunit RelA, treated with TNFα and analysed the levels of cleaved PARP. Expression of exogenous RelA visibly reduced the levels of cleaved PARP observed in cells depleted of TfR1 and treated with TNFα (Figure 6B). The results confirm the role of TfR1 in the modulation of IKK–NF-κB function in cells.

DISCUSSION

The IKK complex controls the activity of a very important family of transcription factors called NF-κB. Given the role of NF-κB in the immune system and in pathological conditions, the IKK complex has become an attractive drug target for pharmaceutical intervention [1]. As such, increasing our understanding of how the IKK complex is regulated would be of great interest. For

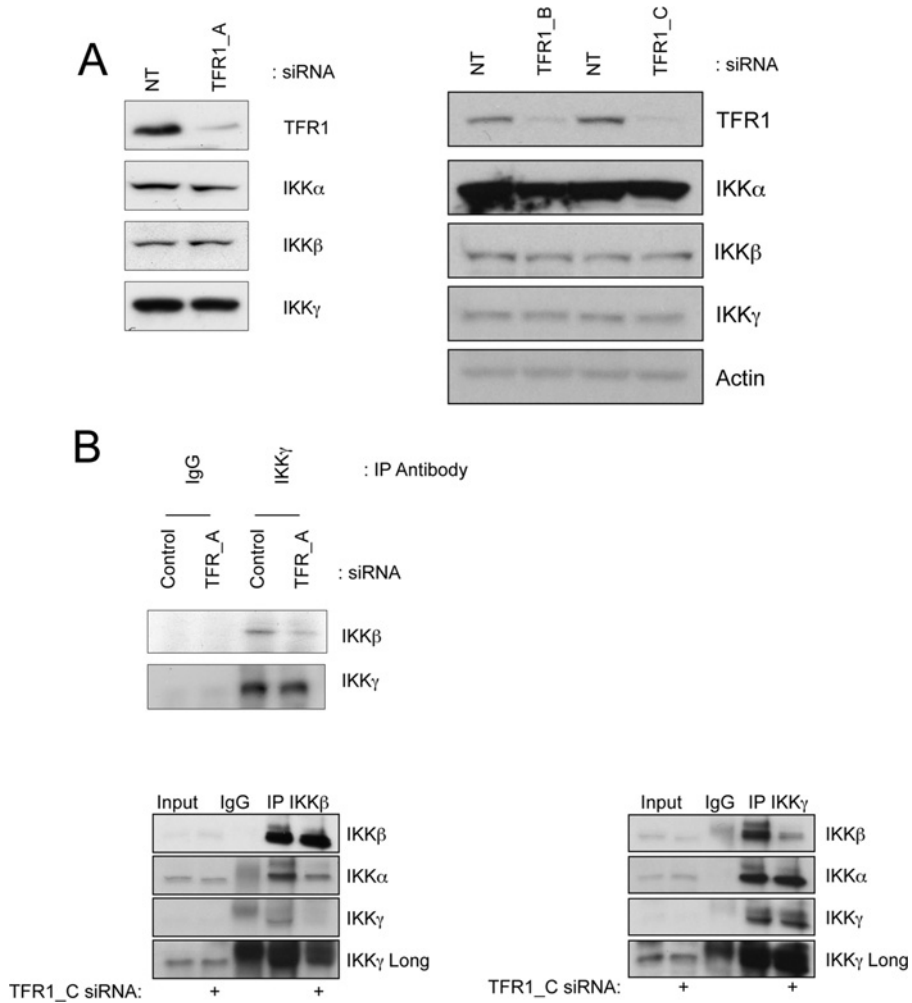

Figure 3 Depletion of TfR1 impairs IKK complex formation

(**A**) U2OS cells were transiently depleted of TfR1 using siRNA. Whole-cell lysates were subjected to immunoblot analysis for the levels of the proteins indicated. (**B**) Whole-cell lysates prepared from U2OS cells treated with non-targeting (NT) siRNA or TfR1 siRNAs were immunoprecipitated with normal rabbit IgG or anti-IKKγ or anti-IKKβ antibodies. Precipitates were resolved by SDS/PAGE and then analysed by Western blotting with anti-IKKα, anti-IKKβ and anti-IKKγ antibodies to assess the integrity of the IKK complex. IP, immunoprecipitation.

this purpose, we performed a quantitative analysis for IKK-interacting proteins using quite stringent purification procedures and identified TfR1 as a novel binding partner for the IKK subunits in cells (Figures 1 and 2). Functionally, we found that TfR1 is required for IKK complex formation and activity (Figures 3–6). As such, depletion of TfR1 results in lower IKK and NF-κB activation and increased sensitivity to apoptosis following TNFα (Figures 4–6).

TfR1 is a type II transmembrane protein that is the primary cell-surface receptor that regulates uptake of iron-bound transferrin by receptor-mediated endocytosis [18,19]. It plays an essential role in iron homoeostasis [18,19].

As iron is essential for cell-cycle progression, TfR1 expression is up-regulated in dividing cells, and has been found to be elevated various types of cancer [17,24]. Given these observations, it has become an attractive target for therapy, with the use of inhibiting antibodies and TfR1–toxin fusion molecules [17,25].

TfR1 has been implicated previously, although indirectly, in NF-κB signalling. Gambogic acid, a ligand for TfR1, has been shown to block TNFα-induced NF-κB signalling. This may be through disruption of IKKβ signalling [26], or perhaps covalent modification of the IKKβ subunit of IKK [27], but the exact mechanism remains unclear. In the present study, we have shown that TfR1 is a novel IKK-interacting protein. We have demonstrated that TfR1 can regulate NF-κB by altering the activity of the IKK complex. Loss of TfR1 alters the activity and integrity of the complex, which subsequently compromises the expression of NF-κB-target genes. These functional results are consistent with previous results using gambogic acid [26,28,29]. Although we could not find an *in vitro* interaction between the cytoplasmic tail of TfR1 and IKK, these proteins do interact in cells, and it suggests that TfR1 interacts with the IKK complex through a different domain, or via an intermediate protein. Interestingly, TfR1 was identified as an interacting protein of IKKε [30], a related IKK that has also been shown recently to control canonical IKK activity [31,32].

Studies using gambogic acid have shown that HSP90 could be involved in the control of IKK activity [33]. Zhang et al. [33] have shown that HSP90 is inhibited by gambogic acid, leading to reduced expression of HSP90 and IKKs. We did not find any changes in the levels of IKK subunits in the absence of TfR1 (Figure 3A). However, it is still possible that TfR1–IKK complex assembly could be mediated by changes in HSP90.

The control of IKK by TfR1 raises the interesting possibility that NF-κB can respond to iron changes in the cells. Previous studies have shown that iron chelation leads to NF-κB inhibition

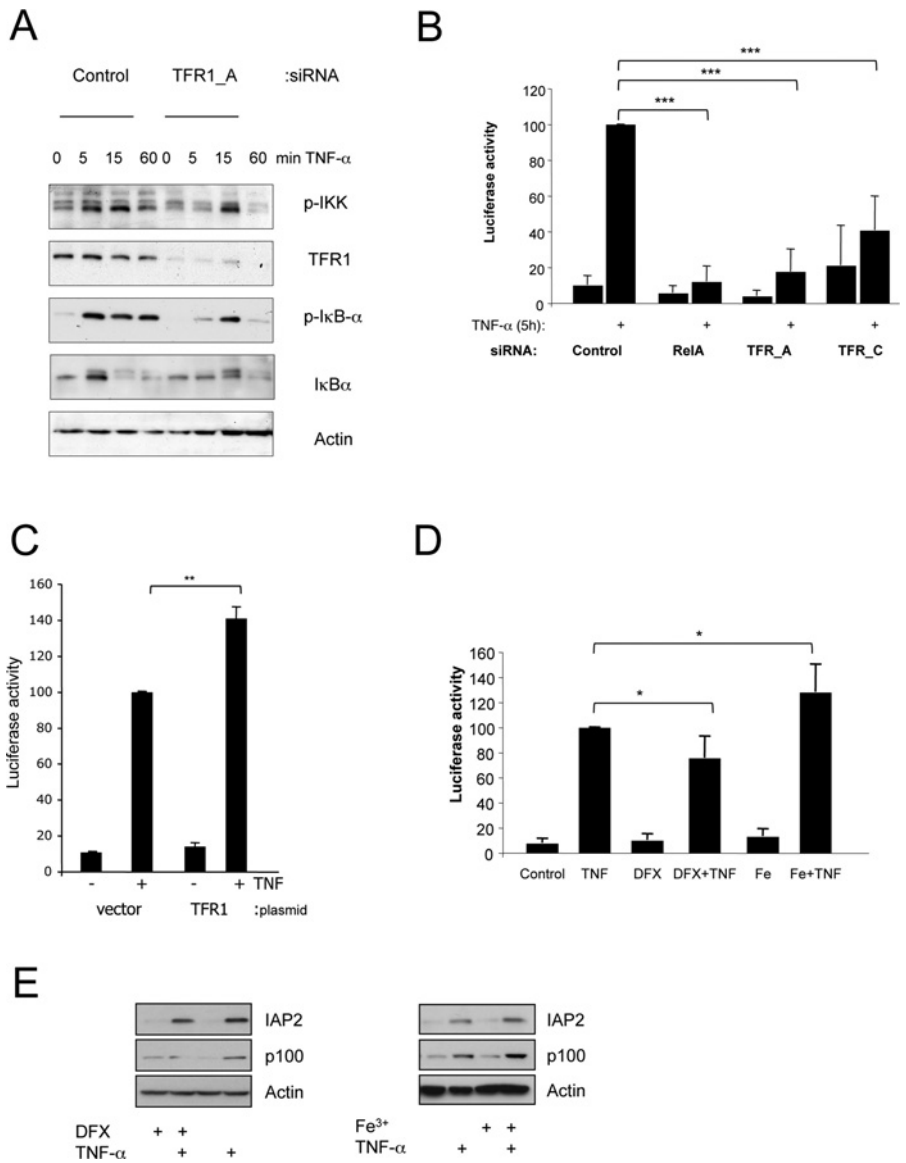

Figure 4 Depletion of TfR1 or iron impairs IKK and NF-κB activity

(**A**) U2OS cells were depleted of TfR1 using siRNA and subsequently treated with 10 ng/ml TNFα for the times indicated. Whole-cell lysates were subjected to immunoblot analysis for the levels of the proteins indicated. (**B**) U2OS-NF-κB luciferase reporter cells were transfected with the siRNAs indicated and treated with 10 ng/ml TNFα for 5 h before harvest. Results are means $\pm$ S.E.M. for at least three independent experiments expressed as fold activation/repression relative to TNFα-treated control siRNA levels. (**C**) U2OS-NF-κB luciferase reporter cells were transfected with 1 μg of control or TfR1 expression constructs before treatment with 10 ng/ml TNFα for 5 h before harvest. Results are means $\pm$ S.E.M. for at least three independent experiments expressed as fold activation/repression relative to controls. (**D**) U2OS-NF-κB reporter luciferase reporter cells were treated with 200 μM DFX or 300 μM iron/2 μM ascorbate 2 h before treatment with 10 ng/ml TNFα for an additional 5 h. Results are means $\pm$ S.E.M. for at least three independent experiments expressed as fold activation/repression relative to control. (**E**) U2OS cells were treated with either DFX or iron as in (**D**), but TNFα was added for 24 h before lysis. Whole-cell lysates were subjected to immunoblot analysis for the levels of the proteins indicated. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

[34,35]; however, the mechanism behind these observations is unknown. Similarly, some studies have shown NF-κB activation in response to iron increases [36]. In the present study, we have shown that both depletion and overexpression of TfR1 changes NF-κB activity in cells, suggesting that TfR1 could mediate iron modulation of NF-κB activity. We have also observed increased NF-κB activity when iron was added to the cells or when TfR1 was overexpressed, demonstrating that NF-κB does sense changes in iron levels in the cell. Of interest, NF-κB can also induce TfR1 expression via regulation of HIF-1α levels [13,23,37], establishing another connection between these molecules. Given our results, it would be interesting to test whether TfR1 depletion alters the levels of HIF-1α following TNFα or other inflammatory signals.

The importance of TfR1-mediated control of IKK–NF-κB activity is demonstrated by the sensitization of cells to TNFα-induced apoptosis (Figure 6). TNFα activates both apoptotic [FADD (Fas-associated death domain)/caspase 8] and anti-apoptotic (NF-κB) pathways in the cell. As such, IKKβ-, IKKγ- or RelA-deficient cells are extremely sensitive to TNFα-induced apoptosis, whereas the normal counterparts are resistant [38–41]. This is also true for the knockout mice for these proteins that are embryonic lethal owing to liver apoptosis, which can be rescued by knockout of the TNFα receptor [39–41]. Our results show that, in the absence of TfR1, IKK–NF-κB is not able to prevent apoptosis following TNFα, and, importantly, this apoptosis can be rescued by increasing the levels of RelA in cells. This indicates that

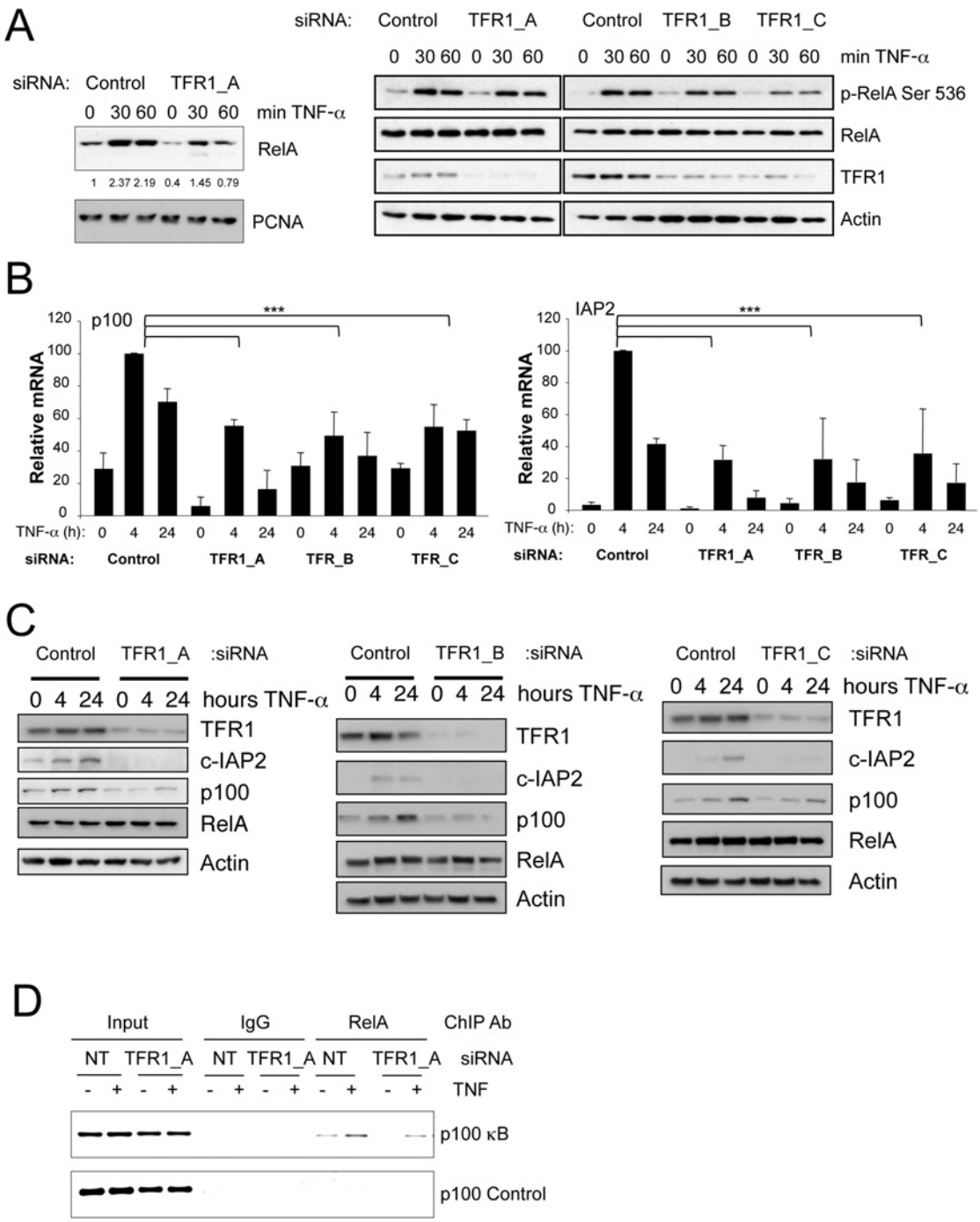

Figure 5 Depletion of TfR1 impairs activation of endogenous NF-κB-target genes

(**A**) Nuclear and cytoplasmic extracts were prepared from control cells or cells depleted of TfR1, and subsequently treated with TNFα for the times indicated. Nuclear extracts were analysed by Western blotting using the antibodies indicated. Whole-cell lysates were analysed for total levels of RelA under the same conditions. Levels of nuclear RelA were quantified and are shown below the RelA panel on the left. (**B**) Quantitative RT–PCR analysis of p100 and c-IAP2 mRNA prepared from U2OS cells depleted of TfR1 and subsequently stimulated with TNFα for the times indicated. ***$P < 0.01$. (**C**) U2OS cells were depleted of TfR1, and subsequently stimulated with TNFα for the times indicated. Whole-cell lysates were subjected to immunoblot analysis for the levels of the proteins indicated. (**D**) ChIP assay showing cross-linking of RelA to the NF-κB consensus sequence and a control region in the p100 promoter, in cells transfected with non-targeting siRNA or cells transfected with siRNA against TfR1, in the presence or absence of 10 ng/ml TNFα stimulation for 1 h. Ab, antibody; NT, targeting.

the effects seen on apoptosis following TfR1 depletion are NF-κB-dependent and not due to a different role of TfR1 in the cell.

As NF-κB has mostly pro-survival functions in advanced tumour stages [42], our results suggest that TfR1 would be a good therapeutic target for cancer types with elevated IKK–NF-κB activity.

In summary, we have demonstrated that TfR1 binds to IKK and controls complex assembly and activity, which has physiological implications for the levels of NF-κB activation following cytokine stimulation. It also provides a mechanism for effects seen using TfR1 inhibitors and modulators of iron levels in cells.

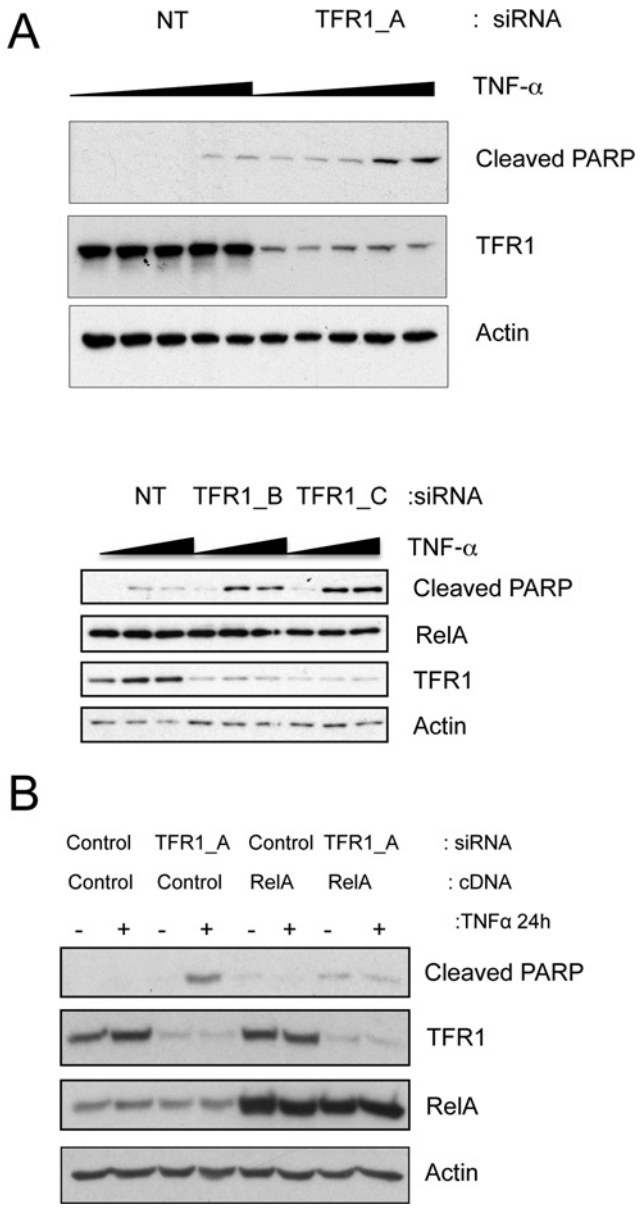

Figure 6 Depletion of TfR1 sensitizes cells to TNFα-induced apoptosis

(**A**) U2OS cells were depleted of TfR1, and subsequently stimulated with increasing concentrations (0, 10, 20, 30 and 40 ng/ml) of TNFα for 24 h. Whole-cell lysates were subjected to immunoblotting analysis for the levels of the proteins indicated. NT, non-targeting. (**B**) U2OS cells were transfected with 1 μg of control or RelA-expression constructs 24 h before TfR1 depletion by siRNA. Cells were treated with 10 ng/ml TNFα for an additional 24 h before lysis. Whole-cell lysates were subjected to immunoblotting analysis for the levels of the proteins indicated.

AUTHOR CONTRIBUTION

Niall Kenneth, Sharon Mudie, Sanne Naron and Sonia Rocha performed experiments. Niall Kenneth, Sharon Mudie and Sonia Rocha analysed the data. Niall Kenneth and Sonia Rocha initiated the study. All authors contributed to writing the paper.

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online data

SUPPLEMENTARY ONLINE DATA

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

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Cells

IKK$\beta^{-/-}$ 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. IKK$\beta^{-/-}$ 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 IKK$\beta^{-/-}$ 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-[$^{13}C_6$]lysine hydrochloride and L-[$^{13}C_6$]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′-GATATAGG-ATCCATGATGGATCAAGCT-3′ and 5′-GGCCAAGCTTAACT-TCCACTACACCT-3′.

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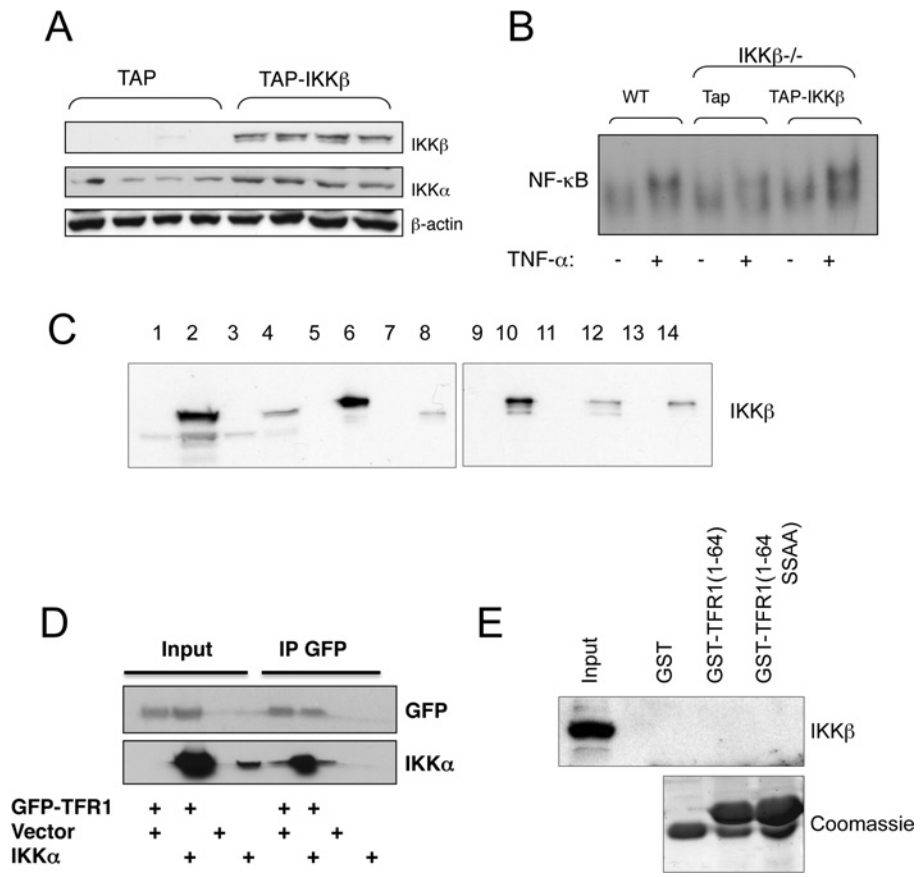

Figure S1 Identification of TFR1 as an IKKβ-interacting protein

(**A**) Characterization of reconstituted IKK$\beta^{-/-}$ 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 IKK$\beta^{-/-}$ MEFs rescues NF-κB DNA-binding activity. IKK wild-type (WT), IKK$\beta^{-/-}$ TAP and IKK$\beta^{-/-}$ 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 IKK$\beta^{-/-}$ 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–IgG beads post-TEV (tobacco etch virus) protease; lane 8, TAP–IKKβ IgG beads post-TEV protease; lane 9, TAP TEV eluate; lane 10, TAP–IKKβ TEV eluate; lane 11, TAP TEV eluate post-calmodulin beads; lane 12, TAP–IKKβ TEV 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 immunoprecitate (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.

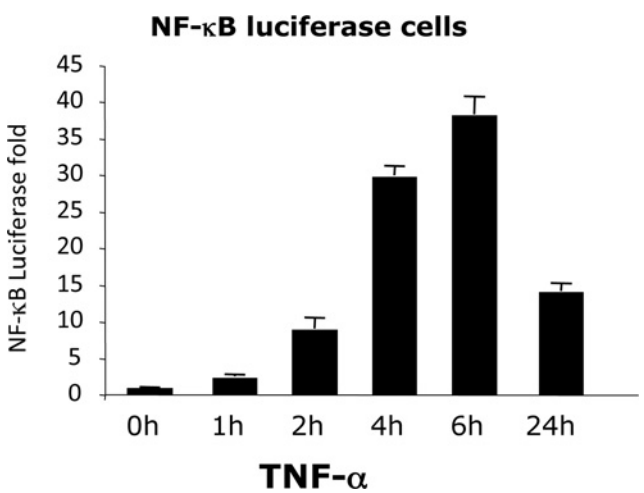

Figure S2 Characterization of U2OS-NF-κB reporter cells

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 $\pm$ S.E.M. for at least three independent experiments expressed as fold activation/repression relative to untreated cells.

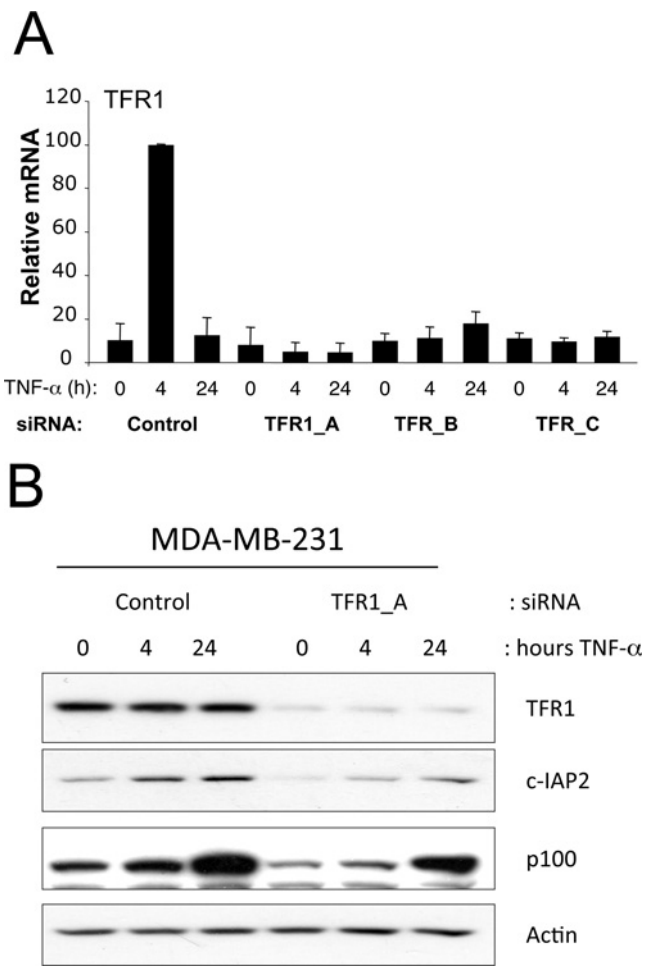

Figure S3 TFR1 depletion impairs NF-κB activity

(**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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