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The kinase IKKα inhibits activation of the transcription factor NF-κB by phosphorylating the regulatory molecule TAX1BP1

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Abstract

In response to stimulation with proinflammatory cytokines, the deubiquitinase A20 inducibly interacts with the regulatory molecules TAX1BP1, Itch and RNF11 to form the A20 ubiquitin-editing complex. However, the molecular signal that coordinates the assembly of this complex has remained elusive. Here we demonstrate that TAX1BP1 was inducibly phosphorylated on Ser593 and Ser624 in response to proinflammatory stimuli. The kinase IKKα, but not IKKβ, was required for phosphorylation of TAX1BP1 and directly phosphorylated TAX1BP1 in response to stimulation with tumor necrosis factor (TNF) or interleukin 1 (IL-1). TAX1BP1 phosphorylation was pivotal for cytokine-dependent interactions among TAX1BP1, A20, Itch and RNF11 and downregulation of signaling by the transcription factor NF-κB. IKKα therefore serves a key role in the negative feedback of NF-κB canonical signaling by orchestrating assembly of the A20 ubiquitin-editing complex to limit inflammatory gene activation.

Inflammation is critical for the host response to injury or infection with microbes. Cells of the innate immune system, such as macrophages, produce proinflammatory cytokines such as tumor necrosis factor (TNF) or interleukin-1β (IL-1β), which have important roles in clearing pathogens and initiating an adaptive immune response. Inflammatory responses are tightly regulated to prevent chronic inflammation, which has been linked to autoimmune diseases and many cancers[1].

Transcription factors of the NF-κB (Rel) family act as master regulators of genes encoding molecules that control innate and adaptive immune responses[2]. NF-κB signaling consists of two distinct pathways: the canonical pathway and the noncanonical pathway. In the canonical NF-κB pathway, heterodimeric NF-κB proteins such as RelA (p65) and p50 are sequestered in the cytoplasm as inactive subunits by inhibitory IκB proteins. A wide variety of stimuli, such as TNF and IL-1, as well as microbial components, including lipopolysaccharide (LPS), trigger signaling pathways that culminate at the level of IκB kinase (IKK)[3]. IKK consists of the catalytic kinase subunits IKKα and IKKβ and the

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AUTHOR CONTRIBUTIONS

N.S., R.P. and N.S.H. did experiments and analyzed data; D.W.A. provided reagents and did bioinformatic analyses; and E.W.H. designed and supervised the experiments, analyzed data and wrote the manuscript.

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regulatory subunit IKKγ (NEMO)[4,5]. IKKβ and IKKγ serve important roles in the activation of the canonical pathway via phosphorylation of the NF-κB inhibitor IκBα, which leads to its ubiquitination and degradation by the 26S proteasome complex[6]. The degradation of IκBα allows NF-κB to enter the nucleus, where it regulates the expression of large numbers of target genes encoding molecules that regulate inflammation and survival.

In the noncanonical NF-κB pathway, various ligands of the TNF superfamily, such as BAFF and CD40L, trigger processing of the precursor protein p100 (NF-κB2) to yield the NF-κB subunit p52 (refs. 7,8). RelB and p52 regulate genes encoding molecules involved in B cell survival and lymphoid organogenesis. IKKα is activated in the noncanonical pathway by the kinase NIK (NF-κB-inducing kinase)[9,10].

Activation of canonical NF-κB signaling is under tight control by many mechanisms, all of which ensure transient NF-κB signaling in response to a specific stimulus. The gene encoding IκBα is a target of NF-κB, and IκBα functions in a negative feedback loop to terminate NF-κB signaling[11]. In addition, the deubiquitinases CYLD and A20 target important signaling intermediates upstream of IκBα to terminate NF-κB activation[12,13]. CYLD and A20 cleave Lys63 (K63)-linked polyubiquitin chains on overlapping substrates, including the ubiquitin ligases TRAF2 and TRAF6 and the adaptor RIP1 (refs. 14–16). However, given the lack of redundancy between CYLD and A20, each of these deubiquitinases probably has a highly specific role *in vivo* in the regulation of inflammatory, apoptotic and developmental signaling pathways[17–20].

Polymorphisms in the gene encoding A20 have been linked to many inflammatory autoimmune diseases in humans[21]. Furthermore, A20-deficient mice succumb to uncontrolled and spontaneous multiorgan inflammation as a result of unchecked NF-κB signaling[22]. Studies indicate that A20 also functions as a tumor-suppressor gene in several subtypes of B cell lymphomas[23,24]. Therefore, understanding the mechanisms of A20-mediated downregulation of NF-κB is of critical importance. A20 has been shown to function as a unique dual-function deubiquitinase and E3 ligase in the TNF receptor pathway[25]. A20 first cleaves K63-linked polyubiquitin chains on RIP1 via the OTU deubiquitinase domain, followed by zinc finger 4–mediated catalysis of K48-linked polyubiquitin chains, to trigger RIP1 degradation[25]. Another important mechanism used by A20 to downregulate NF-κB signaling is the disruption of interactions among the E2 conjugating enzymes Ubc13 and Ubc5hc and E3 ligases TRAF6, TRAF2, cIAP1 and cIAP2 (refs. 26, 27).

It is becoming increasingly clear that A20 requires essential contributions from many other proteins, including the regulatory molecules TAX1BP1, Itch and RNF11 (refs. 26, 28). In response to TNF or IL-1 or bacterial LPS, A20, TAX1BP1, Itch and RNF11 inducibly interact to form the A20 ubiquitin-editing complex[28]. Cells lacking either TAX1BP1, Itch or RNF11 are a phenocopy of A20-deficient cells in their persistent activation of NF-κB signaling[29]. Although the specific contributions of each of these proteins are unclear, TAX1BP1 probably functions as a ubiquitin-binding adaptor molecule for A20 (ref. 30). However, it is unknown what regulates the inducible interactions among A20, TAX1BP1, Itch and RNF11, a key event required for the termination of NF-κB signaling.

Human T cell leukemia virus type I (HTLV-I) is an oncogenic retrovirus etiologically linked to adult T cell leukemia-lymphoma[31]. HTLV-I encodes the oncogenic regulatory protein Tax, which persistently activates NF-κB to transform T cells and maintain the survival of virus-transformed cells[32,33]. Tax activates NF-κB by associating with IKKγ and triggering persistent activation of the IKK subunits[34]. Furthermore, Tax bypasses the negative feedback inhibition of NF-κB by interacting with TAX1BP1 and disabling the function of

the A20 ubiquitin-editing complex[35]. The exact mechanism by which Tax inactivates A20 has remained elusive.

In this report we describe IKKα as a regulator and kinase for TAX1BP1. We found that TAX1BP1 was inducibly phosphorylated at Ser593 and Ser624 in response to stimulation with TNF and IL-1. Phosphorylation of TAX1BP1 was critical for the inducible interactions among proteins of the A20 ubiquitin-editing complex and the termination of TNF- and IL-1-dependent NF-κB signaling.

RESULTS

TNF and IL-1 induce phosphorylation of TAX1BP1 by IKKα

TAX1BP1 has an essential role in the termination of NF-κB signaling by regulating the recruitment and function of A20 (ref. 29). However, it is unclear if TAX1BP1 undergoes any type of post-translational modifications that may regulate its function. In response to stimulation with TNF or LPS, a form of TAX1BP1 that migrated slower was apparent in mouse embryonic fibroblasts (MEFs; Fig. 1a). Treatment of protein lysates with calf intestinal alkaline phosphatase converted the more slowly migrating form of TAX1BP1 to a form that migrated faster (Fig. 1b), which indicated that the shift in mobility was indeed caused by phosphorylation. We also observed phosphorylation of endogenous TAX1BP1 in MEFs labeled with ^{32}P orthophosphate and treated with TNF, but not in unstimulated cells (Fig. 1c). We also observed by immunoblot analysis a change in TAX1BP1 mobility when TAX1BP1 was expressed together with either IKKα or IKKβ, but not when it was expressed together with IKKγ (Fig. 1d). However, phosphorylation of TAX1BP1 was intact in response to TNF and IL-1 in MEFs deficient in IKKβ (*Ikbkb*$^{-/-}$) or IKKγ (*Ikbkg*$^{-/-}$), but not in MEFs deficient in IKKα (*Chuk*$^{-/-}$; called '*Ikka*$^{-/-}$' here; Fig. 1e and Supplementary Fig. 1). Therefore, overexpression of IKKβ probably caused phosphorylation of TAX1BP1 by activating endogenous IKKα. These results suggest that IKKα is essential for TAX1BP1 phosphorylation in response to stimulation with TNF or IL-1.

To determine if IKKα directly phosphorylates TAX1BP1, we did *in vitro* kinase assays with recombinant active IKKα and IKKβ. For substrates, we generated three different fragments of TAX1BP1 spanning the entire protein (amino acids 1–204, 205–599 and 600–747), each fused to glutathione *S*-transferase (GST). As a control for IKKβ phosphorylation, we used IκBα amino acids 1–54 fused to GST, a known phosphorylation substrate of IKKβ[9]. Of the three TAX1BP1 fragments, only the fusion protein containing amino acid 205–599 was efficiently phosphorylated by IKKα (Fig. 1f and data not shown). IKKα, in contrast to IKKβ, 'preferentially' phosphorylated TAX1BP1, whereas IKKβ 'preferentially' phosphorylated IκBα (Fig. 1f). Therefore, IKKα directly phosphorylates TAX1BP1 but IKKβ does not.

We next sought to determine if we could detect a stimulus-dependent interaction between IKKα and TAX1BP1. Endogenous TAX1BP1 interacted with wild-type IKKα, but not with the 'kinase-dead' mutant IKKα (K44M), in *Ikka*$^{-/-}$ MEFs transfected with expression vector encoding wild-type or mutant IKKα and stimulated with TNF or IL-1 (Fig. 1g). Furthermore, wild-type IKKα restored TAX1BP1 phosphorylation in reconstituted *Ikka*$^{-/-}$ MEFs stimulated with TNF or IL-1, but IKKα (K44M) did not (Fig. 1h), which suggested that the catalytic activity of IKKα is necessary for TNF- and IL-1-induced phosphorylation of TAX1BP1.

To identify the TAX1BP1 phosphorylation site(s), we undertook a combination of several approaches. First, we used a bioinformatics approach to identify consensus sites in TAX1BP1 that undergo phosphorylation by IKK. Peptide substrate array profiling has been

done for all IKK family members. Quantification of that profiling has allowed both a positive matrix and negative matrix to be generated. This matrix can enable proteome-wide bioinformatic screening of potential substrates for which the local positive and negative amino acid 'preferences' surrounding the phosphorylation site are known[36,37]. In general, the IKKs tended to use acidic or phosphorylated amino acids both amino (N)-terminal and carboxy (C)-terminal to the phosphorylation site[36,37] (Fig. 2a). Using the matrix generated before for the IKKs, we identified a total of three potential IKK phosphorylation sites in TAX1BP1 at Ser212, Ser254 and Ser593 (Fig. 2a). These potential phosphorylation sites conformed well to known IKK sites; however, because of the rate of false-positive results with this technique, we also generated a series of constructs of TAX1BP1 with deletions at the N and C termini to examine phosphorylation of TAX1BP1 by IKKα. TAX1BP1 constructs truncated at the C terminus (amino acids 1–696 and 1–650) were phosphorylated by TAX1BP1 (Fig. 2b, c). However, further deletion of amino acids from the C terminus abrogated TAX1BP1 phosphorylation, as amino acids 1–504 and 1–360 of TAX1BP1 did not undergo phosphorylation (Fig. 2b, c). These results suggest that TAX1BP1 phosphorylation occurs between amino acids 504 and 650. Thus, IKKα phosphorylates the C terminus of TAX1BP1, which indicates that Ser593 is a potential phosphorylation site.

Phosphorylation of TAX1BP1 at Ser593 and Ser624

Next we undertook an unbiased approach using mass spectrometry to identify TAX1BP1-phosphorylation site(s). We transfected 293T human embryonic kidney cells with expression vector encoding Flag-tagged TAX1BP1 and treated the cells with TNF. We immunoprecipitated TAX1BP1 with antibody to Flag (anti-Flag), separated the proteins by SDS-PAGE and stained gels with silver to visualize TAX1BP1 (Fig. 2d). We excised bands and digested them with trypsin, then enriched the resultant samples for phosphorylated peptides and analyzed them by matrix-assisted laser desorption-ionization quadrupole ion–trap time-of-flight mass spectrometry. Although we did not identify any phosphorylated TAX1BP1 peptides in untreated cells, we identified two overlapping phosphorylated peptides in TNF-treated cells (Fig. 2d). Each contained a single serine at position 593, which confirmed our hypothesis that Ser593 is indeed a TNF-inducible TAX1BP1-phosphorylation site.

Given that result, we used site-directed mutagenesis to substitute that serine residue with an alanine residue (TAX1BP1(S593A)). Unexpectedly, phosphorylation of TAX1BP1, observed as a shift in mobility by immunoblot analysis, was intact when IKKα was expressed together with TAX1BP1(S593A) (Fig. 2e), which suggested additional phosphorylation sites not detected by mass spectrometry. Therefore, we used the NetPhos 2.0 network for the prediction of phosphorylation sites to identify additional sites in the C-terminal region of TAX1BP1. Ser624, Ser641 and Ser652 all received high scores as potential phosphorylation-acceptor sites; these serine residues are all highly conserved in many species (human, mice, rat and zebrafish). To determine if these TAX1BP1 sites may also be phosphorylated, we generated single serine-to-alanine point substitutions as well as double mutants in combination with substitution of Ser593. We transfected the mutants into TAX1BP1-deficient MEFs and treated the cells with either TNF or IL-1 to trigger phosphorylation of TAX1BP1. The inducible mobility shift of TAX1BP1 was lost only whenever the serine at position 624 was substituted with alanine (TAX1BP1(S624A); Fig. 2f), which suggested that phosphorylation of Ser624 was solely responsible for the observed mobility shift of TAX1BP1. We obtained similar results with the TAX1BP1(S624A) mutant after overexpression of IKKα (Supplementary Fig. 2). Phosphorylation-induced shifts in mobility are typically observed when the phosphorylated serine or threonine is adjacent to a proline residue, and indeed Ser624, but not Ser593, had an adjacent proline that was highly conserved (Fig. 2g). Therefore, both Ser593 and Ser624 in TAX1BP1 were phosphorylated

in response to overexpression of IKKα, although only Ser624 contributed to the shift in TAX1BP1 mobility.

Although Ser593 and Ser624 were both phosphorylated after cytokine stimulation, only Ser593 conformed to a consensus IKK site, which thus raised the possibility that a kinase distinct from IKKα phosphorylates TAX1BP1 Ser624. Although the amino acids surrounding TAX1BP1 Ser624 did not conform perfectly to any known consensus phosphorylation motifs, the Scansite motif scanner indicated a putative site for phosphorylation by the kinase Akt1 (RXRXXS/T, where 'X' indicates any amino acid, 'R' indicates arginine and 'S/T' indicates serine or threonine). However, phosphorylation of TAX1BP1 at Ser624 remained intact in MEFs doubly deficient in both Akt1 and Akt2 (Supplementary Fig. 3).

We next examined the interactions between the TAX1BP1- phosphorylation mutants and IKKα in TAX1BP1-deficient MEFs reconstituted with wild-type TAX1BP1, TAX1BP1(S593A), TAX1BP1(S624A) or TAX1BP1(S593A, S624A). Both wild-type TAX1BP1 and TAX1BP1(S593A) interacted with endogenous IKKα after TNF stimulation, but TAX1BP1(S624A) or TAX1BP1(S593A, S624A) did not (Supplementary Fig. 4). Thus, phosphorylation of TAX1BP1 at Ser624 seemed to stabilize the interaction between TAX1BP1 and IKKα, consistent with our findings reported above in which the 'kinase-dead' IKKα mutant did not stably interact with TAX1BP1 (Fig. 1g).

The TAX1BP1 plasmid encoding amino acids 1–747 used in our studies thus far corresponded to TAX1BP1 isoform 2 (in the Universal Protein Resource Knowledgebase–Swiss-Prot database), which lacks 42 amino acids (604–645) present in TAX1BP1 isoform 1 (amino acids 1–789) because of alternative splicing. To confirm that there were no additional phosphorylation sites in the larger isoform, we substituted Ser593 and Ser666 (which correspond to Ser624 in isoform 2) in TAX1BP1 isoform 1 (amino acids 1–789). Indeed, we observed a shift in the mobility of wild-type TAX1BP1 (amino acids 1–789) that was abolished for the TAX1BP1(S593A, S666A) double mutant (Supplementary Fig. 5). Therefore, Ser593 and Ser624 (Ser666 in isoform 1) seemed to be the only IKKα-mediated phosphorylation sites in TAX1BP1. We further confirmed that result by *in vivo* phosphorylation experiments after reconstituting TAX1BP1-deficient MEFs with either wild-type TAX1BP1 or the phosphorylation mutant TAX1BP1(S593A, S624A). Although wild-type TAX1BP1 was considerably phosphorylated after TNF stimulation, no phosphorylation of the TAX1BP1-phosphorylation mutant was evident (Fig. 2h). These results confirmed that Ser593 and Ser624 were the only sites on TAX1BP1 that were phosphorylated after stimulation with TNF.

To more easily monitor phosphorylation of TAX1BP1 at Ser593, we generated an antibody specific for such phosphorylation. We confirmed the specificity of the antibody by overexpressing TAX1BP1 or TAX1BP1(S593A) together with IKKα. The phosphorylation-specific antibody detected only wild-type TAX1BP1, not TAX1BP1(S593A), in the presence of IKKα (Fig. 2i). The phosphorylation-specific antibody also detected endogenous TAX1BP1 in control MEFs treated with TNF (Fig. 2j) or IL-1 (Supplementary Fig. 6), but not in *Tax1bp1*$^{-/-}$ MEFs, thus indicating its specificity. Furthermore, endogenous phosphorylated TAX1BP1 was also detected in primary mouse bone marrow–derived macrophages (BMDMs) treated with either TNF or IL-1 (Fig. 2k).

TAX1BP1 phosphorylation downregulates NF-κB signaling

TAX1BP1 is a critical negative regulator of NF-κB, acting by downregulating polyubiquitination of RIP1 and TRAF6 in the signaling pathways of the TNF receptor and IL-1 receptor, respectively[29,30]. To determine the functional relevance of TAX1BP1

phosphorylation, we reconstituted TAX1BP1-deficient MEFs with either wild-type TAX1BP1 or TAX1BP1(S593A, S624A). TAX1BP1 in either form was typically expressed in reconstituted *Tax1bp1*$^{-/-}$ MEFs in amounts similar to those of endogenous TAX1BP1 in wild-type MEFs (Supplementary Fig. 7). As expected, *Tax1bp1*$^{-/-}$ MEFs showed prolonged degradation of IκBα, phosphorylation of the kinase Jnk and K63-linked polyubiquitination of RIP1 and TRAF6 (Fig. 3a, b). Reconstitution of TAX1BP1-deficient MEFs with wild-type TAX1BP1 restored transient activation of NF-κB, phosphorylation of Jnk and ubiquitination of RIP1 and TRAF6 (Fig. 3a, b). However, the phosphorylation-defective TAX1BP1(S593A, S624A) mutant was impaired in the termination of signaling by NF-κB, phosphorylation of Jnk and ubiquitination of RIP1 and TRAF6 (Fig. 3a, b).

We also did functional studies of primary mouse BMDMs transfected with a single small interfering RNA (siRNA) specific for mouse *Tax1bp1* to silence endogenous expression of TAX1BP1. We then reconstituted the cells with human *TAX1BP1* (encoding either wild-type or phosphorylation-defective mutant TAX1BP1), which contains three mismatches with the mouse siRNA and therefore was not efficiently targeted by the siRNA (data not shown). Knockdown of TAX1BP1 triggered prolonged IκBα degradation in mouse macrophages (Fig. 3c), indicative of persistent NF-κB signaling. Similar to results obtained with MEFs, wild-type TAX1BP1 restored transient NF-κB signaling in macrophages, but the phosphorylation-defective mutant TAX1BP1 did not (Fig. 3c). Collectively, these results suggest that phosphorylation of TAX1BP1 is essential for downregulation of NF-κB signaling.

TAX1BP1 phosphorylation regulates targeting of A20 to TRAFs

Next we examined the role of TAX1BP1 phosphorylation in the recruitment of the A20 ubiquitin-editing complex to substrates. We reconstituted TAX1BP1-deficient MEFs with either wild-type TAX1BP1 or TAX1BP1(S593A, S624A), treated the cells for various times with TNF or IL-1 and monitored protein-protein interactions by immunoprecipitation (Fig. 4a, b). In TAX1BP1-deficient MEFs transfected with empty vector, there was a defect in the TNF- and IL-1-mediated recruitment of Itch, A20 and TAX1BP1 to TRAF2 and TRAF6 that was restored by transfection of wild-type TAX1BP1 (Fig. 4a, b). However, TAX1BP1(S593A, S624A) failed to interact with TRAF2 or TRAF6 and was also impaired in the recruitment of Itch and A20 to the TRAF proteins (Fig. 4a, b). A20 and TAX1BP1 act together to disrupt the interactions between TRAF2-TRAF6 and Ubc13 in signaling via IL-1 and TNF[26]. Indeed, TRAF2 and TRAF6 interacted persistently with Ubc13 in *Tax1bp1*$^{-/-}$ MEFs (Fig. 4a, b). Reconstitution with wild-type TAX1BP1 restored transient TRAF2-TRAF6–Ubc13 interactions, but reconstitution with phosphorylation-defective mutant TAX1BP1 did not (Fig. 4a, b). TNF signaling is known to induce the formation of a membrane-bound signaling complex (complex I) that consists of TRADD, RIP1, cIAP1-cIAP2 and TRAF2-TRAF5 and is essential for activation of NF-κB[38]. Thus, we examined the role of TAX1BP1 phosphorylation in TNF-dependent interactions among TRAF2, RIP1 and cIAP1. TRAF2 interacted persistently with both RIP1 and cIAP1 in TAX1BP1-deficient MEFs treated with TNF. These interactions were rendered transient after reintroduction of wild-type TAX1BP1 but not after reintroduction of phosphorylation-defective mutant TAX1BP1 (Fig. 4a). Thus, TAX1BP1 phosphorylation is essential for the recruitment of TAX1BP1, A20 and Itch to TRAF2 or TRAF6 in response to stimulation with TNF or IL-1, respectively.

TAX1BP1 phosphorylation assembles the A20 protein complex

Next we determined the relative contributions of TAX1BP1 Ser593 and Ser624 to the termination of NF-κB signaling by using single-point mutants for functional studies of *Tax1bp1*$^{-/-}$ MEFs. Wild-type TAX1BP1 abrogated the activation of NF-κB in response to

TNF (Fig. 5a) or IL-1 (Fig. 5b), as measured by an NF-κB luciferase assay. However, single substitution of either Ser593 or Ser624 largely abolished the NF-κB-inhibitory effects of TAX1BP1, whereas combined substitution of both serine residues completely abolished the function of TAX1BP1 (Fig. 5a, b).

We then used immunoprecipitation to determine the role of TAX1BP1 phosphorylation in the assembly of the A20 ubiquitin-editing complex. We immunoprecipitated RNF11 from *Tax1bp1*$^{-/-}$ MEFs reconstituted with empty vector, wild-type TAX1BP1, TAX1BP1(S593A), TAX1BP1(S624A) or TAX1BP1(S593A, S624A) and treated with either TNF or IL-1. We then examined the RNF11 immunoprecipitates for immunoreactivity with anti-Itch, anti-A20 and anti-TAX1BP1. As expected, in TAX1BP1-deficient MEFs, the binding of RNF11 to Itch, A20 or TAX1BP1 was impaired after stimulation with TNF (Fig. 5c) or IL-1 (Fig. 5d), which highlighted the importance of TAX1BP1 as a scaffolding molecule for the A20 ubiquitin-editing complex. Reconstitution of TAX1BP1-deficient MEFs with wild-type TAX1BP1 restored the interactions among RNF11, Itch, A20 and TAX1BP1 (Fig. 5c, d). However, single-mutant TAX1BP1(S593A) or TAX1BP1(S624A) and double-mutant TAX1BP1(S593A, S624A) were each impaired in restoring the assembly of the A20 ubiquitin-editing complex in response to stimulation with TNF or IL-1 (Fig. 5c, d). Collectively, these data suggested that both Ser593 and Ser624 in TAX1BP1 serve essential and nonredundant roles in cytokine-dependent nucleation of the A20 ubiquitin-editing complex.

IKKα is required for assembly of the A20 protein complex

Because IKKα is essential for the phosphorylation of TAX1BP1, which in turn regulates protein-protein interactions between members of the A20 ubiquitin-editing complex, we hypothesized that IKKα-deficient MEFs would show a similar defect in formation of the A20 ubiquitin-editing complex. We stimulated wild-type, *Ikka*$^{-/-}$, *Ikbkb*$^{-/-}$ and *Ikbkg*$^{-/-}$ MEFs with TNF and assessed the recruitment of A20 to RIP1 by immunoprecipitation (Fig. 6a). A20 interacted with RIP1 in response to TNF stimulation in wild-type, *Ikbkb*$^{-/-}$ *and* *Ikbkg*$^{-/-}$ MEFs but not in *Ikka*$^{-/-}$ MEFs (Fig. 6a). Furthermore, TNF-dependent interactions between TAX1BP1 and Itch were impaired in *Ikka*$^{-/-}$ MEFs (Fig. 6b). We also confirmed those results in wild-type MEFs transfected with a pool of siRNAs specific for IKKα. In agreement with the results obtained with IKKα-deficient MEFs, knockdown of IKKα abrogated the recruitment of A20 to RIP1 in response to stimulation with TNF (Fig. 6c). Furthermore, silencing of IKKα impaired the IL-1-dependent interactions among TAX1BP1, A20 and Itch (Fig. 6d). We also did IKKα-knockdown studies with primary mouse macrophages to examine TNF- and IL-1-mediated NF-κB signaling events. As expected, TNF triggered transient K63-linked polyubiquitination of RIP1 and degradation of IκBα in the presence of control siRNA (Fig. 6e). However, siRNA-mediated silencing of IKKα elicited persistent RIP1 polyubiquitination and IκBα degradation (Fig. 6e), which emphasized the importance of IKKα in terminating NF-κB signaling in primary macrophages. We obtained similar results for the IL-1 receptor pathway, as IKKα silencing triggered persistent K63-linked polyubiquitination of TRAF6 and degradation of IκBα (Fig. 6f). Thus, IKKα served an essential role in the negative feedback of NF-κB signaling by facilitating TAX1BP1-mediated assembly of the A20 ubiquitin-editing complex (Supplementary Fig. 8).

Downregulation of NF-κB signaling by phospho-mimetic TAX1BP1

Thus far our results had defined an NF-κB negative feedback loop initiated by IKKα phosphorylation of TAX1BP1 that was essential for the assembly of the A20 ubiquitin-editing complex. As TAX1BP1 is downstream of IKKα, we hypothesized that a TAX1BP1 mutant that mimics phosphorylated TAX1BP1 (phospho-mimetic TAX1BP1) would bypass

the requirement of IKKα in the A20 negative feedback loop. Thus, we substituted the serine residues at positions 593 and 624 with glutamic acid (to mimic phosphorylated serine) to generate the phospho-mimetic mutant TAX1BP1(S593E, S624E). We reconstituted *Tax1bp1*$^{-/-}$ MEFs with wild-type TAX1BP1, TAX1BP1(S593A, S624A) or TAX1BP1(S593E, S624E), then transfected the cells with control siRNA with scrambled sequence or IKKα-specific siRNA and stimulated them with TNF or IL-1. As expected, the phosphorylation-defective mutant TAX1BP1(S593A, S624A) was impaired in terminating NF-κB signaling, whereas the phospho-mimetic mutant TAX1BP1(S593E, S624E) resembled wild-type TAX1BP1 in restoring transient activation of NF-κB (Fig. 7a, b). In the absence of IKKα, wild-type TAX1BP1 was unable to undergo phosphorylation and was therefore impaired in the ability to turn off NF-κB after stimulation with TNF or IL-1 (Fig. 7a, b). However, the phospho-mimetic mutant TAX1BP1(S593E, S624E) retained its ability to inhibit NF-κB in the absence of IKKα, as observed by transient degradation of IκBα (Fig. 7a, b). Therefore, the phospho-mimetic mutant bypassed the requirement for IKKα to inhibit NF-κB signaling.

HTLV-I Tax inhibits phosphorylation of TAX1BP1

TAX1BP1 was originally identified in a yeast two-hybrid screen as a protein that interacts with the HTLV-I Tax oncoprotein[39,40]. Studies have indicated that Tax counteracts the adaptor function of TAX1BP1 to promote persistent NF-κB signaling[26,35]; however, it has remained unclear if Tax affects the phosphorylation of TAX1BP1. Therefore, we transfected MEFs with plasmids encoding TAX1BP1 and Tax, then stimulated the cells with TNF or IL-1 to induce phosphorylation of TAX1BP1. As expected, TAX1BP1 was phosphorylated after stimulation with TNF or IL-1, as shown by its shift in mobility by immunoblot analysis; however, Tax completely blocked TAX1BP1 phosphorylation (Fig. 8a, b). To address the mechanism of inhibition, we examined TAX1BP1-IKKα interactions in the presence or absence of Tax. Stimulation with either TNF or IL-1 promoted interaction between TAX1BP1 and IKKα that was impaired in Tax-expressing cells (Fig. 8c). Therefore, Tax prevented the recruitment of IKKα to TAX1BP1 and the subsequent phosphorylation of TAX1BP1 in cells stimulated with TNF or IL-1. As HTLV-I infects mainly T lymphocytes *in vivo*, we next examined TAX1BP1 phosphorylation in a panel of HTLV-I-transformed T cell lines, as well as the Jurkat Tax Tet-on human T lymphocyte cell line that conditionally expresses Tax. Notably, TAX1BP1 phosphorylation was evident in TL-OM1 cells (a human lymphocytic adult T cell leukemia cell line that lacks Tax expression) but not in the Tax-expressing HTLV-I-transformed human T lymphocyte cell lines MT-2 and C8166 (Fig. 8d). Furthermore, treatment of Jurkat Tax Tet-on cells with the phorbol ester PMA in combination with the calcium ionophore ionomycin induced phosphorylation of TAX1BP1, but this did not occur when Tax expression was activated by treatment with doxycycline (Fig. 8d). Therefore, TAX1BP1 phosphorylation was inhibited by Tax in T cells and in HTLV-I transformed cell lines that expressed Tax despite the persistent NF-κB signaling in such cells.

A published study has indicated that siRNA-mediated silencing of human *TAX1BP1* in 293T cells impairs Tax-induced NF-κB activation[41], although the mechanism remains unclear. As we had found that the phospho-mimetic mutant TAX1BP1(S593E, S624E) bypassed the requirement for IKKα to terminate NF-κB signaling (Fig. 7), we next examined the effect of that mutant on Tax-mediated NF-κB activation. We reconstituted TAX1BP1-deficient MEFs with wild-type TAX1BP1 or TAX1BP1(S593E, S624E) and analyzed them by Tax-driven NF-κB luciferase assays. Indeed, activation of NF-κB by Tax was impaired in the absence of TAX1BP1 (Fig. 8e). Furthermore, Tax was unable to activate NF-κB in the presence of TAX1BP1(S593E, S624E) (Fig. 8e). This result

suggested that negative feedback inhibition by TAX1BP1(S593E, S624E) may have overridden the activation of NF-κB by Tax.

DISCUSSION

Our results have demonstrated the importance of TAX1BP1 phosphorylation in the termination of TNF- and IL-1-mediated NF-κB signaling. TAX1BP1 has two C-terminal ubiquitin-binding Cys_2His_2 zinc finger motifs that are essential for inhibition of NF-κB[30]. TAX1BP1 inducibly interacts with the HECT domain–containing E3 ligase Itch via two C-terminal PPXY motifs (where 'X' is any amino acid) positioned in the center of each of the zinc-finger domains[35]. We hypothesize that TAX1BP1 is normally in an inactive conformation whereby the zinc-finger domains and associated PPXY motifs are inaccessible to cofactors such as Itch, RNF11 and A20 as well as polyubiquitinated substrates. However, phosphorylation of TAX1BP1 by IKKα probably triggers a conformational change in TAX1BP1 that exposes the zinc fingers and PPXY motifs to allow the recruitment of Itch, RNF11 and A20 and the concomitant recognition of substrates. The scaffolding function of TAX1BP1 is strictly dependent on IKKα-mediated phosphorylation of TAX1BP1. This mechanism of autoregulation ensures that IKK is activated only transiently by proinflammatory cytokines and raises the possibility of dysregulation of TAX1BP1 phosphorylation in inflammatory disorders and cancers in which NF-κB is commonly persistently activated.

The HTLV-I oncoprotein Tax promotes persistent NF-κB signaling through constitutive activation of IKKα and IKKβ[33]. Therefore, it was unexpected that Tax selectively blocked phosphorylation of TAX1BP1 by IKKα yet promotes IKKα-mediated phosphorylation of NF-κB2 in the noncanonical pathway[42]. Tax blocks the interactions of A20 with its substrates RIP1, Ubc13 and TRAF6 (refs. 26,35). As TAX1BP1 phosphorylation seems to be upstream of all these events, Tax inhibition of TAX1BP1 phosphorylation is probably the initial and defining event in the inactivation of A20. Also, because Tax blocked cytokine-induced interactions between TAX1BP1 and IKKα, it is plausible that Tax relocalizes TAX1BP1 to a distinct subcellular compartment inaccessible to IKKα or, alternatively, Tax may compete with IKKα for binding to TAX1BP1. Further studies are necessary to elucidate the precise role of TAX1BP1 in the activation of NF-κB by Tax.

As IKKα is a critical component of the noncanonical NF-κB pathway, it was unexpected that it also has a prominent role in the regulation of the canonical NF-κB pathway. However, it is known that extensive crosstalk exists between the canonical and noncanonical NF-κB pathways. For example, TRAF3-deficient cells have higher expression of NIK and constitutive activation of the noncanonical pathway, as well as enhanced activation of canonical NF-κB signaling[43]. Thus, TRAF3 negatively regulates IKK complexes in both canonical and noncanonical NF-κB pathways. A published study has demonstrated that IKKα phosphorylates NIK in negative feedback regulation of the noncanonical pathway[44], which supports the idea that IKKα has important roles in terminating both the canonical and noncanonical pathways. Understanding how IKKα is regulated in the canonical NF-κB pathway will require further investigation.

Given the essential role of IKKα, but not of IKKβ, in phosphorylating TAX1BP1, it is notable that IKKα has been suggested to be a negative regulator of canonical NF-κB signaling and inflammation. Macrophages from *Ikka*$^{-/-}$ mice or knock-in mice expressing inactive IKKα show greater production of proinflammatory cytokines as a result of enhanced IKKβ kinase activation and IκBα degradation[45,46]. Thus, the catalytic activity of IKKα is essential for the resolution of inflammatory signaling. IKKα has been shown to phosphorylate PIAS1, the inhibitor of activated transcription factor STAT1, as a mechanism

for inhibiting nuclear NF-κB and downregulating proinflammatory signaling[47]. Therefore, an important and nonredundant function of IKKα is to turn off inflammatory signaling pathways by phosphorylating multiple substrates that together coordinate the repression of NF-κB signaling.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureimmunology/.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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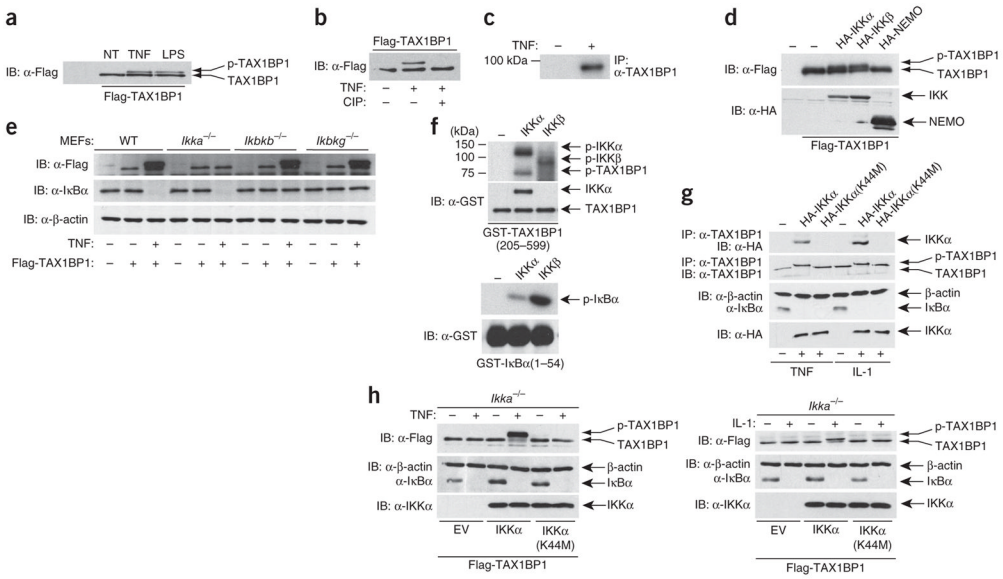

Figure 1.

Phosphorylation of TAX1BP1 in response to proinflammatory stimuli. (**a**) Immunoblot analysis (IB) of lysates of MEFs transfected with expression vector for Flag-tagged TAX1BP1 (Flag-TAX1BP1), then left untreated (NT) or treated for 30 min with TNF or LPS (10 ng/ml). α-, anti-; p-, phosphorylated. (**b**) Immunoblot analysis of lysates of MEFs treated with calf intestinal alkaline phosphatase (CIP) and left unstimulated (−) or stimulated for 30 min (+) with TNF (10 ng/ml). (**c**) *In vivo* kinase assay of MEFs labeled with ^{32}P-orthophosphate and left untreated (−) or treated for 15 min (+) with TNF (10 ng/ml), followed by immunoprecipitation of proteins from lysates with anti-TAX1BP1 and autoradiography for visualization of phosphorylated TAX1BP1. kDa, kilodaltons. (**d**) Immunoblot analysis of lysates of 293T cells transfected with expression vector for Flag-TAX1BP1 and left untransfected (−) or transfected with expression vector for hemagglutinin (HA)-tagged IKKα, IKKβ or IKKγ (above lanes). (**e**) Immunoblot analysis of lysates of wild-type (WT), *Ikka*$^{-/-}$, *Ikbkb*$^{-/-}$ or *Ikbkg*$^{-/-}$ MEFs left untransfected (−) or transfected (+) with expression vector for Flag-TAX1BP1 and left untreated (−) or treated (+) for 15 min with TNF. Anti-β-actin serves as a loading control throughout. (**f**) *In vitro* kinase assay of recombinant active IKKα or IKKβ, with a fusion of GST and TAX1BP1 amino acids 205–599 (GST-TAX1BP1(205–599)) or IκBα amino acids 1–54 (GST-IκBα (1–54)) as the substrate. Far left (−), GST fusion proteins alone; bottom blots in each, immunoblot analysis of GST fusion proteins with anti-GST. (**g**) Immunoassay of TAX1BP1 interactions in *Ikka*$^{-/-}$ MEFs transfected with expression vectors for HA-IKKα or HA-IKKα (K44M) and left untreated or treated for 15 min with TNF or IL-1, detected in lysates after immunoprecipitation (IP) with anti-TAX1BP1 (top two blots) by immunoblot analysis with anti-HA or anti-TAX1BP1; below, immunoblot analysis of total cell lysates with anti-HA, anti-IκBα or anti-β-actin. (**h**) Immunoblot analysis of lysates of *Ikka*$^{-/-}$ MEFs transfected with empty vector (EV) or expression vectors for Flag-TAX1BP1, plus HA-IKKα or HA-IKKα (K44M), then left untreated (−) or treated (+) for 15 min with TNF (left) or IL-1 (right); blots were probed with anti-Flag, anti-β-actin, anti-IκBα or anti-IKKα. Data are representative of at least three experiments.

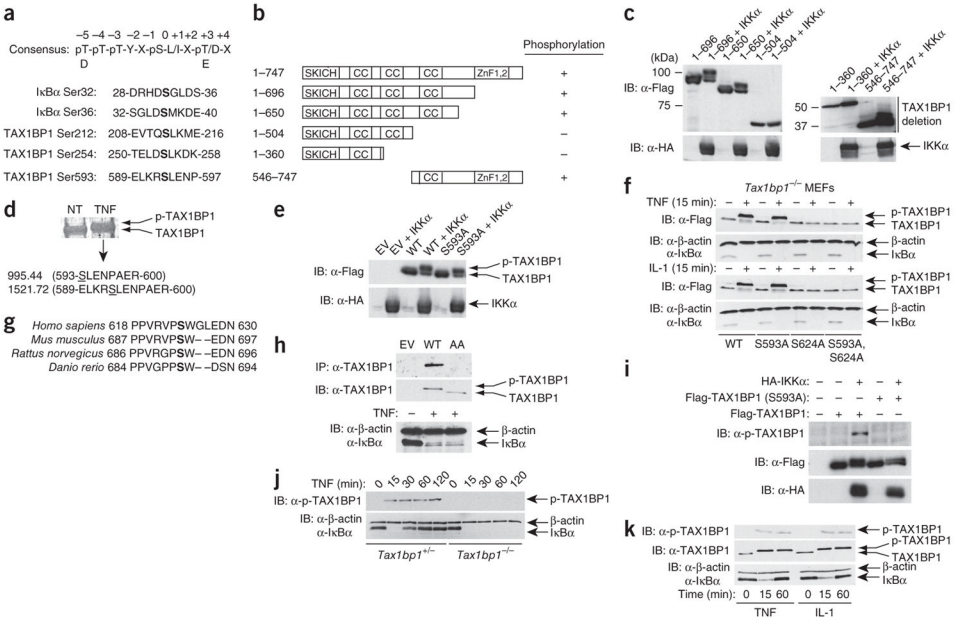

Figure 2.

Phosphorylation of TAX1BP1 on Ser593 and Ser624 in response to stimulation with TNF or IL-1. (**a**) Consensus IKK-phosphorylation sites in IκBα (Ser32 and Ser36, **S**) and putative IKK-phosphorylation sites in TAX1BP1 (Ser212, Ser254 and Ser593, **S**). Top, consensus phosphorylation sites, with phosphorylated serine (pS) at position 0 and acidic amino acids aspartic acid (D) upstream at position −5 and glutamic acid (E) downstream at position +3. pT, phosphorylated threonine; L/I, leucine or isoleucine; pT/D, phosphorylated threonine or aspartic acid; Y, tyrosine; X, any amino acid. (**b**) TAX1BP1 deletion mutants (left): SKICH, SKIP (skeletal muscle and kidney enriched inositol phosphatase) carboxyl homology domain; CC, coiled coil domain; ZnF, zinc-finger domain; far left, amino acids. Right, phosphorylation results. (**c**) Immunoblot analysis of lysates of 293T cells transfected with expression vector for Flag-tagged TAX1BP1 deletion mutants in **b**, with or without HA-tagged IKKα (above lanes). (**d**) Silver-stained gel of 293T cells transfected with expression vector for Flag-TAX1BP1 and left untreated or treated for 30 min with TNF (top), and identification of phosphorylated peptides in those cells by matrix-assisted laser desorption-ionization quadrupole ion–trap time-of-flight (bottom): far left, observed mass; right, amino acid sequence (phosphorylated residues underlined). (**e**) Immunoblot analysis of lysates of 293T cells transfected with empty vector or expression vector for wild-type TAX1BP1 or TAX1BP1 (S593A), with or without IKKα (above lanes). (**f**) Immunoblot analysis of lysates of *Tax1bp1*$^{-/-}$ MEFs transfected with expression vector for wild-type or mutant TAX1BP1 (below blots) and left untreated or treated with TNF or IL-1 (above lanes); blots were probed with anti-Flag, anti-β-actin or anti-IκBα. (**g**) Sequence alignment of the phosphorylation site of TAX1BP1 (**S**; human Ser624) in various species. (**h**) *In vivo* kinase assay of *Tax1bp1*$^{-/-}$ MEFs reconstituted with empty vector or expression vector for Flag-tagged wild-type TAX1BP1 or TAX1BP1(S593A, S624A) (AA), labeled with ^{32}P-orthophosphate and left untreated or treated for 15 min with TNF (10 ng/ml), followed by immunoprecipitation and autoradiography (as in Fig. 1c); blots were probed with anti-TAX1BP1 (after immunoprecipitation with anti-TAX1BP1) or anti-β-actin and anti-IκBα (total lysates). (**i**) Immunoblot analysis of lysates of 293T cells transfected with various expression vectors (above lanes), probed with antibody specific for TAX1BP1 phosphorylated at Ser593 (α-p-TAX1BP1), anti-Flag or anti-HA. (**j**) Immunoblot analysis of lysates of *Tax1bp1*$^{+/-}$ and *Tax1bp1*$^{-/-}$ MEFs treated for 0–120 min with TNF, probed with the phosphorylation-

specific antibody in **i**, anti-IκBα or anti-β-actin. (**k**) Immunoblot analysis of lysates of mouse BMDMs treated for 0–60 min with TNF or IL-1, probed with the phosphorylation-specific antibody in **j**, anti-TAX1BP1, anti-IκBα or anti-β-actin. Data are representative of at least three experiments.

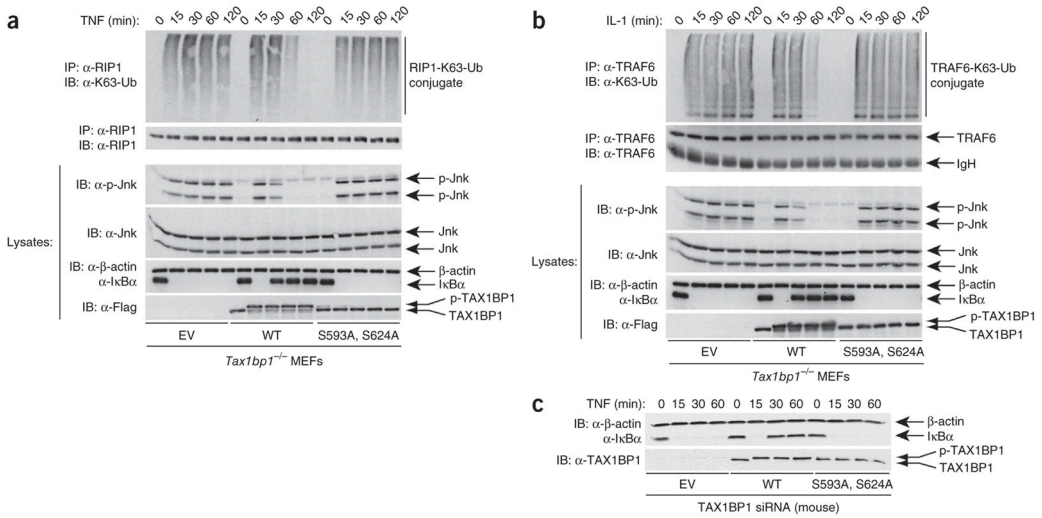

Figure 3.

Phosphorylation of TAX1BP1 is essential for the termination of NF-κB signaling, Jnk phosphorylation and RIP1 ubiquitination. (**a**, **b**) Immunoassay of lysates of *Tax1bp1*$^{-/-}$ MEFs transfected with empty vector or expression vector for Flag-tagged wild-type TAX1BP1 or Flag-TAX1BP1(S593A, S624A), then treated for 0–120 min with TNF (**a**) or IL-1 (**b**), assessed after immunoprecipitation with anti-RIP1 by immunoblot analysis with antibody specific for K63-linked ubiquitin (K63-Ub) and anti-RIP1 (**a**) or after immunoprecipitation with anti-TRAF6 by immunoblot analysis with antibody specific for K63-linked ubiquitin and anti-TRAF6 (**b**). Below (Lysates), immunoblot analysis of total cell lysates with antibodies along left margin. (**c**) Immunoblot analysis of lysates of mouse BMDMs transfected with siRNA specific for mouse *Tax1bp1*, together with empty vector or expression vector for Flag-tagged wild-type human TAX1BP1 or TAX1BP1(S593A, S624A), and treated for 0–60 min with TNF, probed with anti-TAX1BP1, anti-IκBα or anti-β-actin. Data are representative of at least three experiments.

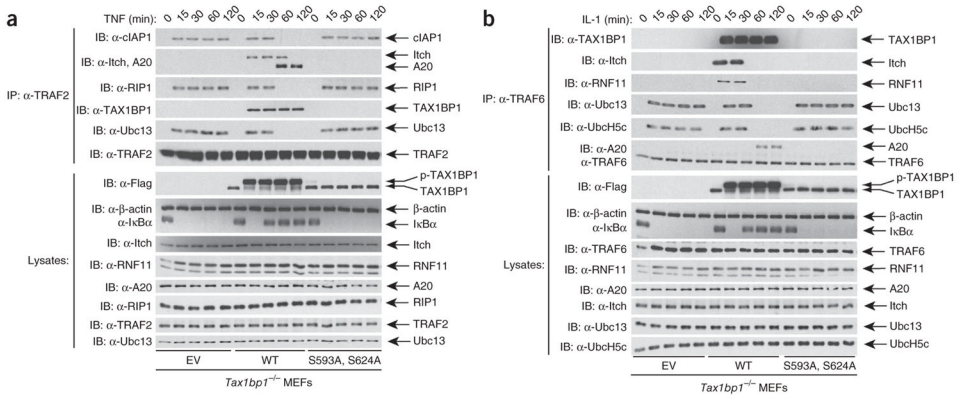

Figure 4.

Phosphorylation of TAX1BP1 regulates recruitment of the A20 ubiquitin-editing complex to TRAF2 and TRAF6. Immunoassay of lysates of *Tax1bp1*$^{-/-}$ MEFs transfected with empty vector or expression vector for Flag-tagged TAX1BP1 or TAX1BP1(S593A, S624A), then treated for 0–120 min with TNF (**a**) or IL-1 (**b**), assessed after immunoprecipitation with anti-TRAF2 (**a**) or anti-TRAF6 (**b**) by immunoblot analysis with antibodies along left margins (**a**, **b**). Below (Lysates), immunoblot analysis of total cell lysates with antibodies along left margins. Data are representative of at least three experiments.

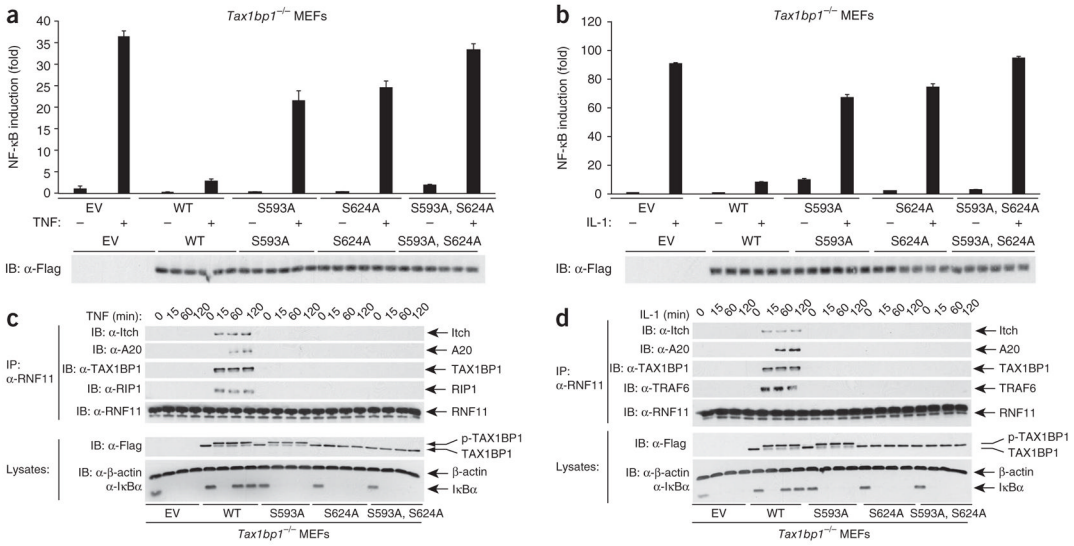

Figure 5.

Phosphorylation of TAX1BP1 is required for assembly of the A20 ubiquitin-editing complex. (**a**, **b**) NF-κB luciferase activity in lysates of *Tax1bp1*$^{-/-}$ MEFs transfected with an NF-κB firefly luciferase reporter and a renilla luciferase vector reporter, plus empty vector or expression vector for Flag-tagged wild-type TAX1BP1, TAX1BP1(S593A), TAX1BP1(S624A) or TAX1BP1(S593A, S624A), then treated for 8 h with TNF (**a**) or IL-1 (**b**); results are presented relative to renilla luciferase activity. Below, immunoblot analysis of the cells above with anti-Flag. (**c**, **d**) Immunoassay of lysates of *Tax1bp1*$^{-/-}$ MEFs transfected with empty vector or expression vector for Flag-tagged wild-type TAX1BP1, TAX1BP1(S593A), TAX1BP1(S624A) or TAX1BP1(S593A, S624A), then treated for 0–120 min with TNF (**c**) or IL-1 (**d**), assessed after immunoprecipitation with anti-RNF11 by immunoblot analysis with antibodies along left margins. Below (Lysates), immunoblot analysis of total cell lysates with antibodies along left margins. Data are representative of at least three experiments (error bars (**a**, **b**), s.e.m. of triplicates).

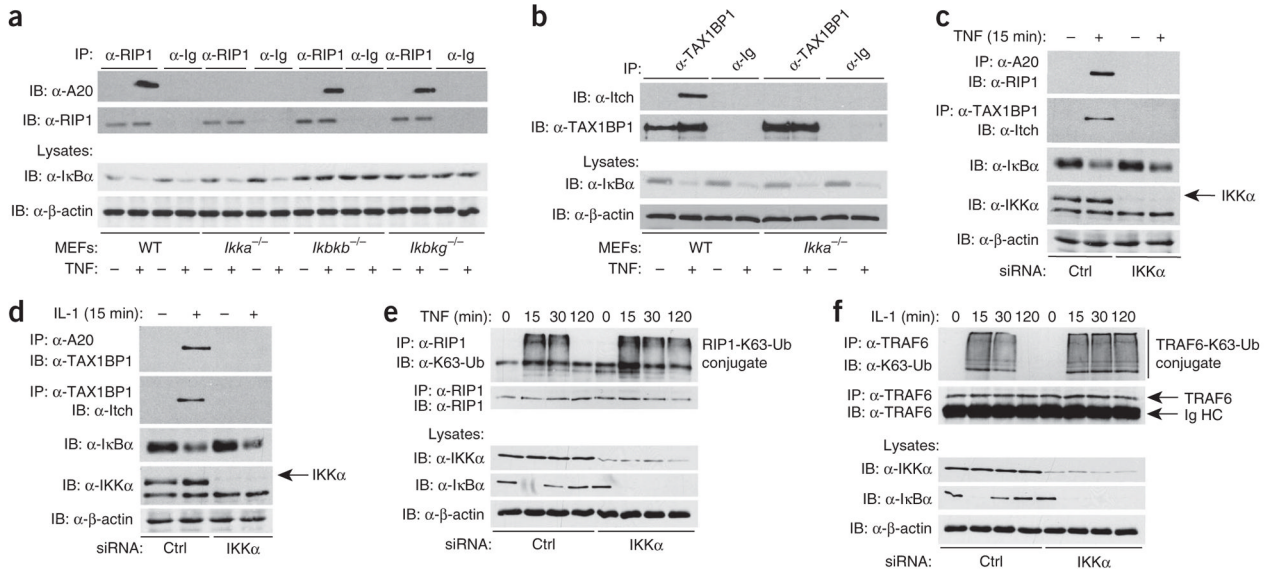

Figure 6.

IKKα is essential for assembly of the A20 ubiquitin-editing complex and termination of NF-κB signaling. (**a**) Immunoassay of lysates of wild-type, *Ikka*$^{-/-}$, *Ikbkb*$^{-/-}$ and *Ikbkg*$^{-/-}$ MEFs, left untreated or treated for 15 min with TNF, assessed after immunoprecipitation with anti-RIP1 or antibody to isotype-matched control immunoglobulin (α-Ig) by immunoblot analysis with anti-A20 or anti-RIP1. (**b**) Immunoassay of lysates of wild-type and *Ikka*$^{-/-}$ MEFs treated as in **a**, assessed after immunoprecipitation with anti-TAX1BP1 or control antibody (as in **a**) by immunoblot analysis with anti-Itch or anti-TAX1BP1. Below (Lysates; **a**, **b**), immunoblot analysis of total cell lysates with anti-IκBα and anti-β-actin. (**c**, **d**) Immunoassay of lysates of wild-type MEFs transfected with control siRNA (Ctrl) or IKKα-specific siRNA and treated with TNF (**c**) or IL-1 (**d**), assessed after immunoprecipitation with anti-A20 or anti-TAX1BP1 by immunoblot analysis with antibodies along left margins. Anti-IκBα, anti-IKKα or anti-β-actin performed on total cell lysates (**c**, **d**). (**e**, **f**) Immunoassay of lysates of mouse BMDMs treated for 0–120 min with TNF (**e**) or IL-1 (**f**), assessed after immunoprecipitation with anti-RIP1 by immunoblot analysis with antibody specific for K63-linked ubiquitin and anti-RIP1 (**e**) or after immunoprecipitation with anti-TRAF6 by immunoblot analysis with antibody specific for K63-linked ubiquitin or anti-TRAF6 (**f**). Below (Lysates), immunoblot analysis of total cell lysates with anti-IKKα, anti-IκBα or anti-β-actin. Ig HC (**f**), immunoglobulin heavy chain. Data are representative of at least three experiments.

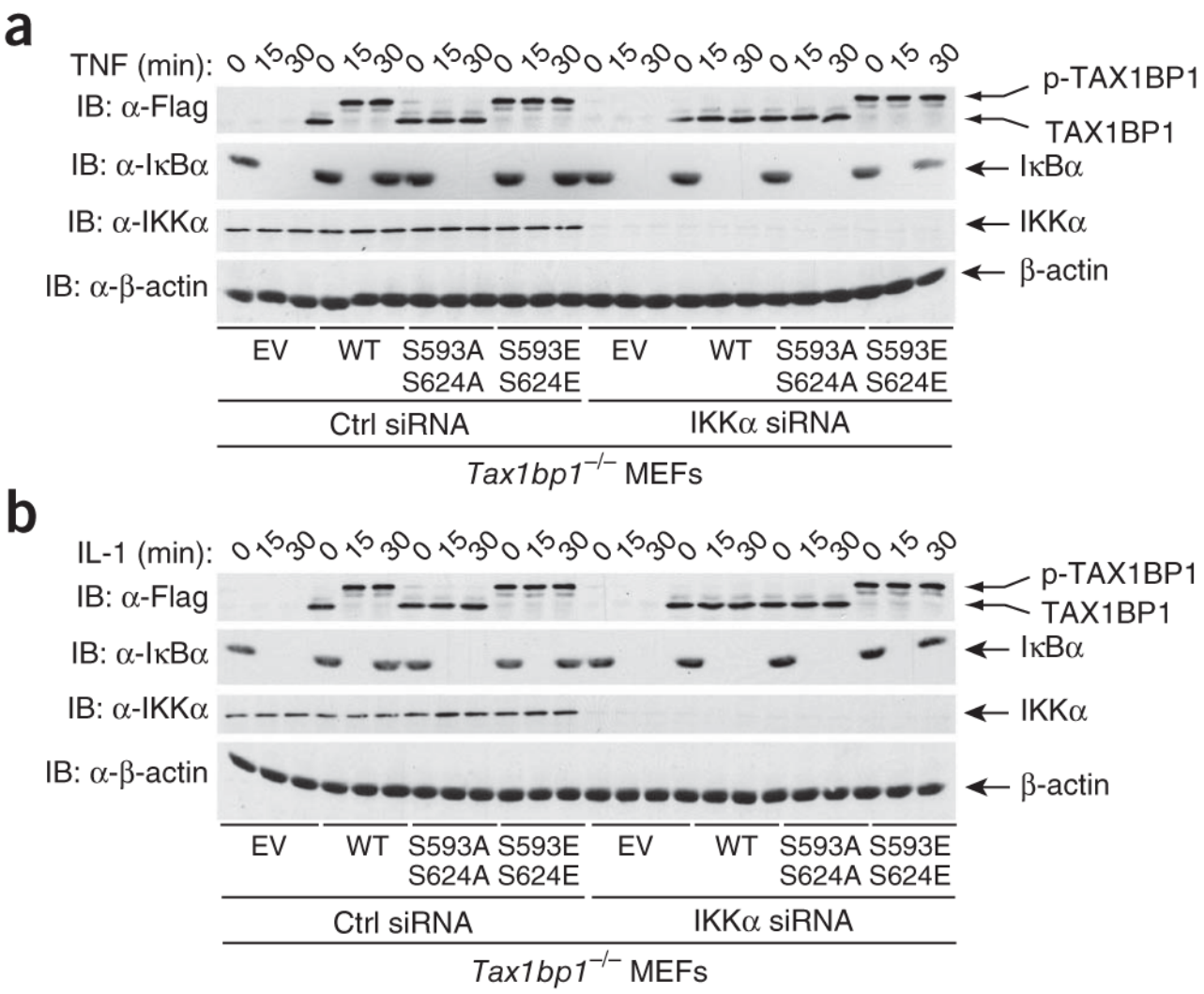

Figure 7.

Phospho-mimetic TAX1BP1 bypasses the requirement for IKKα in terminating NF-κB signaling. Immunoblot analysis of lysates of *Tax1bp1*$^{-/-}$ MEFs transfected with empty vector or expression vector for Flag-tagged wild-type TAX1BP1, TAX1BP1(S593A, S624A) or TAX1BP1(S593E, S624E), together with control siRNA or IKKα-specific siRNA, then treated for 0–30 min with TNF (**a**) or IL-1 (**b**); blots were probed with anti-Flag, anti-IκBα, anti-IKKα or anti-β-actin. Data are representative of two experiments.

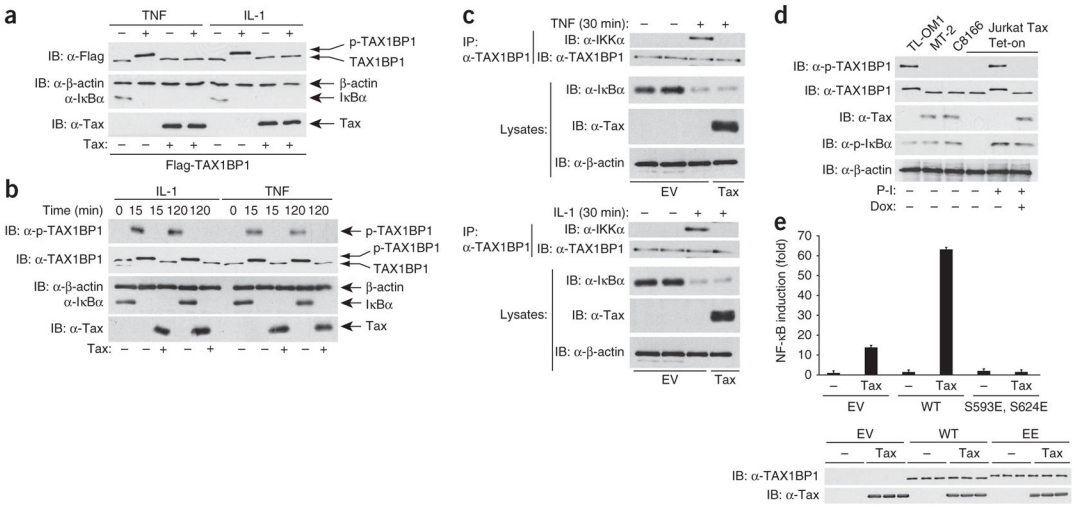

Figure 8.

HTLV-I Tax blocks the phosphorylation of TAX1BP1. (**a**) Immunoblot analysis of lysates of wild-type MEFs transfected with expression vector for Flag-tagged TAX1BP1 plus plasmid encoding Tax, then treated with TNF or IL-1, probed with anti-Flag, anti-β-actin plus anti-IκBα or anti-Tax. (**b**) Immunoblot analysis of lysates of wild-type MEFs transfected with empty plasmid or plasmid encoding Tax, then treated for 0–120 min with IL-1 or TNF, probed with antibody specific for TAX1BP1 phosphorylated at Ser593, anti-TAX1BP1, anti-β-actin plus anti-IκBα or anti-Tax. (**c**) Immunoassay of lysates of wild-type MEFs transfected with empty plasmid or plasmid encoding Tax, then treated for 30 min with TNF or IL-1 (above lanes), assessed after immunoprecipitation with anti-TAX1BP1 by immunoblot analysis with anti-IKKα or anti-TAX1BP1. Below (Lysates), immunoblot analysis of total cell lysates with anti-IκBα, anti-Tax or anti-β-actin. (**d**) Immunoblot analysis of lysates of the adult T cell leukemia cell lines TL-OM1, MT-2 and C8166 and of Jurkat Tax Tet-on cells left untreated (−) or treated (+) for 15 min with PMA and ionomycin (P-I) with (+) or without (−) treatment for 16 h with doxycycline (Dox; 1 μg/ml); blots were probed with antibody specific for TAX1BP1 phosphorylated at Ser593, anti-TAX1BP1, anti-Tax, antibody to phosphorylated IκBα or anti-β-actin. (**e**) NF-κB luciferase assay of lysates of *Tax1bp1*$^{-/-}$ MEFs transfected with an NF-κB firefly luciferase reporter and a renilla luciferase vector reporter, plus empty vector or expression vector for Flag-tagged wild-type TAX1BP1 or TAX1BP1(S593E, S624E) (EE), with (Tax) or without (−) plasmid encoding Tax; results are presented relative to renilla luciferase activity. Below, immunoblot analysis with anti-TAX1BP1 or anti-Tax. Data are representative of at least three experiments (error bars (**e**), s.e.m. of triplicates).