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by

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Running title: Phosphorylation sites in TAK1

Summary.

The protein kinase TAK1, which has been implicated in the activation of MAP kinase cascades and the production of inflammatory mediators by lipopolysaccharide (LPS), interleukin 1 (IL-1) and tumour necrosis factor (TNF), comprises the catalytic subunit complexed to the regulatory subunits, turned TAK1-binding subunit 1 (TAB1) and either TAB2 or TAB3. We previously identified a feedback control mechanism by which p38α MAP kinase down-regulates TAK1 and showed that p38α MAP kinase phosphorylates TAB1 at Ser423 and Thr431. Here, we identify two IL-1-stimulated phosphorylation sites on TAB2 (Ser372 and Ser524) and three on TAB3 (Ser60, Thr404 and Ser506) in human IL-1R cells and mouse embryonic fibroblasts (MEFs). Ser372 and Ser524 of TAB2 are not phosphorylated by pathways dependent on p38α/β MAP kinases, ERK1/2 and JNK1/2. In contrast, Ser60 and Thr404 of TAB3 appear to be phosphorylated directly by p38α MAPK, while Ser506 is phosphorylated by MAPKAP-K2/MAPKAP-K3, which are protein kinases activated by p38α MAPK. Studies using TAB1-/- MEFs indicate important roles for TAB1 in recruiting p38α MAPK to the TAK1 complex for the phosphorylation of TAB3 at Ser60 and Thr404 and to inhibit the dephosphorylation of TAB3 at Ser506. TAB1 is also required to induce TAK1 catalytic activity, since neither IL-1 nor TNFα were able stimulate detectable TAK1 activity in TAB1-deficient MEFs. Surprisingly, the IL-1 and TNFα-stimulated activation of MAP kinase cascades and IκB kinases were similar in TAB1-/-, MEKK3-/- and wild type MEFs, suggesting that another MAP3K may mediate the IL-1/TNFα-induced activation of these signaling pathways in TAB1-/- and MEKK3-/- MEFs.

Key Words: TGFβ-activated protein kinase 1 (TAK1), TAK1-binding protein 1 (TAB1), Interleukin-1 (IL-1), mitogen-activated protein (MAP) kinases, NFκB, tumour necrosis factor α (TNFα).

Introduction

The uncontrolled production of tumour necrosis factor α (TNFα) and other pro-inflammatory cytokines is a major cause of chronic inflammatory diseases, such as rheumatoid arthritis (RA) and inflammatory bowel disease (IBD). TAK1 is a mitogen-activated protein kinase kinase kinase (MAP3K), which becomes activated when cells are stimulated with bacterial lipopolysaccharide (LPS) or the pro-inflammatory cytokines tumour necrosis factor α (TNFα) or interleukin-1 (IL-1) [1-3]. Along with other MAP3Ks, such as MEKK3 [4], TAK1 is thought to play a key role in the production of TNFα and other inflammatory mediators by activating several mitogen-activated protein kinases (MAPKs), termed p38α MAPK, JNK1/JNK2 and ERK1/ERK2, and the transcription factor NFκB [3, 5-7], via the signalling pathways shown in Fig 1.

The native forms of TAK1 comprise the catalytic subunit complexed to a regulatory subunit TAB1, which is an inactive pseudophosphatase structurally related to members of the MPP family of protein serine/threonine phosphatases [8], and either of two structurally related proteins, termed TAB2 and TAB3 [9-11]. The activation of TAK1 by LPS or IL-1 is thought to be triggered by the formation of Lys63-linked polyubiquitinated TRAF6, which binds to the C-terminal Nuclear Zinc Finger (NZF)-motifs of TAB2 and TAB3, leading to the auto-phosphorylation and activation of TAK1 [3]. We have previously identified a feedback control loop in which p38α MAPK suppresses the activation of TAK1, and which correlates with the phosphorylation of TAB1 at Ser423 and Thr431 [12]. This feedback loop is prevented by the p38α MAPK inhibitor SB 203580 [13], and is not observed in p38α MAPK-deficient fibroblasts [12]. For this reason, the inhibition of p38α MAPK causes the hyper-activation of TAK1 and hence the hyper-activation of JNK. This may contribute to side effects of p38α MAPK inhibitors, such as hepatotoxicity [14], which have prevented these drugs from progressing to later stage clinical trials for the treatment of chronic inflammatory diseases.

Agonists that trigger the phosphorylation of TAB1 also induce the phosphorylation of the TAB2 and TAB3 regulatory subunits, as judged by a decrease in the electrophoretic mobility of these proteins that is reversed by incubation with a protein serine/threonine phosphatase [10, 11]. This decreased mobility is partially (TAB2) or completely (TAB3) prevented by SB 203580, indicating that the p38α MAPK-induced feedback control of TAK1 may involve the phosphorylation of TAB2/TAB3 as well as TAB1 [11]. Here we demonstrate that TAB2 and TAB3 are phosphorylated at multiple sites in human IL-1R cells and mouse embryonic fibroblasts (MEFs) in response to IL-1. We also identify three IL-1-stimulated sites on TAB3 whose phosphorylation is dependent on p38α MAPK activity and two phosphorylation sites on TAB2 that are phosphorylated by p38α MAPK-independent mechanisms. Studies with TAB1-deficient fibroblasts suggest that TAB1 plays several roles in the regulation of the TAK1 complex, namely to recruit p38α MAPK to the TAK1 complex for the phosphorylation of TAB3, to suppress the dephosphorylation of TAB3, and to induce TAK1 catalytic activity. We find that IL-1 and TNFα do not activate TAK1 in either TAB1-deficient MEFs or MEKK3-deficient MEFs, despite the unimpaired activation of MAP kinase cascades in these cells

Materials and Methods.

Materials. PD 184352 [15] synthesised by an improved method [16] and BIRB 0796 [17] synthesised from 4,4-dimethyl-3-exopentanenitrile [18] were provided by Dr Natalia Shpiro, College of Life Sciences, University of Dundee. SB 203580 was purchased from Promega, mouse IL-1α and IL-1β and human IL-β from Sigma, LPS from Invivogen, and glutathione Sepharose from GE healthcare.

DNA cloning. TAB2 and TAB3 were cloned and inserted into pGEX6P-1 as described previously [11]. Both constructs were digested with BamH1 and Not1 and ligated into the same sites in pEBG6P to form pEBG6P-TAB2 and pEBG6P-TAB3, respectively, for transfection into IL-1R cells Mutations in phosphorylation sites in TAB2 ([S372A], [S524A] and [S582A]) and TAB3 ([S60A], [T404A] and [S506A])

were created following the Quick Change method (Stratagene), but using KOD Hot Start Polymerase (Novagen). DNA encoding TAK1 was amplified from IMAGE EST 3906837 with Expand HiFi (Roche) using standard methods and subcloned into the BamH1 site in pEBG6P.

Antibodies. The phosphopeptides YIAAS*PPNTD, RKLS*MGSDD and LKRSNS*ISQIP, corresponding to residues 368-377, 521-529 and 576-587 of TAB2, YMEYHS*PDDNR and YMEYHS*PEDNR corresponding to residues 55-65 of human and mouse TAB3, respectively, LYTATT*PPSSS and KYQRSSS*SGSDD corresponding to residues 399-409 and 500-511 of TAB3, respectively, and IQTHMT*NNKGS corresponding to residues 182-192 of TAK1 (where S* and T* correspond to phosphoserine and phosphothreonine), were synthesized by Dr Graham Bloomberg (University of Bristol, UK), coupled to both bovine serum albumin and keyhole limpet haemocyanin and injected into sheep at Diagnostics Scotland (Edinburgh, UK). The antibodies were affinity purified from the anti-sera on CH-Sepharose to which the relevant phosphorylated peptide had been coupled covalently, and used for immunoblotting at 3 μg/ml in the presence of 30 μg/ml of the unphosphorylated peptide immunogen to neutralize any antibodies that recognize the unphosphorylated form of the protein. His-tagged full length MEKK3 expressed from a baculovirus vector in insect Sf21 cells was also injected into sheep and the antisera affinity purified against purified MEKK3 immobilised on CH-Sepharose and used for immunoblotting at 1 μg/ml. The antibodies that recognise TAB3, TAK1, TAB1, TAB1 phosphorylated at Ser423 and TAB1 phosphorylated at Ser 438 were raised in sheep and affinity purified as described previously [12]. Antibodies recognising all forms of JNK1/2, ERK1/ERK2, IκBα, MAPKAP-K2, MAPKAP-K2 phosphorylated at Thr334, the active phosphorylated forms of p38α MAPK and ERK1/ERK2 and c-Jun phosphorylated at Ser63 were from Cell Signaling Technologies. An antibody recognising the phosphorylated forms of JNK1 and JNK2 was purchased from Biosource, and anti-TAB2 and anti-TAK1 from Santa Cruz.

Generation of MAPKAP-K2/MAPKAP-K3 double knock-out mice.

Mice were maintained under specific pathogen free conditions and work carried out in accordance with UK legislation. MAPKAP-K3 gene targeted ES cells were generated by standard techniques in 129Sv ES cells. Exon 3 encoding subdomains III and IV, which form the major part of the ATP-binding site of MAPKAP-K3, was replaced with a neomycin cassette, These ES cells were used to produce MAPKAP-K3 knockout mice using established protocols [19]. Full details of the generation of these mice will be described elsewhere. MAPKAP-K2/3 double knockout mice were obtained by inter-crossing with MAPKAP-K2 knockout mice [20], and the mice genotyped by PCR based methods from ear biopsies.

Generation of Cre-conditional MEKK3 knockout mice. Genomic DNA encoding exons 9-16 of the MEKK3 gene from 129Sv wild-type mouse DNA was cloned into the Bluescript targeting vector. The neomycin resistant gene (PGKneo) flanked by FRT sites was cloned in reverse orientation into the intron flanked by exons 11 and 12. Lox P sites were introduced 3' to the neo resistance gene in the intron between exon 11 and 12 and the intron flanked by exons 13 and 14. The linearized targeting vector was electroporated into 129Sv embryonic stem (ES) cells. Homologous recombination in three ES cell clones was verified by PCR and Southern blotting. Flp recombinase was used to excise the neomycin resistance gene and confirmed by PCR. ES cells were injected into C57/Bl6 blastocysts and founder males crossed with 129Sv females. Heterozygotes were crossed and germ line transmission verified by Southern blot and PCR. Mouse embryo fibroblasts were isolated from E14.5 embryos and immortalized fibroblasts isolated by serial passage. Adenovirus encoding Cre recombinase was used to infect fibroblasts for deletion of exons 12 and 13 which encode kinase subdomains I-V within the MEKK3 coding sequence. Deletion of exons 12 and 13 was confirmed by PCR. MEKK3 mRNA having exons 12 and 13 deleted was detected following fibroblast treatment with Cre recombinase. A truncated MEKK3 protein was detected by immunoblotting (see Results) and demonstrated to be kinase inactive by in vitro kinase assay of immunoprecipitated protein.

Cell culture, stimulation and lysis.

Human embryonic kidney (HEK) 293 cells stably expressing the IL-1 receptor, termed IL1-R cells (provided by Tularic Inc., USA), immortalised mouse embryonic fibroblasts (MEFs) deficient in JNK1 and JNK2 (JNK1-/-/JNK2-/-) [21], immortalized TAB1-/- MEFs [5] or immortalized MEKK3-/- MEFs were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) heat inactivated foetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. Primary MEFs were isolated from E13.5 embryos of the MAPKAP-K2/MAPKAP-K3 double knock-out mice as reported previously for MSK-deficient MEFs [19], and cultured for up to five passages as described above for immortalised MEFs. After 6 h or 16 h prior to stimulation with human IL-1β in IL-1R cells or murine IL-1α, IL-1β, or sorbitol in mouse cells, the medium was removed and replaced with DMEM from which FCS had been omitted. 1 h prior to stimulation with agonists, aliquots of concentrated (10-20 mM) solutions SB 203580, PD 184352, or BIRB 0796 dissolved in dimethylsulphoxide (DMSO) was added to the culture medium to achieve the final concentrations indicated under Results. The equivalent volume of DMSO was added to control cells. Cells were lysed in ice cold lysis buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 10 mM sodium β-glycerolphosphate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 1% (v/v) Triton X-100, 0.1% (v/v) 2-mercaptoethanol, and Complete™ proteinase inhibitor cocktail (Roche: one tablet per 50 ml buffer)). Lysates were centrifuged at 13 000 × *g* for 10 min at 4 °C and the supernatants used immediately or snap frozen in liquid nitrogen and stored in aliquots at −20 °C until use. Protein concentrations were determined according to Bradford.

Transfection of IL-1R cells. Cells were transfected at 40–50% confluency using polyethyleneimine. To express TAK1 complexes for purification and mass spectrometric analysis of TAB3 and TAB2, a single 15 cm dish of IL-1R cells was transfected with DNA encoding GST-TAK1 (2 μg), GST-TAB1 (8 μg), and either GST-TAB3 or GST-TAB2 (20 μg). In order to test the specificities of the TAB3 or

TAB2 phospho-specific antibodies, 10 cm dishes of IL-1R cells were transfected with DNA encoding GST-TAK1 (2.5 μg), TAB1 (2.5 μg) and wild-type or mutant GST-TAB2 or GST-TAB3 (10 μg).

Purification of complexes. To purify GST-tagged TAK1 complexes, a 100 μl of a 50% slurry of glutathione-Sepharose 4B was incubated for 2 h at 4°C with 8 mg cell extract protein. After brief centrifugation, the supernatant was removed and the beads washed with 4 ml of 50 mM Tris-HCl pH 7.5, 250 mM NaCl, 0.03 % (w/v) Brij-35, 0.2 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM benzamidine, and 0.1% (v/v) 2-mercaptoethanol, followed by two further washes with 1 ml of 50 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.2 mM PMSF, and 1 mM benzamidine. The beads were then resuspended in 100 μl of the same buffer and the GST-TAK1 complexes digested for 16 h at 4°C with 4 μg of PreScission protease to release the TAK1 complexes from the beads. They were then denatured in LDS, subjected to SDS-PAGE and stained with colloidal Coomassie Blue.

Immunoprecipitation of TAK1 complexes. The endogenous TAB1-TAK1-TAB3 or TAB1-TAK1-TAB2 complexes were immunoprecipitated from 3mg IL-1R cell extract protein or 2mg MEF cell extract protein using an anti-TAB1 antibody [12]. 3 mg of cell extract protein was incubated for 2 h at 4°C with 10 μg of anti-TAB1 antibody coupled with 10 μl of Protein G-Sepharose. The immunoprecipitates were washed twice with 1 ml of lysis buffer containing 0.25 M NaCl, followed by two washes with 1 ml of 50 mM Tris-HCl pH 7.5, 50 mM NaCl and 0.1% (v/v) 2-mercaptoethanol.

Measurement of TAK1 activity. TAK1 activity in TAB1 immunoprecipitates was measured by the activation of MKK6 and coupled to the activation of p38α MAPK . The active p38α MAPK generated in this reaction was then quantitated in a second assay by the phosphorylation of myelin basic protein. This coupled assay has been detailed and validated previously [12].

Results.

Expression of TAB2 and TAB3

In order to identify phosphorylation sites on TAB2 and TAB3, we first expressed these proteins in HEK 293 cells that stably express the IL-1 receptor, termed IL-1R cells, alone or in the presence of the other subunits of the TAK1 complex. These studies revealed that the expression of either TAB3 or TAB2 was greatly enhanced when they were co-expressed with TAK1 and TAB1 (data not shown). The co-expression of vectors encoding GST-fusions of TAB3, TAK1 and TAB1 or TAB2, TAK1 and TAB1 in a single 15 cm dish of cells generated sufficient amounts of TAB3 and TAB2 (Fig 2A) for the phosphorylated peptides in each protein to be identified by tryptic digestion and mass spectrometry.

Identification of phosphorylation sites on TAB2.

Nine tryptic phosphopeptides were detected by precursor ion scanning mass spectrometry (MS), all of which were identified (Fig 2B). Phosphopeptides 1a and 1b both comprised residues 369-382 of TAB2 phosphorylated at Ser372, these peptides only differing by the presence of methionine sulphoxide in 1b compared to unoxidised methionine in 1a. Peptides 1a and 1b arise from a chymotryptic-like cleavage of the Tyr-Ile bond between residues 368 and 369 of TAB2. Phosphopeptides 5a and 5b both comprised residues 363-382 of TAB2 phosphorylated at Ser372, these peptides also only differing by the presence of methionine sulphoxide in 5b compared to unoxidised methionine in 5a. Phosphopeptide 2 comprised residues 355-369 and contained one phosphate, but which of the six serine residues and three threonine residues was phosphorylated could not be identified. Phosphopeptides 3a and 3b both comprised residues 580-596 of TAB2, these peptides again differing by the presence of methionine sulphoxide in 3b compared to unoxidised methionine in 3a. Phosphopeptides 3a and 3b were not phosphorylated at Ser588, indicating that the site(s) of phosphorylation was (were) residues 580, 582 or 584. Phosphopeptide 4b was an oxidised form of phosphopeptide 4a comprising residues 522-541 of TAB2.

MS data showed that these peptides were not phosphorylated at Thr533, indicating that the site(s) of phosphorylation was(were) Ser524 and/or Ser527.

Identification of phosphorylation sites on TAB3.

Nine tryptic phosphopeptides were detected by precursor ion-scanning mass spectrometry (Fig 2C), eight of which were identified. Phosphopeptides 1a and 1b both comprised residues 53-65 of TAB3 phosphorylated at Ser60, these peptides only differing by the presence of methionine sulphoxide in 1b compared to unoxidised methionine in 1a. Peptide 3, resulting from an incomplete tryptic cleavage of the Arg-Met bond between residues 65 and 66, comprised residues 53-68 with both methionine residues oxidised to the sulphoxide derivative, and was also phosphorylated at Ser60.

Peptides 2a and 2b both corresponded to residues 395-412 of TAB3, these peptides containing one and two phosphorylated residues respectively, which were not located at Ser407, Ser408, Ser409 or Ser411. This narrowed down the potential sites of phosphorylation to Ser398, Tyr400, Thr401,Thr403 or Thr404. Peptides 2a* and 2b* also corresponded to singly and doubly phosphorylated forms of the peptide comprising residues 395-412. However their molecular masses were 203 Da greater than peptides 2a and 2b, respectively, which equates to the mass predicted for an N-acetyl-hexosamine residue, probably an O-GlcNAc residue, attached covalently to a serine or threonine residue in the peptide.

Peptide 4 was found to comprise residues 504-523 phosphorylated at one residue, which was not Thr515. This indicated that the phosphorylation site(s) was(were) located at Ser504, Ser505, Ser506, Ser507 or Ser509.

The phosphopeptide indicated by a double asterisk (Fig 2C) could not be identified by mass spectrometry.

Production and specificity of phospho-specific antibodies.

Phospho-specific antibodies were raised against the two phosphopeptides containing the sites of phosphorylation that were identified definitively by mass spectrometry, namely residues Ser60 on TAB3 and Ser372 on TAB2. In addition, we

also raised a phospho-specific antibody capable of recognising TAB3 if it were phosphorylated at Thr404. This residue was selected as the likely site of phosphorylation in peptide 2a/2b (Fig 2C) because MAPKs are proline-directed protein kinases and Thr404, not only lies in a Thr-Pro sequence but is located in a position equivalent to Ser372 of TAB2. We also raised phospho-specific antibodies that should recognise TAB3 phosphorylated at Ser506 and TAB2 phosphorylated at either Ser 524 or Ser582, which were among the potential sites of phosphorylation in phosphopeptide 4 from TAB3 (Fig 2C) and phosphopeptides 4a/4b and 3a/3b from TAB2 (Fig 2B), respectively. These serine residues were selected as likely sites of phosphorylation because they are located in Arg-Xaa-Xaa-Ser sequences, which are targeted by many protein kinases.

All six phospho-specific antibodies that were generated recognised GST-TAB2 or GST-TAB3 purified from transfected IL-1R cells, but did not recognise mutant forms of GST-TAB2 or GST-TAB3 in which the particular phosphorylation site had been changed to Ala (Figs 2D and 2E). These experiments established that TAB3 was phosphorylated at Ser60, Thr404 and Ser506 and TAB2 at Ser372, Ser524 and Ser582, at least when overexpressed in IL-1R cells, and that each antibody recognised only a single phosphorylated residue in each protein.

Effect of IL-1β on the site-specific phosphorylation of TAB2.

The phosphorylation state of TAB2 in the endogenous TAK1 complex was analysed after its immunoprecipitation with the TAK1 complex by anti-TAB1. These studies showed that TAB2 became phosphorylated at Ser372 and Ser524 in response to IL-1β. Phosphorylation was maximal after about 10 min and sustained for at least 30 min (Fig 3A). The phosphorylation of TAB2 at Ser372 or Ser524 was not prevented by 5 μM SB 203580 (Fig 3B) or by 0.1 μM BIRB 0796 (data not shown), which are structurally unrelated and relatively [13, 22] and highly [17, 22] specific inhibitors of p38α MAPK, although the phosphorylation of TAB1 at Ser423, which is known to be phosphorylation by p38α MAPK in cells [12], was blocked by these compounds as expected (Fig 3B and data not shown). Moreover, the phosphorylation

of TAB2 at Ser372 or Ser524 was not prevented by the exquisitely specific MKK1 inhibitor PD 184352 [22, 23] in the absence or presence of SB 203580 (Fig 3B), excluding the involvement of ERK1/ERK2, or kinases activated by ERK1/ERK2, in the phosphorylation of these residues. PD 184352 prevented the IL-1-stimulated phosphorylation of ERK1/ERK2, as expected (Fig 3B).

If the concentration of BIRB 0796 in the culture medium is increased from 0.1 μM to 10 μM, it not only suppresses the activity of p38α MAPK and p38β MAPK, but also the MAPKs, termed JNK1 and JNK2 [24]. Preincubation of the IL-1R cells with 10 μM BIRB 0796 suppressed the IL-1-induced phosphorylation of TAB2 at Ser372, as well as c-Jun at Ser63 (a physiological substrate of JNKs), but did not affect the phosphorylation of Ser524 (Fig 4A), indicating that Ser372 and Ser524 are phosphorylated by distinct protein kinases and that Ser372 might be phosphorylated by a JNK isoform(s). However, the IL-1-stimulated phosphorylation of Ser372 in immortalised embryonic fibroblasts from JNK1-/-/JNK2-/- double knockout mice was similar to that observed in wild type fibroblasts (Fig 4B). Moreover, the IL-1-induced phosphorylation of TAB2 at Ser372 was suppressed by 10 μM BIRB 0796 in the JNK1-/-/JNK2-/- fibroblasts (Fig 4C). Since fibroblasts do not express the JNK3 isoform, these results demonstrate that 10 μM BIRB 0796 prevents the phosphorylation of Ser372 in JNK-deficient fibroblasts (and presumably also in wild type fibroblasts) by inhibiting a protein kinase distinct from JNK.

The phospho-specific antibody that recognises TAB2 phosphorylated at Ser582 in transfected cells (Fig 2D) did not recognise the endogenous TAK1 complex in either unstimulated or IL-1-stimulated IL-1R cells.

Effect of IL-1β on the site-specific phosphorylation of TAB3.

The phosphorylation of TAB3 in the endogenous TAK1 complex was studied after immunoprecipitating the TAK1 complex from extracts of IL-1R cells with anti-TAB1. These studies showed that TAB3 was minimally phosphorylated at Ser60, Thr404 and Ser506 in unstimulated cells, but these residues became phosphorylated in response to IL-1β, with maximal phosphorylation after 20 min (Fig 5A). Moreover,

the IL-1-induced phosphorylation of all three sites was suppressed by either 5 μM SB 203580 or 0.1μM BIRB 0796. At the same concentrations, these compounds inhibited the phosphorylation of TAB1 at Ser423, a known physiological substrate for p38α MAPK (Fig 5B).

The experiments presented in Fig 5B demonstrated that Ser60, Thr404 and Ser506 are phosphorylated by p38α MAPK either directly or indirectly by another protein kinase that is activated by p38α MAPK. Since p38α MAPK is a proline-directed protein kinase and Ser60 and Thr404 are followed by proline, it seems likely that these sites are phosphorylated directly by p38α MAPK. However Ser506 is not followed by proline, but lies in a Hyd-Xaa-Arg-Xaa-Xaa-Ser- motif reminiscent of the consensus sequence for phosphorylation by MAPKAP-K2 and MAPKAP-K3 [25], two closely related protein kinases that are activated by p38α MAPK in vivo. We therefore studied the effect of IL-1 using fibroblasts from mice that do not express MAPKAP-K2 and MAPKAP-K3. These experiments revealed that the IL-1-stimulated phosphorylation of Ser506 did not occur in these cells (Fig 5C), indicating that MAPKAP-K2 and/or MAPKAP-K3 are likely to mediate the phosphorylation of TAB3 at this site in wild type cells. The expression of p38α MAPK is reduced in MAPKAP-K2/MAPKAP-K3-deficient cells [26], which accounts for the reduced IL-1 stimulated phosphorylation of p38α MAPK observed in our knockout cells. Consistent with this, the IL-1 stimulated phosphorylation of TAB3 at Ser60 and Thr404, which is probably catalysed by p38α MAPK, was reduced, but not abolished, in the MAPKAP-K2/MAPKAP-K3-deficient cells (Fig 5C).

IL-1 stimulated phosphorylation of TAB3 in TAB1-deficient fibroblasts.

It has been reported that the IL-1-stimulated activation of MAP kinase cascades and NFκB in embryonic fibroblasts from TAB1-/- cells is similar to wild-type cells [5], which was confirmed in the present study (Fig 6A). It had been inferred from these observations that TAB1 is not required for the IL-1-stimulated activation of TAK1, which seemed to offer the opportunity of studying how the p38α MAPK-dependent phosphorylation of TAB3 regulates the activity of TAK1

independently of the phosphorylation of TAB1. The results of these experiments are presented in Fig 6B. Interestingly, these studies revealed that, although the expression of TAB3 in TAB1-/- and wild type cells was similar, the IL-1-induced phosphorylation of TAB3 at Ser60 and Thr404 was greatly reduced in the TAB1-/- cells. IL-1 still stimulated the phosphorylation of TAB3 at Ser506 in TAB1-/- cells but, in contrast to wild type fibroblasts, phosphorylation of this site was transient. The IL-1 induced phosphorylation of MAPKAP-K2 was similar in wild type and TAB1-/- cells. The lack of expression of TAB1 in the TAB1-/- cells explains why IL-1-induced phosphorylation of TAB1 at Ser423 was not detected (Fig 6B).

TAK1 is not activated by IL-1 or TNFα in TAB1-/- MEFs.

Since the IL-1 stimulated phosphorylation of TAB3 at Ser60 and Thr404 did not occur, and the phosphorylation of Ser506 was reduced in TAB1-/- fibroblasts, it was of interest to investigate how the feedback control of TAK1 by p38α MAPK was affected in these cells. However, this experiment could not be performed because, surprisingly, the TAK1 immunoprecipitated from the fibroblasts of TAB1-/- cells did not have detectable activity, whether or not the cells were stimulated with IL-1 (Fig 7A) or tumour necrosis factor α (TNFα) (Fig 7B). We also assessed the phosphorylation of the TAK1 catalytic subunit at Thr187. The phosphorylation of this residue correlates with activation and is thought to be an autophosphorylation event catalysed by TAK1 itself [27]. Although no TAK1 activity could be detected in the immunoprecipitates, some residual IL-1 (Fig 7C) or TNFα (Fig 7D) stimulated phosphorylation of Thr187 appeared to be present, although it was reduced compared to that observed in wild-type cells.

IL-1 induced signalling in MEKK3-deficient fibroblasts.

The observation that no TAK1 activity could be detected when this protein kinase was immunoprecipitated from IL-1 or TNFα-stimulated TAB1-/- fibroblasts, raised the question of why the IL-1/TNFα-stimulated activation of MAP kinase cascades was normal in TAB1-/- cells. One possibility was that MEKK3 could substitute for TAK1 in TAB1-/- fibroblasts, because it has been reported that the IL-1

or LPS-stimulated activation of p38α MAPK, JNK and NFκB [4] or the TNFα-stimulated activation of NFκB [28] does not occur in MEKK3-/- cells. We therefore generated mice expressing a truncated, but inactive form of MEKK3 and generated immortalised embryonic fibroblasts from these MEKK3 -/- mice as well as control wild-type mice (see Methods and Fig 8A). However, in contrast to the earlier reports [4, 28], we found that IL-1 (Fig 8B) or TNFα (Fig 8C) activated p38α MAPK, JNK and ERK1/ERK2 and induced the degradation of IκBα similarly in MEKK3-deficient and wild-type MEFs. These cells express a truncated form of MEKK3 (Fig 8C), which is catalytically inactive (results not shown).

Identification of the protein kinases that phosphorylate TAB1 at Ser438.

We have reported previously that LPS, IL-1, TNF and several cellular stresses induce the phosphorylation of TAB1 at Ser438 and that this is catalysed by a protein kinase(s) distinct from p38α MAPK, because phosphorylation is not suppressed by SB 203580 or in p38α MAPK or in p38α MAPK-/- fibroblasts [12]. Ser438 is followed by a proline residue, implying that it is phosphorylated by a proline-directed protein kinase, such as another MAPK. Indeed, we have reported previously that TAB1 can be phosphorylated at Ser438 in vitro by ERK2, JNK1, JNK2 and all four isoforms of p38 MAPK [12].

In order the identify the protein kinase that phosphorylates TAB1 at Ser438 in cells, we studied the effect of the MKK1 inhibitor PD 184352 in wild type fibroblasts and fibroblasts that do not express JNK1 or JNK2. These experiments revealed that the IL-1α stimulated phosphorylation of TAB1 at Ser438 was partially reduced in the JNK1-/-/JNK2-/- fibroblasts, partially suppressed by PD 184352 in the wild type fibroblasts, and greatly reduced by PD 184352 in the JNK1-/-/JNK2-/- fibroblasts (Fig 9A). Similar results were obtained if the phosphorylation of Ser438 was induced by osmotic shock instead of IL-1 (Fig 9B). These experiments indicate that the phosphorylation of Ser438 in MEFs is catalysed by both ERK and JNK isoforms and that TAB1 is therefore targeted by three different MAPKs in MEFs. However, we have been unable, thus far, to detect a significant difference in IL-1-stimulated TAK1

activation between JNK1-/-/JNK2-/- MEFs and wild type MEFs (data not shown). The role of Ser438 phosphorylation has therefore still to be determined.

Discussion.

Our earlier studies had identified a feedback control mechanism by which p38α MAPK down-regulated TAK1 and which correlated with the phosphorylation of TAB1 at Ser423 and Thr431 [12] and a decrease in the electrophoretic mobilities of TAB2 and TAB3 that could be reversed by protein phosphatase treatment [11]. In the present study we sought to identify the phosphorylation sites on TAB2 and TAB3 in order to evaluate their importance in the feedback control of TAK1.

Earlier work had also shown that the LPS-, IL-1- or osmotic shock-induced decrease in the electrophoretic mobility of TAB2, was only partially prevented by SB 203580 or in p38α MAPK-deficient fibroblasts, indicating that TAB2 was likely to be phosphorylated in cells by p38α MAPK and at least one other protein kinase [11]. Here, we identified two IL-1-stimulated phosphorylation sites on TAB2 in IL-1R cells, Ser372 and Ser524, whose phosphorylation was not prevented by specific inhibition of p38α MAPK (Figs 3 and 4) and which may underlie the p38α MAPK-insensitive decrease in the electrophoretic mobility of TAB2 noted previously [11].

Ser372 of TAB2 is followed by a proline residue, but several proline-directed protein kinases known to be activated by IL-1 do not appear to phosphorylate Ser372. Thus the MKK1 inhibitor PD 184352 (Fig 1) did not prevent the IL-1-induced phosphorylation of Ser372 in the presence or absence of SB 203580 (Fig 3) and IL-1 stimulated the phosphorylation of Ser372 in JNK-deficient cells in a similar manner to wild type cells (Fig 4). Moreover, IL-1 did not activate ERK5 in IL-1R cells (results not shown). It would therefore appear that IL-1 activates a proline-directed Ser372 kinase that has yet to be identified, perhaps p38γ MAPK and/or p38δ MAPK, whose activities are suppressed by high concentrations of BIRB 0796 [24].

In contrast to Ser372, Ser524 is not followed by proline and its phosphorylation was unaffected by PD 184352, SB 203580 and low or high

concentrations of BIRB 0796. These observations indicate that this residue is phosphorylated by an IL-1-stimulated protein kinase that does not lie downstream of ERK1/2, JNK1/2 or p38 MAPK isoforms.

The residue(s) in TAB2 that is (are) targeted by the p38 MAPK pathway remain(s) to be identified, but one site could be Ser582, which became phosphorylated when the TAK1 complex was overexpressed in IL-1R cells (Fig 2D). Although the phosphorylation of the endogenous TAB2 at this site was not detected in IL-1-stimulated IL-1R cells, we have observed the LPS-induced phosphorylation of Ser582 in murine RAW macrophages (results not shown). Moreover, the LPS-induced phosphorylation of Ser582 in RAW cells was prevented by SB 203580, indicating that the phosphorylation of this site may underlie the SB 203580-sensitive decrease in the electrophoretic mobility of TAB2. Ser582 is not followed by a proline residue and is presumably phosphorylated by a protein kinase that is activated by p38α MAPK. Ser582 lies in a Leu-Xaa-Arg-Xaa-Xaa-Ser-Ile sequence, which is an optimal consensus for phosphorylation by MAPKAP-K2/MAPKAP-K3 [25], but further studies will be needed to establish whether these protein kinases mediate the phosphorylation of Ser582 in LPS-stimulated macrophages.

We identified three residues on TAB3, namely Ser60, Thr404 and Ser506 (Fig5 and 6) that became phosphorylated in response to IL-1, two of which (Thr404 and Ser506) were located in positions equivalent to Ser372 and Ser524 of TAB2 (Fig 10A). However, in contrast to the phosphorylation sites on TAB2, the phosphorylation of Thr404 and Ser524 of TAB3, as well as Ser60, was prevented by either of two structurally unrelated inhibitors of p38α MAPK, SB 203580 and BIRB 0796. The phosphorylation of these sites presumably accounts for the previously observed IL-1- or LPS-dependent reduction in the electrophoretic mobility of TAB3, which was prevented by SB 203580 and did not occur in p38α MAPK-/- fibroblasts [11]. Ser60 and Thr404 are followed by proline, and since MAPKs normally phosphorylate Ser-Pro and Thr-Pro sequences, these residues are likely to be phosphorylated directly by p38α MAPK in cells. However, Ser506 is not followed by

a proline residue, suggesting that it is not phosphorylated directly by p38α MAPK, but by another protein kinase that is activated by p38α MAPK. This led us to discover that the IL-1-stimulated phosphorylation of Ser506 did not occur in fibroblasts that do not express MAPKAP-K2 and MAPKAP-K3 (Fig5C). Thus these protein kinases are likely to mediate the IL-1 induced phosphorylation of Ser506 in wild-type fibroblasts. The protein kinases that are likely to phosphorylate the different sites on TAB2 and TAB3 in fibroblasts are summarized in Fig 10B.

We [12] and others [29] have shown that p38α MAPK interacts strongly with a region near the C-terminus of TAB1 and that, in contrast to other MAP kinases, including the very closely-related p38β MAPK, it can be pulled down on glutathione-Sepharose together with GST-TAB1 [12]. This observation suggested that one role of TAB1 is to recruit p38α MAPK to the TAK1 complex [8], a hypothesis now supported by the finding that the IL-1-stimulated phosphorylation of TAB3 at Ser60 and Thr404 is greatly reduced in TAB1-/- fibroblasts (Fig 6B). This implies that the phosphorylation of TAB3 is dependent on the recruitment of p38α MAPK to the TAB1 component of the TAK1 complex.

The IL-1 induced, MAPKAP-K2/MAPKAP-K3-catalysed phosphorylation TAB3 at Ser506 was still observed in TAB1-/- fibroblasts, but was far more transient than in wild type fibroblasts (Fig 6B). The structure of TAB1 has revealed that it is an inactive pseudophosphatase of the MPP family of serine/threonine-specific protein phosphatases and we have suggested that a further role of TAB1 may be to interact with phosphorylated residues in the TAK1 complex, thereby protecting them against dephosphorylation [8]. The failure of TAB1 to protect Ser506 from dephosphorylation in TAB1-deficient fibroblasts could account for its transient phosphorylation compared to the more sustained activation seen in wild type cells, and may also contribute to the lack of phosphorylation at Ser60 and Thr404.

TAK1 is widely believed to be a “master” kinase that switches on MAP kinase cascades and NFκB in response to IL-1, LPS and TNFα, because the activation of these signalling pathways is abolished or reduced in TAK1-/- cells [5-7]. Like the

knock-out of TAK1, the knock-out of TAB1 is embryonic lethal [5, 30] and the generation of active TAK1 requires its co-expression with TAB1 [9, 31-33] or fusion to the TAK1-binding C-terminal domain of TAB1 [33, 34]. These studies implied an essential role for TAB1 in activating the TAK1 complex. However, more recently, the IL-1- and TNFα-induced activation of MAP kinase cascades and NFκB were reported to be unaffected by siRNA knock-down of TAB1 in HeLa cells, which reduced the level of expression of this protein by 80%, whereas siRNA knockdown of TAK1 did inhibit the activation of these signaling pathways [35]. Similarly, the IL-1- or TNFα-induced activation of JNK and IKK, or IL-1-, TNFα– or LPS-stimulated NFκB gene transcription, were not impaired in TAB1-/- MEFs, although they were suppressed in TAK1-deficient MEFs [4]. It had been inferred from the latter study that TAB1 was dispensable for the activation of TAK1 by IL-1 and TNFα in these cells. However, consistent with the earlier reports that TAB1 is required for TAK1 catalytic activity, we found here that IL-1 and TNFα were unable to stimulate detectable TAK1 activity in the same TAB1-/- MEFs (Fig 7). The simplest explanation that can account for these observations is that a protein kinase distinct from TAK1 activates MAP kinase cascades and IKK in TAB1-/- fibroblasts. However, we cannot exclude the possibility that TAK1 is active in TAB1-/- cells, but is unstable without TAB1 so that activity disappears during its immunoprecipitation from the cell extracts before it can be assayed. Alternatively, the TAK1 in TAB1-/- cells may have trace residual activity (5% or less) that would be difficult to detect in our assay, this slight activity nevertheless being sufficient to sustain normal downstream signalling. Another possible explanation of the data is that the lack of TAB1 prevents TAK1 from activating MKK6 in vitro, but does not prevent TAK1 from activating another protein(s), such as another MAP3K, required to switch on MAP kinase cascades and NFκB in response to IL-1 and TNFα. This scenario, or an essential role for TAK1 that does not require its protein kinase activity, could explain why downstream signalling does not occur in TAK1-/- MEFs, but is similar in

TAB1-/- and wild type MEFS. It is also possible that, in the absence of TAK1, TAB1 and/or TAB2/TAB3 become dominant negative inhibitors of another protein kinase required for IL-1-induced "down-stream" signalling.

It has been reported that TAK1 co-operates with MEKK3 in mediating TNFα-induced activation of NFκB [36], raising the possibility that MEKK3 mediates IL-1- and TNFα-stimulated "down-stream" signalling in TAB1-/- MEFs. Indeed, it has been reported that the IL-1 or LPS-stimulated activation of p38α MAPK, JNK and NFκB [4], or the TNFα-stimulated activation of NFκB [28], does not occur in MEKK3-/- MEFs. However, using MEFs that express a truncated, but catalytically inactive form of MEKK3, we found that the IL-1 and TNFα stimulated activation of p38α MAPK, JNK and ERK1/ERK2 and the degradation of IκBα occurred similarly in the MEKK3-deficient and wild-type MEFs (Fig 8). Apoptosis signal-regulating kinase 1 (ASK1) is another MAP3K that may mediate "down-stream" signalling, since it is activated in response to LPS and LPS-induced activation of p38α MAPK is suppressed in ASK1-/- splenocytes and dendritic cells [37]. However, LPS-stimulated activation of JNK and IKK was similar in ASK1-/- and wild type splenocytes and dendritic cells [37]. Therefore ASK1 may therefore not be the MAP3K that mediates IL-1-induced activation of JNK and IKK in MEFs. Taken together, the present study suggests that further work is needed to clarify which MAP3K mediate the IL-1-stimulated activation of MAP kinase cascades and IKK in TAB1-/- and wild type MEFS.

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Figure Legends

Fig 1. Pro-inflammatory protein kinase cascades whose activation by IL-1 requires the expression of the TAK1 catalytic subunit. IKKβ and JNK control the transcription of genes encoding inflammatory mediators by activating the NFκB and AP1 transcription factors, respectively, whereas ERK1/2, p38α MAPK and MAPKAP-K2/MAPKAP-K3 (MK2/MK3) exert their effects at the post-transcriptional level**.** The LPS-induced production of TNFα is suppressed by compounds, such as SB 203580 and BIRB 0796 that inhibit p38α MAPK or in MAPKAP-K2 knock-out mice [20]. These compounds show efficacy in animal models of RA [38], and several have entered clinical trials. The LPS-induced secretion of TNFα is also suppressed in COT/Tpl2-deficient macrophages [39] and COT/Tpl2-deficient mice are protected against TNFα-induced IBD [40]. The absence of COT/Tpl2, or pharmacological inhibition of its substrates MKK1/MKK2 by the drug PD 184352 [15, 22], prevents the activation of ERK1/ERK2 [39] and the conversion of pre-TNFα to the mature, secreted form of this cytokine [41]. In contrast, the p38α MAPK-dependent pathway suppresses the production of pre-TNFα, probably by inhibiting the translation of its mRNA into protein [38, 42]. The feedback control of TAK1 by p38α MAPK is also indicated. Abbreviations:- COT, the Cancer Osaka Thyroid oncogene, also called Tpl2, tumour progression locus 2; ERK, extracellular signal regulated kinase; IKK, IκB kinase, JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MKK, MAPK kinase; MAPKAP, MAPK-activated; NFκB, nuclear factor regulating the κ-immunoglobulin gene in B-cells; TAK1, TGFβ-activated kinase-1; TNFα, tumour necrosis factor α.

Fig. 2. The expression of TAK1 complexes in IL-1R cells, mass spectrometry of TAB2 AND TAB3, and characterisation of phospho-specific antibodies. (**A**) DNA vectors encoding GST-TAK1 and GST-TAB1 were co-expressed in a single 15 cm dish of IL-1R cells together with a vector encoding GST-TAB2 or GST-TAB3. 24h after transfection, the cells were lysed, centrifuged and the GST-fusion proteins

purified from the extracts by affinity chromatography on glutathione Sepharose. The GST-tag was removed from each protein by cleavage on the beads with PreScission protease, releasing the purified TAB1-TAK1-TAB2 or TAB1-TAK1-TAB3 complexes. These were denatured in SDS, subjected to SDS/PAGE and stained with colloidal Coomassie Blue. The identities of each band were established by excising them from the gel and subjecting them to tryptic mass fingerprinting. The TAK1 catalytic subunit is resolved into three discreet bands. (**B, C**) TAB2 (B) or TAB3 (C) were digested with trypsin and analysed by liquid chromatography-mass spectrometry (LC-MS) with precursor 79 scanning on an Applied Biosystems 4000 Q-TRAP system [43]. Selected peptides were also analysed by Multiple Reaction Monitoring (MRM). The *y*-axis denotes ion intensity in counts per second (cps) and the *x*-axis the mass/charge ratio (m/z). Masses shown are those obtained from the negative mode Enhanced Resolution spectra and are all determined from the 2- charge state. Residues in which methionine has become oxidised to methionine sulphoxide are denoted by m. Abbreviation:- HxNAc, N-Acetyl-hexosamine residue. The ion denoted by a double asterisk was not identified. **(D, E)** The TAB1-TAK1-TAB2 (D) and TAB1-TAK1-TAB3 (E) complexes were expressed in IL-R cells, purified from 0.5 mg lysate protein, and subjected to SDS-PAGE as in A. After transfer to PVDF membranes, they were immunoblotted with six different phospho-specific antibodies to detect the particular sites of phosphorylation indicated (lanes 1 and 2 in D and E). Six different TAK1 complexes were also purified in which the one relevant phosphorylation site in TAB2/TAB3 had been mutated individually to Ala and immunoblotted as described above to assess the specificities of each phospho-specific antibody (see lanes 3 and 4 of D and E).

Fig. 3. Effect of interleukin 1β on the site-specific phosphorylation of TAB2.

(A) IL-1R cells were serum starved for 6 h, then stimulated for the times indicated with IL-1β (5 ng/ml) and lysed. The TAK1 complex was immunoprecipitated from the cell extracts (2 mg extract protein) with an anti-TAB1 antibody, denatured in

LDS, subjected to SDS-PAGE and immunoblotted with phospho-specific antibodies that recognise the phosphorylated residues indicated and with further antibodies that recognize the TAB2 and TAB1 proteins. **(B)** Same as A, except that after serum deprivation the cells were incubated for 1 h in the presence (+) or absence (-) of 5 μM SB 203580 or 2 μM PD 184352 before stimulation for 20 min with (+) or without (-) IL-1β. Further aliquots of the cell extracts from B were immunoblotted (without immunoprecipitation) using antibodies that recognize TAB1 phosphorylated at Ser423 and the active, phosphorylated forms of ERK1/ERK2.

Fig. 4. Effect of IL-1β on the phosphorylation of TAB2 in wild type and JNK-deficient cells. The results shown are representative of several independent experiments. **(A)** The experiment was carried out in IL-1R cells as in Fig 3 , except that before stimulation with IL-1β, the cells were incubated for 1 h with (+) or without (-) 10 μM BIRB 0796. Further aliquots of the cell extracts were immunoblotted (without immunoprecipitation) using antibodies that recognize c-Jun phosphorylated at Ser63. **(B)** Immortalised mouse embryonic fibroblasts from wild type (WT) and JNK1-/-/JNK2-/-) mice were serum starved for 6 h, then stimulated for 10 or 20 min with 10 ng/ml IL-1β and lysed. The TAK1 complex was immunoprecipitated from the cell extracts with an anti-TAB1 antibody and immunoblotted with antibodies that recognize TAB2 phosphorylated at Ser372 or an antibody that recognizes the TAB2 protein. Further aliquots of the cell extracts were immunoblotted (without immunoprecipitation) using an antibody that recognizes the phosphorylated and unphosphorylated forms of the JNK isoforms equally well. **(C)** The fibroblasts from JNK1-/-/JNK2-/- mice were serum starved for 6 h, then incubated for 1 h with (+) or without (-) 10 μM BIRB 0796 before stimulation for 20 min with 10 ng/ml IL-1β followed by cell lysis.

Fig 5. Effect of interleukin 1β on the site-specific phosphorylation of TAB3.

The results shown are representative of several independent experiments.

(A) IL-1R cells were serum starved for 6 h, then stimulated for the times indicated with IL-1β (5 ng/ml). **(B)** Same as A, except that after serum deprivation the cells were incubated for 1 h in the presence (+) or absence (-) of 5 μM SB 203580 or 0.1 μM BIRB 0796 before stimulation for 20 min with (+) or without (-) IL-1β. The TAK1 complex was immunoprecipitated from the cell extracts (2 mg extract protein) with an anti-TAB1 antibody, denatured in LDS, subjected to SDS-PAGE and immunoblotted with phospho-specific antibodies that recognise the phosphorylated residues indicated and with further antibodies that recognise the TAB3 and TAB1 proteins. Further aliquots of the cell extracts from B were immunoblotted (without immunoprecipitation) using antibodies that recognize TAB1 phosphorylated at Ser423. **(C)** Fibroblasts from primary wild type (WT) and MAPKAP-K2-/-/MAPKAP-K3-/- (MK2/MK3-/-) fibroblasts were serum starved for 6 h, then stimulated for the times indicated with 10 ng/ml IL-1α. The TAK1 complex was immunoprecipitated from the cell extracts with anti-TAB3 and immunoblotted as in A. Further aliquots of the cell extracts were immunoblotted (without immunoprecipitation) using antibodies that recognize the active phosphorylated form of p38α MAPK, MAPKAP-K2 phosphorylated at Thr334, and with antibodies that recognize all forms of MAPKAP-K2 and ERK2.

Fig 6. Phosphorylation of TAB3 in wild type and TAB1-deficient fibroblasts. The results shown are representative of several independent experiments. **(A)** Immortalised mouse embryonic fibroblasts from wild type (WT) and TAB1-/- mice were serum starved for 6 h, then stimulated with IL-α (10 ng/ml) for the times indicated. Aliquots of cell extract (30 μg protein) were denatured in LDS, subjected to SDS-PAGE and immunoblotted with antibodies that recognise all forms of ERK1/2 and TAK1 or the phosphorylated (p) forms of TAB1 (pS423), JNK1/2, p38α MAPK, or ERK1/2. (**B**) Same as (A) except that the TAK1 complex was immunoprecipitated from the cell extracts (2 mg extract protein) with an anti-TAB3 antibody, denatured in LDS, subjected to SDS-PAGE, transferred to PVDF membranes and immunoblotted

with phospho-specific antibodies that recognise phosphorylated Ser60, Thr404 and Ser506, and with a further antibody that recognizes the TAB3 protein. The antibody recognising TAB3 phosphorylated at Ser60 also detected a non-specific (NS) band migrating slightly slower than TAB3. Further aliquots of the cell extracts were immunoblotted (without immunoprecipitation) using antibodies that recognise TAB1 phosphorylated at Ser423, the active phosphorylated form of p38α MAPK, MAPKAP-K2 (MK2) phosphorylated at Thr334, and with an antibody that recognizes all forms of ERK2.

Fig 7. IL-1α and TNFα-induced activation of TAK1 in wild type and TAB1 deficient fibroblasts. Immortalized embryonic fibroblasts from wild-type mice (WT, open bars) and TAB1 -/- mice (filled bars) were serum starved for 6 h, then stimulated for the times indicated with 10 ng/ml IL-1α (A) or 10 ng/ml TNFα (B). (**A, B**) The TAK1 complexes were immunoprecipitated from the cell extracts (0.15 mg of extract protein) using anti-TAK1, and TAK1 activity determined as described under Methods. The results are expressed as mU TAK1 activity per mg cell lysate (+/- SD for determinations from three separate immunoprecipitations). Similar results were obtained in four (IL-1) or two (TNFα) independent experiments. (**C, D**) The TAK1 complex was immunoprecipitated from 0.5 mg cell extract protein, denatured in LDS, subjected to SDS-PAGE, transferred to PVDF membranes and immunoblotted with a phospho-specific antibody that recognizes TAK1 phosphorylated at Thr187 (pT187) and with a further antibody that recognizes all forms of TAK1.

Fig 8. Characterisation of IL-1 stimulated signaling in MEKK3-deficient fibroblasts. **(A)** Targeting construct for Cre-conditional MEKK3 inactivation. Further details are given under Methods. **(B,C)** Immortalised MEFs from wild type (WT) and MEKK3 deficient (-/-) mice were serum starved for 16 h, then stimulated with IL-α (10 ng/ml) **(B)** or with 10 ng/ml TNFα **(C)** for the times indicated and lysed. The cells were lysed, centrifuged and the supernatant, termed cell extract,

decanted. The cell extracts were denatured in LDS, subjected to SDS-PAGE and immunoblotted with antibodies that recognize total IκBα, ERK1/2, TAK1 and MEKK3 or the phosphorylated (p) forms of JNK1/2, p38α MAPK, or ERK1/2. Full-length MEKK3 and the truncated form of the protein expressed in the MEKK3-/- cells are indicated.

Fig 9. Phosphorylation of TAB1 at Ser438 in wild type and JNK-deficient fibroblasts. Fibroblasts from immortalized wild type (WT) and JNK1-/-/JNK2-/- fibroblasts (KO) were serum starved for 6 h, incubated for 1 h with (+) or without (-) 2 μM PD 184352, then stimulated for 20 min with 10 ng/ml IL-1α (A) or exposed for 30 min to an osmotic shock (0.5 M sorbitol) (B). The cells were lysed, denatured in LDS, subjected to SDS-PAGE, transferred to PVDF membranes and immunoblotted with antibodies that recognize TAB1 phosphorylated at Ser438 (pS438), the active, phosphorylated forms of ERK1/ERK2, and with antibodies that recognize all forms of ERK1/2 or JNK1/2 equally well. Similar results were obtained in several independent experiments.

Fig 10. Location of the phosphorylation sites in TAB2 and TAB3 and the protein kinases involved in their phosphorylation. (**A**) The sequences were aligned using the clustalX programme [44]. Identical residues are in white letters on a black background, and the phosphorylation sites are indicated by asterisks. (**B**) The IL-1 induced phosphorylation sites on TAB1, TAB2 and TAB3 in IL-1R cells and MEFs, and the protein kinases that phosphorylate them are shown. Ser372 and Ser524 of TAB2 are phosphorylated by different protein kinases, termed PKX and PKY, which are distinct from p38α/β MAP kinases, JNK1/2 and ERK1/2 or protein kinases that are activated by these MAP kinase family members. Ser60 and Thr404 of TAB3 are phosphorylated directly by p38α MAPK and Ser506 by MAPKAP-K2/MAPKAP-K3 (MK2/MK3). TAB1 is phosphorylated by p38α MAPK at Ser423 and Thr431 and by both ERK1/2 and JNK1/2 at Ser438.

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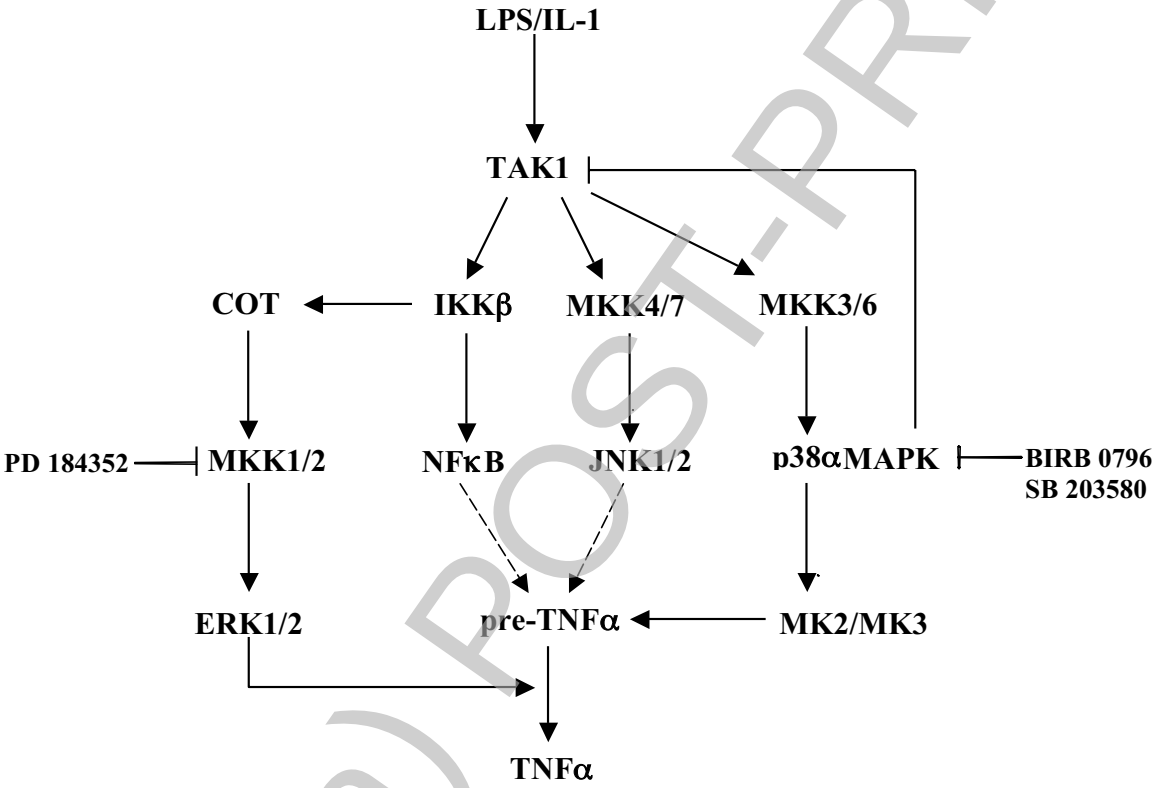
Figure 1



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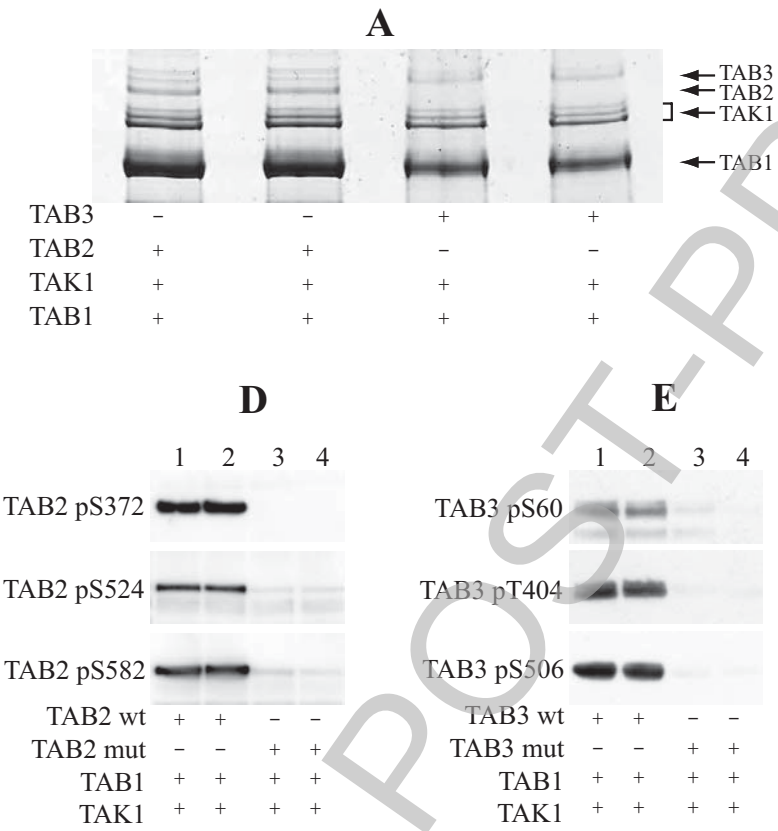
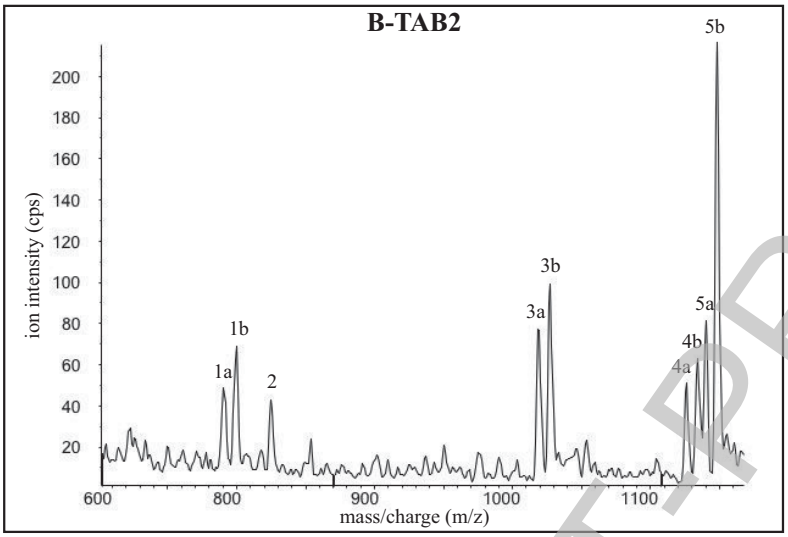
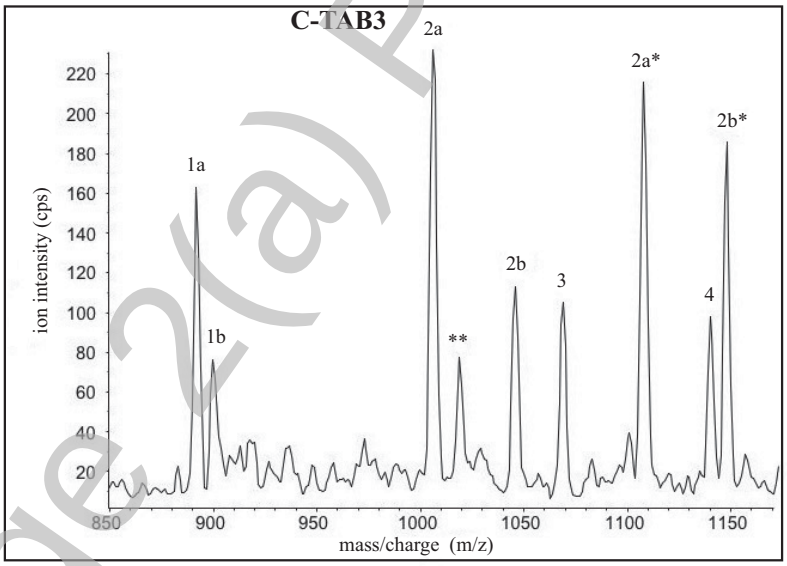
Figures 2A, 2D and 2E



Figures 2B and 2C



Peptide	Residue Numbers	Amino acid sequence	m/z	number of phosphates
1a	369-382	IAApSPPNTDELMSR	789.4	1
1b	369-382	IAApSPPNTDELmSR	797.4	1
2	355-369	TSSTSSSVNSQTLNR	822.9	1
3a	580-596	SNSISQIPSLEEMQQLR	1018.4	1
3b	580-596	SNSISQIPSLEEmQQLR	1026.4	1
4a	522-541	KLSMGSDDAAYTQALLVHQK	1126.5	1
4b	522-541	KLSmGSDDAAYTQALLVHQK	1134.5	1
5a	363-382	NQPTVYIAApSPPNTDELMSR	1140.5	1
5b	363-382	NQPTVYIAApSPPNTDELmSR	1148.5	1



Peptide	Residue Numbers	Amino Acid Sequence	m/z	number of phosphates
1a	53-65	YLYMEYHpSPDDNR	889.9	1
1b	53-65	YLYmEYHpSPDDNR	897.9	1
2a	395-412	NQHSLYTATTPPSSSPSR	1004.0	1
2b	395-412	NQHSLYTATTPPSSSPSR	1043.9	2
2a*	395-412	NQHSLYTATTPPSSSPSR	1105.5	1 + 1HxNAc
2b*	395-412	NQHSLYTATTPPSSSPSR	1145.5	2 + 1HxNAc
3	53-68	YLYmEYHpSPDDNRmNR	1066.5	1
4	504-523	SSSSGSDDYAYTQALLLHQR	1138.0	1

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Figure 3

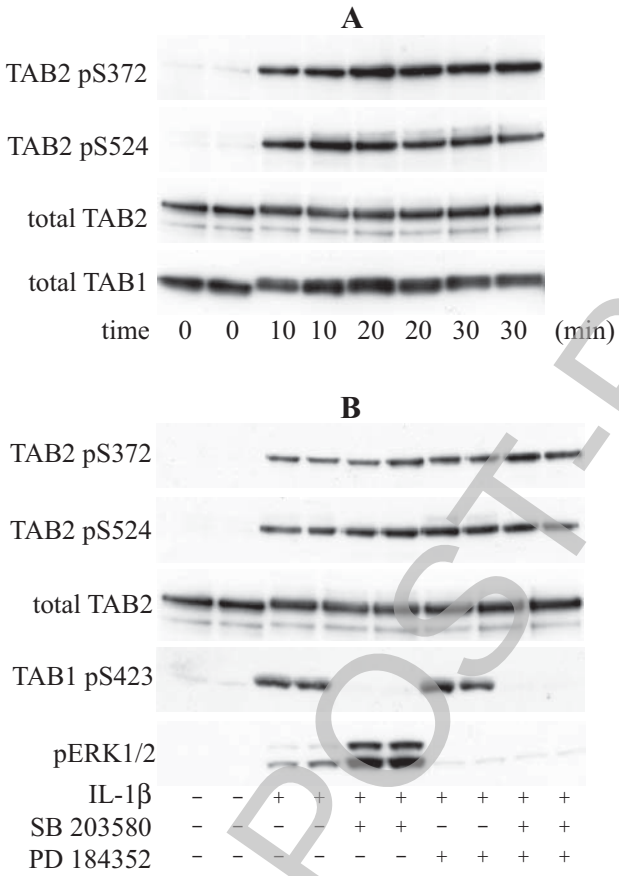

Figure 4

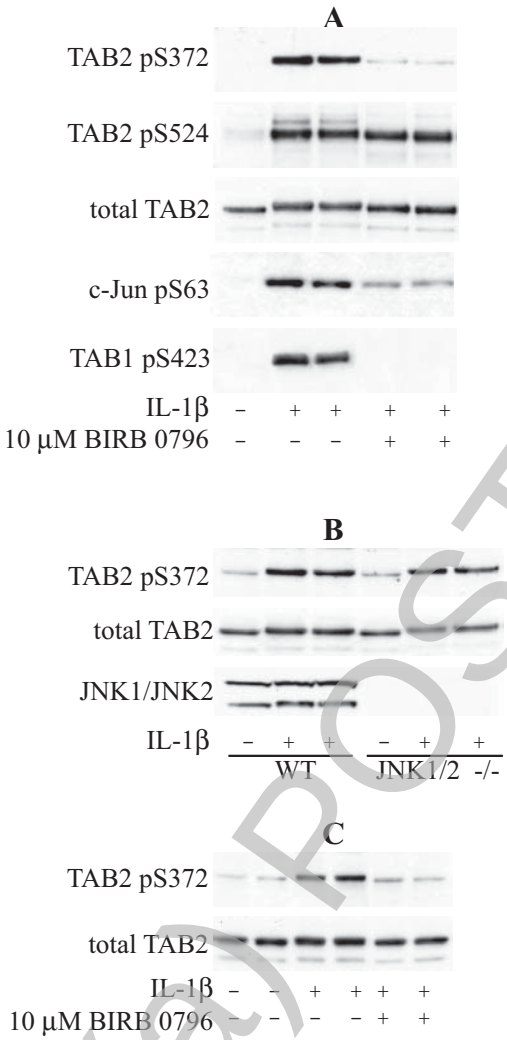

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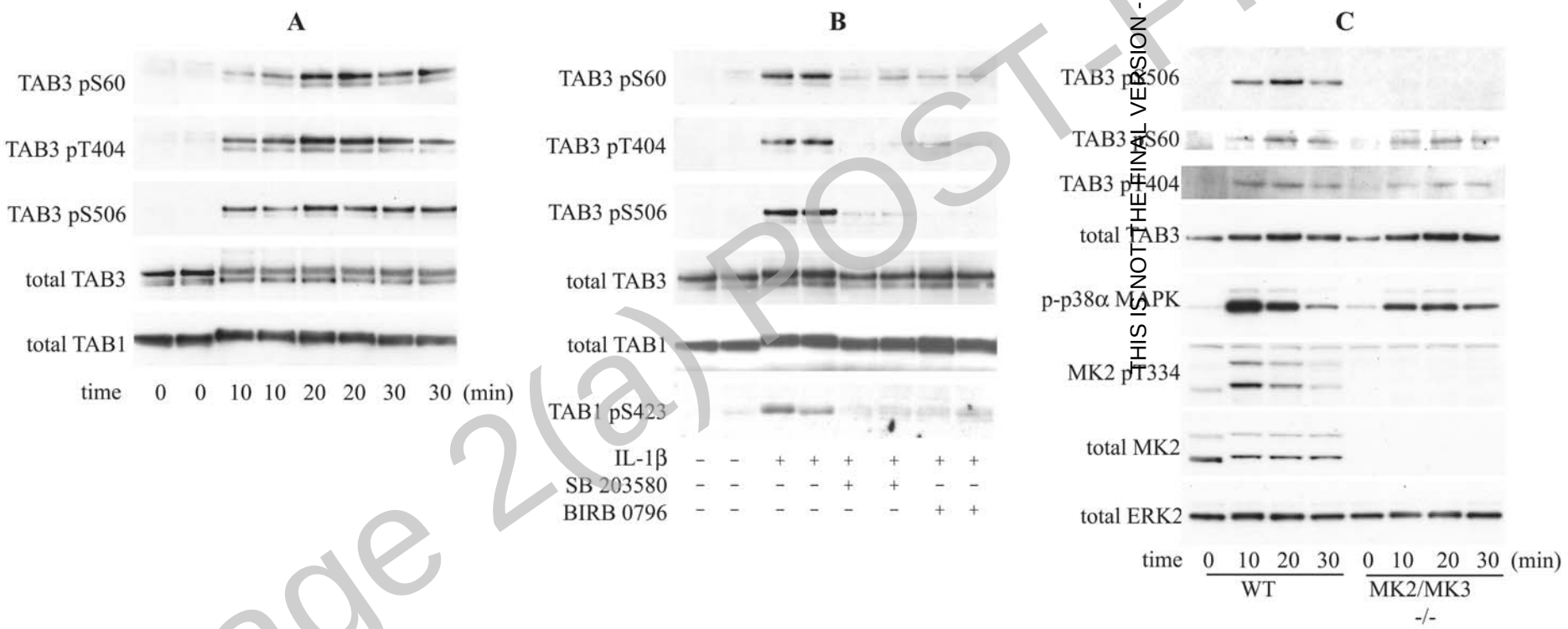
Figure 5



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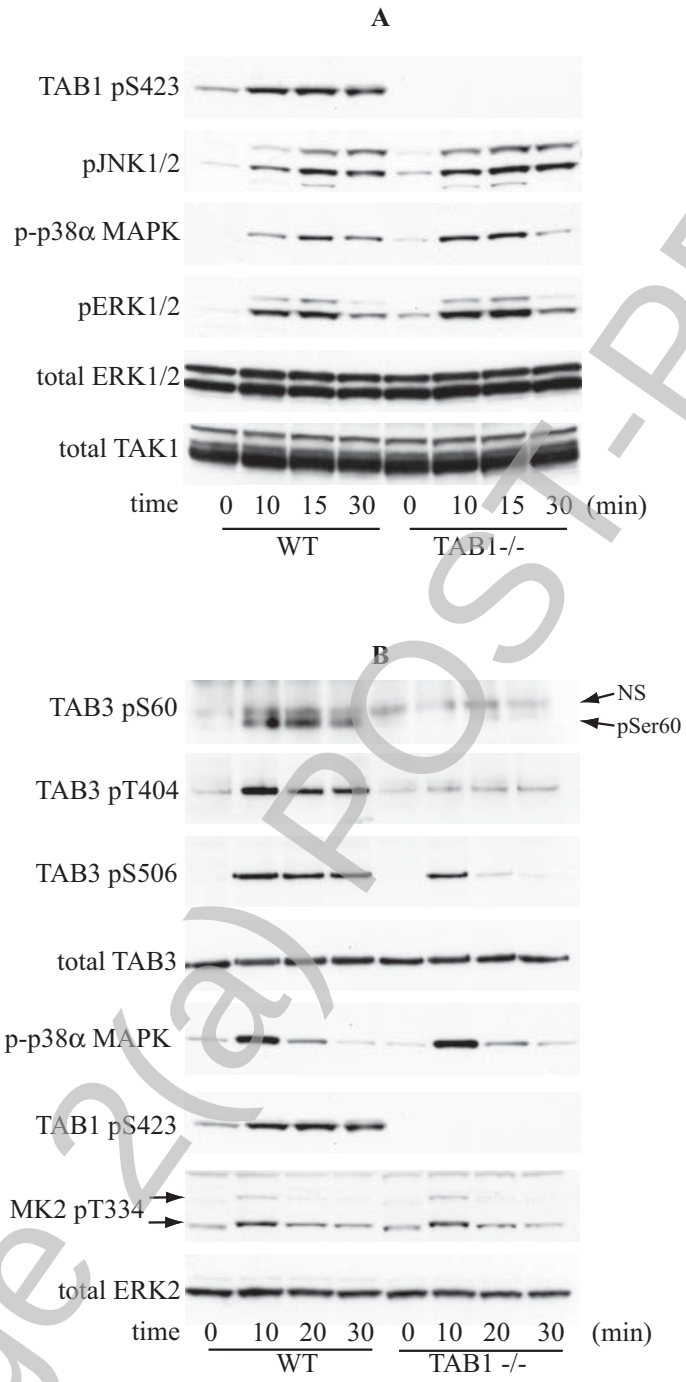

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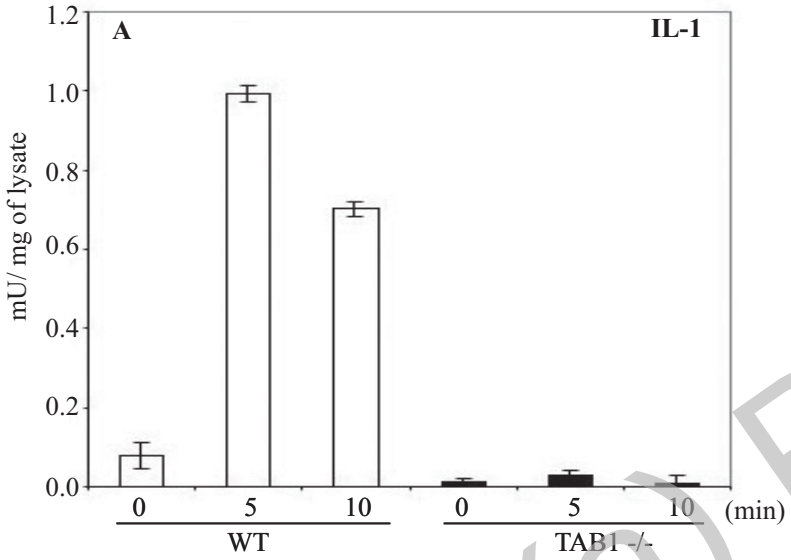
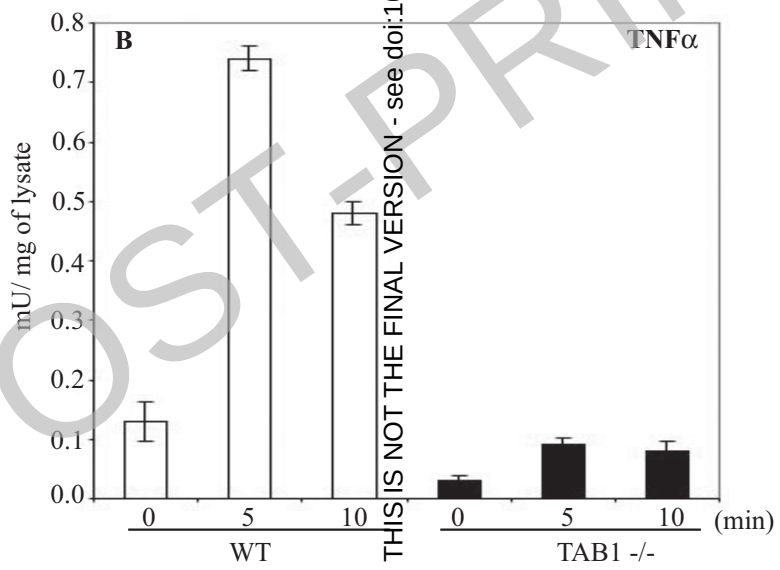
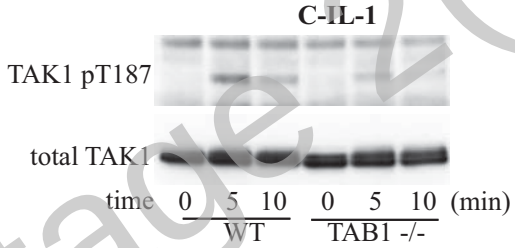
Figure 7







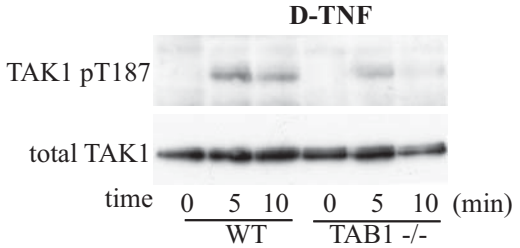
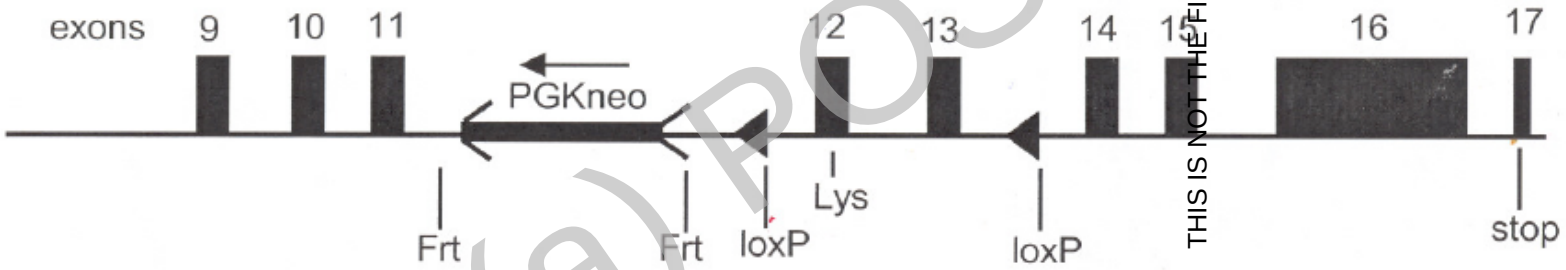


Figure 8A



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Figures 8B and 8C

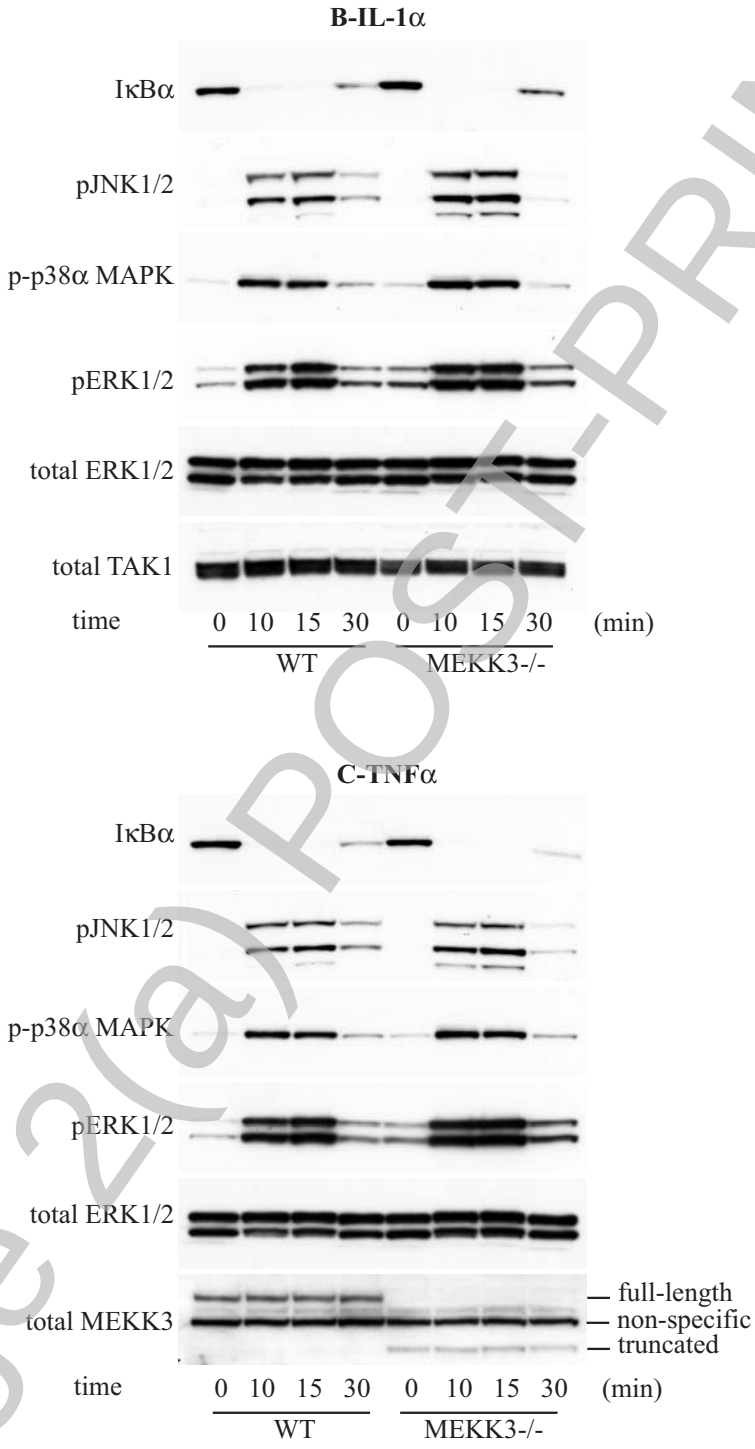

Figure 9

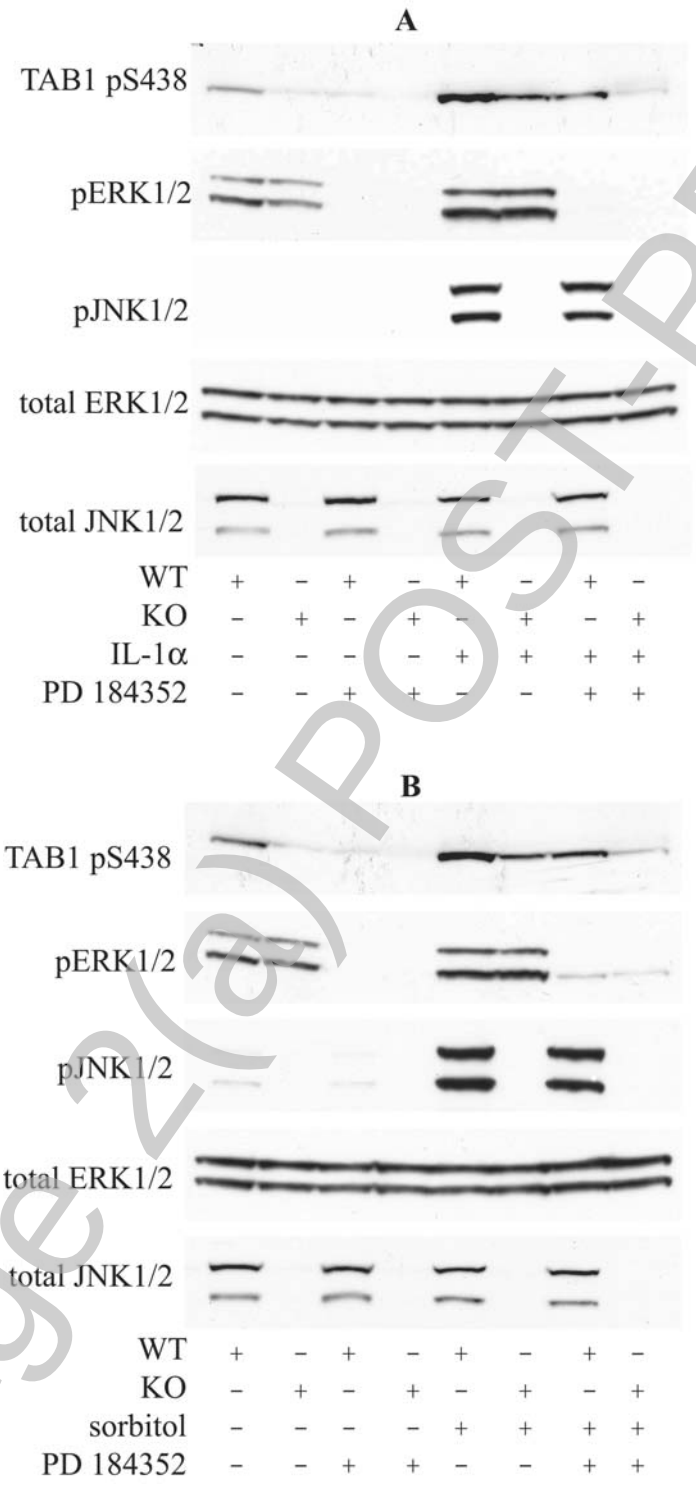

Figure 10