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MEKK3 and TAK1 synergize to activate IKK complex in *Helicobacter pylori* infection ☆

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ABSTRACT

Helicobacter pylori colonises the gastric epithelial cells of half of the world's population and represents a risk factor for gastric adenocarcinoma. In gastric epithelial cells *H. pylori* induces the immediate early response transcription factor nuclear factor of kappa light polypeptide gene enhancer in B-cells (NF-κB) and the innate immune response. We show that *H. pylori* induces in a type IV secretion system-dependent (T4SS) and cytotoxin associated gene A protein (CagA)-independent manner a transient activation of the inhibitor of NF-κB (IκBα) kinase (IKK)-complex. IKKα and IKKβ expression stabilises the regulatory IKK complex subunit NF-κB essential modulator (NEMO). We provide evidence for an intimate mutual control of the IKK complex by mitogen-activated protein kinase kinase kinase 3 (MEKK3) and transforming growth factor β activated kinase 1 (TAK1). TAK1 interacts transiently with the E3 ubiquitin ligase tumor necrosis factor receptor-associated factor 6 (TRAF6). Protein modifications in the TAK1 molecule, *e.g.* TAK1 autophosphorylation and K63-linked ubiquitinylation, administer NF-κB signalling including transient recruitment of the IKK-complex. Overall, our data uncover *H. pylori*-induced interactions and protein modifications of the IKK complex, and its upstream regulatory factors involved in NF-κB activation.

1. Introduction

The human pathogen *Helicobacter pylori* colonises the gastric epithelium and could induce gastric diseases [1]. Virulent strains of the gram-negative, microaerophilic bacterium *H. pylori* carry a *cag* pathogenicity island (*cag*PAI). It encodes the T4SS, which translocates the effector protein CagA and possibly other molecules into epithelial host cells [2].

Infection of the gastric epithelial cell layer by *H. pylori* induces inflammatory responses in the host by releasing chemotactic chemokines which recruit monocytes and granulocytes to the site of infection [3]. Thus, gastric epithelial cells actively participate in inflammation and mucosal immunity during initial and persistent *H. pylori* infection. The host antimicrobial response provoked by colonising *H. pylori* involves NF-κB, which regulates, along with other signalling pathways, the activation of genes involved in the innate immune response. Activation of NF-κB is fast and leads to the release of chemokines, *e.g.* interleukin 8 (IL-8) from colonised epithelial cells [4–8]. Systematic mutagenesis revealed that some *cag*PAI genes were required for induction of IL-8 and also showed that CagA is dispensable for IL-8 release [9]. Further it has been shown that *H. pylori* induces NF-κB activity very fast in a T4SS-dependent, but CagA-independent manner [6,10–13].

The heterodimeric NF-κB transcription factor composed of p50 and RelA becomes translocated into the nucleus in response to *H. pylori* within a few minutes. A prerequisite for nuclear translocation of NF-κB is the phosphorylation of the NF-κB inhibitor IκBα in a consensus sequence (DSGXXS: at S32 and S36) by IKKβ [12–14]. Further, tyrosine phosphorylation of IKKβ (Y199) directed by cellular sarcoma (c-Src) contributes to NF-κB activation [15]. IKKβ is part of the IKK-complex, which contains two highly homologous kinase subunits, IKKα and IKKβ, and the regulatory subunit NF-κB essential modulator (NEMO). Phosphorylation of IκBα is a signal function for K48-linked ubiquitinylation of IκBα by a cullin-RING ubiquitin ligase [16].

Different eukaryotic factors involved in NF-κB activation in *H. pylori*-infection have been described so far, *e.g.* p21-activated kinase1 (PAK1) [14,17], NF-κB-inducing kinase (NIK) [14,17,18], TRAF2 [19] and TRAF6 [18,19], the peptidoglycan recognising nucleotide-binding oligomerisation domain-containing protein 1 (NOD1) [20], TAK1 and myeloid differentiation primary response 88 (MyD88) [19], c-Src [15] and Ca^{2+}/calmodulin-dependent kinase II (CAMKII) [21]. Hitherto, the mechanism of IKK complex regulation in *H. pylori* infection is not resolved [22].

Distinctive microbial macromolecular ligands, such as lipopolysaccharide (LPS) and flagellin, which bind to toll-like receptors (TLRs) might not be responsible for activation of NF-κB, because isogenic T4SS mutant strains of *H. pylori* omit NF-κB activation. Further, muropeptides

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(γ-D-glutamyl-mesodiaminopimelic acid) derived from peptidoglycans, which bind NOD1 protein, might be also not involved in *H. pylori*-induced NF-κB activation [23].

Here, we report that *H. pylori* induces in a T4SS-dependent, but CagA-independent manner a transient activation of the IKK complex. The integrity of the IKK complex is required for phosphorylation of RelA and IκBα, and the IKK complex assembly stabilises the NEMO protein. Further, we provide evidence for an intimate mutual control of the IKK complex by MEKK3 and TAK1. *H. pylori* induces a transient recruitment of TRAF6 to TAK1. Protein modifications of TAK1, *e.g.* autophosphorylation and K63-linked ubiquitinylation, administer NF-κB downstream signalling including transient interaction of the IKK complex with TAK1.

2. Materials and methods

2.1. Cell culture and bacteria

AGS, NCI-N87 (human gastric carcinoma) and SW480 (human colorectal carcinoma) cells (ATCC) were grown in RPMI 1640 medium (PAA Laboratories) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin. Sixteen hours prior to the experiment, the cell medium was replaced with fresh RPMI 1640 supplemented with 0.5% FBS.

H. pylori wild-type (wt) strain P1 and their isogenic mutants P1 *cagA* and P1 *cagT* as well as wild-type (wt) strain G27 and their isogenic mutants G27 *cagA* and G27 *cagE* were cultured for 48–72 h as described previously [24,25]. AGS cells were infected at a multiplicity of infection of 100.

2.2. Antibodies and inhibitors

The description of the antibodies used for the experiments is provided in Supplementary Table S1.

A derivative of (5Z)-7-oxozeaenol was purchased from AnalytiCon Discovery and IKK inhibitor VII was from Calbiochem/Merck. Recombinant human TNFα and IL-1β were purchased from R&D Systems.

2.3. Cell fractionation, immunoprecipitation and immunoblot

Cell lysates were prepared with a modified RIPA buffer as described [26]. For ubiquitin detection, inhibitors of ubiquitin and ubiquitin-like isopeptidases and deubiquitinylases PR-619 (10 μM) (LifeSensors), N-ethylmaleimide (2 mM) and 1,10-phenantroline (2 mM) (Sigma-Aldrich) were added to the lysis buffer. Lysates were boiled with sample buffer (50 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 100 mM DTT, and 0.1% bromphenol blue) for 5 min. Subcellular fractions of AGS cells were prepared with the ProteoExtract kit (Calbiochem/Merck).

For immunoprecipitation, cell lysates were incubated with the antibody overnight at 4 °C. Protein G Dynabeads (Invitrogen Dynal AS) were used to capture the immunocomplexes.

Proteins in the lysates or immunoprecipitates were separated by SDS-PAGE and the gel was electrotransferred onto Immobilon-P transfer polyvinylidene fluoride membranes (Millipore). The blots were incubated with primary antibodies diluted in 5% non-fat milk overnight at 4 °C and subsequently with HRP-conjugated anti-rabbit or anti-mouse secondary antibodies (Jackson ImmunoResearch Laboratories). Immunoblots were developed using the enhanced chemiluminescence detection kit Amersham ECL (GE Healthcare).

2.4. Transient transfection

Cells (~10^5 cells/35 mm dish) were transfected with siRNAs specific to IKKα, IKKβ, NEMO, MEKK3 (Eurogentec) and TAK1 (Santa Cruz Biotechnology) using siLentFect Lipid Reagent (BioRad) in Opti-MEM I culture medium (Life Technologies) supplemented with 5% FCS. A scrambled sequence that does not lead to the specific inhibition of any known cellular mRNA was purchased from Santa Cruz Biotechnology.

For ectopic expression of proteins, AGS cells (2×10^6 cells/100 mm dish) were transfected with pCMV-TAK1, pCMV-TAK1(K63W) and pRK5-HA-Ubiquitin-K63 (Addgene, plasmid 17606, a gift of T. Dawson) by using Effectene transfection reagent (Qiagen).

2.5. RNA isolation and RT-PCR

Total RNA was extracted with the RNeasy Plus Micro kit (Qiagen). cDNA was synthesized from 1 μg of RNA using a random hexamer primer and RevertAid First Strand cDNA Synthesis kit (Fermentas). cDNA was amplified as described [26] using the following primers: 5′-TCCAAAATCAAGTGGGGCGATGCT-3′ (forward) and 5′-CCACCTGGTGCTCAGTGTGACCC-3′ (reverse) for GAPDH; 5′-GCAGAGAGGAGGACCTGTTG-3′ (forward) and 5′-ACTGCTTCAGCCCACACTTT-3′ (reverse); 5′-GAAACAACAGCTGCCTCTCC-3′ (forward) and 5′-TCCTGTACAGCTCCCTTGCT-3′ (reverse); and 5′-GACAAGGCCTCTGTGAAAGC-3′ (forward) and 5′-ATCGATCACCTCCTGTTTGG-3′ (reverse) for IKKα, IKKβ and NEMO, respectively. GAPDH expression served as an internal control. PCR products were visualized by ethidium bromide staining following the agarose gel electrophoresis.

2.6. Transactivation assay

AGS cells were seeded onto 24-well plates at a density of 3.5×10^4 cells per well in Opti-MEM I culture medium supplemented with 5% FCS. The Firefly Luciferase plasmid containing three repeats of the NF-κB binding site from the human immunodeficiency virus was mixed with *Renilla* Luciferase plasmid in a ratio of 60:1 and co-transfected with 50 nM of specific siRNAs against TAK1 or MEKK3 using SureFECT transfection reagent (SABiosciences). Luciferase activity was estimated in crude cell lysates using the Dual-Luciferase Reporter Assay System (Promega) with a Lumat LB 9507 luminometer (Berthold Technologies). The inducible firefly luciferase activity was normalised relative to *Renilla*'s activity, and fold changes in stimulated samples were calculated in comparison to non-stimulated cells.

2.7. Statistical analysis

Statistical analysis of the results was performed using the Student's *t*-test. The data are expressed as the mean fold changes from at least 3 separate experiments ± SEM with the value of the control arbitrarily normalised to 1.

3. Results

3.1. IKK complex activity is required for NF-κB activation

We investigated NF-κB regulation in AGS cells infected with isogenic *H. pylori* P1 strains (wt, *cagA* or *cagT*). CagA represents a virulence factor, which becomes translocated *via* the T4SS, and the CagT protein is required for the assembly of the pilus-like T4SS [27].

Infection of AGS cells with isogenic wt and *cagA*-deficient *H. pylori* induced a strong and transient phosphorylation within the activation loops of IKKα and IKKβ, whereas phosphorylation of NEMO was sustained (Fig. 1A). Infection with the *cagT* mutant did not induce phosphorylation of the IKK complex. Prominent phosphorylation of IKKα/IKKβ and NEMO in TNFα-treated cells was similar to *H. pylori*-infected cells (Fig. 1B). IKK activity is crucial for *H. pylori*-induced NF-κB activation, because the selective ATP-competitive IKK inhibitor VII abrogated IκBα and RelA phosphorylation (Fig. 1C). The integrity of the IKK complex was not affected in response to *H. pylori* infection as demonstrated by co-immunoprecipitation of IKKα/IKKβ with an antibody against NEMO (Fig. 1D).

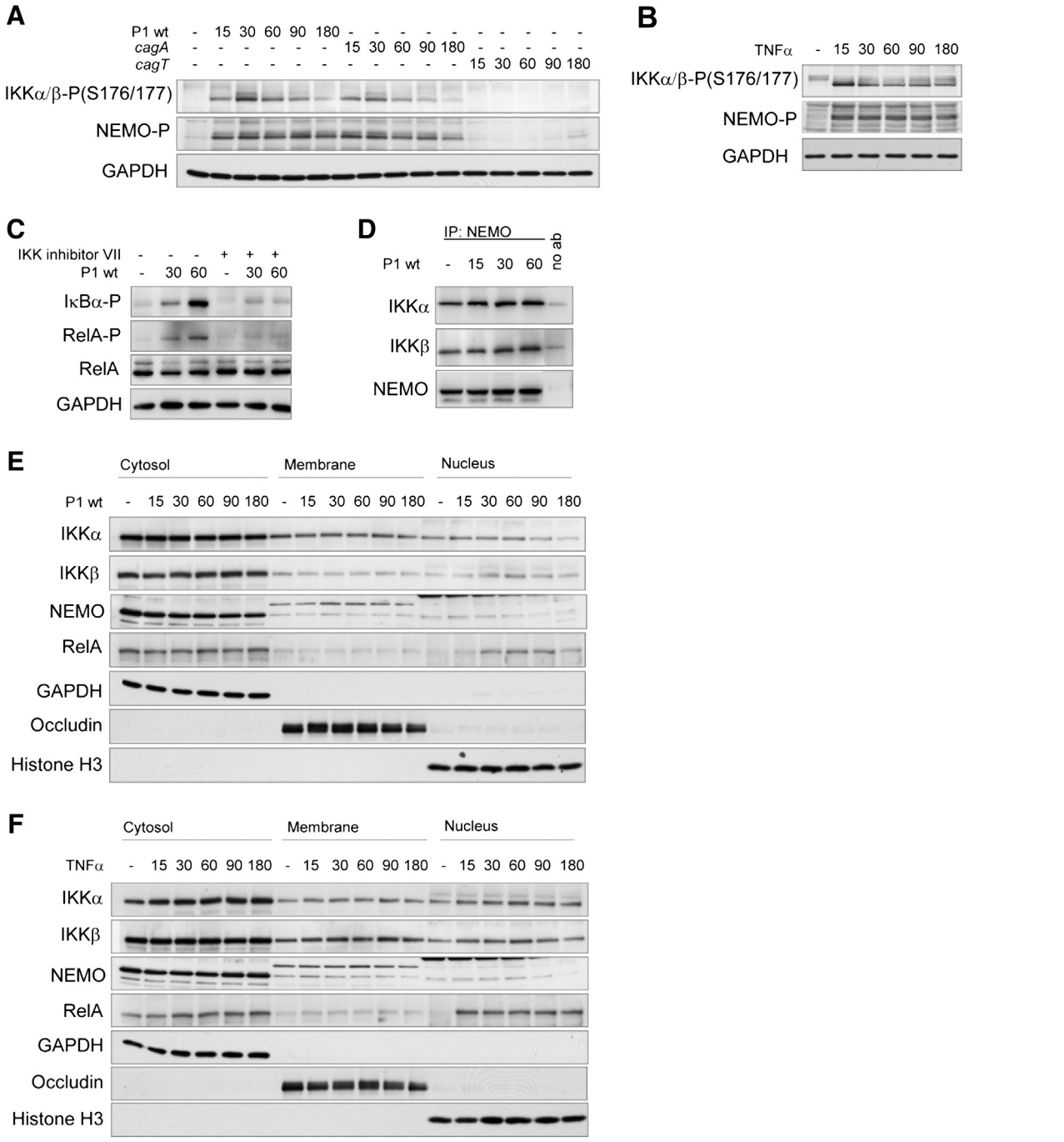

Fig. 1. IKK complex activity is required for NF-κB activation. AGS cells were infected with the *H. pylori* P1 wt strain or with the *cagA* or *cagT* mutants or were stimulated with TNFα for the indicated periods of time. (A and B) Cell lysates were analysed in an immunoblot using the antibodies against the indicated proteins. Immunostaining of GAPDH was performed to show equal protein loading. (C) Cells were treated with 10 μM of IKK inhibitor VII for 30 min prior to infection. (D) Cell lysates were subjected to immunoprecipitation (IP) using anti-NEMO antibody and immunoprecipitates were analysed in an immunoblot. The sample without antibody (no ab) indicates unspecific binding to the beads and serves as a control. (E and F) Subcellular fractions were prepared and analysed in an immunoblot. Immunostainings of GAPDH, occludin and histone H3 were performed to show the purity of the cellular fractions and equal protein amounts in the samples. The blots shown in each panel comprise data from the same experiment. Full-size blots are presented in Supplementary Fig. 4.

To analyse whether differences exist between *H. pylori*-infected and TNFα-stimulated cells regarding the cellular localisation of the activated IKK complex, we studied the abundance of the IKK subunits in cytosolic, membranous and nuclear fractions. As expected, infection with *H. pylori* or TNFα treatment actuated an accumulation of RelA in the nucleus of AGS cells within 15 min, but no changes in the localisation of IKK subunits were detected (Fig. 1E and F).

3.2. Integrity of the IKK complex is required for NF-κB activation

The particular involvement of each IKK subunit in NF-κB activation was investigated by using specific siRNAs. Treatment of AGS cells with different amounts of siRNAs against IKKα, IKKβ or NEMO led to a dose-dependent reduction of IκBα and RelA phosphorylation in *H. pylori* infection (Fig. 2A–C). Herein, knockdown of NEMO led to the

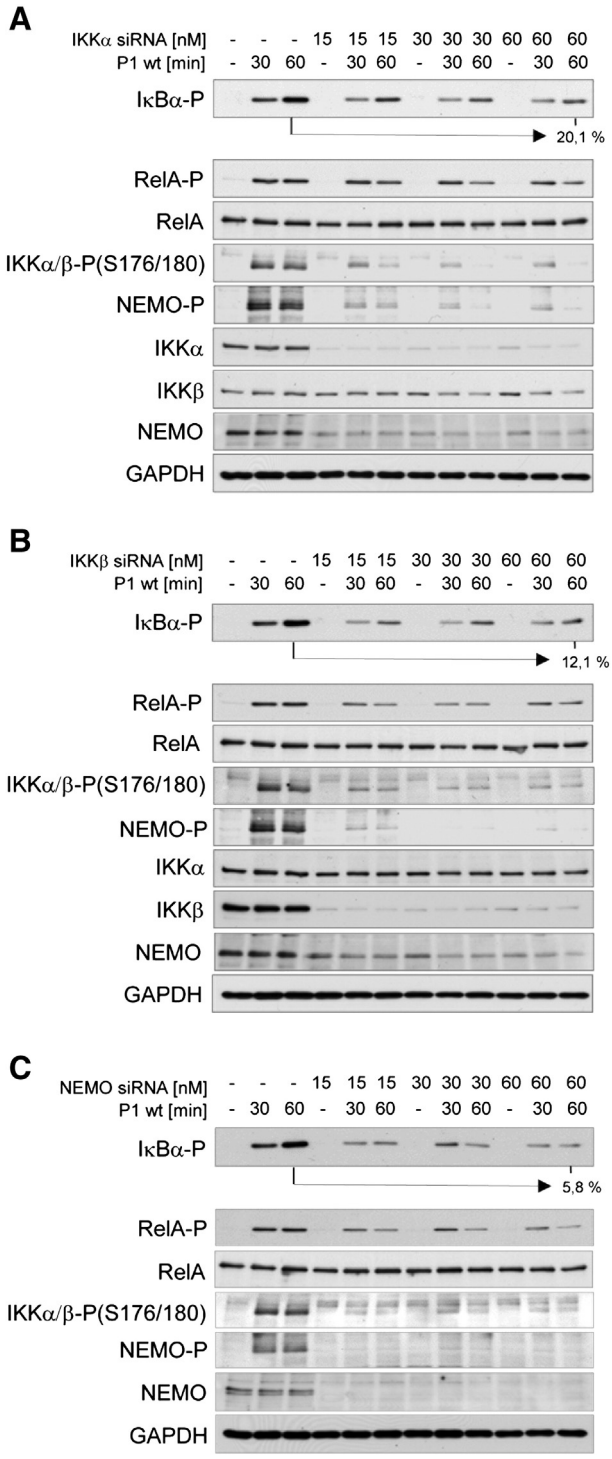

Fig. 2. Integrity of the IKK complex is required for NF-κB activation. siRNA-transfected AGS cells were infected with the *H. pylori* P1 wt strain for 30 or 60 min, and cell lysates were analysed in an immunoblot using antibodies as indicated. 15, 30 and 60 nM of IKKα siRNA (A), IKKβ siRNA (B) or NEMO siRNA (C) were used for transfection. A scrambled siRNA (30 nM) was used as a control. Quantification of immunoblot bands was performed using densitometry and the relative phospho-IκBα/GAPDH ratio is indicated. The blots shown in each panel comprise data from the same experiment. Full-size blots are presented in Supplementary Fig. 5.

most prominent reduction of IκBα phosphorylation (5.8%) (Fig. 2C). Thus, the integrity of the IKK complex is strongly required for its catalytic activity in *H. pylori*-infected cells. Interestingly, the NEMO protein abundance was very low in cells treated with specific siRNAs against IKKα or IKKβ (Fig. 2A and B). Similar data have been observed in *H. pylori*-infected NCI-N87 and SW480 cells (Fig. S1A and B).

3.3. IKKα/β complex assembly stabilises NEMO

We observed a dramatic loss of the NEMO protein in IKKα- and IKKβ-deficient cells (Fig. 3A and B). The effect was most prominent in a double knockdown of IKKα/IKKβ (Fig. 3A). To exclude off-target effects on NEMO, transfection of AGS cells with 3 different siRNAs against each of IKKα or IKKβ were performed showing similar results (Fig. 3B). Quantification of NEMO mRNA by RT-PCR indicated that the decrease of NEMO in cells treated with siRNAs against IKKα or IKKβ was not due to changes at the transcriptional level (Fig. 3C). Thus, IKK complex assembly stabilises the regulatory subunit NEMO.

3.4. MEKK3 and TAK1 are required for efficient activation of the IKK complex

MEKK3 and TAK1 kinases have been shown to transduce signalling from cytokine receptors to IKK complex activity [28]. Infection of AGS cells with P1 wt and *cagA*-deficient mutant of *H. pylori* induced a transient phosphorylation (S166) of MEKK3 kinase (Fig. 4A). The functional consequence of phosphorylation at amino acid S166 in the MEKK3 protein was not clarified so far. Interestingly, we observed also a slight phosphorylation of MEKK3 in the *cagT*-infected cells (Fig. 4A), whereas in TNFα-stimulated cells, no phosphorylation of MEKK3 was detected (Fig. 4B). Inducible phosphorylation of TAK1 at critical sites (T184/T187) in the activation loop peaked at 15 min p.i., whereas the *cagT* mutant did not induce TAK1 phosphorylation (Fig. 4A). Similar to the *H. pylori* strain P1, we observed an inducible phosphorylation of MEKK3 and TAK1 in a T4SS-dependent, but CagA-independent manner by the *H. pylori* G27 strain (Fig. S2A). TNFα treatment resulted also in transient, but less strong TAK1 phosphorylation than infection with *H. pylori* (Fig. 4B). Studying putative differences of TAK1 and MEKK3 phosphorylation in other cell lines, we observed that *H. pylori* induced strong phosphorylation of TAK1 and weak phosphorylation of MEKK3 in NCI-N87 cells, whereas similar phosphorylation of both proteins was observed in infected SW480 cells (Fig. S2B).

A transient depletion of TAK1 or MEKK3 led to a reduction of IKKα/IKKβ phosphorylation (27.8% and 71.1%), and IκBα and RelA phosphorylation in *H. pylori*-stimulated cells (Fig. 4C). Interestingly, depletion of MEKK3 together with TAK1 had an additive suppressive effect on phosphorylation of IKKα/IKKβ (12.5%) (Fig. 4C). Similar data have been received in *H. pylori*-infected NCI-N87 and SW480 cells (Fig. S2C and D). These results suggest that *H. pylori*-induced activation of NF-κB involves both kinases (MEKK3 and TAK1). Even more prominent was the impact of TAK1 or MEKK3 siRNAs on NF-κB transactivation activity in response to *H. pylori* infection and TNFα treatment (Fig. 4D). Pre-treatment of AGS, NCI-N87 and SW480 cells with (5Z)-7-oxozeaenol, a highly potent inhibitor of TAK1 [29], dose-dependently reduced the phosphorylation of IKKα/IKKβ, IκBα and RelA in response to *H. pylori* (Fig. 4E; Fig. S2E and F), in agreement with the data shown above about depletion of TAK1 by RNA interference. The inhibitor did not compromise the viability of *H. pylori* (Fig. 4F). Pre-treatment of AGS cells with the (5Z)-7-oxozeaenol reduced also TNFα- and IL-1β-stimulated phosphorylation of NF-κB effector molecules (Fig. 4G and H).

3.5. TAK1 interacts with the IKK complex

Investigation of TAK1-associated proteins revealed that IKKα and IKKβ were co-immunoprecipitated with TAK1 in an inducible manner in cells infected with P1 wt and *cagA*, but not with the *cagT* strain (Fig. 5A and B). In addition to AGS cells, we observed interaction of IKKs to TAK1 in P1 wt-infected NCI-N87 and SW480 cells (Fig. S3A and B). Thus, *H. pylori* infection induces formation of a protein complex

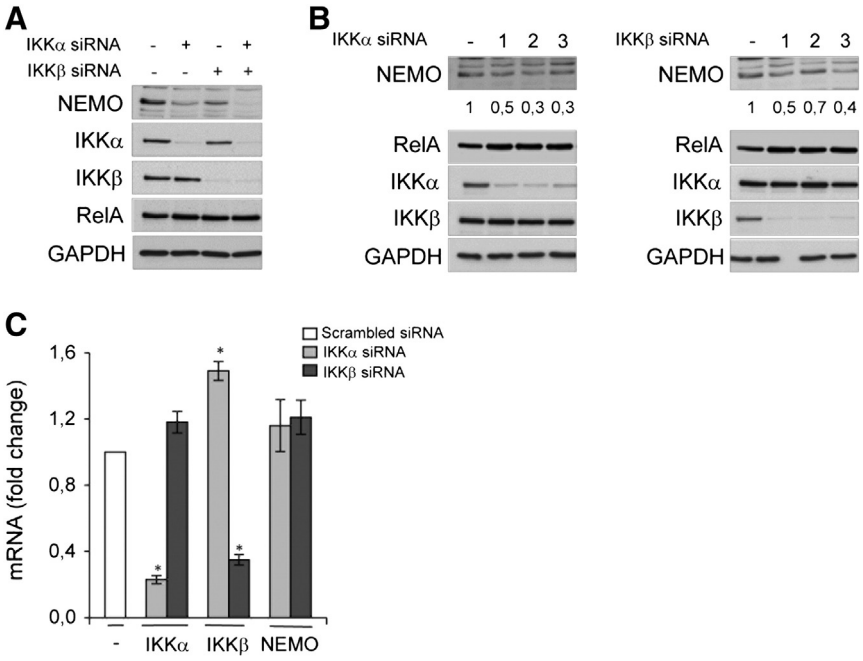

Fig. 3. IKK complex assembly stabilises NEMO. (A and C) AGS cells were transfected for 48 h either with a mixture of 3 different IKKα- or IKKβ-specific siRNAs (10 nM of each, 30 nM in total), or (B) with each of these 3 siRNAs separately (30 nM of each). A scrambled siRNA (30 nM) was used as a control. (A and B) Cell lysates were analysed in an immunoblot using the antibodies as indicated. Immunostaining of GAPDH was performed to show equal protein loading in each lane. The relative changes of NEMO were calculated by comparing the intensity of the protein bands of NEMO *vs.* GAPDH and from cells transfected with siRNAs against IKKα or IKKβ *vs.* scrambled siRNA. (C) Total RNA was isolated and quantitative RT-PCR was performed. $^{*}P < 0.01$ compared with the cells transfected with the scrambled siRNA. The blots shown in each of the panels A and B comprise data from the same experiment. Full-size blots are presented in Supplementary Fig. 6.

composed of TAK1/IKKs in a T4SS-dependent, but CagA-independent manner.

It has been published that CagA might interact with TAK1 [30]. Thus, we analysed the proteins immunoprecipitated by the TAK1 antibody in detail for the presence of CagA. As shown in Fig. 5A, there was CagA protein detected in wt-infected cells after long exposure of the immunoblot. In the *cagA*-infected cells, there was no CagA protein recognised, indicating that the protein band from the wt-infected cells represents the CagA protein. Further, we detected the CagA protein in the *cagT*-infected cells also after long exposure of the immunoblot (Fig. 5B). Of note, the *cagT* mutant expresses the CagA protein, but does not inject CagA into the epithelial cells due to the non-functional T4SS. This indicates that the observed CagA protein in the TAK1 immunoprecipitation from lysates of the *cagT*-infected cells arose from adherent bacteria. When we performed appropriate controls within the experiment, we observed surprisingly the CagA protein in the TAK1 immunoprecipitation from the lysate of the *H. pylori* wt bacteria (only wt + ab), in the absence of an AGS cell lysate (Fig. 5A, lane 12) or from the lysate of the *cagT* bacteria (only *cagT* + ab) (Fig. 5B, lane 13). In the TAK1 immunoprecipitation from the lysate of the *H. pylori cagA* bacteria (only *cagA* + ab) there was no CagA protein detected (Fig. 5A, lane 13). Thus, it is curious that the antibody against TAK1 immunoprecipitated to some extent the CagA protein. This is at least one explanation for the misinterpretation of the data by Lamb et al. [30] where the same antibody from Santa Cruz has been used for TAK1 immunoprecipitation. In a reverse immunoprecipitation using a CagA antibody we could not recognise co-immunoprecipitated TAK1 even after long exposure (Fig. 5C), which supports the data of the TAK1 immunoprecipitation.

3.6. TAK1 associates transiently with TRAF6

TRAF6, an E3 ligase, has been shown to bind to TAK1 (through TAK1 binding protein TAB2) in response to IL-1β and LPS [31]. In line with this, inducible TRAF6 recruitment to TAK1 was observed in *H. pylori*-infected AGS cells (Fig. 6A). Being most prominent at 30 min p.i., TAK1–TRAF6 transient association was detected in the *H. pylori* wt- and *cagA*- but not *cagT*-infected cells (Fig. 6B). Similar to the *H. pylori* strain P1, the G27 strain induced recruitment of TRAF6 to TAK1 and an interaction between TAK1 and IKKα/β in a T4SS-dependent, but CagA-independent manner (Fig. 6E). In addition to AGS cells, we observed recruitment of TRAF6 to TAK1 in P1 wt-infected NCI-N87 and SW480 cells (Fig. S3D and E). We also found an increase of K63-linked ubiquitinylation of TAK1 in AGS cells after overexpression of TAK1 and ubiquitin-K63 (other lysines mutated to arginine) in response to *H. pylori* (Fig. 6C) and IL-1β (Fig. 6D). TAK1 catalytic activity is required for ubiquitinylation of TAK1, because ectopic expression of TAK1(K63W) kinase inactive protein showed no ubiquitinylation in response to IL-1β (Fig. 6D).

4. Discussion

Pathogens often subvert the innate and adaptive immune response by secretion of effector molecules *via* a secretion system directly into the cytosol of target cells, where the effectors can access the host cell signalling. Many proteins delivered by pathogens into host cells affect the regulation of the IKKs, IκBα and the transcription factor RelA [16]. A number of eukaryotic molecules have been described which might contribute to the regulation of NF-κB activation in *H. pylori* infection [22]. Despite these findings, details about the entire mechanism of how *H. pylori* infection promotes the activity of the immediate early response factor NF-κB are still not well understood. Here, we add more facets to the apparent complexity of NF-κB regulation in *H. pylori* infection by analysing the regulation of the IKK complex and identifying functional components within the NF-κB signal transmission (Fig. 7).

H. pylori-induced phosphorylation within the activation loops of IKKα (S176/S180) and IKKβ (S177/S181) requires the functional T4SS, but not CagA (Fig. 1). The phosphorylation causes conformational changes resulting in kinase activation [32]. We show for the first time, that *H. pylori* promotes NEMO phosphorylation at S376 (Fig. 1). In previous studies it has been shown that IKKβ, but not IKKα

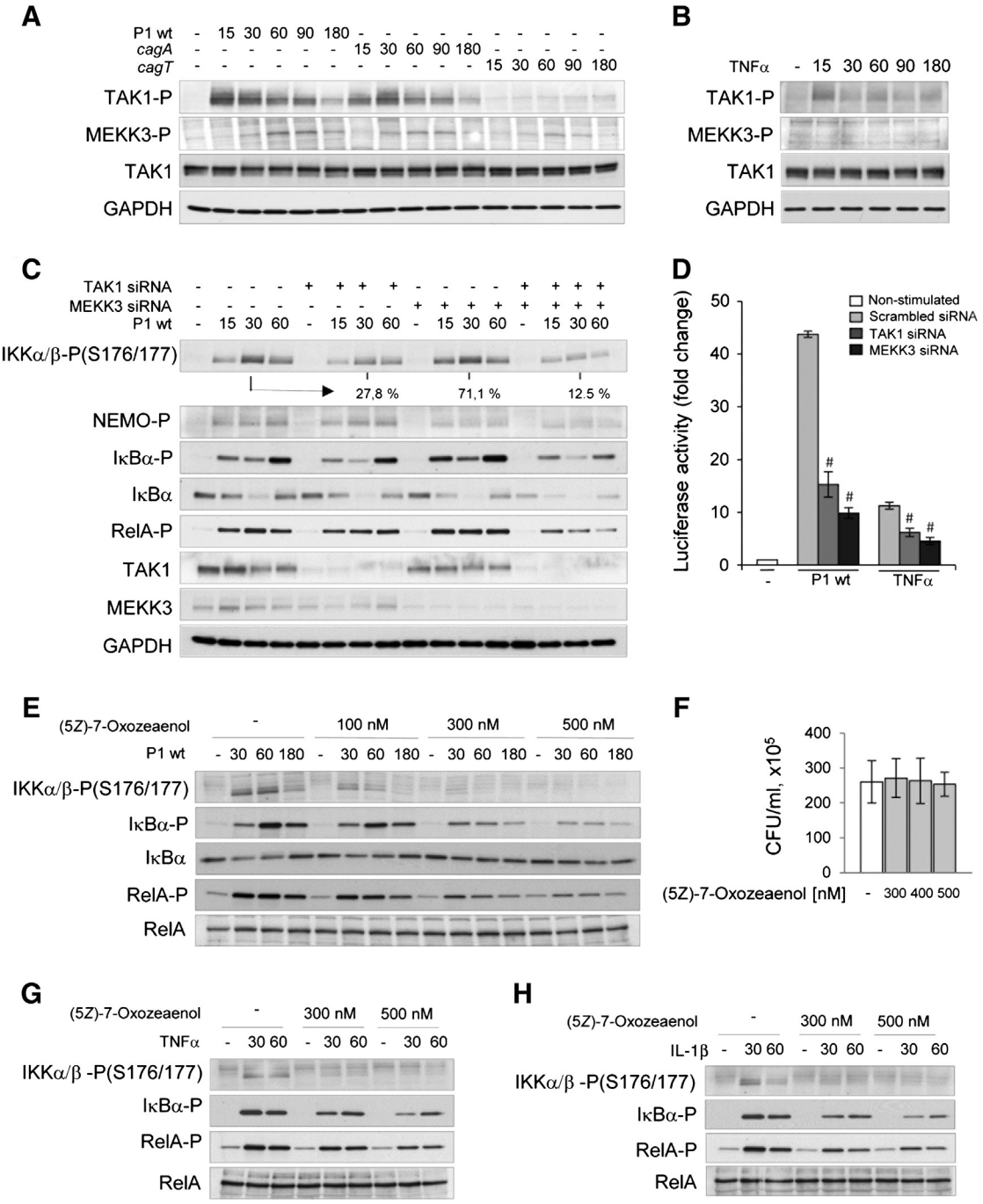

Fig. 4. TAK1 and MEKK3 are required for efficient activation of the IKK complex and NF-κB. (A–C and E, G, and H) AGS cells were infected with the *H. pylori* P1 wt strain or with the *cagA* or *cagT* mutants or were stimulated with TNFα, or IL-1β for the indicated periods of time. Cell lysates were analysed in an immunoblot using the antibodies as indicated. Immunostainings of TAK1, RelA, or GAPDH were performed to show equal protein loading. (C) 48 h prior to infection, cells were transfected with 30 nM TAK1, MEKK3 or scrambled siRNAs. (D) Cells were transfected with Firefly NF-κB and *Renilla* Luciferase plasmids and co-transfected with 50 nM of TAK1, MEKK3 or scrambled siRNAs. 48 h after transfection, cells were infected with *H. pylori* wt or treated with TNFα for 3 h, and a reporter gene assay was performed. $^{\#}P < 0.01$ compared to the scrambled siRNA-transfected cells. (E, G, and H) Cells were treated with increasing doses of TAK1 inhibitor (5Z)-7-oxozeaenol for 30 min prior to stimulation. (F) *H. pylori* P1 wt strain was incubated in RPMI 1640 medium supplemented with increasing amounts of a (5Z)-7-oxozeaenol derivative for 1 h. The bacterial suspension was serially diluted in PBS and plated onto GC agar supplemented with horse serum, vancomycin, trimethoprim, nystatin and vitamins. The bacterial colonies were counted after 3-day growth in standard microaerophilic conditions. Quantification of immunoblot bands was performed using densitometry and the relative phospho-IKK/GAPDH ratio is indicated. The blots shown in each panel (A–C, E, G and H) comprise data from the same experiment. Full-size blots are presented in Supplementary Fig. 7.

phosphorylates NEMO in response to TNFα in murine embryonic fibroblasts [33], and that the phosphorylation of NEMO at orthologous S369 controls intrinsic activity of the IKK complex [34]. Our data indicate that the pre-assembled IKK complex and its subcellular localisation is not affected in *H. pylori* infection. In contrast to Hirata et al. [12], we found no nuclear translocation of IKKα in AGS cells colonised by *H. pylori* (Fig. 1E). Interestingly, we show for the first time that the integrity of the IKK complex is required for NF-κB activation by *H. pylori*, and depletion of NEMO has the most prominent suppressive effect on NF-κB activity. In addition, IKK complex assembly is a prerequisite for the stability of the regulatory subunit NEMO (Figs. 2 and 3).

The regulation of the IKK complex exerts an extraordinary degree of connectivity of central signalling pathways and executes cell type- and stimulus-specific functions. Studying the intricate interactions and

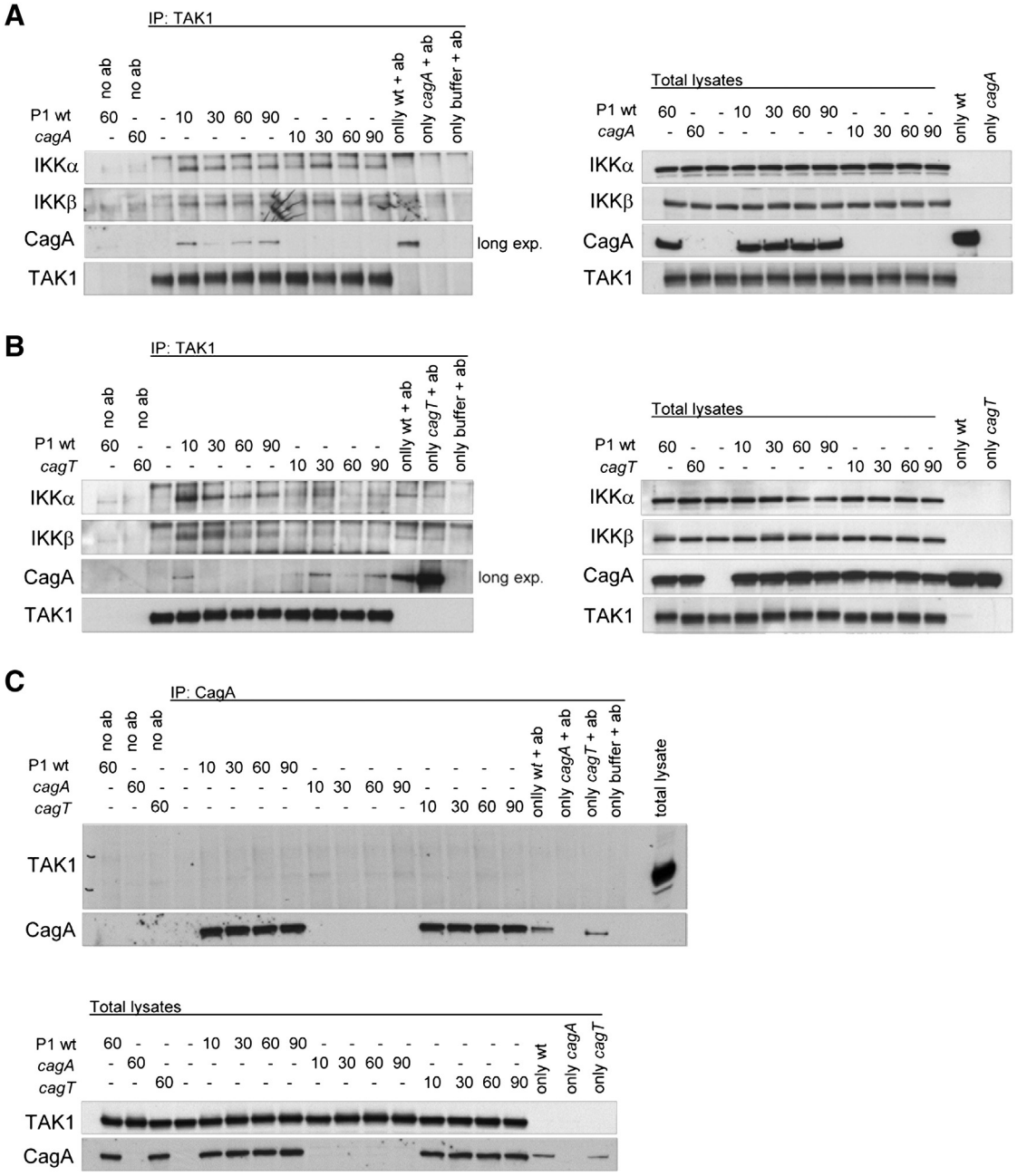

Fig. 5. TAK1 interacts with the IKK complex in a T4SS-dependent manner. AGS cells were infected with the *H. pylori* P1 wt strain or with the *cagA* or *cagT* mutants for the indicated periods of time. Cell lysates were subjected to immunoprecipitation (IP) using anti-TAK1 antibody (A and B) or anti-CagA antibody (C). The samples without antibody (no ab), without AGS cell lysate (only wt + ab; only *cagA* + ab; only *cagT* + ab), or without AGS cell lysate/bacterial lysate (only buffer + ab) indicate for the specificity of the IP and serve as controls. Immunoprecipitates and cell lysates were analysed in an immunoblot using the antibodies as indicated. The blots shown in each panel comprise data from the same experiment. Full-size blots are presented in Supplementary Fig. 8.

modifications of upstream regulatory components, which contribute to IKK activation within *H. pylori*-induced NF-κB signal transmission, we identified the kinases MEKK3 and TAK1. MEKK3 plays an essential role in lysophosphatidic acid-, TNFα- and LPS-induced NF-κB activation [35–37]. It has been suggested by Yao et al. [28] that MEKK3-dependent activation of NF-κB involves NEMO, IKKα and IκBα phosphorylation, leading to subsequent dissociation of IκBα from NF-κB without IκBα degradation. We found that infection with *H. pylori* promoted phosphorylation of MEKK3 at S166, but not at S337 (data not shown), whereas TNFα did not stimulate S166 phosphorylation (Fig. 4). Activating autophosphorylation at S184/S187 within the activation loop of TAK1 was induced in *H. pylori*-infected cells in a T4SS-dependent, but CagA-independent manner, and in TNFα-stimulated cells. Transfection of AGS, NCI-N87 and SW480 cells with TAK1-specific, together with MEKK3-specific siRNAs led to a strong reduction of IKKα/β, IκBα and RelA phosphorylation and suppressed the transactivation activity of RelA following *H. pylori* infection. Thus, both kinases contribute to *H. pylori*-directed activation of NF-κB. The suppression of NF-κB activation was prominent when we used the TAK1 kinase inhibitor (5Z)-7-oxozeaenol (Fig. 4). These data are in accordance with the results of Hirata et al. [19], which have demonstrated that TAK1 is required for *H. pylori*-induced IκBα phosphorylation. In addition to TAK1 which phosphorylates IKKα/β, also PAK1 contributes to IKK activation by phosphorylation of IKKα [14]. Thus, *H. pylori* could induce NF-κB activation by different contributing pathways/factors. Other contributing factors like TRAF2, NIK [18], and MyD88 [19] have been

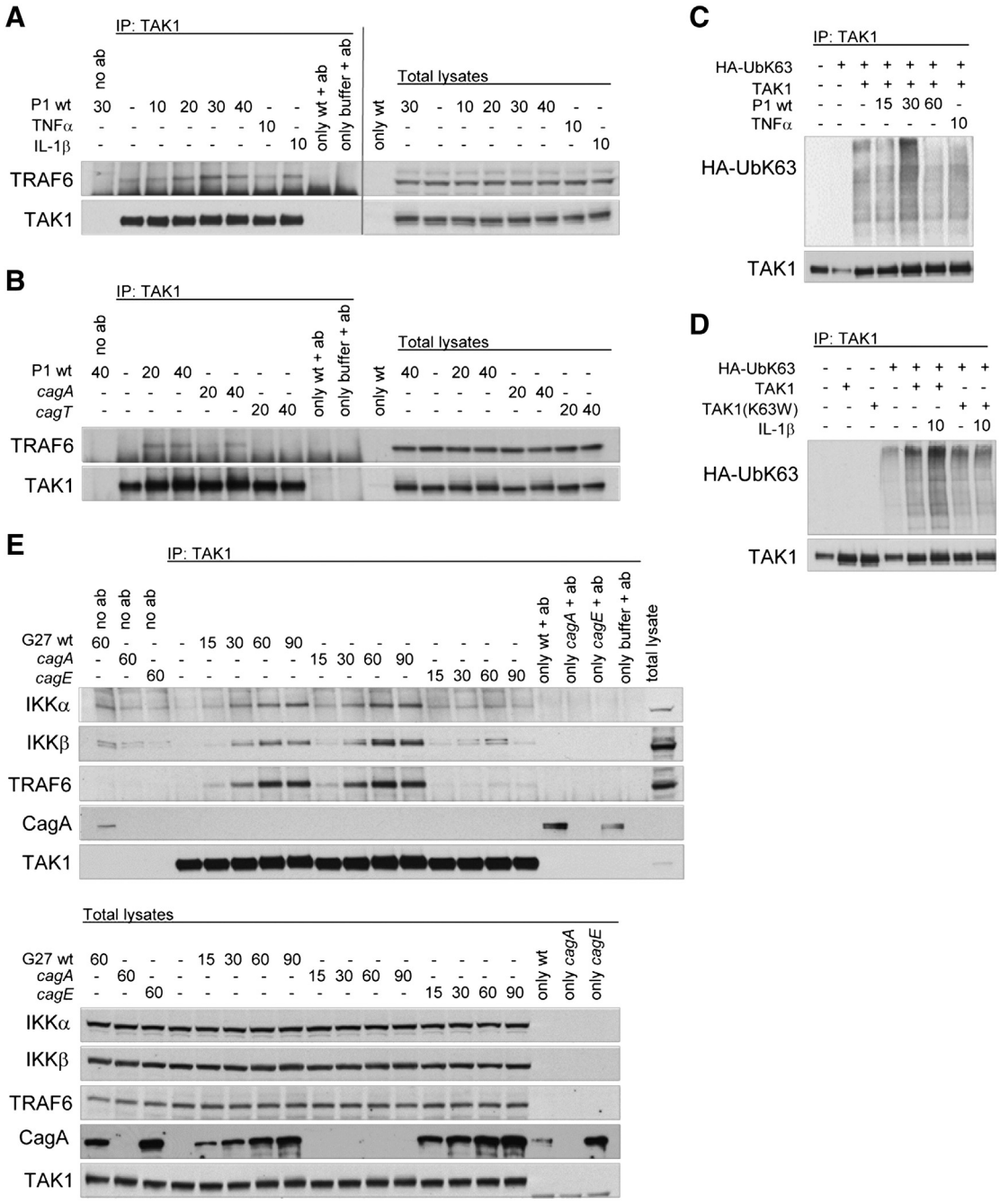

Fig. 6. TAK1 interacts with TRAF6. AGS cells were infected with the *H. pylori* P1 wt strain, the *cagA* or *cagT* mutants (A–C), with the *H. pylori* G27 wt strain, the *cagA* or *cagE* mutants (E), or were stimulated with TNFα or IL-1β for the indicated periods of time. Cell lysates were subjected to immunoprecipitation (IP) using anti-TAK1 antibody. The samples without IP antibody (no ab), without AGS cell lysate (only wt + ab; only *cagA* + ab; only *cagE* + ab), or without AGS cell lysate/bacterial lysate (only buffer + ab) indicate for the specificity of the IP and serve as controls. Immunoprecipitates and cell lysates were analysed in an immunoblot using the antibodies as indicated. (C and D) 24 h prior to infection with the *H. pylori* wt, or treatment with TNFα or IL-1β, cells were transfected either with TAK1 or with its kinase-inactive mutant TAK1(K63W) together with HA-tagged ubiquitin K63 plasmids (3 μg of each). The blots shown in each panel comprise data from the same experiment. Full-size blots are presented in Supplementary Fig. 9.

described previously. Of note, we show now by a variety of experimental approaches, including siRNA interference, that TAK1 is involved in *H. pylori*-induced NF-κB activation; thus, we have to repeal our previous data in which transient transfection of a dominant negative TAK1 cDNA had no impact on NF-κB transactivation [17].

TAK1 transiently associates with the IKKs, in a T4SS-dependent, but CagA-independent manner. The kinetics of the IKK interaction to TAK1 is in front of IκBα and RelA phosphorylation. Further, we provide experimental evidence that CagA does not interact with TAK1 in *H. pylori*-infected cells (Fig. 5).

TAK1 forms a complex with TAB1, TAB2 or the structurally related TAB3 [38], and attracts several additional activators, including apoptosis signal-regulating kinase 1 (ASK1) [39] and TRAF6. TRAF molecules represent a group of structurally similar adaptor proteins, which link the receptors to downstream signalling. TRAF6 transduces signalling from TLRs and the IL-1β receptor by associating with phosphorylated IL-1-receptor associated kinase 1 (IRAK1). TRAF6 is regulated by an E2 enzyme complex (Ubc13)/Uev1a) and is capable of autoubiquitinylation. K63-linked ubiquitin chains of TRAF6 recruit TAB2 and its homologue TAB3, and thereby TAK1 [38]. Similarly to IL-1β receptor/TLR-dependent

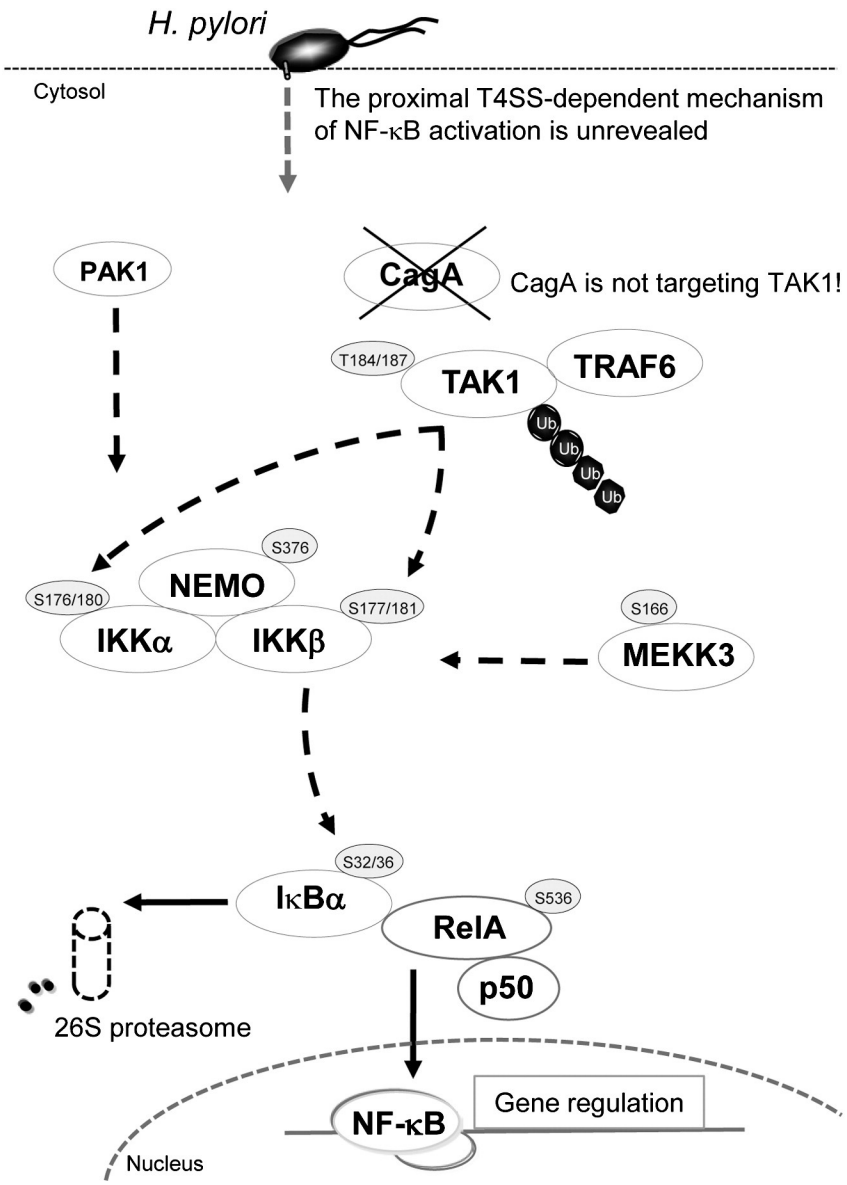

Fig. 7. Activation of IKK complex and NF-κB in *H. pylori* infection. *H. pylori* induces in a T4SS-dependent manner MEKK3 phosphorylation and triggers intricate interactions and protein modifications within the TAK1/TABs complex. Common target of upstream regulatory components and integral part of *H. pylori*-induced NF-κB regulation is the IKK complex. Interactions in the multi-protein complexes coincide with a range of post-translational modifications (phosphorylation and ubiquitinylation) within their members and its effector molecules (see explanation in text). TAK1 is not a target of the *H. pylori* effector protein CagA. TAK1-dependent phosphorylation of IKK promotes subsequent phosphorylation of IKKβ substrate IκBα. Phosphorylation of IκBα leads to its degradation in the 26S proteasome, NF-κB translocation to the nucleus, and transcription initiation. Protein phosphorylation sites are indicated. Further, our previous work showed that PAK1 contributes to the activation of the IKK complex.

signal transmission, *H. pylori* induces transient recruitment of TRAF6 to TAK1 (Fig. 6). *H. pylori* has to hold a functional T4SS, but not CagA, to elicit this effect.

Ubiquitinylation plays an important role in NF-κB signalling. K48-ubiquitinylation is associated with protein degradation and K63-ubiquitinylation serves as regulatory modification [40]. Here, we observed a transient K63-linked ubiquitinylation of TAK1 on *H. pylori* infection (Fig. 6). Putative E3 ligases which ubiquitinylate TAK1 comprise TRAF6 or tripartite motif 8 (TRIM8), which ubiquitinylate TAK1 at K34 [41] and K158 [42], respectively. Eventually, TRAF6 promotes *H. pylori*-induced K63-linked ubiquitinylation of TAK1 when recruited to the TAK1/TABs complex. Since the observed K63-linked ubiquitinylation of TAK1 on *H. pylori* infection is transient (Fig. 6), a putative role of deubiquitinylases remains to be investigated. For the removal of TAK1 ubiquitinylation, it has been described that USP4 deubiquitinylates TAK1 and negatively regulates IL-1β- and LPS-induced NF-κB [43]. In addition, an Itch–CYLD complex promotes the transition of K63-linked ubiquitinylation to K48-linked ubiquitinylation by sequentially cleaving the K63-linked ubiquitin chain and catalysing K48-linked ubiquitinylation of TAK1 [44]. Lysine 72 has been suggested as a site for K48-linked ubiquitinylation of TAK1 [45].

In summary, our study provides evidence for an intimate mutual control of the IKK complex by the kinases MEKK3 and TAK1 in *H. pylori* infection. The findings support a mechanism by which *H. pylori*, through the T4SS but not through CagA, promotes MEKK3 and TAK1 phosphorylation, TAK1 ubiquitinylation and the recruitment of the IKK complex promoting NF-κB signalling in the host cell. In depth clarification of the underlying mechanisms of *H. pylori*-induced NF-κB activation will require further extensive studies to identify the intricate interactions of upstream regulatory factors and the protein modifications important for their biological functions.

Author contributions

M.N. conceived the study and supervised the analysis. O.S. and M.N. wrote the manuscript text. O.S. performed the experiments and analysed the data, and G.M. contributed experimental data. All authors have read and approved the final version of the manuscript.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2014.01.006.

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Supplementary Table S1

Antibody	Source	Manufacturer
CagA	Mouse	Austral Biologicals, San Ramon, CA, USA
Occludin NF-κBp65	Mouse	BD Biosciences Pharmingen, San Jose, CA, USA
Phospho-IκBα (Ser32/36) (5A5) Histone H3 IκBα (44D4) IKKα MEKK3 (D36G5) TAK1 *Phospho-IKKα/β(S176/180) *The antibody detects IKKα and IKKβ Phospho-IKKα/β(S176/177) (C84E11) Phospho-IKKγ(S376) Phospho-NF-κB p65(S536) Phospho-TAK1(T184/187)	Mouse Rabbit	Cell Signalling Technology Inc., Danvers, MA, USA
GAPDH IKKβ	Mouse	Millipore, Temecula, CA, USA
IKKγ (B-3) TRAF6 Phospho-MEK kinase-3(S166) TAK1 (for immunoprecipitation)	Mouse Rabbit	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA
HA	Mouse	SIGMA-ALDRICH CHEMIE GmbH, Steinheim, Germany

Appendix A. Supplementary data

Legends to supplementary figures

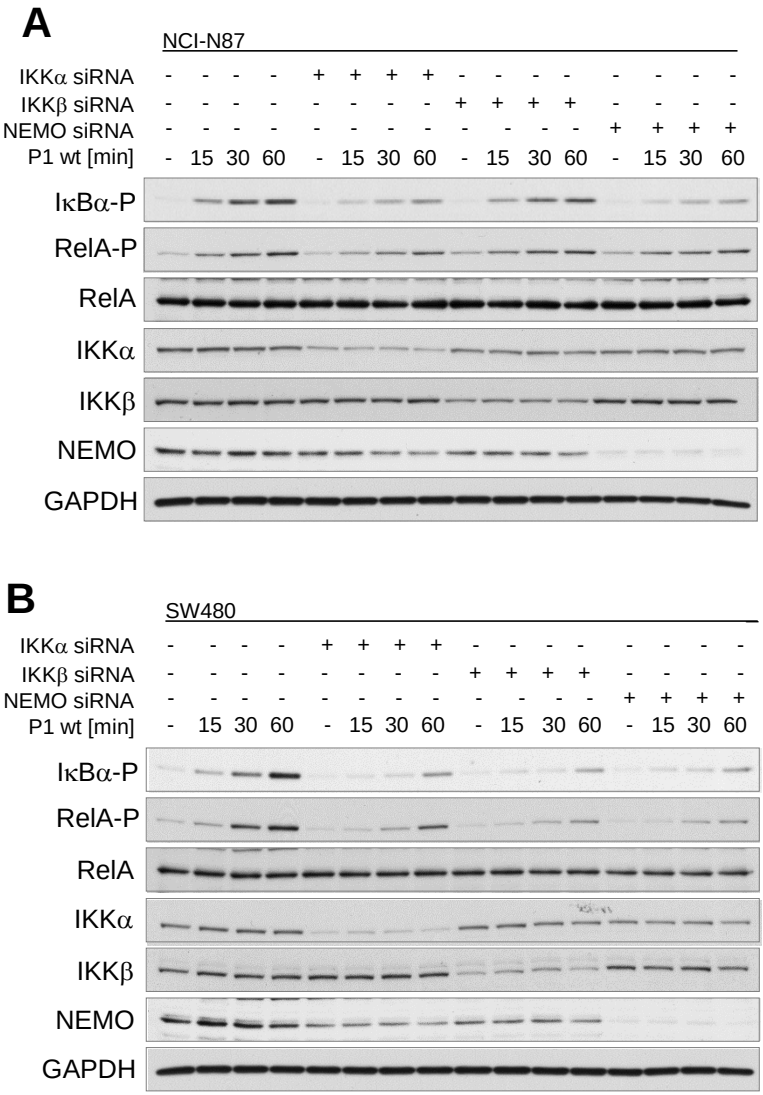
Supplementary Fig. S1. Integrity of the IKK complex is required for NF-κB activation. siRNA-transfected or mock-treated NCI-N87 (A) or SW480 (B) cells were infected with the *H. pylori* P1 wt strain for the indicated periods of time, and cell lysates were analysed by immunoblot using antibodies as indicated. 30 nM of each of IKKα, IKKβ or NEMO siRNA were used for transfection. A scrambled siRNA (30 nM) was used as a control. Immunostaining of GAPDH was performed to show equal protein loading in each lane. The blots shown in each panel comprise data from the same experiment.

Supplementary Fig. S2. TAK1 and MEKK3 are required for efficient activation of the IKK complex and NF-κB. (A) AGS cells were infected with the *H. pylori* G27 wt strain or with the *cagA* or *cagE* mutants for the indicated periods of time. NCI-N87 (B, C and E) and SW480 (B, D and F) were infected with the *H. pylori* P1 wt strain or were stimulated with TNFα, or IL-1β for the indicated periods of time. Cell lysates were analysed by immunoblot using the antibodies as indicated. Immunostainings of GAPDH were performed to show equal protein loading. (C, D) 48 h prior to infection, indicated cells were transfected with 30 nM TAK1, MEKK3 or scrambled siRNAs. (E, F) Cells were treated with increasing doses of TAK1 inhibitor (5Z)-7-Oxozeaenol derivative for 30 min prior to infection. Quantification of immunoblot bands was performed using densitometry and the relative phospho-IKK/GAPDH ratio is indicated. The blots shown in each panel comprise data from the same experiment.

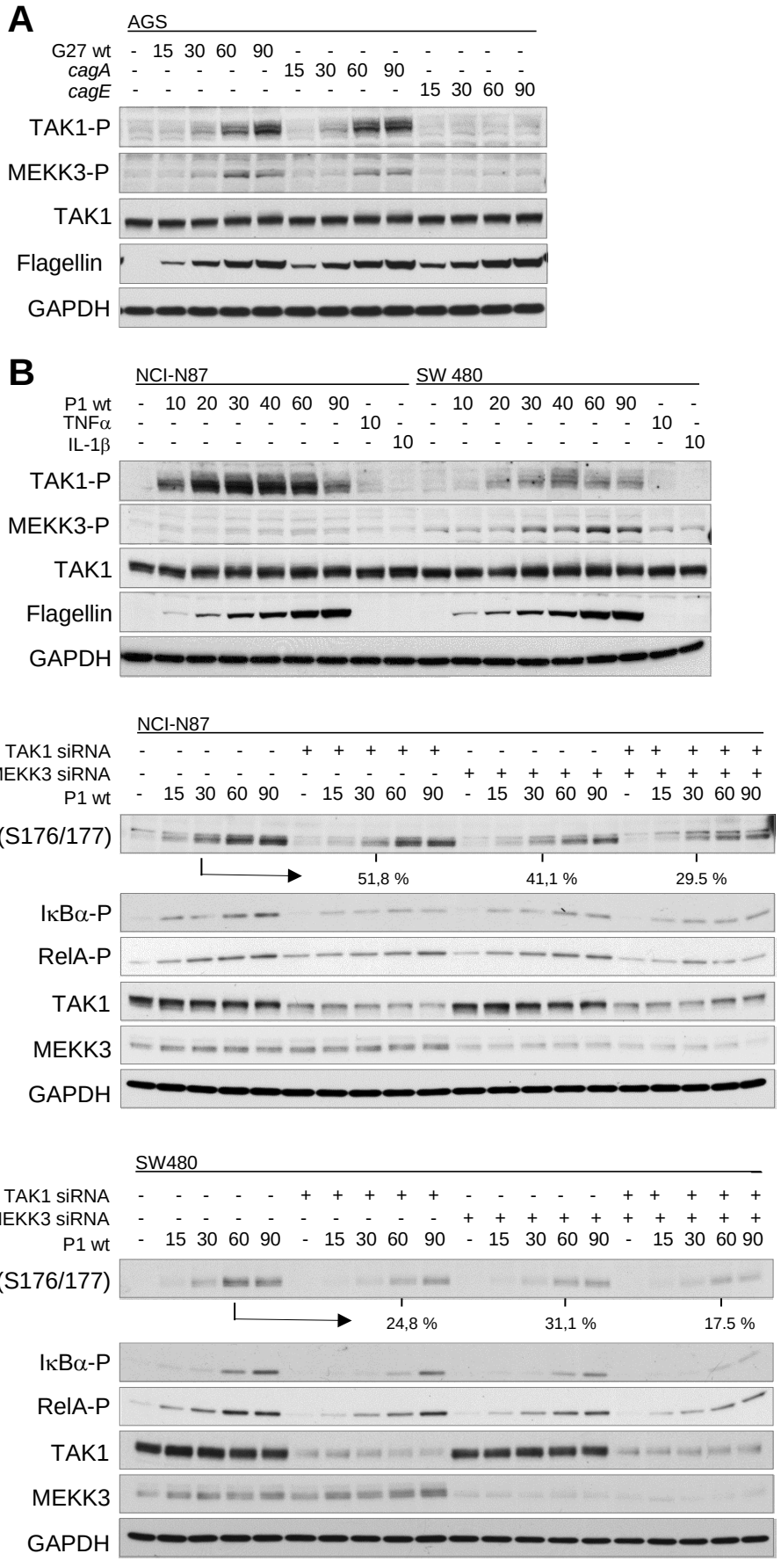
Supplementary Fig. S3. TAK1 interacts with the IKK complex and TRAF6 in a T4SS-dependent manner. NCI-N87 cells (A, C) and SW480 cells (B, D) were infected with the *H. pylori* P1 wt

strain for the indicated periods of time. Cell lysates were subjected to immunoprecipitation (IP) using anti-TAK1 antibody. The samples without antibody (no ab), without eukaryotic cellular lysate (only wt + ab), or without eukaryotic cellular lysate/bacterial lysate (only buffer + ab) indicate for the specificity of the IP and serve as controls. Immunoprecipitates and lysates of the infected cells or only the bacterial (only wt) lysate were analysed by immunoblot using the antibodies as indicated. The blots shown in each panel comprise data from the same experiment.

Supplementary Figure S1

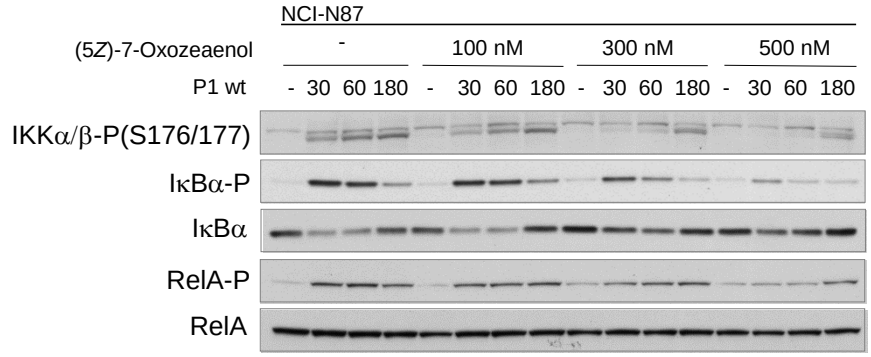


Supplementary Figure S2



Supplementary Figure S2

E



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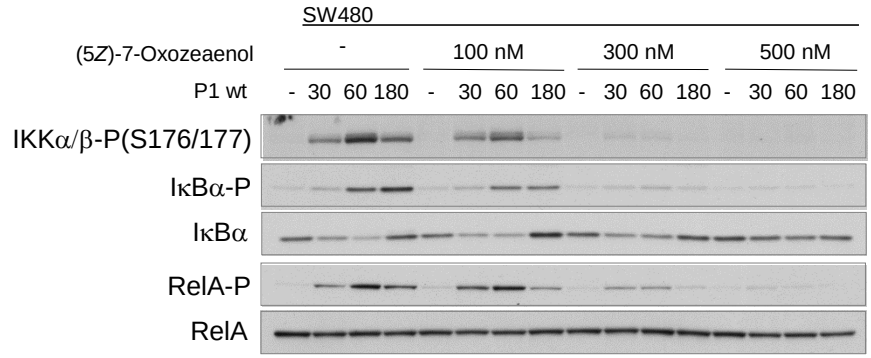
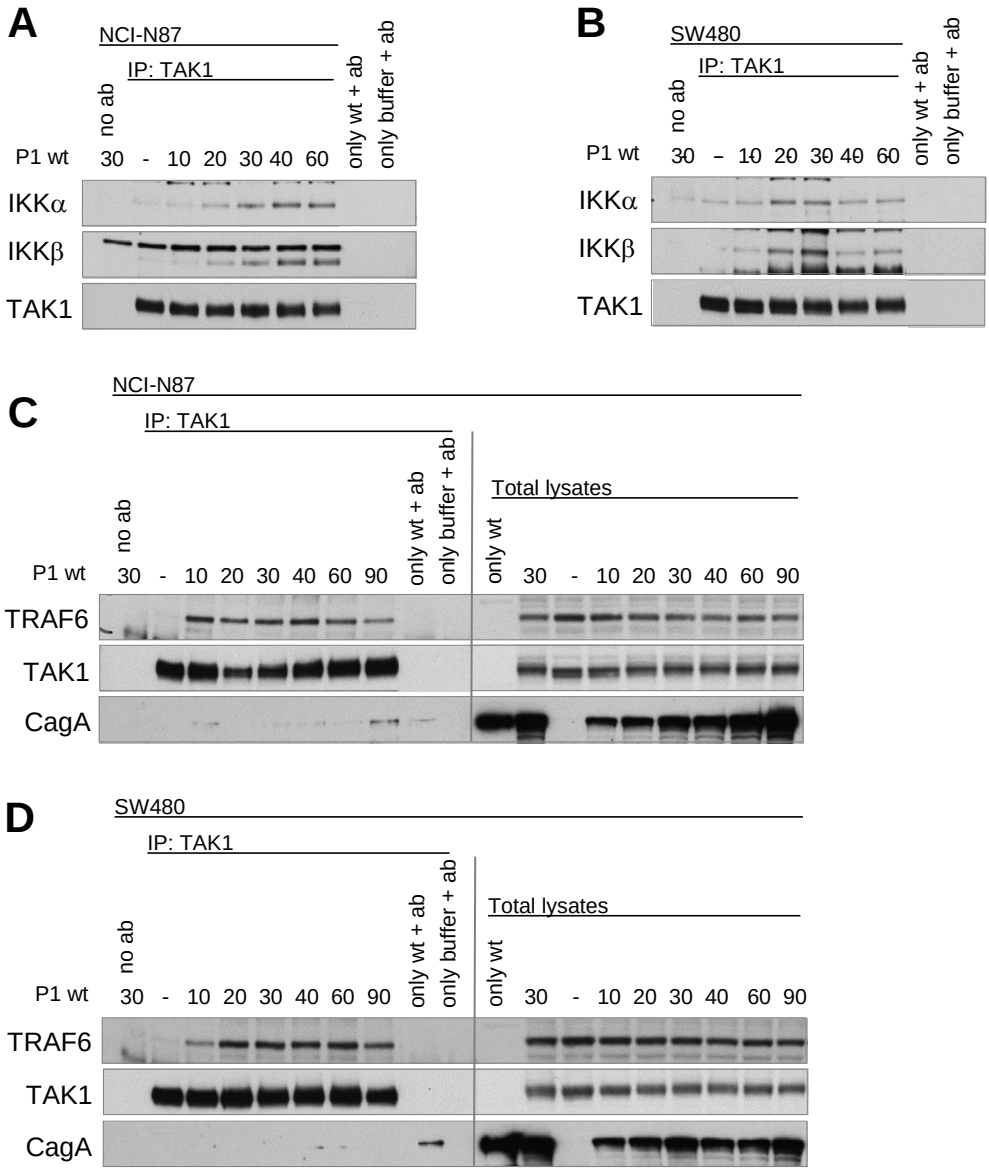
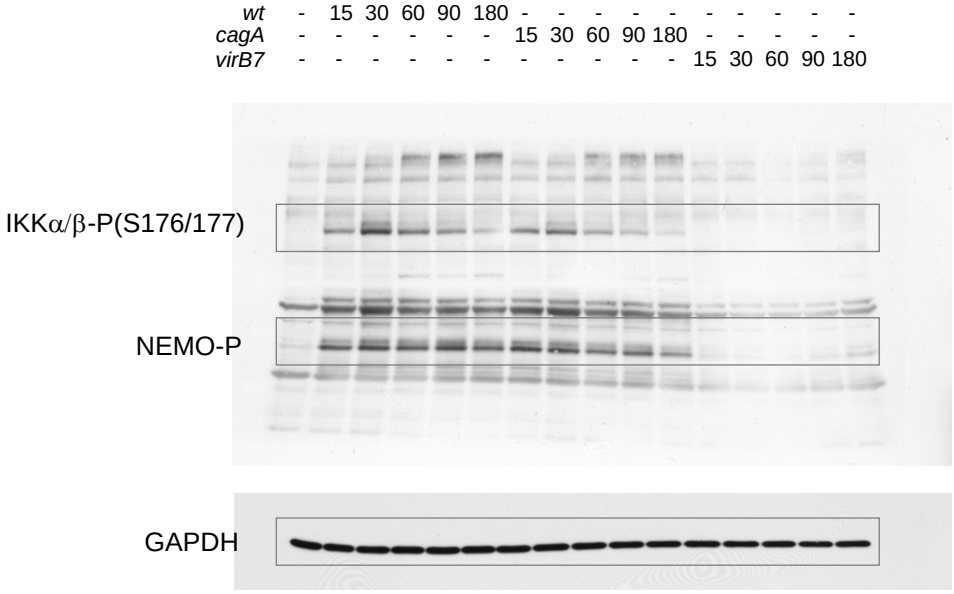


Supplementary Figure S3



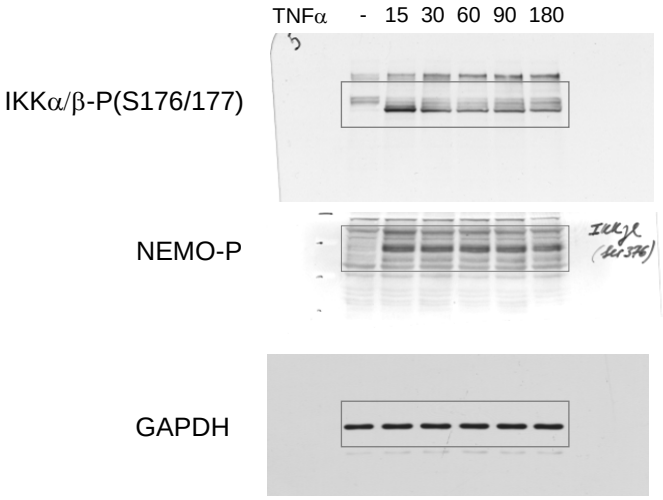
Supplementary Figure S4

a

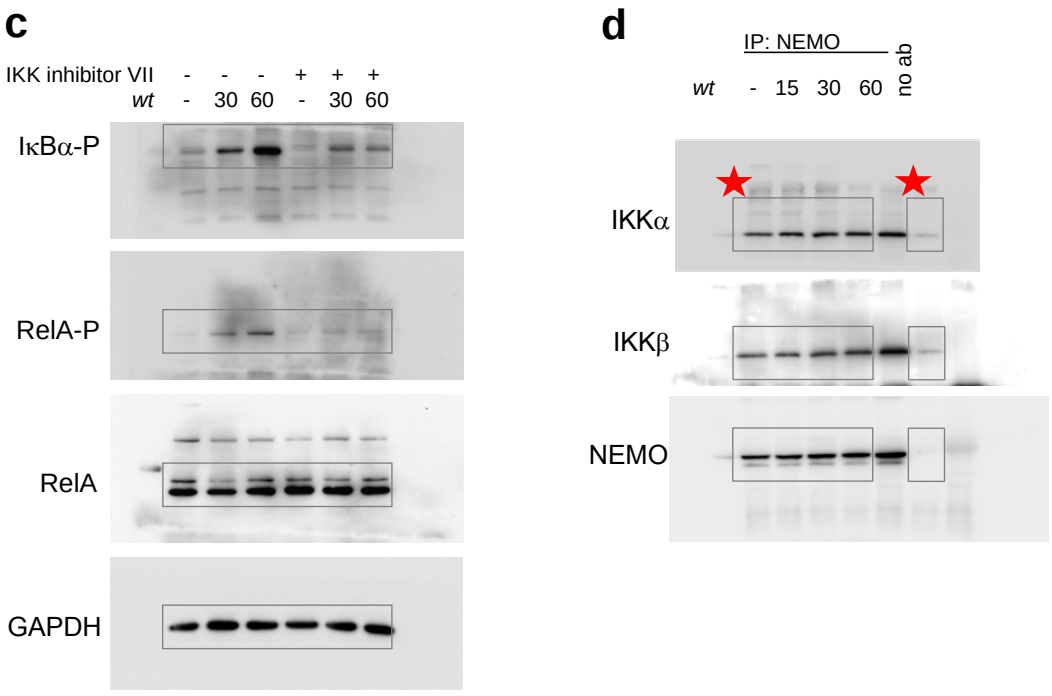


Supplementary Figure S4

b

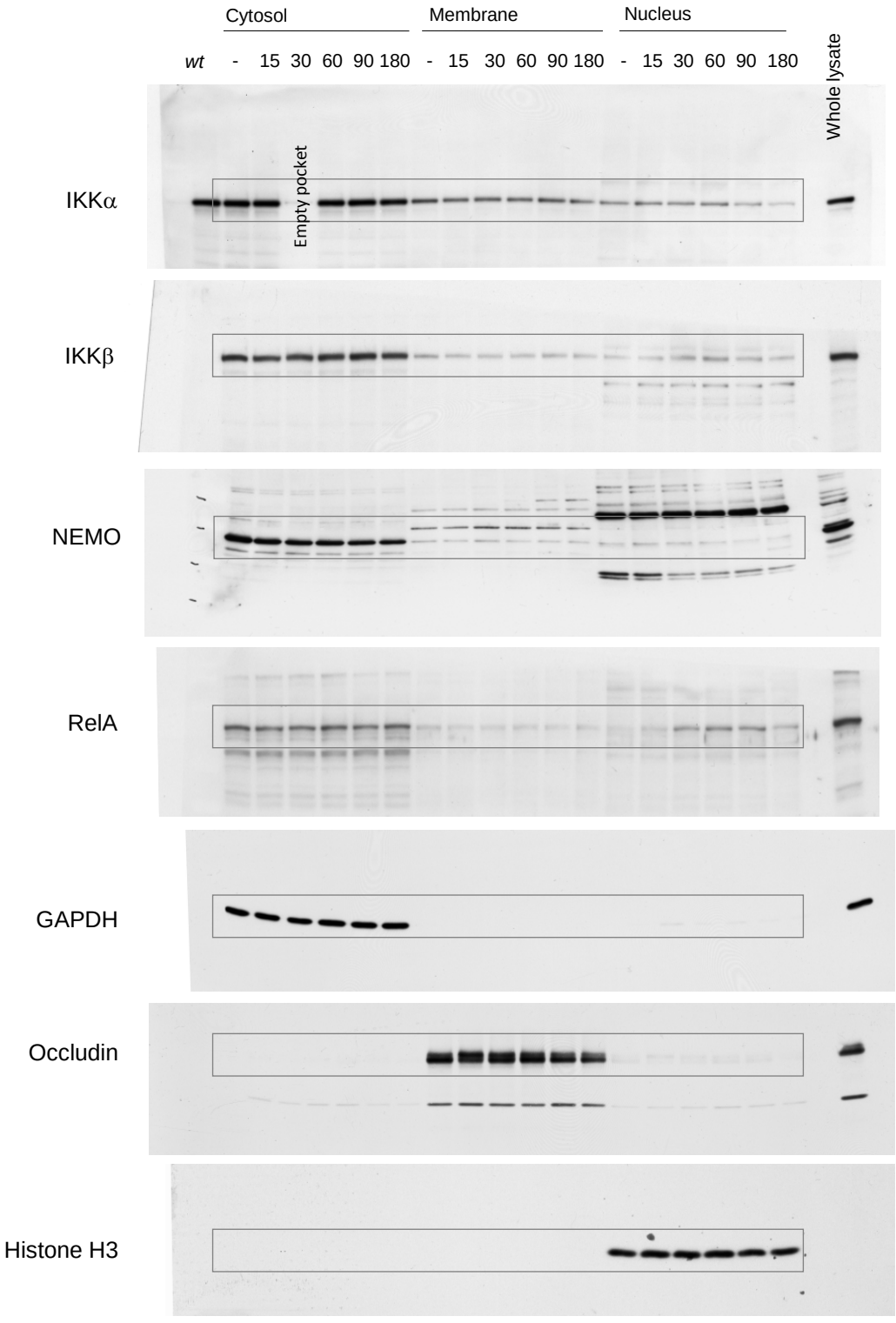


Supplementary Figure S4



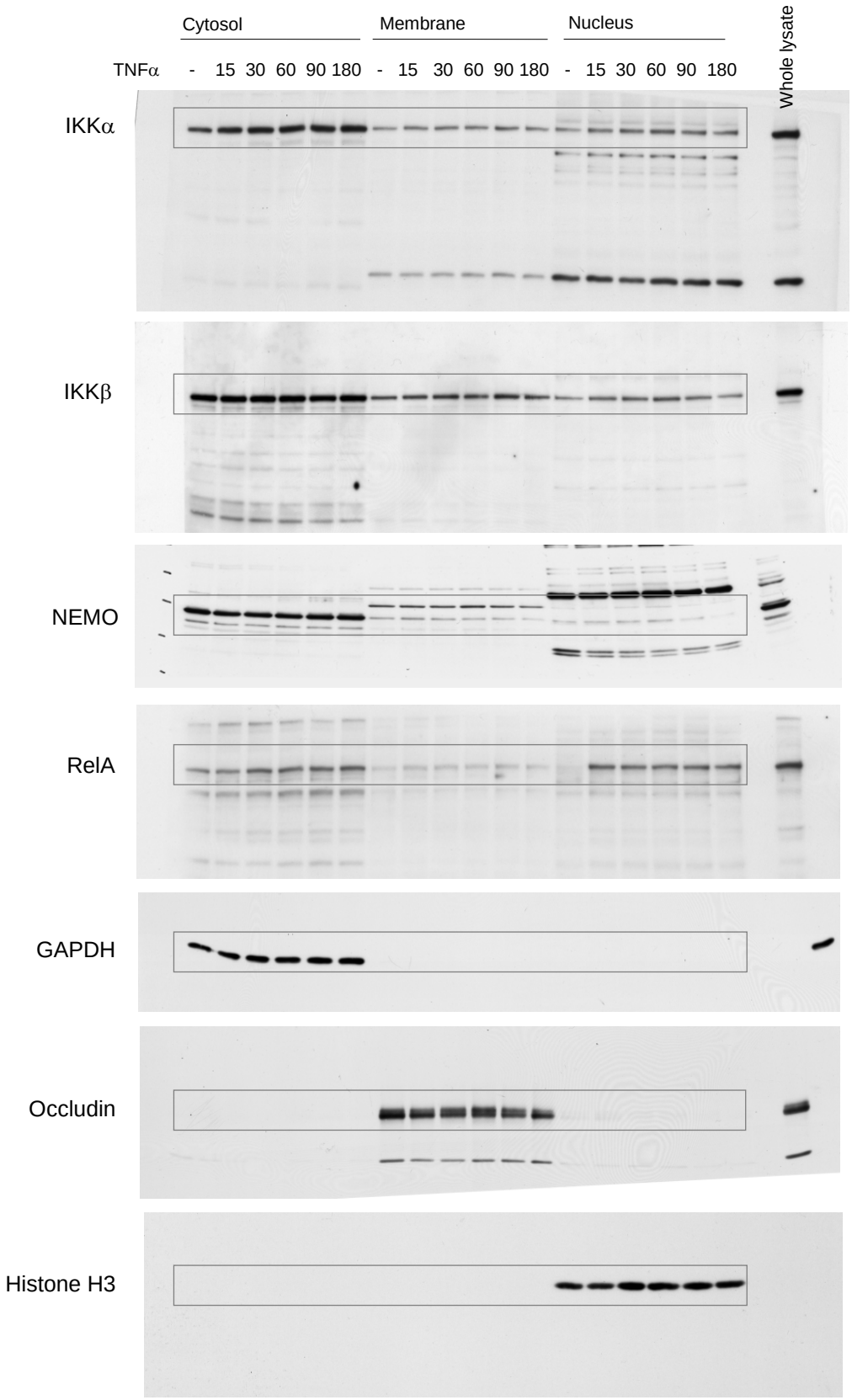
Supplementary Figure S4

e



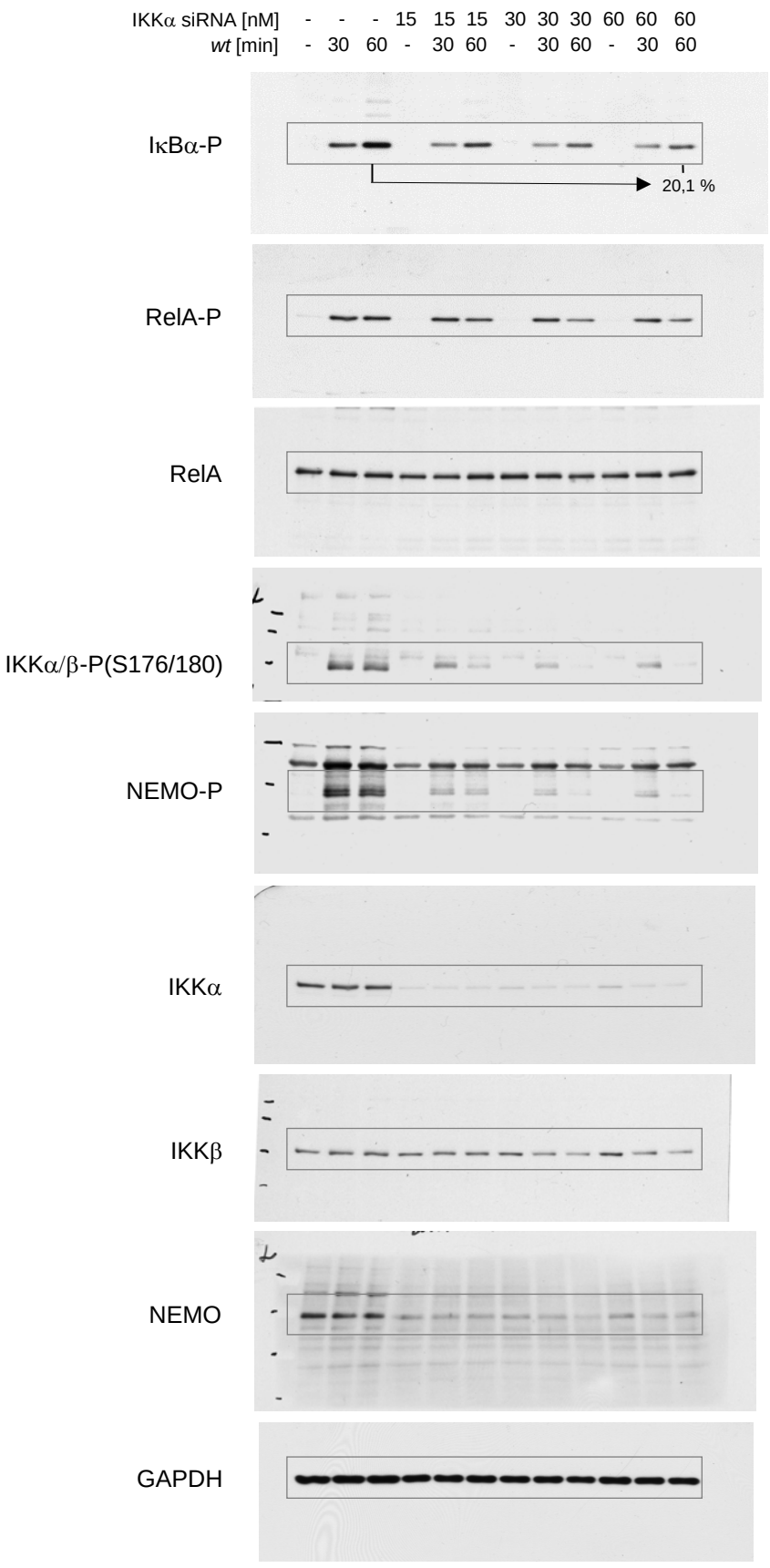
Supplementary Figure S4

f



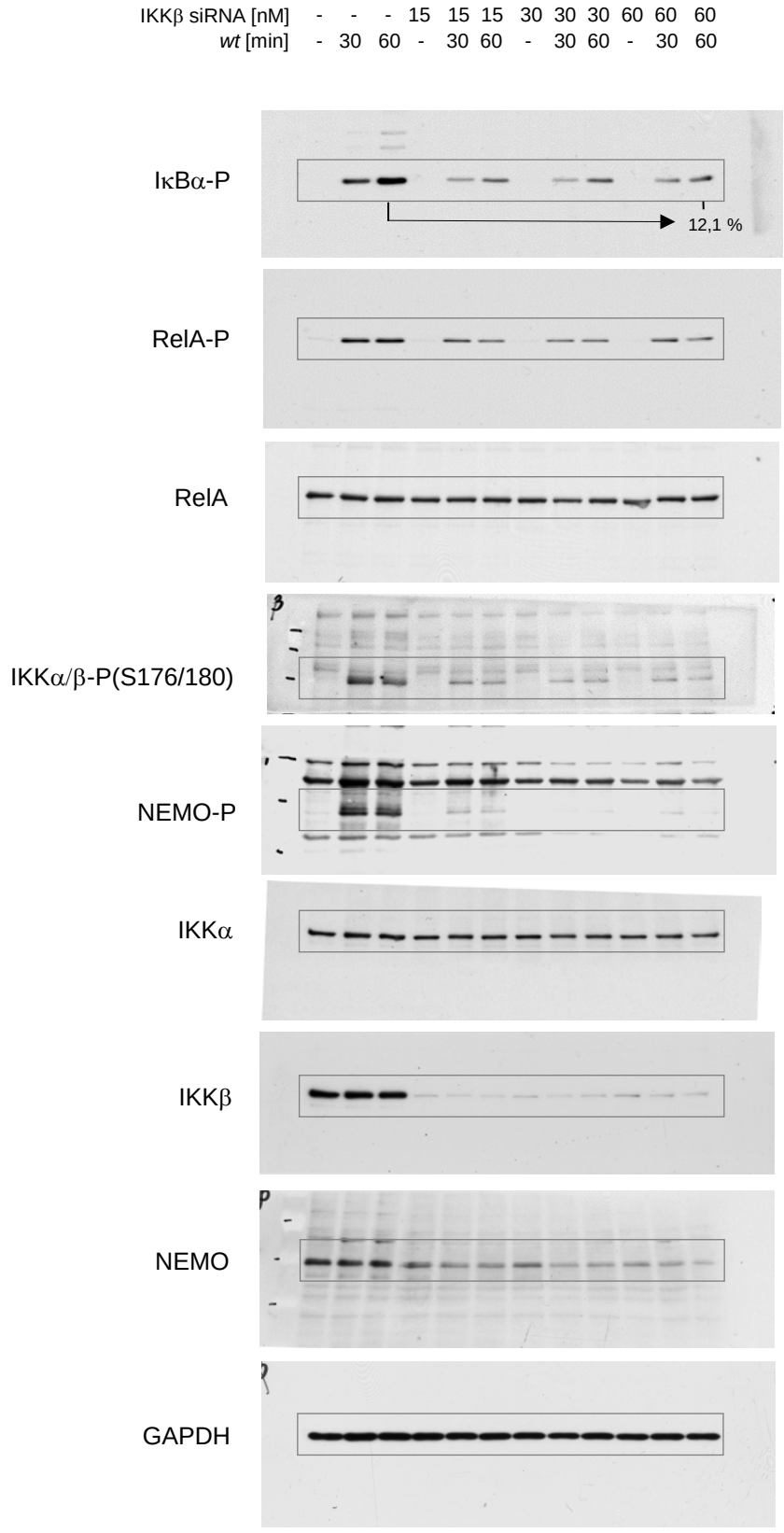
Supplementary Figure S5

a



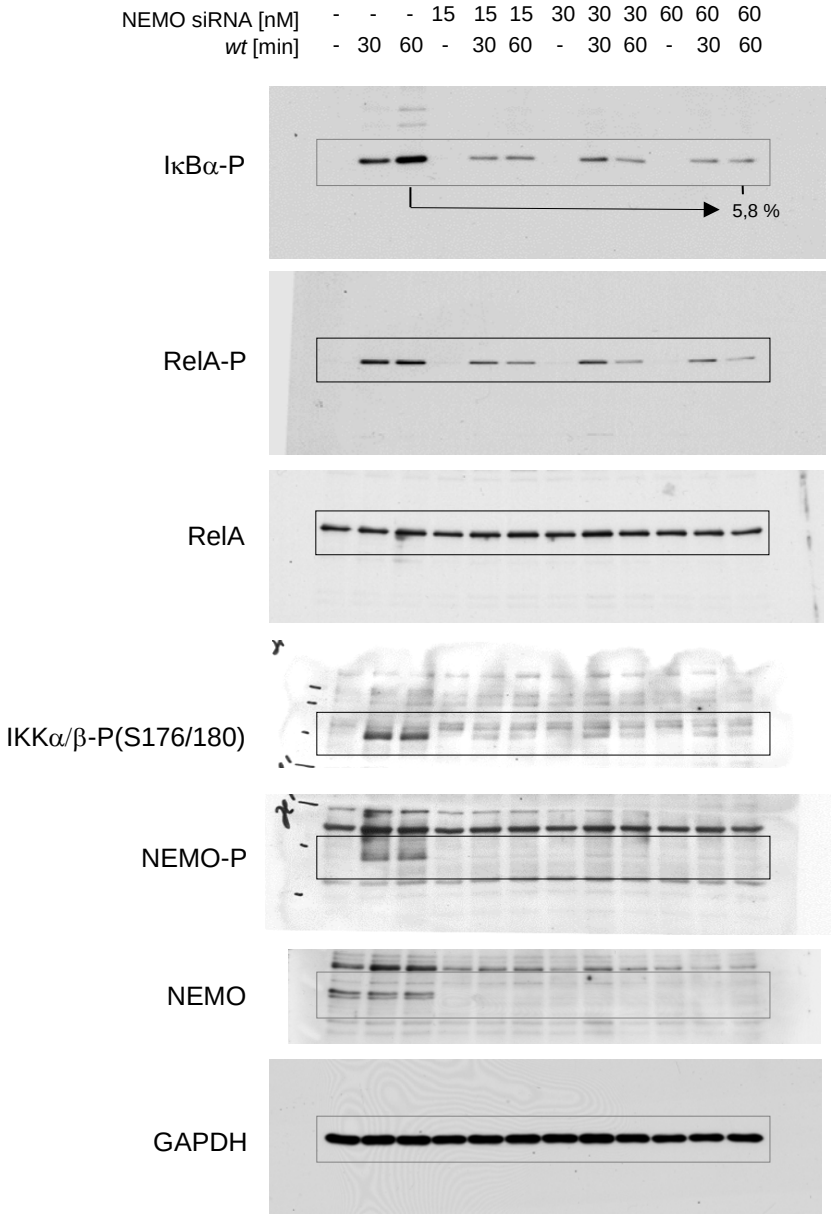
Supplementary Figure S5

b

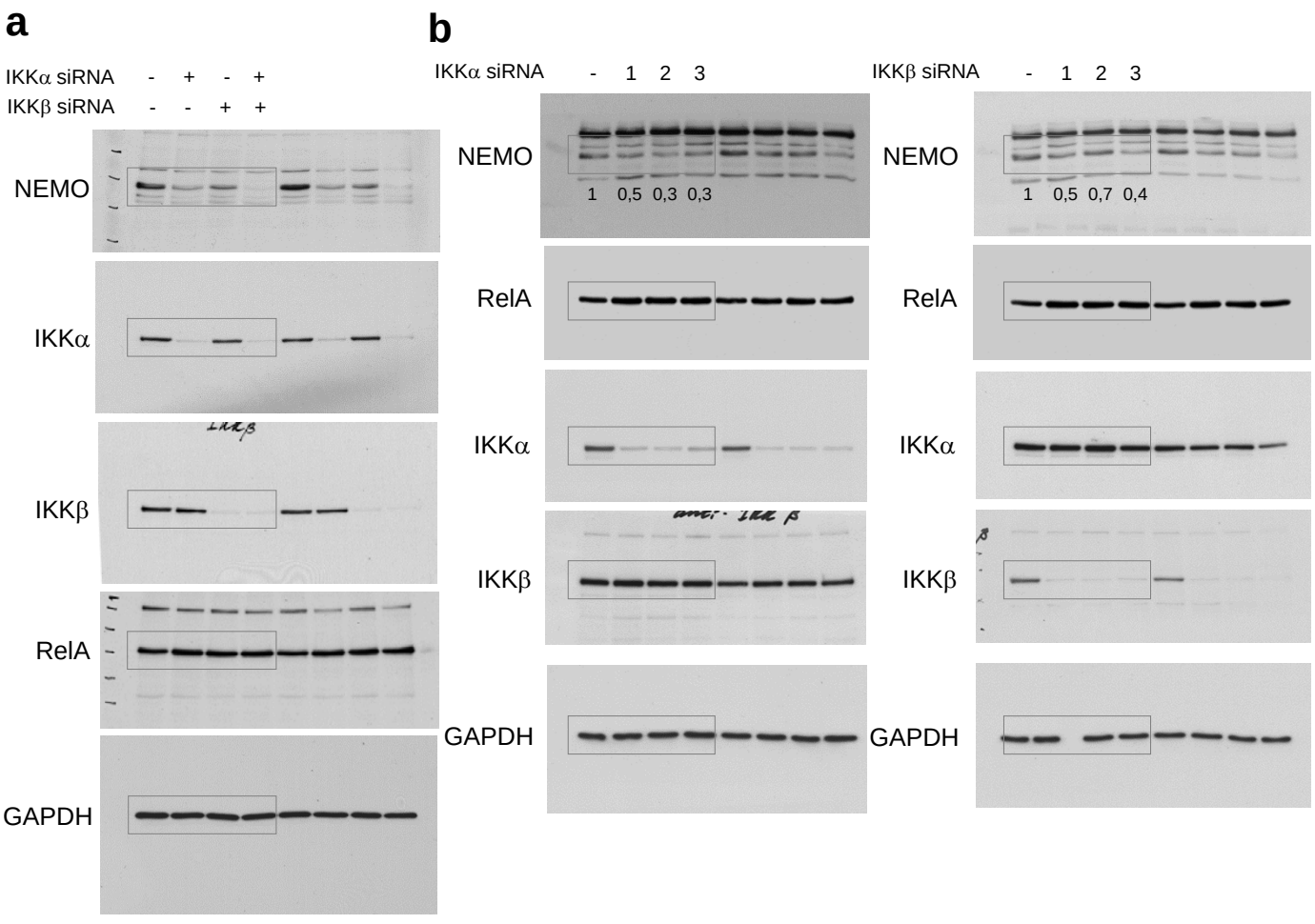


Supplementary Figure S5

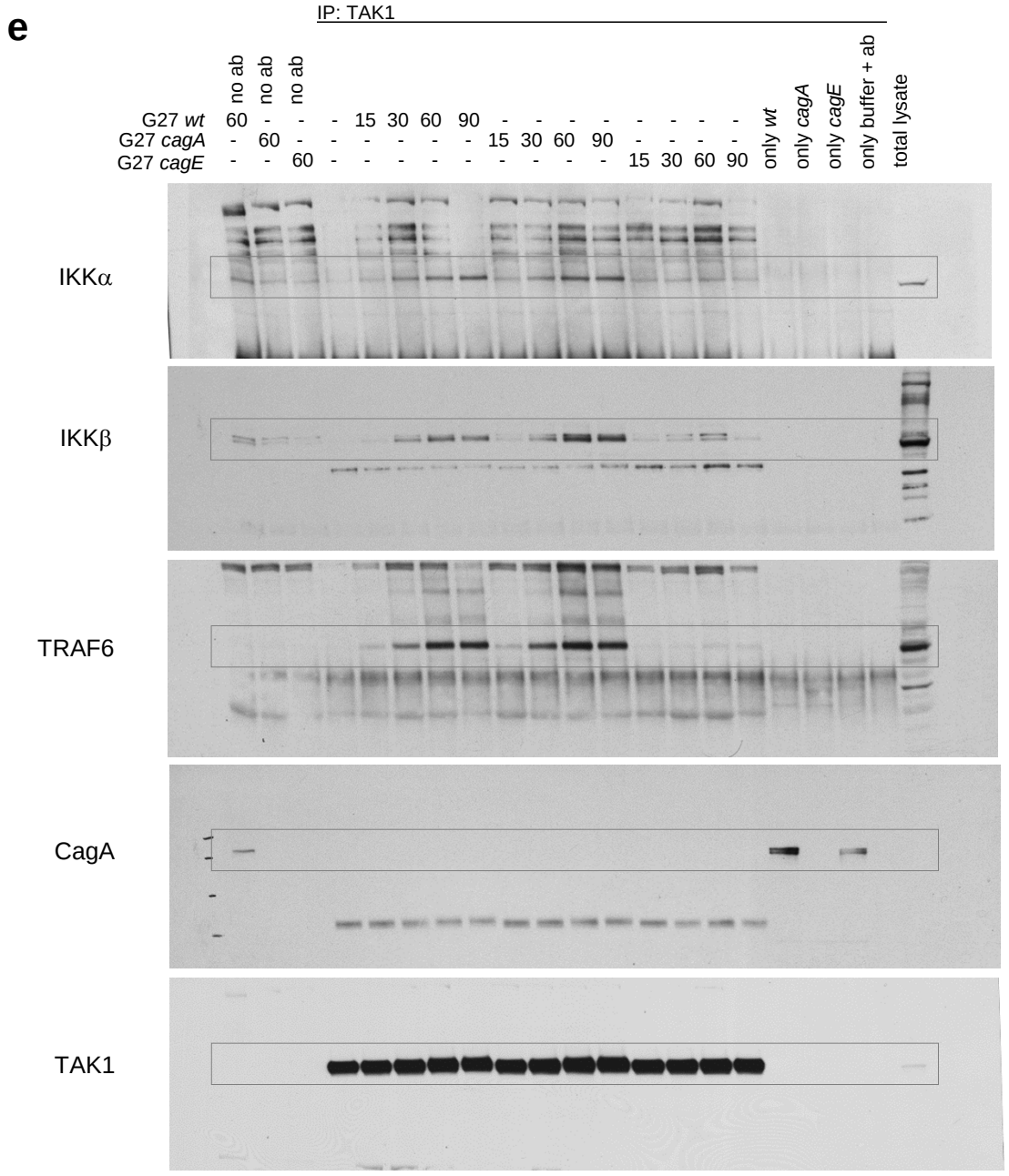
c



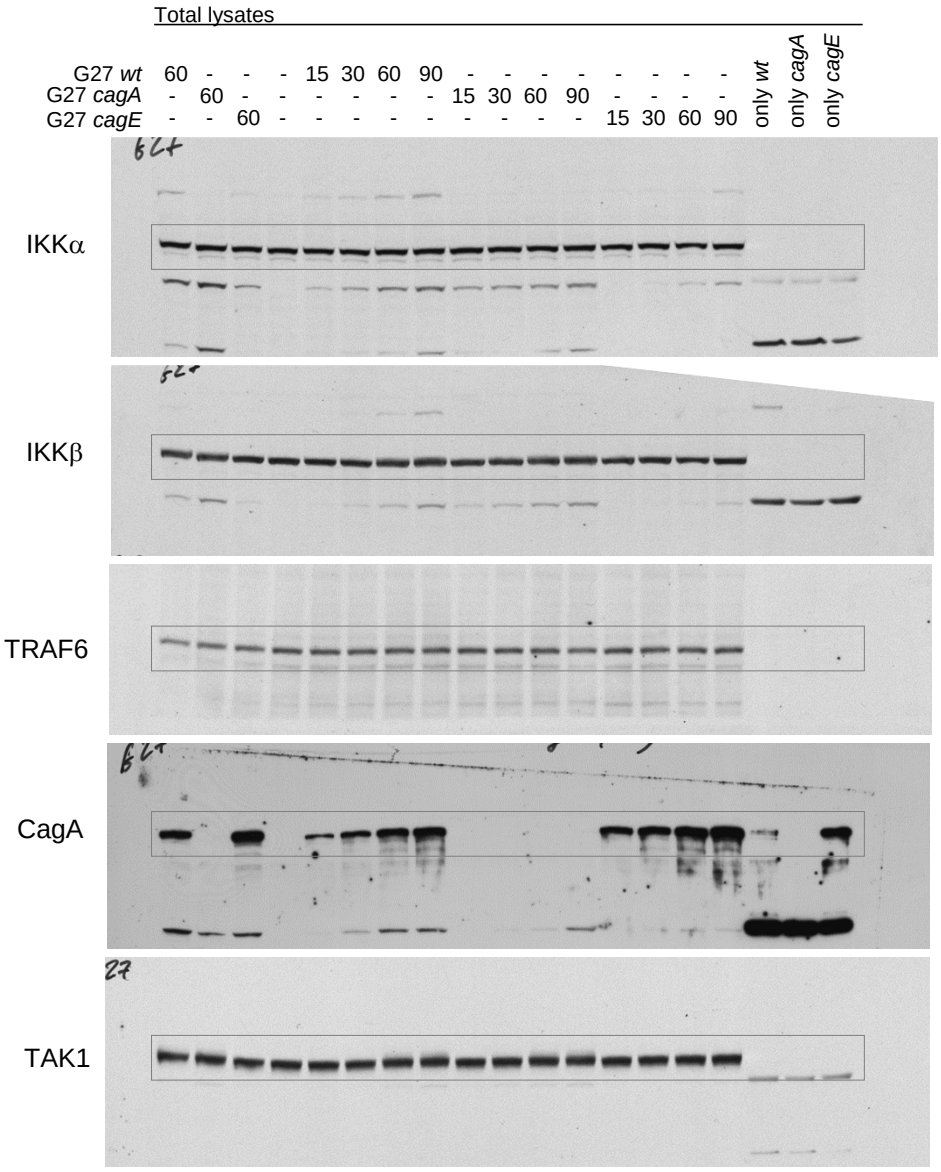
Supplementary Figure S6



Supplementary Figure S6

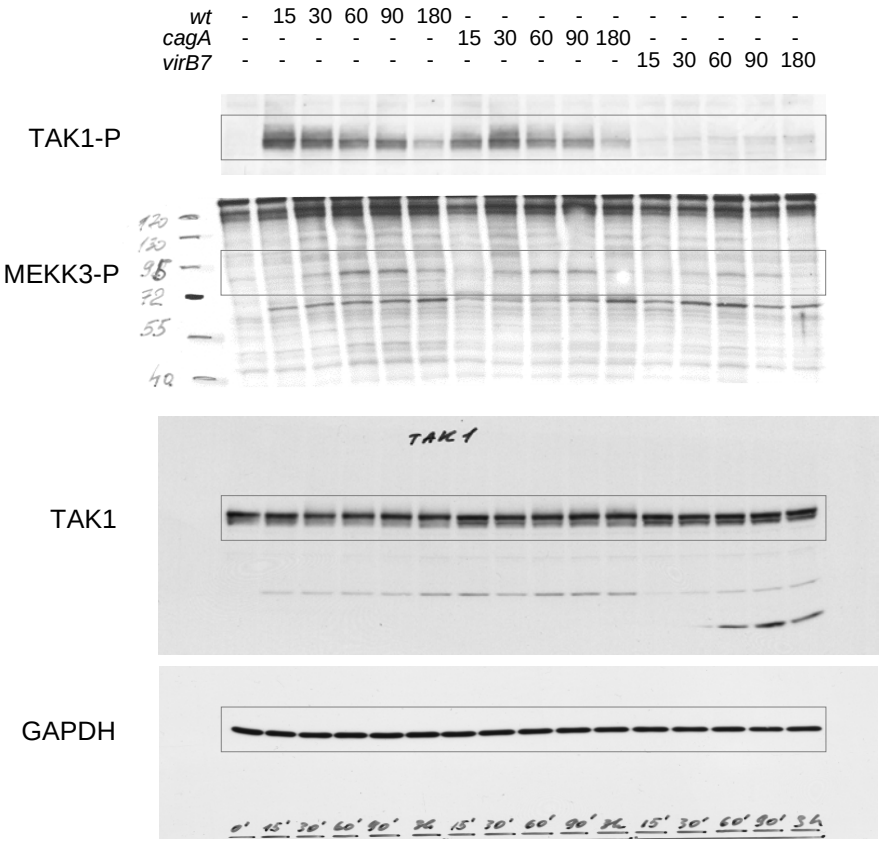


Supplementary Figure S6



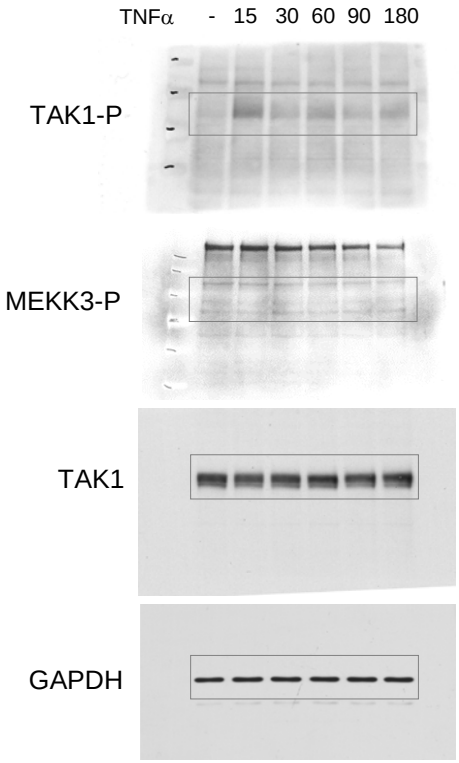
Supplementary Figure S7

a



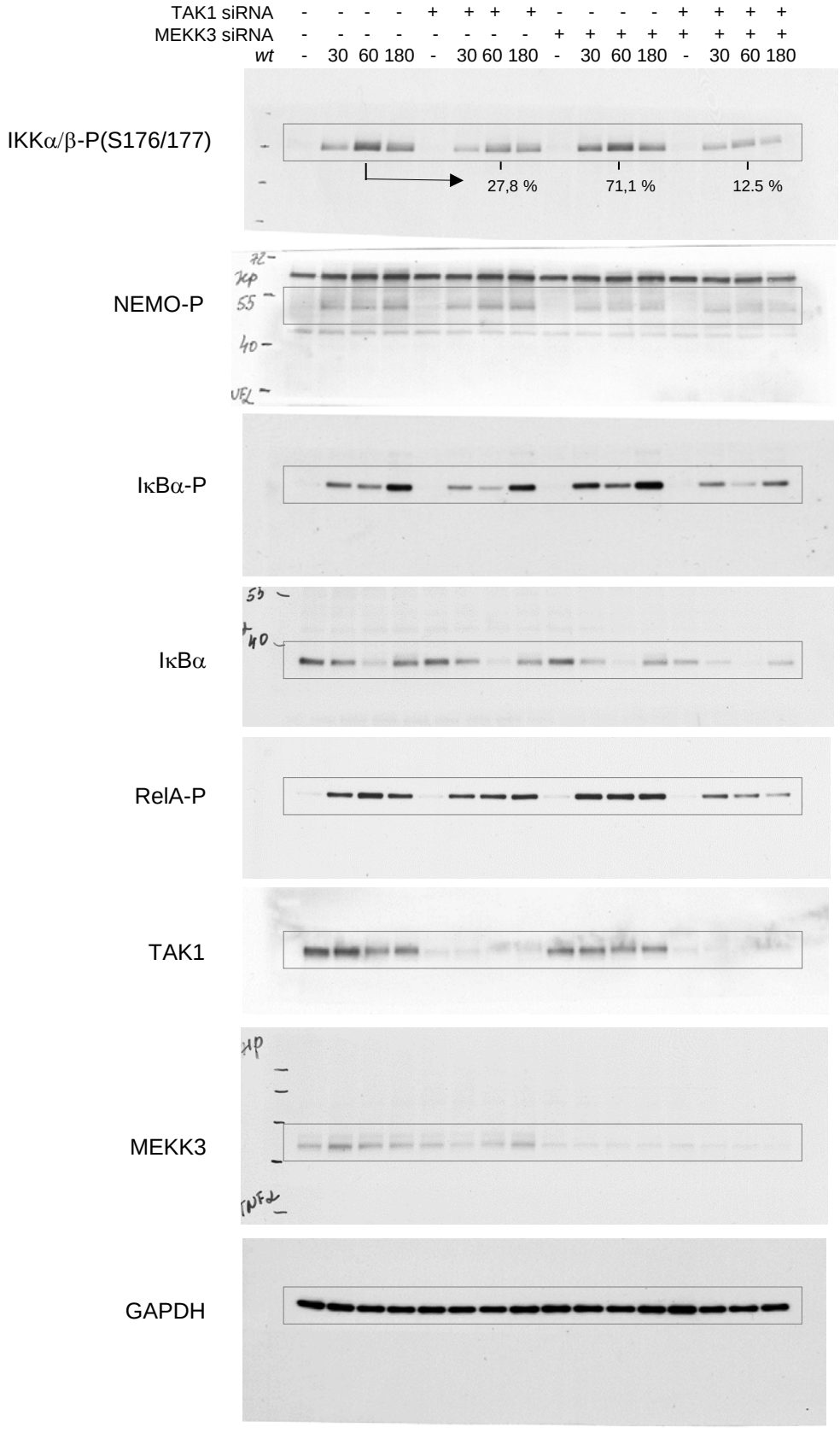
Supplementary Figure S7

b



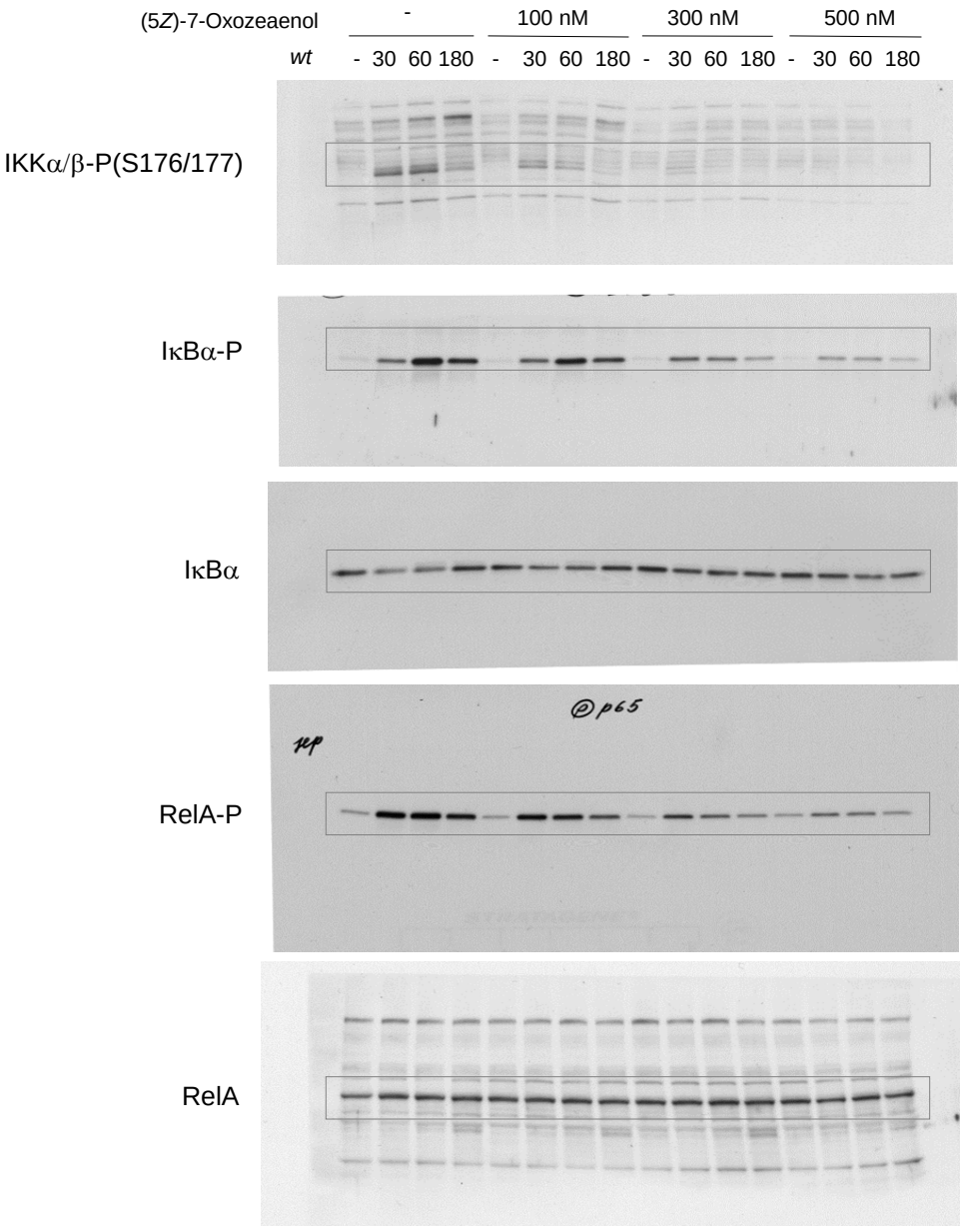
Supplementary Figure S7

c

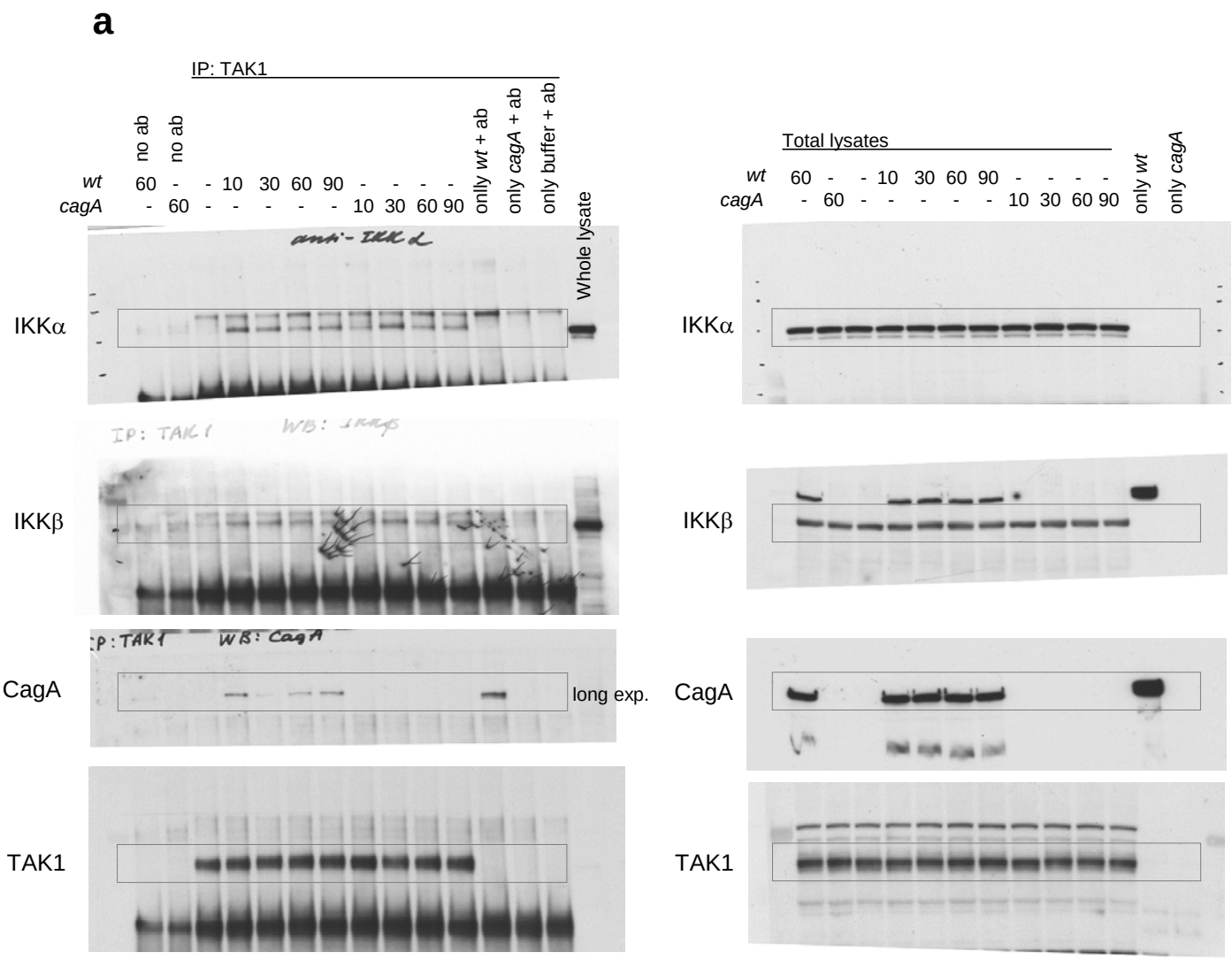


Supplementary Figure S7

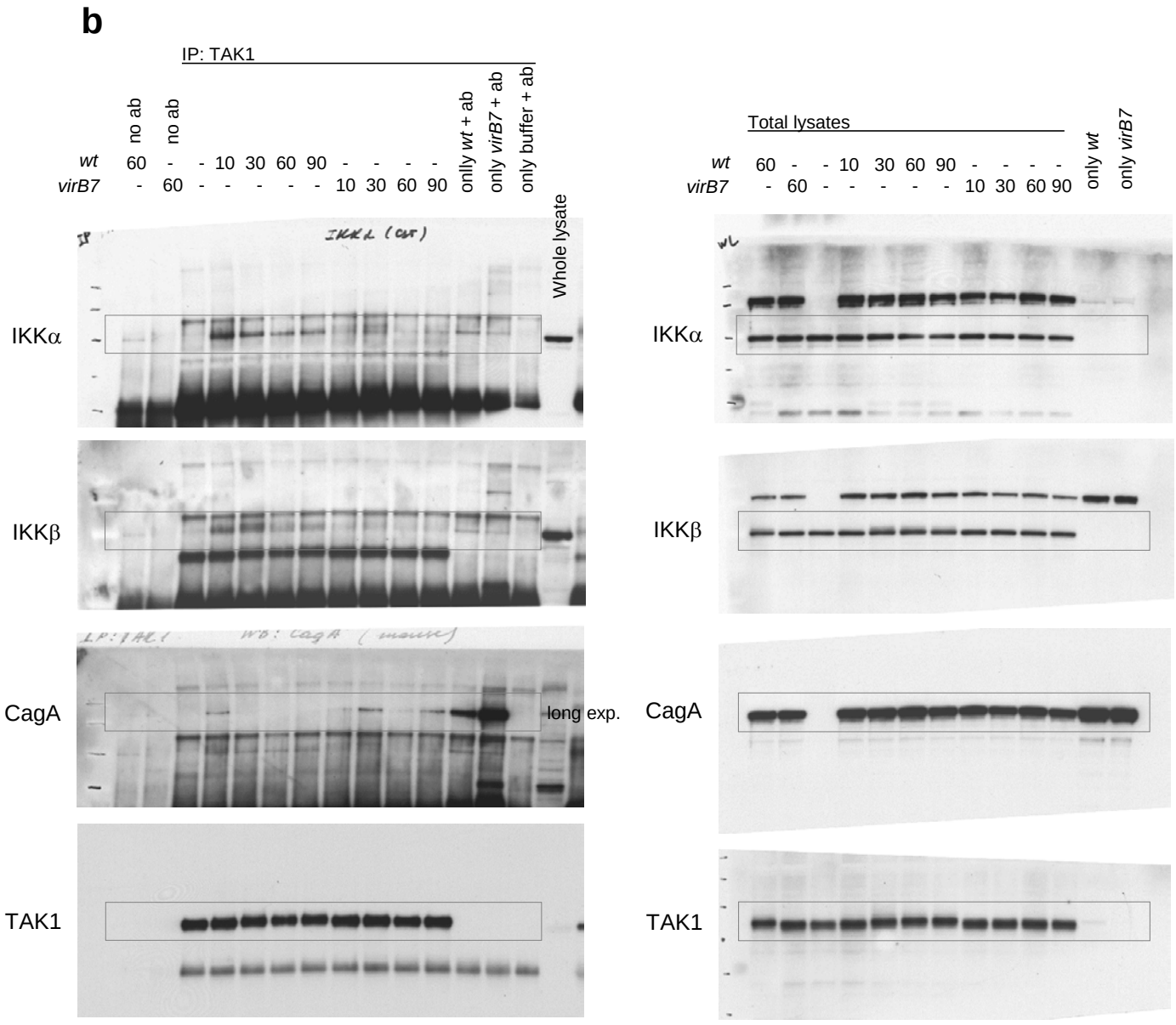
e



Supplementary Figure S8

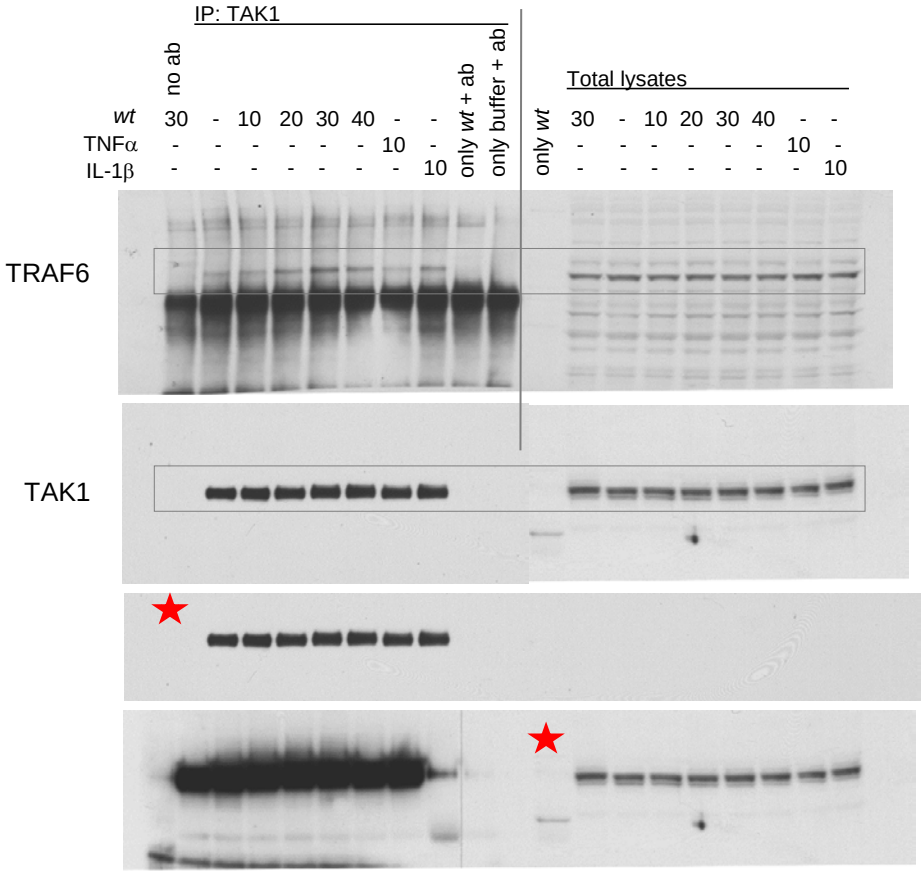


Supplementary Figure S8



Supplementary Figure S9

a



Supplementary Figure S9