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The role of TBK1 and IKK ϵ in the expression and activation of Pellino 1.

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Running title: The role of TBK1 and IKK ϵ in the expression and activation of Pellino 1.

Summary.

Mammalian Pellino isoforms are phosphorylated by IRAK1/IRAK4 *in vitro*, converting them into active E3 ubiquitin ligases. Here we report a striking enhancement in both transcription of the gene encoding Pellino 1 and Pellino 1 protein expression when murine BMDM are stimulated with LPS or poly(I:C). This induction occurs via a TRIF-dependent, IRAK-independent pathway and is prevented by inhibition of the IKK-related protein kinases, TBK1 and IKK ϵ . Pellino 1 is not induced in IRF3^{-/-} BMDM, and is only reduced slightly in type 1 interferon receptor^{-/-} BMDM, identifying Pellino 1 as a new IRF3-dependent gene. We also identify Pellino 1 in a two-hybrid screen using IKK ϵ as bait, and show that IKK ϵ /TBK1 activate Pellino 1 *in vitro* by phosphorylating Ser76, Thr288 and Ser293. Moreover, we show that the E3 ligase activity of endogenous Pellino 1 is activated in LPS or poly(I:C)-stimulated macrophages. This occurs more rapidly than the increase in Pellino 1 mRNA and protein expression, is prevented by inhibition of IKK ϵ /TBK1 and is reversed by phosphatase treatment. Thus IKK ϵ /TBK1 mediate the activation of Pellino 1's E3 ligase activity as well as inducing the transcription of its gene and protein expression in response to TLR3 and TLR4 agonists.

Abbreviations.

BMDM, bone marrow-derived macrophages; IKK ϵ , IkappaB kinase ϵ ; IFN β , interferon β ; IL-1, interleukin-1; IRAK, interleukin receptor associated kinase; IRF3, interferon regulatory factor 3; LPS, lipopolysaccharide; LTA, lipoteichoic acid; MCSF, macrophage colony-stimulating factor; MyD88, myeloid differentiation factor 88; NF κ B, nuclear factor kappa B; RIP1, receptor-interacting protein; TBK1, TANK-binding kinase 1; TLR, toll-like receptor; TRIF, TIR-domain-containing adaptor-inducing interferon- β .

Keywords: E3 ligase, toll-like receptor (TLR), TRAF-interacting factor (TRIF), interleukin receptor associated kinase (IRAK), ubiquitin, interferon regulatory factor 3 (IRF3).

Introduction.

During infection by bacteria or viruses, components of these pathogens bind to toll-like receptors (TLRs) in immune cells. This triggers the activation of signalling pathways that control the production of inflammatory mediators and interferons to combat the invading pathogen [1]. The receptors for the pro-inflammatory cytokine interleukin-1 (IL-1) and all TLRs except for TLR3, signal via an adaptor protein, termed myeloid differentiation factor 88 (MyD88) [2], which recruits the protein kinases interleukin receptor associated kinase (IRAK) 1 and IRAK4 [3, 4]. This enables IRAK4 to activate IRAK1 [5] and IRAK2 [6] which are thought to initiate “downstream” signalling events. In contrast, TLR3 signals via a distinct adaptor, termed TIR-domain-containing adaptor-inducing interferon- β (TRIF), while TLR4 is unique in requiring both MyD88 and TRIF for signalling [7-9]. The TRIF-dependent pathway can either signal via Receptor-Interacting Protein 1 (RIP1), which leads to activation of the transcription factor NF κ B [10, 11], or via I κ B kinase ϵ (IKK ϵ) and TANK-binding kinase 1 (TBK1) [12, 13], the protein kinases that activate the transcription factor Interferon Regulatory Factor 3 (IRF3). IRF3 then stimulates transcription of the gene encoding interferon β (IFN β) [14-16].

Pellino was originally identified as a protein that interacts with Pelle, the orthologue of the only IRAK in *Drosophila* [17], and the three mammalian isoforms, Pellino 1, Pellino 2 and Pellino 3 [18, 19] were subsequently shown to interact with and to be phosphorylated by IRAK1 and IRAK4 [20-22]. More recently, the IRAK1 and IRAK4-catalysed phosphorylation of Pellino isoforms was found to greatly enhance their ability to function as E3 ubiquitin ligases and to produce unanchored Lys63-linked polyubiquitin chains in the presence of the E2 conjugating enzyme Ubc13-Uev1a [21, 23]. IRAK1 and IRAK4 phosphorylate Pellino 1 at 10 serine/threonine residues *in vitro*, but full activation of the E3 ligase activity of Pellino 1 can be achieved by phosphorylation of any one of the three key activating residues, Ser76, Thr288 and Ser293 or by the combined phosphorylation of three other residues, Ser 78, Thr80 and Ser82 [23]. Moreover, the co-transfection of vectors encoding IRAK1 and Pellino 2 into IRAK1-null cells led to the Lys63-linked polyubiquitylation of IRAK1, but not if wild type IRAK1 was replaced by a catalytically inactive mutant or if Pellino 2 was replaced by an E3-ligase defective mutant of this protein [21]. Taken together, these experiments indicated that, in co-transfection experiments, IRAK1 phosphorylates and activates Pellino isoforms, which then polyubiquitylate IRAK1. This suggested that one or more Pellino isoforms may contribute to the Lys63-linked polyubiquitylation of IRAK1, which occurs within minutes when mammalian cells are stimulated with IL-1 [24, 25].

More recently, mice were generated that do not express Pellino 1. Interestingly, these mice did not display any defect in the activation of the canonical IKKs (IKK α and IKK β) or binding of NF κ B to DNA in response to IL-1 in MEFs or to TLR agonists that signal via MyD88 in splenocytes. Instead, the genetic deficiency of Pellino 1 was reported to attenuate the activation of the canonical IKKs and binding of NF κ B to DNA in MEFs and splenocytes in response to the ligands poly(I:C) and lipopolysaccharide (LPS) that activate TLR3 and TLR4 respectively. The Pellino 1^{-/-} mice also produced reduced serum levels of TNF α , IL-6 and IL-12 following an intra-peritoneal injection of poly(I:C) or LPS, explaining their resistance to poly(I:C) or LPS-induced septic shock. These studies demonstrated a key non-redundant role for Pellino 1 in TLR3 and TLR4-mediated innate immunity [26].

Until now, the lack of any antibodies capable of detecting the endogenous Pellino isoforms has hampered the study of how these E3 ligases are regulated. Here, we describe an antibody that recognises Pellino 1 specifically and exploit it to show that the expression of the Pellino 1 protein is greatly enhanced when bone marrow derived macrophages (BMDM) are stimulated with LPS or poly(I:C). We demonstrate that this occurs via a TRIF-dependent pathway that requires the IKK-related protein kinases, IKK ϵ and TBK1, and the transcription factor interferon-regulatory factor 3 (IRF3), but not the type 1 interferon receptor. We also show that LPS or poly(I:C) rapidly activate the E3 ligase activity of the endogenous Pellino 1 in macrophages and demonstrate that this is mediated by phosphorylation catalysed by IKK ϵ /TBK1. Taken together, our results establish that TBK1 and IKK ϵ stimulate both the activation of Pellino 1's E3 ligase activity and, via IRF3, the transcription of its gene in response to agonists that activate TLR3 and TLR4.

Experimental.

Materials.

The IRAK4 inhibitor [27, 28] was synthesised by Dr Natalia Shpiro, MRC Protein Phosphorylation Unit, University of Dundee, UK and the structures of the TBK1/IKK ϵ inhibitors MRT67307 [29] and BX795 [30] have been presented. Both compounds were dissolved and stored at -20 °C as 10 mM solutions in DMSO. Poly(I:C) was purchased from Invitrogen, LPS (*E. coli* strain O5:B55), poly(I:C) and R848 were from Alexis Biochemicals, CpG [ODN 1826], IL-1, LPS and Flag-Ubiquitin were from Sigma, IFN β was from PBL Interferon Source. The source of the LPS and poly(I:C) used in each experiment are indicated in the figure legends.

DNA constructs.

Pellino 1, Pellino 2 and Pellino 3 were subcloned into the pCMV5 vector by *EcoRI/BamHI* with an in frame Flag epitope at the N-terminus. pSUPER-GFP-Pellino 1 shRNA[216] and pSUPER-GFP-Pellino1 shRNA[1239] target the sequences [agaccagcatagcatatca] and [tcaaggaccttagactaa] in the human Pellino 1 coding regions respectively [31]. pSUPER-GFP-mPellino1 shRNA[216] and pSUPER-GFP-mPellino 1 shRNA[1239] targets the sequences [ggaccagcatagcatatca] and [ccaaggaccttagactag] in the mouse Pellino 1 coding region, respectively. All plasmid constructs were sequenced to verify DNA integrity. Other DNA and expression vectors have been described previously [21, 23].

Protein expression and purification.

GST-IRAK1 and GST-IRAK4 expressed in insect cells and GST-Pellino 1, GST-Ubc13-Uev1a and His₆-UBE1 expressed in *E. coli* were purified as described previously [21, 23, 32]. His₆-tagged TBK1 and His₆-tagged IKK ϵ were expressed and purified as described [30]. The GST-tag was removed from Pellino 1, Ubc13 and Uev1a by cleavage with PreScission Protease. The protein phosphatase encoded by bacteriophage λ gt10 was obtained from New England Biolabs, and ubiquitin was purchased from Sigma.

Production and characterisation of a Pellino 1-specific antibody.

The lack of antibodies capable of detecting the endogenous levels of any Pellino isoform in cells has hampered the study of these E3 ubiquitin ligases. A polyclonal antibody was raised against Pellino 1 in rabbits by Biogenes (Germany) using as an antigen the peptide [CPDQENHPSKAPVKY] corresponding to residues 4-17 of Pellino 1 preceded by a cysteine residue. The cysteine was used to couple the peptide to keyhole limpet haemocyanin prior to injection. The antibody was purified from the anti-sera by affinity chromatography using the antigen peptide coupled to Sepharose. The peptide antibody recognised Pellino 1, but not Pellino 2 and Pellino 3 in transfected HEK293 cells (Fig 1A). The antibody also recognised a ~50 kDa protein in murine RAW264.7 extracts and this band was no longer detected after the level of Pellino 1 had been “knocked down” by expression of two different shRNAs targeting Pellino 1 (Fig 1B). These shRNAs had previously been found to be effective in “knocking down” transiently-transfected Pellino 1 in human HEK293 cells (Fig 1C). These studies demonstrated that the ~50 kDa protein in RAW264.7 cell extracts was indeed Pellino 1.

Other antibodies.

Anti-Flag and anti- α tubulin were purchased from Sigma, anti-ubiquitin was from DakoCytomation, anti-actin was from Santa Cruz, anti-Ser(P)396 of IRF3 and anti-Tyr(P)701 of STAT1 were from Cell Signalling and a rabbit secondary antibody conjugated to HRP (horseradish peroxidase) was from Pierce.

Knock-out mice.

MyD88^{-/-} and TRIF^{-/-} mice [7] and their wild type littermates were kindly provided by Shizuo Akira, Department of Host Defence, University of Osaka, Japan. Bones from IRF3 knockout mice and C57BL/6J control mice were provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan via Christophe Desmet, University of Liege, Belgium. Bones from mice lacking expression of the type 1 interferon receptor (IFN $\alpha\beta$ R^{-/-} mice) were provided by Anne O'Garra, National Institute for Medical Research, London, UK.

Cell culture and transfection.

RAW264.7 and HEK293 cells were obtained from the American Type Culture Collection (Manassas, VA, U.S.A.) and were cultured at 37 °C with DMEM containing 10 % FBS and antibiotics (100 Units/ml penicillin and 100 µg/ml streptomycin) in a humidified atmosphere containing 5% CO₂. HEK293 cells were transiently transfected with 1 µg of DNA per ml of cell culture medium by a modified calcium phosphate method [33]. The cells were harvested 36 h post transfection and lysed with ice-cold lysis buffer [21].

Primary bone marrow-derived macrophages were isolated from mice [34]. Cells were maintained on bacterial grade plates for 1 week in DMEM supplemented with 10 % heat inactivated FBS, 2 mM L-glutamine, 100 Units/ml penicillin G, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin and 5 ng/ml macrophage colony-stimulating factor. Adherent cells were then replated on tissue culture plastic dishes in fresh media and used 24 hours after replating.

Wild type, MyD88^{-/-} [35], TRIF^{-/-} [36] and MyD88^{-/-}/TRIF^{-/-} [7] immortalised bone marrow cell lines were generated as described in [37] and maintained in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 2% (v/v) Foetal Calf Serum, 0.03% Primatone, supplemented with IL-6 and stem cell factor. To initiate experiments, the immortalised bone marrow cells were collected, washed and re-plated in 60 mm Petri dishes at a density of 0.5 x 10⁵. The cells were then incubated for 4-5 days with IMDM supplemented with 2% (v/v) FCS and 30% (v/v) L929-conditioned medium to differentiate them into macrophages.

shRNA mediated knockdown of Pellino 1.

RAW264.7 macrophages were transfected with shRNAs (shRNA[216] and shRNA[1239]) contained within the pSUPER-GFP vector (Oligoengine) that “knocks-down” the expression of Pellino 1 in cells. The shRNAs were co-transfected with pCMV5-Pellino 1 into HEK293 cells by a modified calcium phosphate method as described above or transfected into RAW264.7 macrophages using lipofectamine (Invitrogen), according to the manufacturer's instructions. Transfected RAW264.7 macrophages expressing the plasmids were selected and grown in the presence of G418 [500 µg/ml].

Real time PCR.

Total RNA was extracted from 1x10⁶ RAW264.7 cells using Qiagen RNeasy kit according to the manufacturer's instructions. 5 µg of total RNA was reverse transcribed into cDNA for 1 h at 50 °C using oligo dT primer and reverse transcriptase in the presence of RNase inhibitor. Transcribed cDNA template (50 ng) was incubated with 200 nM primers in a total volume of 20 µl using KAPA SYBR FAST qPCR kit. The following primers were used: Pellino 1-F [ctgctgaaaccagatcagctcagcagag]; Pellino 1-R [ccgagctgcattgatctctctgtcttaaagc]; GAPDH-F [ccatgccatcactgccaccagaa]; GAPDH-R [gtccaccacctgttgctgtagccg].

Immunoprecipitation of Pellino 1.

Cell lysate protein (2 mg) was incubated for 16 h at 4°C with 1 µg of anti-Pellino 1 antibody attached to Protein G-Sepharose (7.5 µl packed beads) on a rotating wheel. The beads were collected by brief centrifugation, washed three times in 0.5 ml of 50 mM Tris-HCl pH 7.5, 1 % (w/v) Triton-X100, 0.05 % (v/v) 2-mercaptoethanol, 0.2 M NaCl and once in 50 mM Tris-HCl pH 7.5, 5 mM MgCl₂.

Analysis of phosphorylation by Mass Spectrometry and Edman sequencing.

Phosphorylated proteins were separated by SDS-PAGE, stained with Colloidal Coomassie Blue and digested with trypsin [38]. An aliquot of the digest was analysed by LC-MS with multistage activation on a ThermoElectron LTQ-orbitrap system coupled to a Proxeon easy nLC system. The resultant MS/MS spectra were searched by using Mascot (Matrixscience) run on a local server, allowing for phosphoserine/phosphothreonine and phosphotyrosine. The mass tolerances used were +/- 20 ppm. Edman degradation of ³²P labeled peptides was performed by solid phase sequencing as described previously [39].

Results.

Inflammatory stimuli induce the expression of Pellino 1 via a TRIF dependent pathway.

Pellino was originally identified as a protein that interacts with the IRAK subfamily of protein kinases (see Introduction). We therefore initially stimulated primary BMDM with the TLR4 agonist LPS, which signals through the adaptor protein MyD88 and the IRAK kinases, as well as via the adaptor TRIF. Stimulation with this agonist was found to induce a striking increase in the expression of the Pellino 1 protein, beginning after 2 h and reaching a maximum level after 4-6 h (Top panels of Figs 2A and 2B). This level of expression was maintained for at least 24 h (results not shown).

To investigate whether the induction of Pellino 1 resulted from the activation of the MyD88-dependent or the TRIF-dependent signalling pathway, we repeated the experiments using primary BMDM from MyD88^{-/-} or TRIF^{-/-} mice. These experiments showed that the LPS-stimulated increase in the expression of Pellino 1 protein was similar in MyD88^{-/-} or wild type BMDM, but almost abolished in BMDM from TRIF^{-/-} mice (Top panel of Figs 2A and 2B). These experiments indicated that the induction of Pellino 1 was mediated via the TRIF signalling pathway, which is not thought to involve the IRAK subfamily of protein kinases [40].

We then stimulated primary BMDM with poly(I:C), a TLR3 agonist that only signals through the adaptor TRIF. Stimulation with poly(I:C) also strongly induced the expression of Pellino 1, in a manner similar to LPS, but with slightly delayed kinetics, beginning at 3 h and increasing up to 6 h. As expected, induction by poly(I:C) did not occur in TRIF^{-/-} BMDM and was unaffected in MyD88^{-/-} BMDM (middle panels Figs 2A and 2B).

Stimulation of primary BMDM with lipoteichoic acid (LTA), a TLR2/6 agonist that signals only through the adaptor MyD88 induced a weaker and slower increase in Pellino 1 expression, which was only revealed by a much longer exposure of the immunoblots. As expected, the weak LTA-induced expression of Pellino 1 was abolished in MyD88^{-/-} BMDM but unaffected in TRIF^{-/-} BMDM (bottom panel of Figs 2A and 2B).

We confirmed these findings using immortalised MyD88^{-/-} and TRIF^{-/-} macrophage cell lines, and an immortalised macrophage cell line lacking expression of both MyD88 and TRIF. These experiments showed that the induction of Pellino 1 after 6 h was considerably decreased in the TRIF^{-/-} cells and completely abolished in the TRIF^{-/-}/MyD88^{-/-} double knockout cells (Fig 2C). Taken together, these experiments demonstrate that signaling downstream of TRIF is the major contributor to the induction of Pellino 1 by LPS, but that the MyD88 pathway is also capable of inducing Pellino 1 expression to a small extent.

We also examined the induction of Pellino 1 by the TLR7 agonist R848, the TLR9 agonist CpG and IL-1 in the RAW264.7 macrophage-like cell line. These agonists, which all signal solely through the MyD88 pathway did not cause a significant increase in the expression of Pellino 1 expression (Fig 2D).

The induction of Pellino 1 is prevented by inhibitors of TBK1 and IKK ϵ and is dependent on the presence of IRF3.

Signaling via TRIF leads to the activation of the transcription factor IRF3 and the production of IFN β via a pathway that requires the IKK-related protein kinases, TBK1 and IKK ϵ , or to the activation of the transcription factor NF κ B by a pathway that is independent of the IKK-related kinases [29]. To examine which arm of the TRIF signalling pathway was required for the induction of Pellino 1 we utilized MRT67307, a potent and specific inhibitor of TBK1 and IKK ϵ that completely suppresses the phosphorylation of IRF3 (Fig 3A) and the production of IFN β , without suppressing the activation of NF κ B by ligands that activate TLR3 or TLR4 [29]. These studies showed that MRT67307 completely blocked the induction of Pellino 1, indicating a requirement for TBK1/IKK ϵ kinase activity (Fig 3A).

We then examined whether the LPS-induced increase in Pellino 1 observed at the protein level resulted from an increase in the Pellino 1 mRNA transcript. These experiments revealed that the mRNA encoding Pellino 1 was greatly elevated after 6 h (Fig 3B). Inhibition of IKK ϵ /TBK1 did not affect the decay in the levels of mRNA that had been elevated by LPS (Fig 3C), indicating that the TRIF-TBK1/IKK ϵ -dependent pathway activates transcription of the Pellino 1 gene.

To investigate whether the induction of Pellino 1 occurred through activation of the transcription factor IRF3, or another transcription factor activated downstream of TBK1/IKK ϵ , we used primary BMDM isolated from IRF3^{-/-} mice (Fig 3D). These experiments revealed that the LPS- or poly(I:C)-stimulated induction of Pellino 1 was abolished in BMDM from IRF3^{-/-} mice (Fig 3E).

The finding that Pellino 1 was not induced in IRF3^{-/-} macrophages did not exclude the possibility that IRF3 stimulated the production of interferon β , which then stimulated the transcription of Pellino 1. To address this issue, we carried out experiments with BMDM from mice that do not express the receptor for type 1 interferons and therefore fail to respond to interferon β , as judged by the failure to stimulate STAT1 phosphorylation (Figs 3F). Stimulation of these cells with poly(I:C) or LPS showed that the induction of Pellino 1 was only reduced slightly relative to BMDM from control mice (Fig 3G). This small reduction reflects the ability of IFN β to stimulate a weak induction of Pellino 1, by a mechanism that is independent of TBK1 and IKK ϵ (Supplementary Fig 1).

Taken together, our experiments demonstrate that the TRIF-dependent signaling pathway activates TBK1/IKK ϵ , which then phosphorylate IRF3, stimulating its translocation to the nucleus where it stimulates transcription of the genes encoding interferon β and Pellino 1. Interferon β can then induce a modest further increase in Pellino 1 transcription via the JAK-STAT1 pathway.

TBK1 and IKK ϵ phosphorylate and activate the E3 ligase activity of Pellino 1 *in vitro*.

The finding that TBK1 and IKK ϵ control the expression of Pellino 1 was interesting because, in a separate study, we had identified Pellino 1 as an IKK ϵ -interacting protein in a yeast two-hybrid screen using IKK ϵ as bait. Four positive clones were identified as Pellino 1 after 10⁶ yeast colonies were screened from a human foetal brain cDNA library, which encoded residues 12-268, 13-266, 1-283, 1-309 of Pellino 1. This region of Pellino 1 contains the Forkhead-associated (FHA) domain, a phosphoserine/phosphothreonine-binding motif that is thought to interact with the phosphorylated form of IRAK1. Since IKK ϵ and TBK1, like IRAK1, are phosphorylated at multiple serine and threonine residues in LPS-stimulated macrophages [41], this raised the possibility that Pellino 1 might also be a substrate of IKK ϵ and/or TBK1.

The phosphorylation of Pellino 1 by IRAK4 *in vitro* can be monitored by a decrease in its mobility on SDS-PAGE (Fig 4A, lane 6). We found that incubation with MgATP and TBK1 (Fig 4A) or IKK ϵ (Fig 4B) produced a similar decrease in the electrophoretic mobility of Pellino 1 (Fig 4A and 4B, lane 2). Pre-incubation with MRT67307 completely blocked the TBK1 or IKK ϵ -induced decrease in the mobility of Pellino 1 (Figs 4A and 4B, lane 3) without affecting the IRAK4-induced "band shift" (Fig 4A, lane 7). Conversely, pre-incubation with an IRAK4 inhibitor, prevented the IRAK4-catalysed "band-shift" of Pellino 1 (Fig 4A, lane 8), but not the TBK1 or IKK ϵ -catalysed decrease in mobility (Figs 4A and 4B, lane 4).

The E3 ligase activity of bacterially expressed Pellino 1 can be studied *in vitro* by its ability to form free Lys63-linked polyubiquitin chains, not anchored to any other protein, in a ubiquitylation assay containing the E2-conjugating enzyme Ubc13-Uev1a [21, 23]. Using this assay, we showed that the phosphorylation of Pellino 1 by TBK1 (Fig 4C) or IKK ϵ (Fig 4D) greatly enhanced the E3 ubiquitin ligase activity of Pellino 1, in a similar manner to that reported previously for IRAK1 or IRAK4 [21]. The unphosphorylated form of Pellino 1 only induced the formation of small ubiquitin oligomers after 10 or 30 min, but much larger, more slowly migrating polyubiquitin chains were formed over the same period following phosphorylation by TBK1 or IKK ϵ (Figs 4C and 4D).

To establish that the activation of Pellino 1 had resulted from phosphorylation, we stopped the TBK1 and IKK ϵ -catalysed phosphorylation with MRT67307 and showed that treatment with the protein serine/threonine phosphatase produced by bacteriophage λ gt10 increased the electrophoretic mobility of Pellino 1 to that of the unphosphorylated protein and abolished its E3 ligase activity (Figs 5A and 5B). Moreover, incubation of TBK1 or IKK ϵ with MRT67307 followed by incubation with Pellino 1 and MgATP did not cause any enhancement of the E3 ligase activity of Pellino 1 (Figs 5C and 5D). Taken together, these experiments demonstrated that the activation of Pellino 1 by TBK1/IKK ϵ had resulted from phosphorylation and not a protein-protein interaction between TBK1/IKK ϵ and Pellino 1.

TBK1 and IKK ϵ phosphorylate the key activating sites on Pellino 1 *in vitro*.

We have previously identified 10 Ser/Thr residues on Pellino 1 that are phosphorylated by IRAK1 and IRAK4 *in vitro*. Mutagenesis studies showed that the phosphorylation of any one of the three residues Ser76, Ser288 or Thr293 was capable of fully activating the E3 ligase activity of Pellino 1, and full activation could also be achieved

by the combined phosphorylation of residues Ser78, Thr80 and Ser82 [23]. A Pellino 1 mutant in which all 10 phosphorylation sites have been changed to Ala (termed 10A Pellino 1) cannot therefore be phosphorylated or activated by IRAK1 (Fig 6A, lane 6). This mutant could also not be phosphorylated by TBK1 (Fig 6A, lane 5) implying that sites on Pellino 1 phosphorylated by TBK1 must overlap with those phosphorylated by IRAK1 and IRAK4.

To identify the sites on Pellino 1 phosphorylated by TBK1, we phosphorylated Pellino 1 with this protein kinase or IRAK1 in the presence of Mg- $[\gamma^{32}\text{P}]\text{ATP}$. Following digestion with trypsin, the tryptic phosphopeptides were separated by reverse phase HPLC (Figs 6B and 6C). The major phosphopeptides were identified by mass spectrometry and the phosphorylation sites were identified by solid phase sequencing (Fig 7). The results, summarized in Supplementary Table 1, showed that the major sites phosphorylated by TBK1 were Ser76, Thr80, Thr288 and Ser293, which include the three key activating sites (Ser76, Thr288 and Ser293). We have shown previously that the phosphorylation of any one of these residues triggers the full activation of the E3 ligase activity of Pellino 1 (see Introduction).

In Fig 6C peak 2 contained a diphosphorylated peptide, phosphorylated at Ser76 and Thr80, while peak 4 contained a peptide phosphorylated at Ser76 alone (Fig 7). Peaks 5 and 6 contained diphosphorylated peptides, which were phosphorylated at Thr288 and Ser293 (Fig 7). In contrast the slightly later-eluting tryptic peptides 5 and 6 generated after phosphorylation by IRAK1 (Fig 6B) were phosphorylated at Thr288 alone. Peptide 6 from the IRAK1 digest also contained a peptide phosphorylated at Thr127, a site not phosphorylated by TBK1. Analysis of the earlier eluting peptides 1-4 confirmed that, in addition to Ser76 and Thr80, IRAK1 also phosphorylates Ser82, a residue not phosphorylated by TBK1.

TBK1 and IKK ϵ phosphorylate and activate Pellino 1 in LPS or poly(I:C)-stimulated macrophages.

To investigate whether TBK1 and IKK ϵ could activate the E3 ligase activity of endogenous Pellino 1 by phosphorylation in cells, we stimulated the RAW264.7 macrophage cell line with LPS or poly(I:C) and then measured the E3 ligase activity of Pellino 1 after immunoprecipitating it from the cell extracts. The E3 ligase activity of Pellino 1 was low in unstimulated cells but, following stimulation with LPS, an increase in activity was detectable after 15 min, which reached a maximum after 30 min (Fig 8A). Stimulation with poly(I:C) also led to an increase in the activity of Pellino 1, with maximal activation observed after 45 min (Fig 9A). The LPS or poly(I:C) induced activation of Pellino 1 was abolished by incubating the Pellino 1 immunoprecipitates with phage λ phosphatase, and this could be prevented by NaF, a general inhibitor of protein Ser/Thr phosphatases (Fig 8B and 9B). Pre-treatment of the macrophages with the TBK1/IKK ϵ inhibitor MRT67307 prevented the LPS or poly(I:C)-stimulated activation of Pellino 1 as well as the phosphorylation of IRF3 at Ser396 (Fig 8C and 9C), consistent with activation being catalysed by TBK1/IKK ϵ .

The results presented above were consistent with the endogenous Pellino 1 E3 ubiquitin ligase being activated by a TBK1/IKK ϵ -catalysed phosphorylation event, but did not establish whether this occurred as a consequence of the phosphorylation of Pellino 1 itself or an associated protein(s). In contrast to the decreased electrophoretic mobility that accompanies the phosphorylation of Pellino 1 by TBK1/IKK ϵ or IRAK1/IRAK4 *in vitro*, activation of endogenous Pellino 1 by LPS or poly(I:C) did not appear to alter its electrophoretic mobility in RAW264.7 macrophages, at least as judged by immunoblotting. This may be explained by the inability of phosphorylated Pellino 1 to be recognised by the Pellino 1 antibody. However, we did notice that, after stimulation of RAW264.7 macrophages for 30-60 min with LPS there was a small, but consistent decrease in the level of Pellino 1 detected by immunoblotting. Since one explanation for this apparent decrease was that unmodified Pellino 1 had been converted to a number of slower migrating phosphorylated species, each of which was present at too low an abundance to be detected by immunoblotting, we incubated Pellino 1 immunoprecipitates with phage λ phosphatase. This restored the level of detected endogenous Pellino 1 to that present in unstimulated cells, but did not affect the level of endogenous Pellino 1 detected in unstimulated cells (Fig 8D). This experiment provides further evidence that the LPS-induced activation of the E3 ubiquitin ligase activity of endogenous Pellino 1 results from the direct phosphorylation of the protein.

To identify the residues that became phosphorylated in response to LPS, we immunoprecipitated endogenous Pellino 1 from LPS-stimulated RAW264.7 macrophages,

digested with trypsin and analysed the resulting tryptic phosphopeptides using an orbitrap velos mass spectrometer. All the endogenous Pellino 1 protein was immunoprecipitated from the cell extracts, as judged by immunoblotting, but we were unable to detect Coomassie blue-staining band corresponding to Pellino 1 after SDS-PAGE. This observation indicates that Pellino 1 is expressed at extremely low levels in unstimulated macrophages. Coupled with low levels of phosphorylation of individual serine/threonine residues in endogenous Pellino 1 and the fact that phosphorylation of any one of three sites induces the full activation of Pellino 1 *in vitro*, this may explain why we have been unable, thus far, to identify phosphorylation sites in the endogenous protein.

Discussion.

In this paper, we show that the expression of the Pellino 1 protein is greatly increased by TLR3 and TLR4 agonists by a TRIF-TBK1/IKK ϵ -IRF3-dependent pathway. Interferon β , which is also induced by this pathway, stimulated a smaller increase in Pellino 1 expression by a TBK1/IKK ϵ -independent pathway, creating a positive autocrine loop, which may make a minor contribution to the overall increase in Pellino 1 expression. Why such a striking increase in the level of Pellino 1 is needed is currently unknown, but since Pellino 1 is an IRF3-regulated gene, we speculate that this may be required to regulate the production of interferon β and/or other IRF3/interferon-regulated genes. To address this important question we have recently generated knock-in mice in which wild type Pellino 1 is replaced by an E3-ligase deficient mutant.

The expression of IKK ϵ is also increased by LPS and poly(I:C), as well as by other MyD88-dependent TLR agonists, and for this reason has also been called IKK i (IKK inducible). We have observed that the time course of induction of IKK ϵ in macrophages is slightly delayed compared to the induction of Pellino 1 (results not shown) and occurs via a different pathway, because it is not blocked by the IKK ϵ /TBK1 inhibitors BX795 [42] or MRT67307 [29]. Therefore, the induction of Pellino 1 is not a consequence of the increased expression of IKK ϵ or vice versa.

The structure of Pellino 1 consists of a Forkhead-associated (FHA) domain and a "wing appendage" that loops out from the FHA domain [43] and a C-terminal RING (Really Interesting New Gene) domain that carries the E3 ubiquitin ligase activity. FHA domains binds to phospho-serine and phospho-threonine residues in proteins, which may explain why wild type IRAK1, which phosphorylates itself at several residues, but not a catalytically inactive mutant of IRAK1, interacts with Pellino isoforms in co-transfection experiments [22]. Our yeast two-hybrid screening using IKK ϵ as bait revealed that this protein kinase also interacts with the FHA domain of Pellino 1, but further work is needed to establish whether this interaction is phosphorylation-dependent.

The finding that IKK ϵ interacts with Pellino 1 led us to discover that IKK ϵ and TBK1 phosphorylate Pellino 1 *in vitro*, activating its E3 ligase activity. Phosphorylation occurred at the same three key activating sites that are phosphorylated by IRAK1 or IRAK4 *in vitro*, namely Ser76 in the "wing appendage" and Thr288 and Ser293 at the start of the RING domain [23]. Studies on the specificity of IKK ϵ using small synthetic peptide substrates have shown that this protein kinase preferentially phosphorylates Ser residues in which Leu, Ile, Met or Phe are preferred at the n+1 position, Tyr, Phe or Trp at the n+3 position and Tyr, Phe, Pro or Met at the n-2 position, where n is the site of phosphorylation [44]. The sequence surrounding Thr288 (Phe-Asn-Thr-Leu-Ala-Phe) conforms to this consensus, while the sequences surrounding Ser 76 (Gln-His-Ser-Ile-Ser-Tyr) and Ser293 (Phe-Pro-Ser-Met-Lys-Arg) each contain two of the three preferred residues. The phosphorylation of Pellino 1 by IKK ϵ /TBK1 may also be enhanced by interaction between the FHA domain of Pellino 1 and the phosphorylated forms of these protein kinases. Whether TBK1 or IKK ϵ plays the major role in activating Pellino 1's E3 ligase activity is unclear, since the pharmacological inhibitor MRT67307 used in this study does not discriminate between these closely related kinases.

Pellino 1 $^{-/-}$ mice were reported to produce less tumour necrosis factor α (TNF α) and IL-6 *in vivo* after injection of poly(I:C) or LPS, which may explain why these mice are resistant to poly(I:C) or LPS-induced septic shock [26]. Embryonic fibroblasts and splenocytes from the Pellino 1 $^{-/-}$ mice showed decreased activation of the canonical IKKs and decreased binding of NF κ B to DNA in response to poly(I:C) or LPS. They also produced decreased levels of the mRNAs encoding TNF α and IL-12 in response to LPS or poly(I:C) in

MEFs and dendritic cells and decreased secretion of IL-12 in LPS or poly(I:C)-stimulated BMDM [26]. From these and other experiments these investigators concluded that Pellino 1 is required for the TRIF-dependent activation of NF κ B and that it plays a non-redundant function in this pathway that cannot be compensated for by either Pellino 2 or Pellino 3. However, we have found that pharmacological inhibition of TBK1/IKK ϵ , which blocks the poly(I:C)- or LPS-stimulated induction of Pellino 1 (Fig 3), as well as the activation of its E3 ligase activity (Figs 8 and 9), does not inhibit the poly(I:C) or LPS-stimulated activation of IKK α /IKK β , the phosphorylation of their substrate p105 or NF κ B-dependent gene transcription in RAW264.7 macrophages [29]. Therefore, either the requirement of Pellino 1 for the TRIF-dependent activation of NF κ B is cell-specific, or Pellino 1 has a function that is independent of its E3 ligase activity. To address this issue and physiological roles of the E3 ligase activity of Pellino 1, we have recently generated knock-in mouse in which wild type Pellino 1 is replaced by an E3 ligase-deficient mutant.

The finding that LPS or poly(I:C) activate Pellino 1's E3 ligase activity via TBK1/IKK ϵ and not via IRAK1/IRAK4 was unexpected and raises the question of how Pellino 1 and other Pellino isoforms are activated by agonists that signal via MyD88. We have recently observed that the IL-1 stimulated activation of the E3 ligase activity of endogenous Pellino 1 was not suppressed by pharmacological inhibition of TBK1/IKK ϵ (E.Goh and P.Cohen, unpublished experiments). Whether this is catalysed by IRAK1 and/or IRAK4 or another protein kinase(s) will require further analysis. Nevertheless our results clearly indicate that Pellino isoforms are activated by at least two classes of protein kinases *in vivo*.

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Figure Legends.

Figure 1. Characterisation of a Pellino 1-specific antibody. (A) HEK293 cells were transfected with plasmids encoding Flag-Pellino 1, Flag-Pellino 2 or Flag-Pellino 3. Cell lysates were subjected to SDS-PAGE and immunoblotted with antibodies that recognise Pellino 1, Flag and actin. (B) Murine RAW264.7 macrophages were transfected with plasmids shRNA[216] or shRNA[1239] or empty vector, and the cells then selected with G418. The efficiency of “knock-down” of the endogenous mouse Pellino 1 was assessed by immunoblotting of cell lysates with the anti-Pellino 1 antibody. (C) The experiment was carried out as in B, except that HEK293 cells were transiently co-transfected with shRNA[216] or shRNA[1239] and Flag-human Pellino 1. “Knock-down” of the transfected Pellino 1 was measured by immunoblotting of the cell lysates with an anti-Flag antibody.

Figure 2. Inflammatory stimuli induce the expression of Pellino 1 predominately via a TRIF-dependent pathway. (A and B) Primary BMDM derived from (A) wild type (TRIF^{+/+}) and TRIF^{-/-} mice or (B) wild type (MyD88^{+/+}) and MyD88^{-/-} mice, were stimulated with 100 ng/ml LPS (Alexis Biochemicals), 10 µg/ml poly(I:C) (InvivoGen) or 2 µg/ml LTA for the times indicated. The lysate from 10⁵ cells was resolved by SDS-PAGE, transferred to polyvinylidene fluoride membranes and immunoblotted with antibodies that recognise Pellino 1 or α tubulin. (C) Immortalised macrophages from wild type, MyD88^{-/-}, TRIF^{-/-} and MyD88^{-/-}/TRIF^{-/-} double “knock-out” mice were stimulated for 6 h with 100 ng/ml LPS (Sigma) and immunoblotted with anti-Pellino 1 and anti-actin antibodies. (D) The RAW264.7 macrophage-like cell line was treated for the times indicated with 100 ng/ml LPS (Sigma), 100 µg/ml poly(I:C) (Alexis Biochemicals), 10 µM R848, 5 µM CpG or 50 ng/ml IL-1 β . The cell lysates were subjected to SDS-PAGE and immunoblotted as in C.

Figure 3. The induction of Pellino 1 is prevented by inhibition of TBK1 and IKK ϵ and requires IRF3.

(A) Primary wild type BMDM were pre-treated for 1 h without (-) or with (+) 2 µM MRT67307, then stimulated for the times indicated with 100 ng/ml LPS (Alexis Biochemicals). Cell lysates were subjected to SDS-PAGE and then immunoblotted with antibodies recognising Pellino 1, IRF3 phosphorylated at Ser-396 and α tubulin. (B) Total mRNA from RAW264.7 macrophages treated for 6 h with or without 100 ng/ml LPS (Sigma) was reverse transcribed. The cDNA was subjected to real-time PCR using gene-specific primers for Pellino 1. The level of Pellino 1 is expressed relative to the level of Pellino 1 mRNA in unstimulated cells using glyceraldehydes 3-phosphatase dehydrogenase (GAPDH) as a reference standard. The results show triplicate determinations from two independent experiments. (C) RAW264.7 macrophages were stimulated for 6 h without (open triangles) or with (open and closed circles) 100 ng/ml LPS (Sigma). Transcription was then inhibited by the addition of Actinomycin D to a final concentration of 5 µg/ml (arrow, Act) with (closed circles) or without (open circles) 1 mM BX795, an equally potent but slightly less specific inhibitor of TBK1/IKK ϵ . The decay of mRNA was then monitored at the times indicated. Cells were harvested, the mRNA was isolated and reverse transcribed, and the resulting cDNA was quantitated as in B. The cDNA was measured as in B. The results shown are the mean (\pm SD) from duplicate samples and similar results were obtained in three separate experiments. (D) Primary BMDM from IRF3^{+/+} and IRF3^{-/-} mice were lysed, and lysates equivalent to 10⁷ cells were subjected to SDS-PAGE and immunoblotted for the presence of IRF3 or α tubulin as a loading control. (E) BMDM from IRF3^{+/+} and IRF3^{-/-} mice were stimulated with 100 ng/ml LPS (Alexis Biochemicals), or 10 µg/ml poly(I:C) (InvivoGen) for the times indicated. Following cell lysis, an aliquot of each lysate was subjected to SDS-PAGE, transferred to polyvinylidene fluoride membrane and immunoblotted with antibodies that recognise Pellino 1 or α tubulin. (F) IFN α β R^{+/+} or IFN α β R^{-/-} BMDMs were stimulated for 30 min with 500 U/ml interferon (IFN) β and cell lysates were subjected to SDS-PAGE and immunoblotted for STAT1 α and STAT1 β phosphorylated at Tyr701 or α tubulin. (G) BMDM from IFN α β R^{+/+} or IFN α β R^{-/-} mice were stimulated for 6 h with 100 ng/ml LPS or 10 µg/ml poly(I:C) (InvivoGen). The cell lysates were subjected to SDS-PAGE and immunoblotted with antibodies that recognise Pellino 1 or α tubulin.

Figure 4. TBK1 and IKK ϵ phosphorylate Pellino 1 and enhance its E3 ubiquitin ligase activity. (A) Pellino 1 (1 μ M) was incubated for 30 min at 30 °C with MgATP in the presence (+) or absence (-) of 2 U/ml IRAK4 or 2 U/ml TBK1 in 50 mM Tris/HCl (pH7.5) which had been preincubated in the absence (-) or presence (+) of 2 μ M MRT67307 or 10 μ M IRAK4 inhibitor. The reactions were terminated by denaturation in SDS, subjected to SDS-PAGE, and the gel was stained with Coomassie Blue. The positions of unphosphorylated and phosphorylated (p) forms of Pellino 1 are indicated. (B) The experiment was carried out as in as A, except that IKK ϵ was used instead of TBK1. (C and D) Pellino 1 (1 μ M), Ubc13-Uev1a (1 μ M), ubiquitin (0.1 mM), MgCl₂ (5 mM) and ATP (2mM) were incubated for 30 min at 30 °C without (-) or with (+) 2U/ml TBK1 (C) or 2U/ml IKK ϵ (D) and the ubiquitylation reactions initiated by the addition of E1 enzyme (0.1 μ M). The reactions were stopped after a further 0, 10, 30 or 60 min by the addition of SDS, subjected to SDS-PAGE, transferred to polyvinylidene fluoride membranes and immunoblotted with an anti-ubiquitin antibody. For definition of enzyme Units see [30].

Figure 5. The E3 ligase activity of Pellino 1 is regulated by reversible phosphorylation. (A and B, top panels) Pellino 1 (1 μ M) was incubated with MgATP in 50 mM Tris/HCl (pH 7.5) without (-) or with (+) 2U/ml TBK1(A) or 2 U/ml IKK ϵ (B) as in Fig 5. After 30 min the reactions were supplemented with (+) or without (-) MRT67307 to a final concentration of 2 μ M to inhibit TBK1/IKK ϵ . The reactions were then incubated for 30 min without (-) or with (+) bacteriophage λ -phosphatase (100 units) in a total volume of 0.02 ml. The gels were stained with Coomassie Blue and the position of phosphorylated (p-) Pellino 1 is indicated. (A and B bottom panels) The reactions were carried out as in the top panels, except that the E3 ubiquitin ligase activity of each sample was assessed by incubation for 10 min at 30 °C with Ubc13-Uev1a, E1 enzyme and ubiquitin followed by immunoblotting with anti-ubiquitin as in Figs 4C and 4D. (C and D) The reactions were carried out as in Figs 4C and 4D, except that where indicated, TBK1 or IKK ϵ were incubated with 2 μ M MRT67307 before the addition of Pellino 1, to prevent phosphorylation. To aid clarity, Figs 5A, B, C and D do not show the whole gel, but only the region with polyubiquitylated species of >50 kDa.

Figure 6. Comparison of the phosphorylation of Pellino 1 by TBK1 and IRAK1.

(A) The reaction was carried out as in Fig 4C and 4D but with either 1 μ M wild type (WT) Pellino 1 (lanes 1-3) or a 1 μ M 10Ala (10A) Pellino 1 (residues 70, 76, 78, 80, 82, 86, 125, 127, 288 and 293 all changed to Ala) (lanes 4-6), and with either 2 U/ml TBK1 or IRAK1. Samples of the reactions were subjected to SDS-PAGE and either immunoblotted with anti-ubiquitin (top panel), or stained with Coomassie Blue (bottom panel). To aid clarity, the figure does not show the whole gel, but only the region with polyubiquitylated species of >50 kDa. (B and C) Separation of tryptic phosphopeptides from phosphorylated Pellino 1. ³²P – labelled Pellino 1 obtained by incubation for 30 min at 30 °C with Mg- $[\gamma^{32}\text{P}]\text{ATP}$ and either (B) IRAK1 or (C) TBK1 was subjected to SDS-PAGE and the gel was stained with Coomassie Blue. The Pellino 1 was excised, digested with trypsin and subjected to reverse phase-HPLC on a Vydac C18 column equilibrated in 0.1% (vol/vol) trifluoroacetic acid. The column was developed with an acetonitrile gradient in 0.1% (vol/vol) trifluoroacetic acid. ³²P-radioactivity in arbitrary units (au) is shown by the full line, and the acetonitrile gradient is indicated by the diagonal broken lines.

Figure 7. Identification of the sites of Pellino 1 phosphorylated by TBK1.

The indicated ³²P-labelled phosphopeptides from Fig 6C were subjected to solid-phase sequencing to identify the cycles of Edman degradation at which ³²P-radioactivity (filled bars) was released from the phosphopeptides present in these fractions. The sequences of each peptide are shown using the single letter code for amino acids.

Figure 8. The LPS-stimulated activation of Pellino 1 is prevented by inhibition of TBK1/IKK ϵ and reversed by phosphatase treatment.

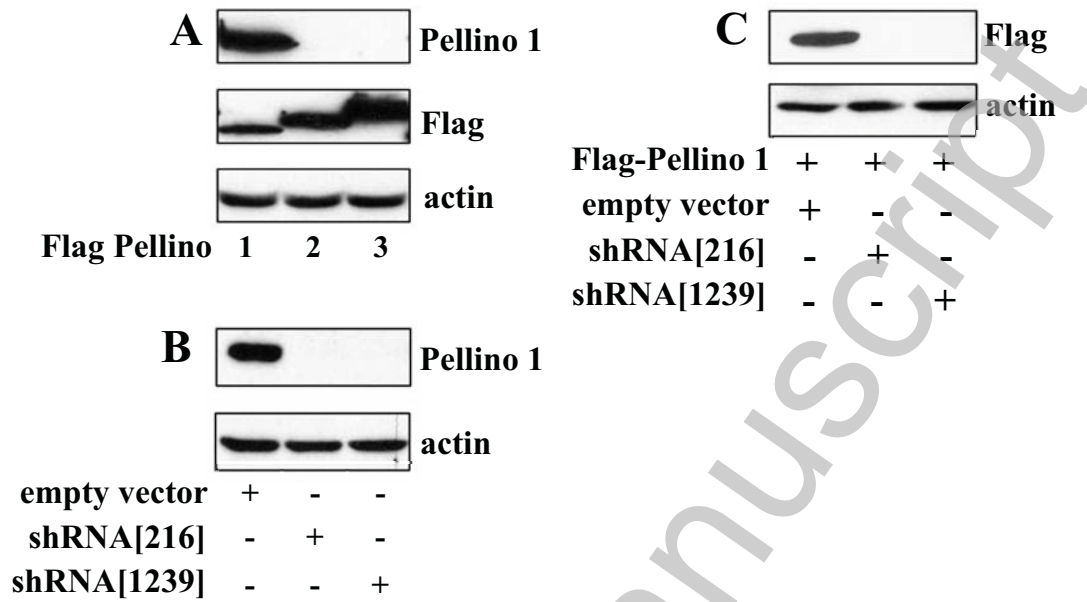
(A) RAW264.7 macrophages were stimulated with 100 ng/ml LPS (Alexis Biochemicals) for the times indicated. Pellino 1 was immunoprecipitated (see Experimental) and incubated for 60 min at 30°C in 0.02 ml of 50 mM Tris-HCl pH 7.5, 5 mM MgCl₂, UBE1 (0.1 μ M), Ubc13-Uev1a (0.05 μ M), Flag-ubiquitin (0.05 mM), ATP (2 mM) in the presence of the non-specific protein kinase inhibitor Staurosporine (75 μ M) to prevent any phosphorylation and

activation of Pellino 1 during the assay by protein kinases present in the immunoprecipitates as trace contaminants. The reactions were terminated by denaturation in SDS, and immunoblotted with an anti-Flag antibody (Top panel). Cell lysates were subjected to SDS-PAGE and immunoblotted with antibodies that recognise, Pellino 1 or IRF3 phosphorylated at Ser396 (bottom two panels). **(B)** RAW264.7 macrophages were stimulated with LPS for 30 min or left unstimulated (0 min) and Pellino 1 was immunoprecipitated as in A. The immunoprecipitates were then incubated for 30 min at 30°C with (+) or without (-) 100 units of phage λ phosphatase in the presence (+) or absence (-) of 50 mM NaF. After washing three times in 50 mM Tris-HCl pH7.5-5 mM MgCl₂, the immunoprecipitated Pellino 1 was assayed for E3 ubiquitin ligase activity as in A and cell lysates immunoblotted with the antibodies used in A. **(C)** As in B except that the RAW264.7 macrophages were incubated for 1 h without (-) or with (+) 2 μ M MRT67307, before stimulation with LPS. To aid clarity, Figs 8A, B and C do not show the whole gel, but only the region with polyubiquitylated species of >150 kDa. **(D)** RAW264.7 macrophages were stimulated with 100 ng/ml LPS (Sigma) for 60 min and the cells lysed in the absence of any phosphatase inhibitor. Cell lysate (20 μ g protein) was then incubated for 30 min at 30°C with (+) or without (-) 300 units of phage λ phosphatase in the presence of 1 mM MnCl₂. The reactions were terminated in SDS, subjected to SDS-PAGE and immunoblotted with antibodies that recognise Pellino 1 or actin. The experiment was carried out three times with similar results.

Figure 9. The poly(I:C)-stimulated activation of Pellino 1 is prevented by inhibition of TBK1/IKK ϵ and reversed by phosphatase treatment.

(A) Same as Fig 8A except that RAW264.7 macrophages were stimulated with 50 ng/ml poly(I:C) (InvivoGen) for the times indicated. **(B)** Same as Fig 8B except RAW264.7 macrophages were stimulated for 45 min with poly(I:C). **(C)** As in B except that the RAW264.7 macrophages were incubated for 1 h without (-) or with (+) 5 μ M MRT67307, before stimulation with poly(I:C). To aid clarity, Figs 9A, B and C do not show the whole gel, but only the region with polyubiquitylated species of >150 kDa.

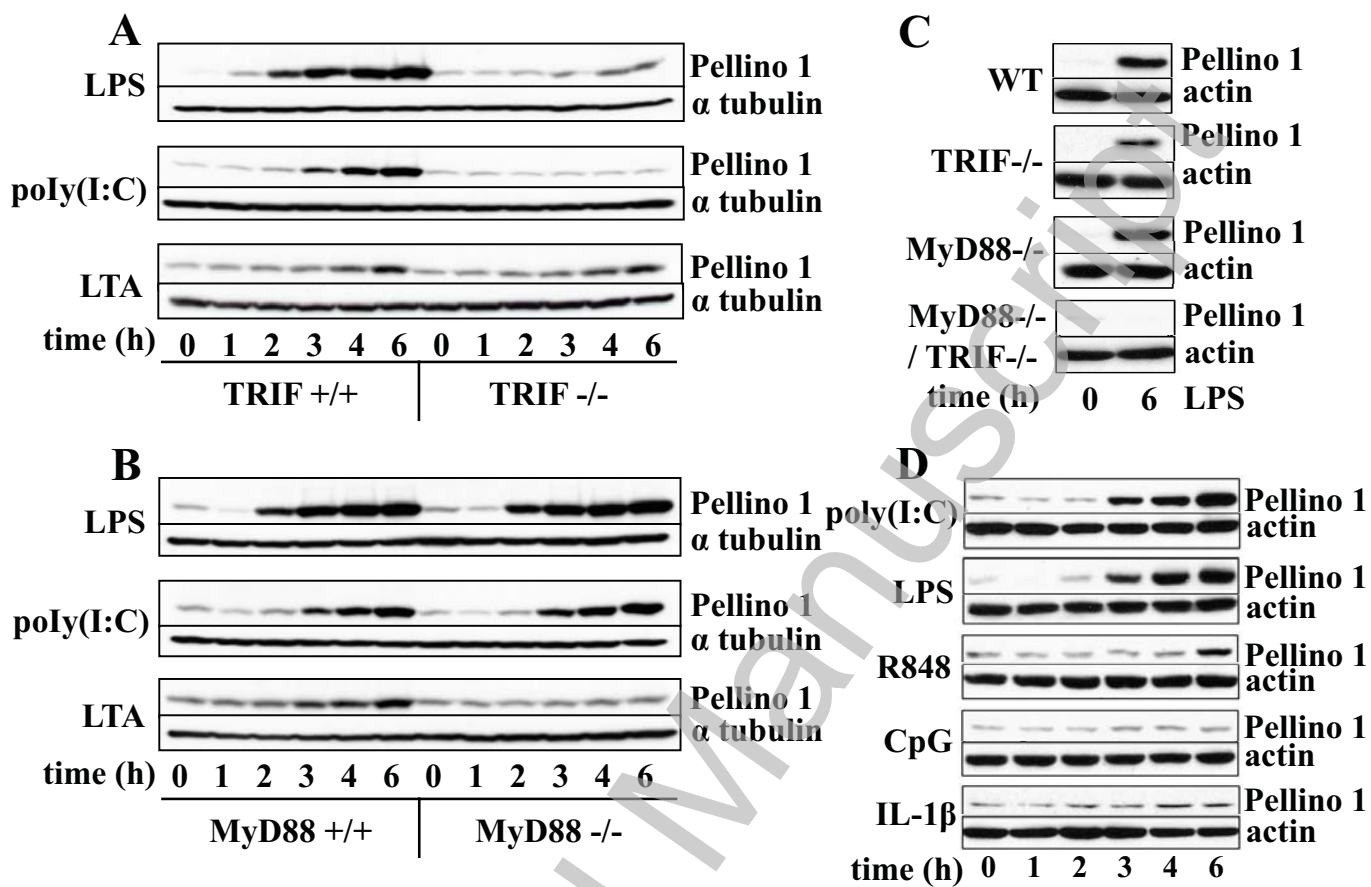
Figure 1



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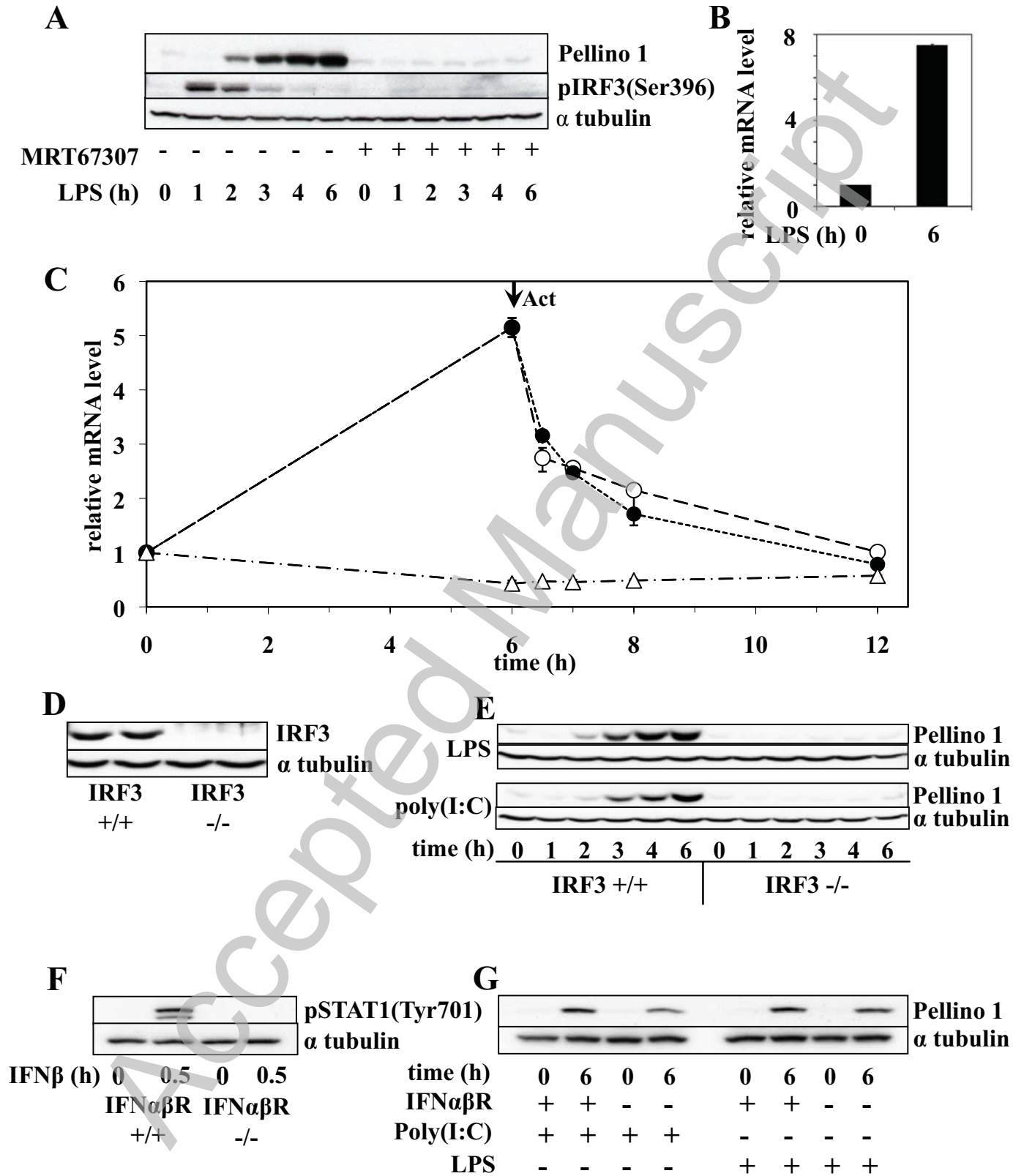
Figure 2



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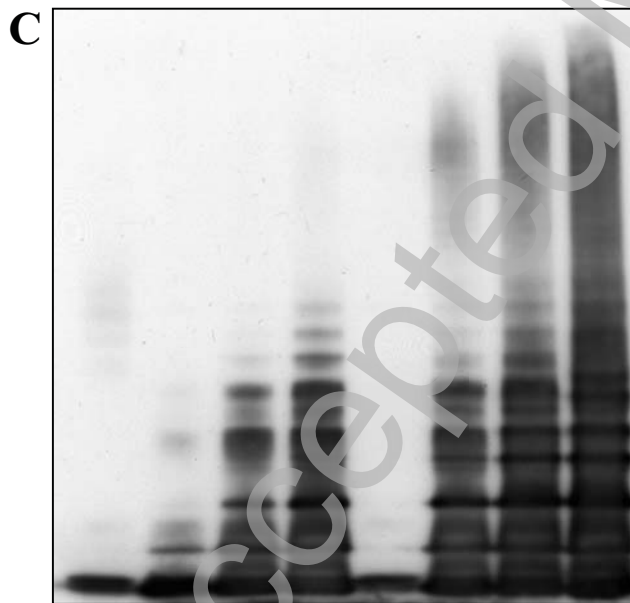
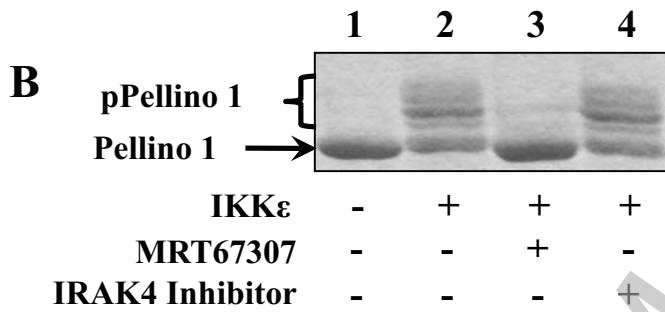
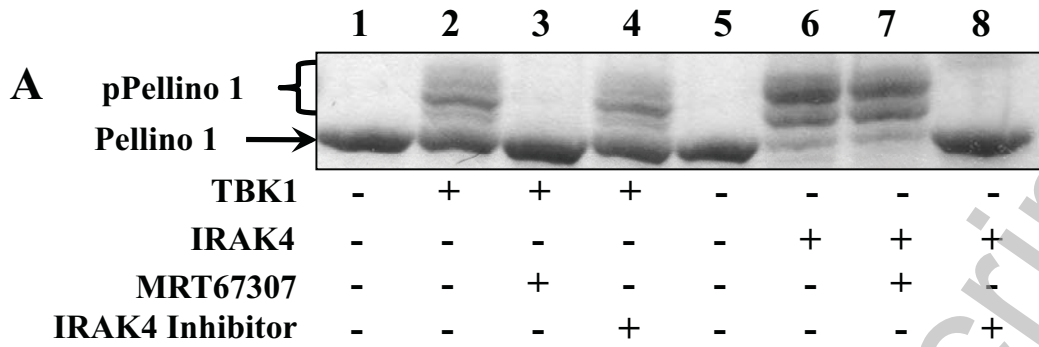
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Figure 3

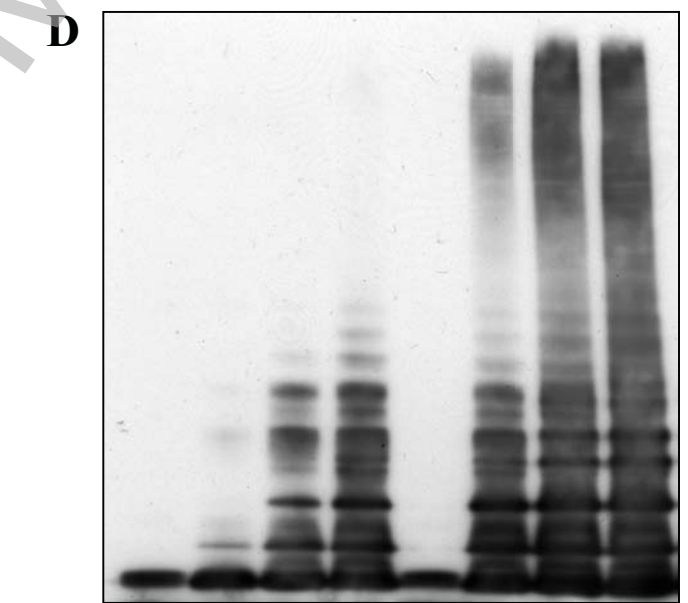


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Figure 4



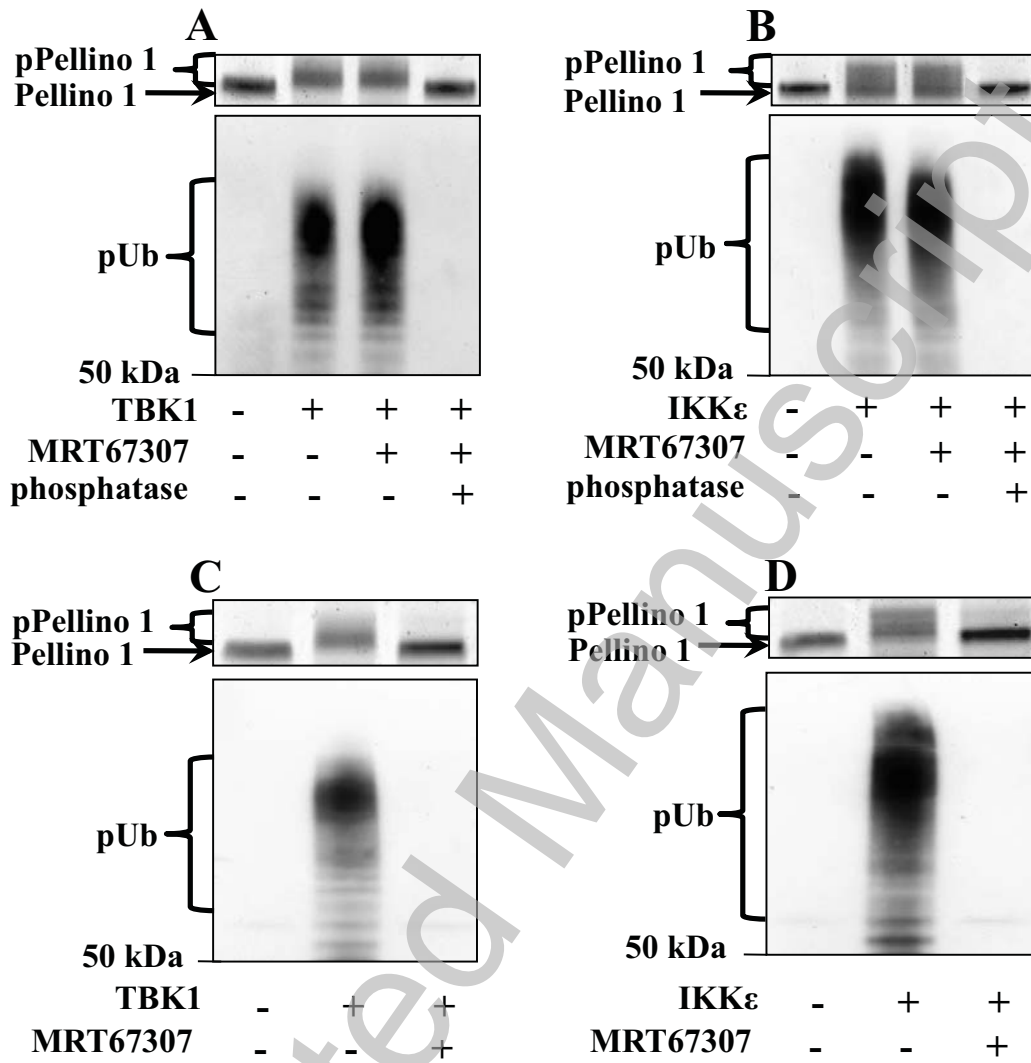
TBK1	-	-	-	-	+	+	+	+
time (min)	0	10	30	60	0	10	30	60



IKKε	-	-	-	-	+	+	+	+
time (min)	0	10	30	60	0	10	30	60

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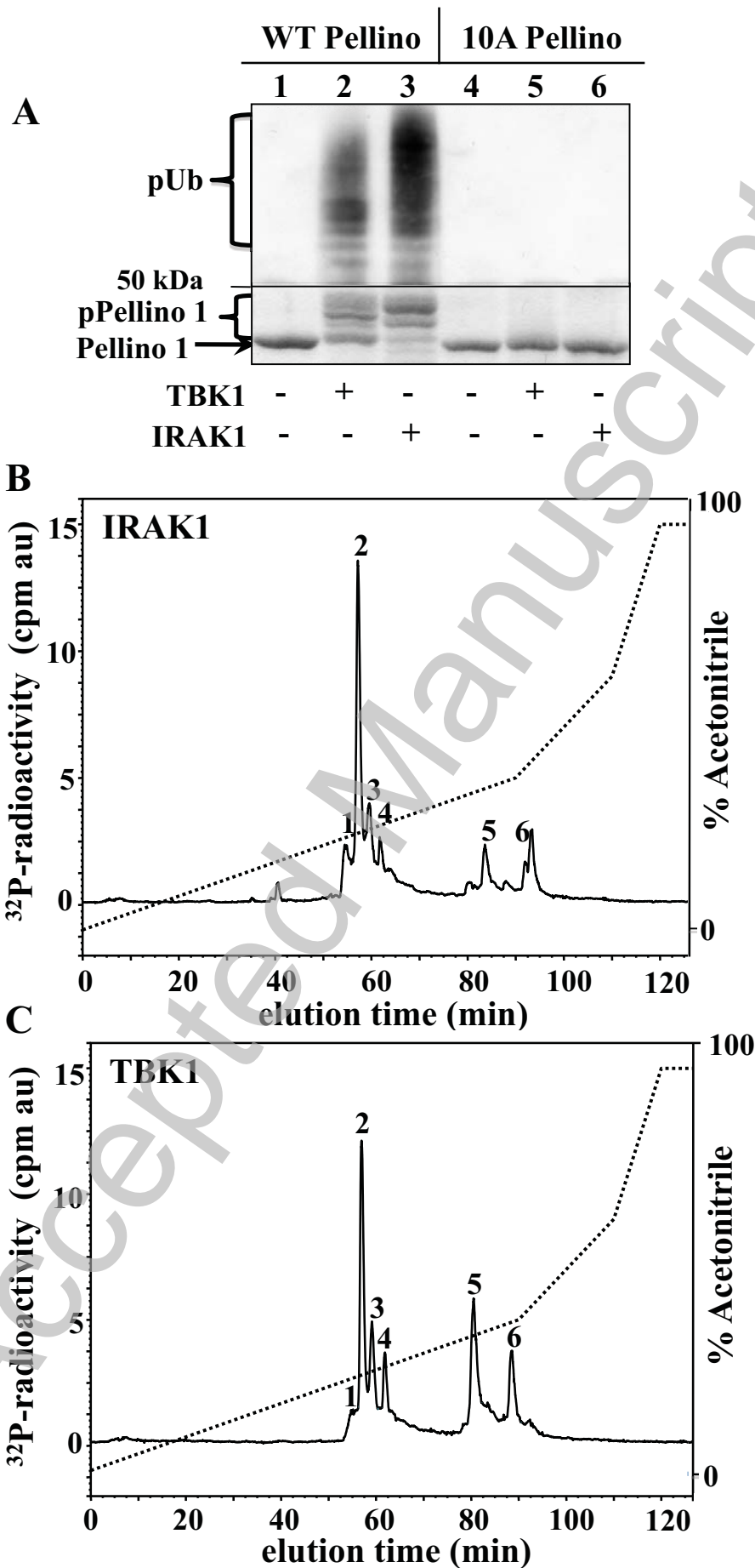
Figure 5



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Figure 6



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Figure 7

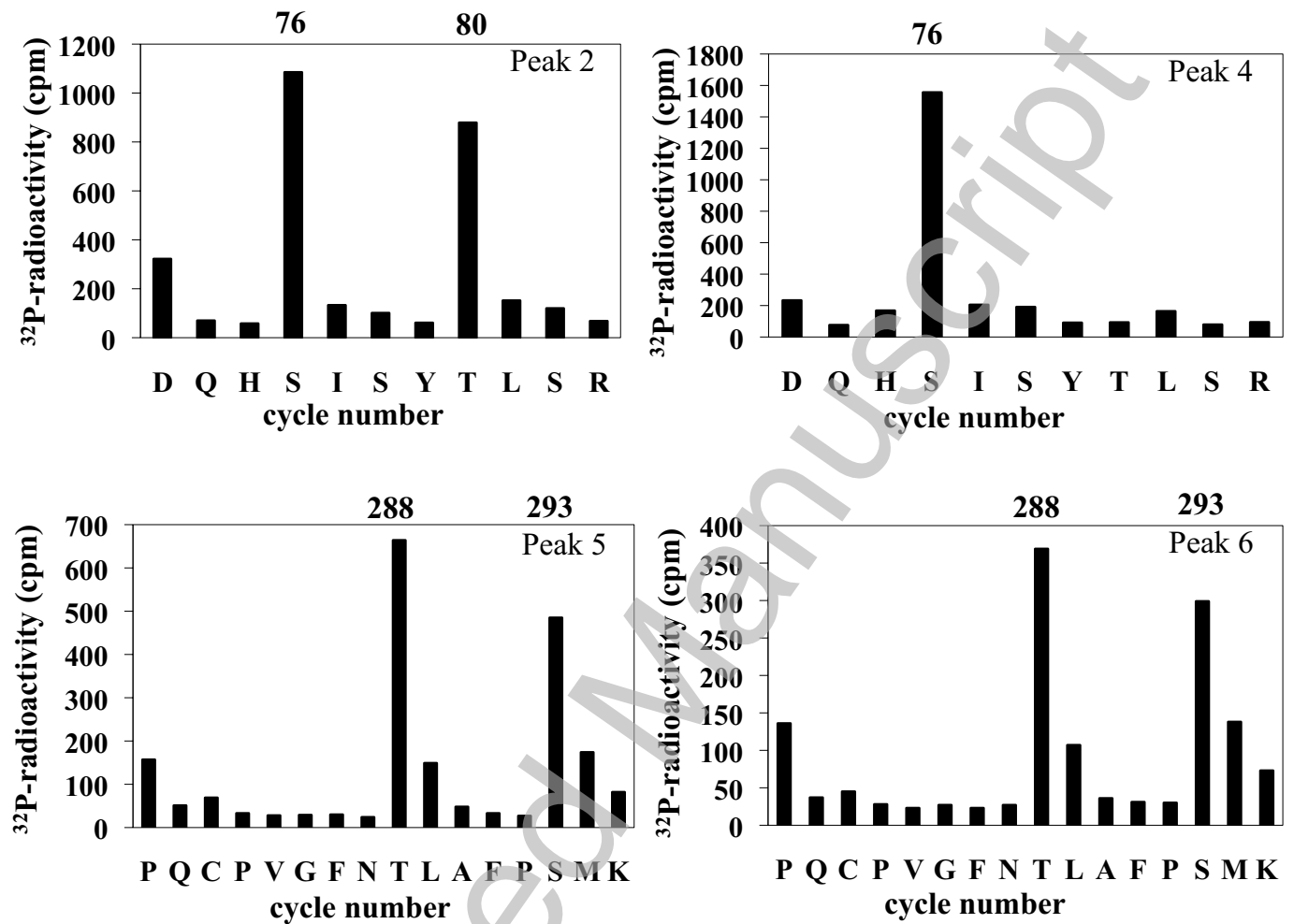
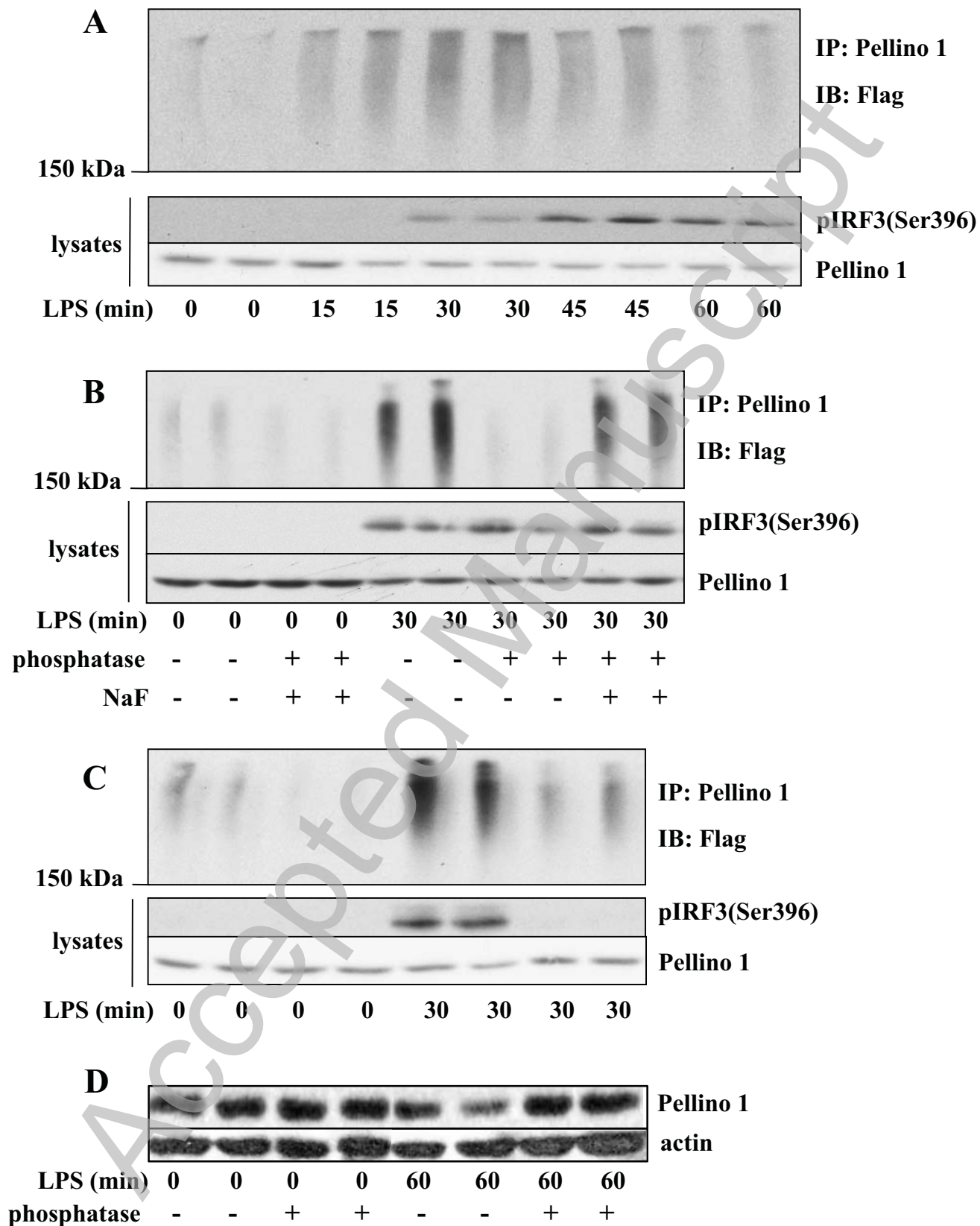
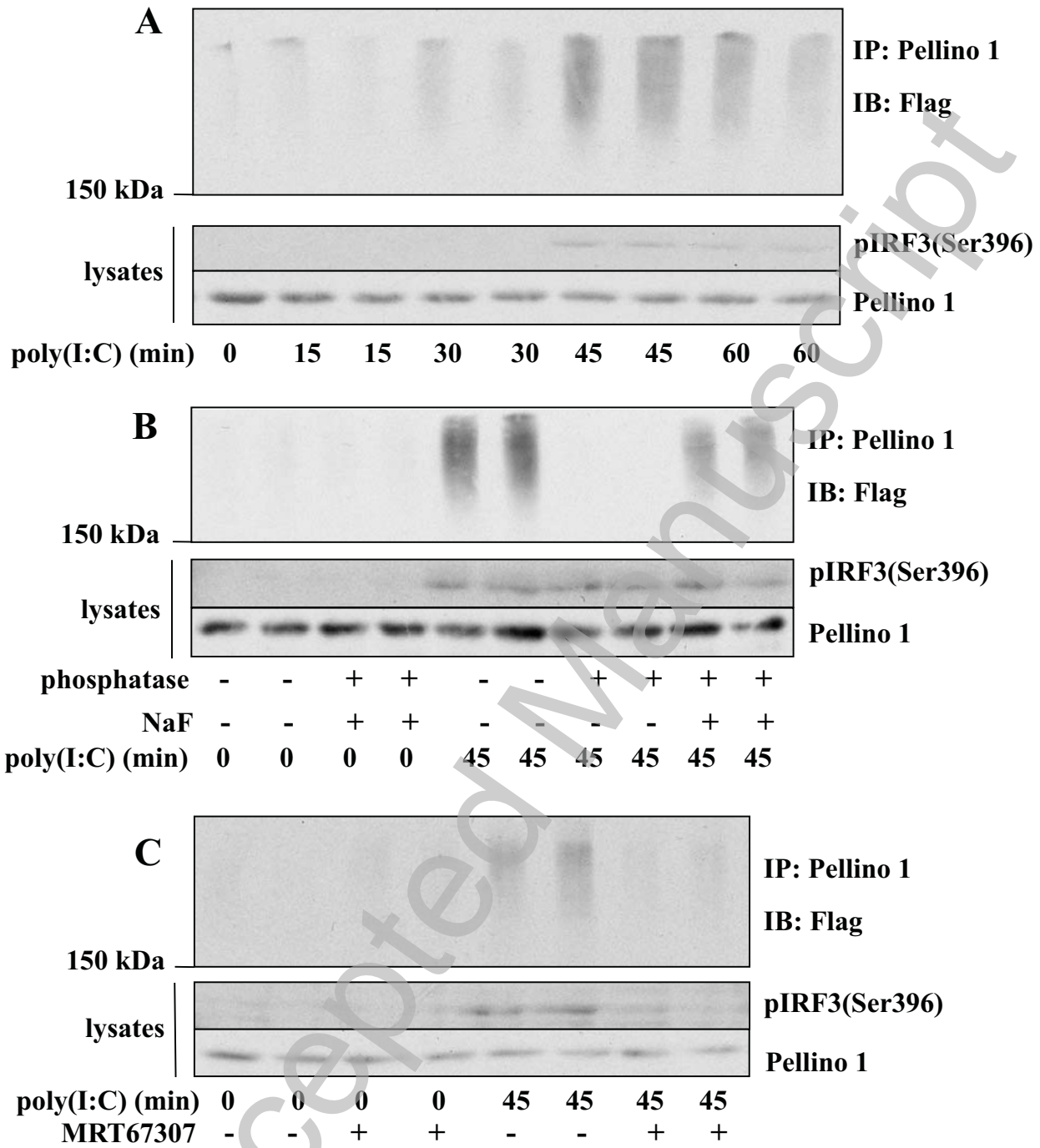


Figure 8



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Figure 9



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