

Regulation and function of TPL-2, an I κ B kinase-regulated MAP kinase kinase kinase

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The I κ B kinase (IKK) complex plays a well-documented role in innate and adaptive immunity. This function has been widely attributed to its role as the central activator of the NF- κ B family of transcription factors. However, another important consequence of IKK activation is the regulation of TPL-2, a MEK kinase that is required for activation of ERK-1/2 MAP kinases in myeloid cells following Toll-like receptor and TNF receptor stimulation. In unstimulated cells, TPL-2 is stoichiometrically complexed with the NF- κ B inhibitory protein NF- κ B1 p105, which blocks TPL-2 access to its substrate MEK, and the ubiquitin-binding protein ABIN-2 (A20-binding inhibitor of NF- κ B 2), both of which are required to maintain TPL-2 protein stability. Following agonist stimulation, the IKK complex phosphorylates p105, triggering its K48-linked ubiquitination and degradation by the proteasome. This releases TPL-2 from p105-mediated inhibition, facilitating activation of MEK, in addition to modulating NF- κ B activation by liberating associated Rel subunits for translocation into the nucleus. IKK-induced proteolysis of p105, therefore, can directly regulate both NF- κ B and ERK MAP kinase activation via NF- κ B1 p105. TPL-2 is critical for production of the proinflammatory cytokine TNF during inflammatory responses. Consequently, there has been considerable interest in the pharmaceutical industry to develop selective TPL-2 inhibitors as drugs for the treatment of TNF-dependent inflammatory diseases, such as rheumatoid arthritis and inflammatory bowel disease. This review summarizes our current understanding of the regulation of TPL-2 signaling function, and also the complex positive and negative roles of TPL-2 in immune and inflammatory responses.

Keywords: ABIN-2; COT; IKK; MAP3K8; MAP kinase; TLR; TPL-2; NF- κ B; p105

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Discovery of *Tpl2* oncogene and initial characterization

The serine/threonine kinase TPL-2, also known as COT and MAP3K8, was independently discovered in three different laboratories in the early 1990s. Initially, Miyoshi *et al.* [1] identified *Cot* (cancer Osaka thyroid) as an oncogene, using DNA isolated from a human thyroid carcinoma cell line, to transform the SHOK hamster embryonic cell line *in vitro*. The rat homolog of *Cot*, called *Tpl2* (tumor progression locus-2), was subsequently identified as a target for provirus integration in Moloney murine leukemia virus (MoMuLV)-induced T-cell lymphomas and demonstrated to transform NIH 3T3 fibroblasts *in vitro* [2]. More recently, MoMuLV in-

sertion into the murine *Tpl2* locus was also found in two genome-wide screens for oncogenes using genetically sensitized mouse strains [3, 4]. It has also been reported that the murine *Tpl2* locus is a site of Mouse Mammary Tumor Virus (MMTV) proviral integration associated with the induction of mammary carcinomas in mice [5]. (For simplicity, the different mammalian homologs will be referred to as *Tpl2* in this review.) Proviral activation of *Tpl2* oncogenicity consistently results in production of TPL-2 proteins truncated at the C terminus compared to the wild-type protein (generally termed TPL-2 Δ C in this review), suggesting important roles of the C terminus in regulation of TPL-2 oncogenic activity (Figure 1). Consistent with this hypothesis, generation of transgenic mice expressing rat TPL-2 or TPL-2 Δ C in their T cells has revealed that C-terminal deletion is essential for TPL-2 to induce the formation of T cell lymphoblastic lymphomas [6].

Tpl2 is expressed in cells as 58 and 52 kDa protein



Figure 1 TPL-2 structure and phosphorylation sites. The *Tpl2* gene encodes two proteins, full-length M1-TPL-2 (p58) and M30-TPL-2 (p52). M30-TPL-2 is translated from the same mRNA transcript as M1-TPL-2 by alternative translational initiation at methionine 30 (M30, black arrowhead). The TPL-2 kinase domain (KD) is located in the centre of the protein, flanked by N-terminal and C-terminal regions with largely unknown functions. C-terminal truncation, however, results in a protein (TPL-2ΔC) with increased kinase-specific activity, suggesting that this region may inhibit TPL-2 kinase activity [6]. Furthermore, a proposed degron sequence (435–457, shaded box) is located within the C terminus and confers destabilizing properties to full-length TPL-2 [8]. Consequently, TPL-2ΔC has increased protein stability and is expressed at higher levels. The positions of oncogenic truncations in TPL-2 identified in MoMuLV- and MMTV-induced murine tumors (424), human TPL-2 (COT) in transformed SHOK cells (397) and in a human lung adenocarcinoma (421) are indicated by red arrowheads. Several phosphorylation sites in TPL-2 have been identified by mass spectrometry [54]. Two of these sites, T290 and S400, are known to regulate TPL-2 MEK kinase activity *in vivo*. T290 phosphorylation may also regulate TPL-2 release from its binding partner p105 (see Figure 4). The physiological significance of the sites shown in italics is not yet known.

isoforms due to alternative translational initiation at methionine 1 (M1) or methionine 30 (M30) [7]. Both M1- and M30-TPL-2 proteins are predominantly localized in the cytoplasm [1]. The weak transforming activity associated with full-length TPL-2 (i.e., not C-terminally truncated) in SHOK cells is predominantly due to M1-TPL-2 [7]. Thus, the presence of an intact N terminus and the absence of the C terminus appear to be necessary for optimal oncogenic transformation by TPL-2. The precise role of the N-terminal 29 amino acids in TPL-2 transformation is unknown. However, removal of the C-terminal domain appears to activate transforming potential of TPL-2 by two mechanisms. First, C-terminal truncation increases the specific kinase activity of TPL-2, and it has been suggested that the C terminus may modulate TPL-2 catalytic activity by folding back onto the kinase domain [6, 8]. Second, C-terminal truncation removes a degron sequence (amino acids 435–457) that promotes proteolysis of TPL-2 by the proteasome [8]. Consequently, TPL-2ΔC is expressed at higher levels in cells than TPL-2.

Surprisingly, although multiple studies have demonstrated that *Tpl2* can function as an oncogene in rodent cells, in some situations, *Tpl2* appears to act as a tumor suppressor. For example, *Tpl2*^{-/-} mice bred onto an MHC Class I-restricted T-cell antigen receptor (TCR) transgenic background develop T-cell lymphomas due to hyper-responsiveness of CD8⁺ T cells following TCR stimulation [9]. Furthermore, a recent study demonstrated that *Tpl2*^{-/-} mice have a significantly increased incidence of skin tumors in a two-stage skin carcinogenesis model, which correlated with increased inflammation and production of proinflammatory cytokines [10].

Tpl2 can clearly function as an experimental oncogene

in mice and rats, and in some conditions as a tumor suppressor. However, it is less clear whether TPL-2 is important in etiology of human cancers. DNA sequencing analyses have demonstrated that TPL-2 is not mutated in the majority of human cancers. Amongst 477 unique primary tumor samples in the Sanger Institute COSMIC database that have been analyzed for somatic mutations in *Tpl2*, only a single brain tumor sample from a patient with glioblastoma multiforme was found to have a somatic A to T mutation at residue 387 (Parsons *et al.* [11] and <http://www.sanger.ac.uk/genetics/CGP/cosmic/>). It is not known whether this mutation contributed to tumor development or progression. In addition, one study reported a *Tpl2* point mutation in a primary human lung adenocarcinoma, which results in the production of a C-terminally truncated protein [12]. However, analyses of other lung cancer cells suggested that this is a very rare event. *Tpl2* overexpression may be important in oncogenesis, and has been reported both in breast tumors [13] and in a small panel of large granular lymphocyte proliferative disorders [14]. Thus, while somatic mutation of *Tpl2* does not appear to be an important event in the development of human cancer, increased TPL-2 protein expression may sometimes contribute to oncogenesis or tumor progression.

Regulation of signaling pathways by TPL-2

While the potential of truncated TPL-2 to behave as a transforming oncoprotein kinase was clear, the physiological function of wild-type TPL-2 was initially less evident. Early northern blot analyses of rat tissues demonstrated that *Tpl2* mRNA is expressed at highest levels

in the spleen, thymus and lungs, with low levels in the brain, testis and liver [2, 15]. A later study also detected *Tpl2* mRNA in the brain, intestine, kidney and skeletal muscles, suggesting that *Tpl2* may be widely expressed [16]. Interestingly, *in situ* hybridization of adult mouse tissues could only identify *Tpl2* mRNA in granular duct cells in the submandibular glands, serous cells in the parotid gland, peptic cells in gastric glands and goblet cells in colonic glands [17]. (The failure of *in situ* hybridization to detect *Tpl2* mRNA expression in other tissues suggests this may be a less sensitive technique than northern blotting.) These latter results raise the possibility that TPL-2 might regulate secretory pathways and/or innate immune responses in the gastrointestinal tract.

In the mid 1990s, DNA sequence homology comparisons revealed that TPL-2 kinase domain is related to the MAP3 kinases STE11 and MEK kinase [18, 19], suggesting that TPL-2 might regulate MAP kinase signaling pathways. Consistent with this hypothesis, TPL-2 overexpression in COS-7 and 3T3 cells activates ERK, JNK, p38 γ and ERK5 MAP kinases [18-20]. Immunoprecipitated TPL-2 phosphorylates MEK-1, MKK-4 (also known as SEK-1), MEK-5 and MKK-6, suggesting that TPL-2 functions directly as a MAP3 kinase [19, 20]. This was subsequently confirmed using recombinant TPL-2 purified from lysates of baculovirus-infected insect cells and recombinant MEK protein as a substrate [21]. Overexpression of TPL-2 also activates NFAT (nuclear factor of activated T cells) in Jurkat T cells and induces IL-2 (interleukin-2) production [22]. IL-2 induction by TPL-2 has also been suggested to involve activation of NF- κ B transcription factors via the related MAP3 kinase NIK [23, 24]. Transfection experiments in Jurkat T cells indicate that TPL-2 may control NF- κ B activity by modulating the transactivation potential of the RelA transcription factor [25, 26]. In addition, overexpression experiments in COS-7 cells demonstrate that TPL-2, which is complexed with the NF- κ B inhibitory protein NF- κ B1 p105 [27] (see section 3), may regulate NF- κ B activation by inducing p105 proteolysis, releasing associated Rel subunits for translocation into the nucleus [28].

Overexpression of kinases can result in artifactual phosphorylation of cellular proteins, and it remained unclear for several years whether TPL-2 really regulated the activation of so many downstream signaling pathways under physiological conditions. This was clarified through the generation of a *Tpl2*^{-/-} mouse strain by the Tsichlis laboratory. *Tpl2*^{-/-} mice display no overt phenotype, are of normal size and weight and have a normal lifespan under pathogen-free conditions [29]. The development of immune cells (T cells, B cells, dendritic cells (DC), natural killer cells and macrophages) occurs

normally in the absence of TPL-2. Surprisingly, despite the results obtained with overexpressed TPL-2 in T-cell lines, *Tpl2*^{-/-} CD4⁺ T cells produce similar amounts of IL-2 to WT cells after TCR stimulation [29, 30], indicating that TPL-2 is not important for the physiological regulation of IL-2 production. LPS-induced activation of NF- κ B and proteolysis of p105 is also normal in *Tpl2*^{-/-} macrophages, suggesting that TPL-2 may not be critical for TLR4 activation of NF- κ B. However, TPL-2 is only associated with a small fraction of total cellular p105. It therefore remains possible that TPL-2 regulates the proteolysis of this pool of p105, which is likely to contribute to only a fraction of total NF- κ B activity. In addition, phosphorylation of RelA on S276 (a critical regulatory site [31]) is dependent on TPL-2 in primary fibroblasts stimulated with TNF [32]. It is therefore possible that TPL-2 contributes to NF- κ B activation in a cell type- and stimulus-specific fashion. The mechanism by which endogenous TPL-2 regulates RelA phosphorylation has not yet been established.

Analysis of *Tpl2*^{-/-} macrophages revealed an essential function for TPL-2 in LPS activation of MEK-1/2 and ERK-1/2, but not of p38, JNK or NF- κ B. Furthermore, LPS stimulation activates the MEK kinase activity of TPL-2 [33, 34]. TPL-2 is also required for TLR2, TLR9 and TNF activation of ERK in macrophages [35-37] and CD40 activation of ERK in B cells [35]. In other cell types, the function of TPL-2 may not be restricted to the regulation of the ERK MAP kinase pathway. Consistent with this idea, TPL-2 is required for optimal activation of ERK and JNK in embryonic fibroblasts after TNF or IL-1 β stimulation, and for maximal p38 activation in LPS- and CpG-stimulated DC [32, 36]. These phenotypes may result directly from TPL-2 signaling, which can phosphorylate and activate MKK-4 and MKK-6, the activators of JNK and p38 MAP kinases, respectively [19, 20].

Together, analyses of primary cells from *Tpl2*^{-/-} mice indicate that the major physiological function of TPL-2 is to regulate ERK MAP kinase activation in immune responses following stimulation of receptors of the TLR and TNF-R families. However, TPL-2 may also regulate the activation of other MAP kinases and NF- κ B in a cell type- and stimulus-specific fashion.

Regulation of TPL-2 signaling by NF- κ B1 p105

An important step toward our current understanding of the regulation of TPL-2 function was the identification of NF- κ B1 p105 as a TPL-2-interacting protein in a yeast two-hybrid screen [28]. TPL-2 binds stoichiometrically with the C-terminal half of NF- κ B1 p105 via two distinct interactions. The TPL-2 C terminus (residues

398-467) binds to a region adjacent to the ankyrin repeat region on p105, while the TPL-2 kinase domain interacts with the p105 death domain (Figure 2) [38]. The binding site on p105 for the TPL-2 C terminus is a conserved helical domain (residues 497-539) that is required for p105 dimerization [38]. This region is within a “processing inhibiting domain” (PID; residues 474-544), which regulates processing of p105 to p50 by the proteasome [39]. It has been proposed that p105 dimerization via this domain blocks processing, possibly due to the increased size of the dimer preventing entry into the proteasome [40]. Therefore, efficient binding of TPL-2 to p105 may require p105 dimerization, and be restricted to a pool of p105 that cannot be processed to p50. While all detectable cellular TPL-2 in unstimulated cells is associated with p105, it is important to note that the majority of total cellular p105 (> 95% in macrophages) is not complexed with TPL-2, but presumably is associated with Rel proteins [28, 41].

NF-κB1 p105 regulates two aspects of TPL-2 function. First, p105 is essential to maintain TPL-2 stability. Steady-state levels of TPL-2 are very low in p105-

deficient *Nfκb1*^{-/-} cells [34, 38], and consequently LPS activation of MEK and ERK is substantially reduced in bone-marrow-derived macrophages (BMDM) generated from *Nfκb1*^{-/-} mice [34]. Since the proposed degron sequence of TPL-2 is located within one of the binding sites for p105, it is possible that this interaction stabilizes TPL-2 by covering the degron [8]. Second, direct interaction of p105 with the kinase domain of TPL-2 inhibits TPL-2 MEK kinase activity by preventing access to MEK [34, 38]. TPL-2 is actively prevented from phosphorylating MEK in unstimulated BMDM, since it is all associated with p105 [41]. However, following LPS stimulation, TPL-2 MEK kinase activity increases, indicating that TPL-2 is no longer subject to p105 inhibition [34]. Indeed, depleting p105 from cell lysates by immunoprecipitation reveals that LPS stimulation induces the release of a fraction of TPL-2 from p105. TPL-2 MEK kinase activity is restricted to this p105-free pool [34, 41].

Like other IκB proteins [42, 43], the signal-induced proteolysis of NF-κB1 p105 is regulated by the IκB kinase complex (IKK), which phosphorylates S927 and

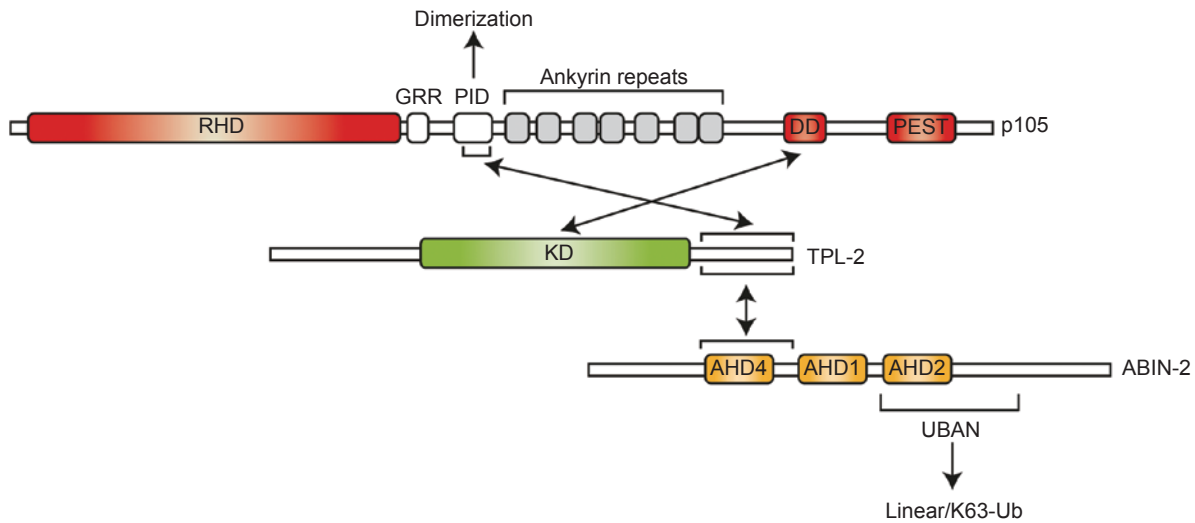


Figure 2 TPL-2 interactions with NF-κB1 p105 and ABIN-2. In unstimulated cells, all detectable TPL-2 is complexed with NF-κB1 p105 and ABIN-2. The TPL-2 kinase domain (KD) directly interacts with the death domain (DD) of p105. This regulates TPL-2 MEK kinase activity by blocking access of the substrate to the active site. The TPL-2 C terminus (398-467) interacts with a region (497-539) of p105 within the “processing inhibitory domain” (PID; 474-544) [39], which also mediates p105 dimerization [38]. These two distinct interactions contribute to a very strong association between TPL-2 and p105, and dissociation of recombinant TPL-2/p105 complex produced in insect cells requires high concentrations of urea (8 M) [21]. The importance of TPL-2 regulation by p105 is highlighted by the dysregulated TPL-2 MEK kinase activity and tumorigenic potential of C-terminal truncated TPL-2, which lacks one of the binding sites for p105 (see text for details). The same region of TPL-2 that mediates binding to the PID of p105 is also the principal interaction site with ABIN-2. The binding site on ABIN-2 (194-250) contains ABIN-homology domain (AHD) 4 (203-220), which is also present in ABIN-1 and ABIN-3 [100]. ABIN-2 interaction is critical to maintain TPL-2 protein stability, and steady-state levels of TPL-2 are dramatically reduced in cells deficient in ABIN-2. RHD: Rel homology domain, GRR: glycine-rich region, PEST: Domain rich in proline (P), glutamate (E), serine (S) and threonine (T), UBAN: ubiquitin-binding in ABIN and NEMO.

S932 in the p105 PEST region (see Figures 3 and 4). These phosphorylations induce the binding of SCF ^{β TrCP} E3 ligase, which catalyzes the subsequent K48-linked polyubiquitination and proteolysis of p105 by the pro-

teasome [42, 44, 45]. This predominantly leads to the complete degradation of p105, rather than processing to p50 [42, 44]. As a consequence of signal-induced p105 degradation, associated Rel subunits are released to regu-

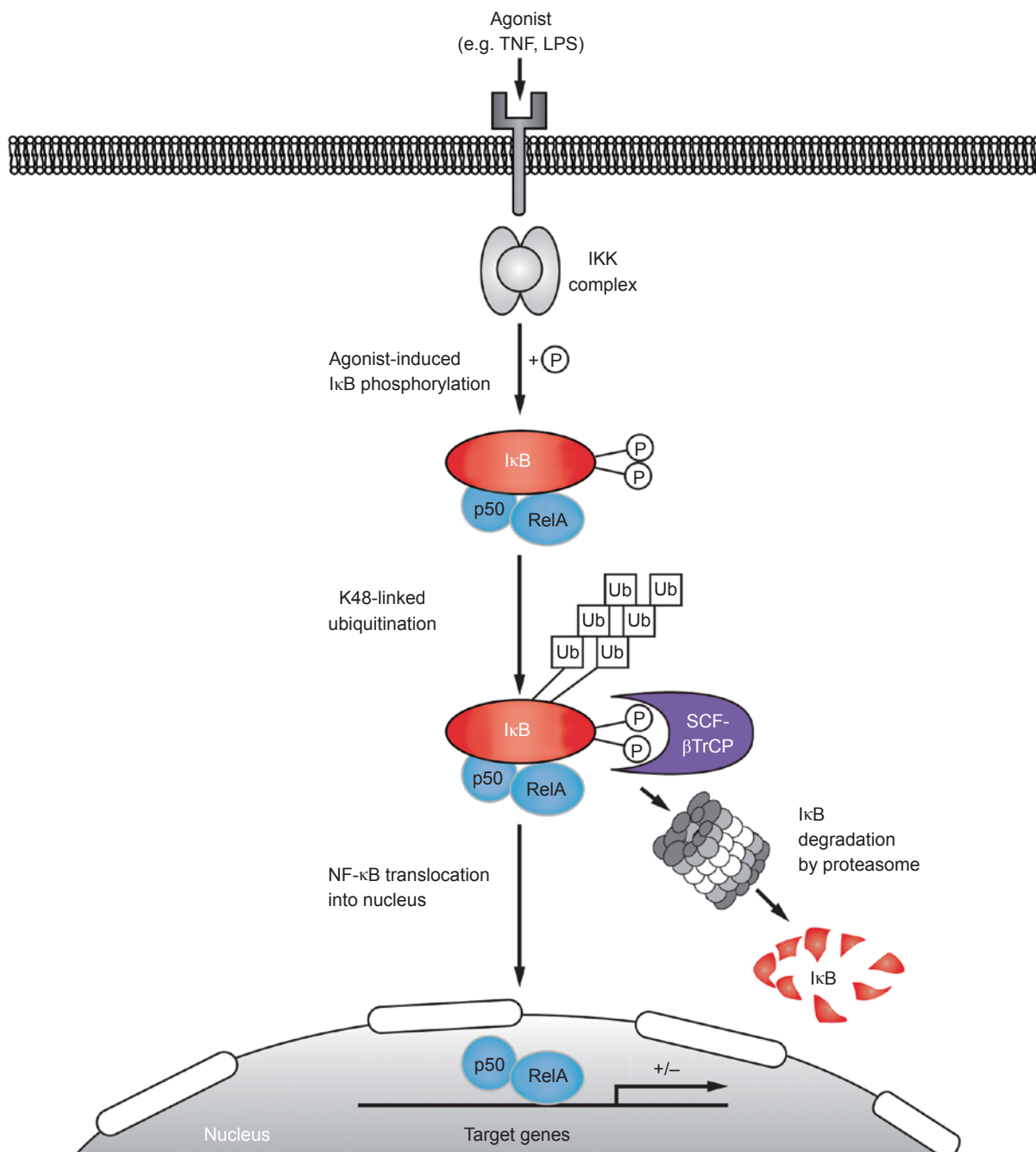


Figure 3 Canonical pathway of NF- κ B activation. Various stimuli activate the classical NF- κ B pathway. Agonist stimulation induces adapter recruitment to the cognate receptor, which then activates the IKK complex by a process that involves linear and K63-linked ubiquitination, as well as phosphorylation (reviewed in Skaug *et al.* [101], Vallabhapurapu and Karin [102]). IKK phosphorylates two N-terminal residues in I κ B α , creating a binding site for the SCF ^{β TrCP} ubiquitin E3 ligase complex, which attaches K48-linked ubiquitin chains to two adjacent lysine residues, targeting I κ B α for proteasomal degradation. Associated NF- κ B dimers are consequently liberated to translocate to the nucleus and modulate target gene expression.

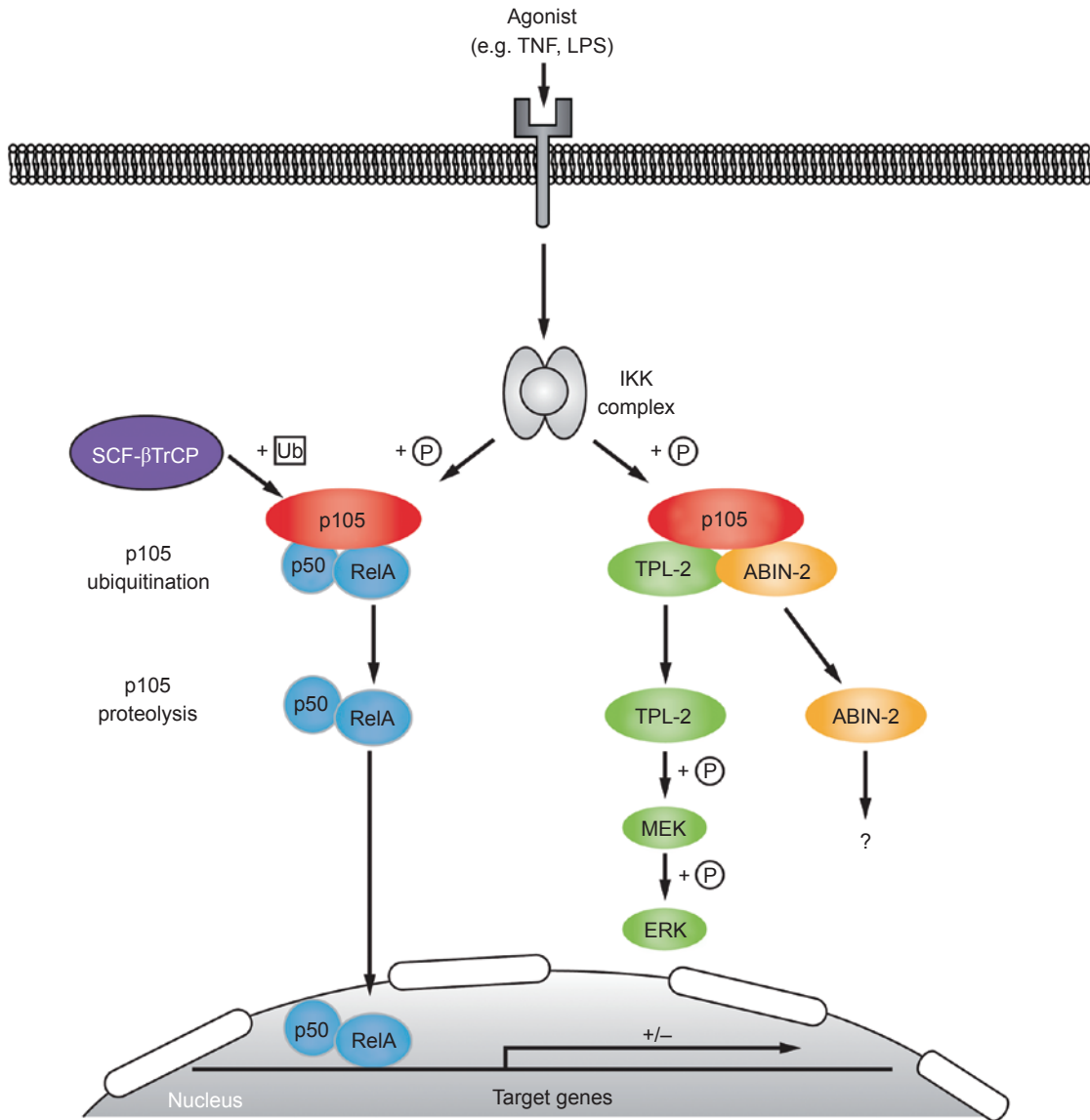


Figure 4 Regulation of TPL-2 through IKK-induced p105 proteolysis. TPL-2 is confined to a cytoplasmic complex with the NF- κ B1 precursor protein p105, and the ubiquitin-binding protein ABIN-2. NF- κ B1 p105 has multiple functions. First, it serves as a precursor molecule for the NF- κ B1 p50 subunit, which is generated by limited proteolysis (processing) of p105 by the proteasome, which removes p105's C-terminal half [33]. Second, p105 functions as a classical κ B, retaining associated NF- κ B subunits in the cytoplasm. Cellular stimulation with various agonists, such as TLR ligands, IL-1 β , TNF and CD40L, induces the formation of receptor proximal complexes that trigger activation of the MAP3 kinase TAK1 [101]. Activation loop phosphorylation of IKK2 by TAK1, in turn, activates IKK2 to phosphorylate the target residues S927 and S932 in p105, creating a binding site for the SCF ^{β TrCP} ubiquitin E3 ligase complex. K48-linked ubiquitination of p105 by SCF ^{β TrCP} triggers its complete degradation by the 26S proteasome. IKK-induced proteolysis of p105 releases associated NF- κ B dimers, which then translocate to the nucleus and modulate expression of target genes, similar to activation of NF- κ B dimers in the classical pathway (see Figure 3). Stimulus-induced p105 proteolysis also couples activation of NF- κ B pathways to MAP kinase signaling by releasing TPL-2 from p105 inhibition. After liberation from p105, TPL-2 directly phosphorylates MEK and thereby activates downstream ERK MAP kinase signaling. Based on immunoblotting of total cell lysates, it has been suggested that LPS stimulation results in the selective dissociation of M1-TPL-2 from p105 [34]. However, immunoblotting of p105-depleted lysates indicates that both M1 and M30 TPL-2 are actually released from p105 after LPS stimulation [41], although the relative contribution of M1 and M30 TPL-2 to MEK phosphorylation in LPS-stimulated macrophages is not known. Activation of TPL-2 MEK kinase activity additionally requires trans-/autophosphorylation of S400 by an unknown kinase and autophosphorylation at T290 in the TPL-2 activation loop. Proteasomal degradation of p105 also releases ABIN-2 from association with p105 and TPL-2. The function and downstream targets of ABIN-2 are not known.

late NF- κ B target genes in the nucleus. A recent study has demonstrated that IKK-induced p105 proteolysis is necessary for optimal TCR-induced NF- κ B activation in CD4⁺ T cells and mature CD4⁺ T-cell helper function [30].

In macrophages, pharmacological inhibition of the proteasome blocks both TPL-2 release from p105 and ERK activation following LPS stimulation [41, 46], raising the possibility that IKK-induced p105 proteolysis might also regulate these processes. Consistent with this hypothesis, LPS-induced release of TPL-2 from p105, and its subsequent activation of MEK and ERK, are blocked by expression of p105^{SSAA}, which is resistant to IKK-induced proteolysis due to mutation of the IKK target serines to alanine. Furthermore, LPS activation of ERK in macrophages is dependent on IKK2 catalytic activity. Thus, IKK-induced p105 proteolysis is essential for LPS activation of TPL-2, and for TPL-2-dependent activation of ERK following TNF stimulation [46]. It is likely that this IKK-induced p105 proteolysis also facilitates TPL-2 phosphorylation of MEK following stimulation with other TLR ligands, IL-1 β and CD40, which each activate ERK via TPL-2.

Regulation of TPL-2 signaling by phosphorylation

Similar to other MAP3 kinases [47], TPL-2 signaling function is regulated by phosphorylation. Activation of TPL-2 requires phosphorylation of T290 in the activation loop of its kinase domain (Figure 1) [48], which may also regulate the association of TPL-2 with p105 [49]. Based on experiments testing the effect of high concentrations of the IKK2 inhibitor PS-1145 in RAW264.7 macrophages, it was originally suggested that TPL-2 T290 phosphorylation is controlled by IKK2 [50]. However, lower concentrations of PS-1145 that effectively block IL-1 β -induced I κ B α degradation do not affect TPL-2 T290 phosphorylation, suggesting that this is an “off-target” effect of the inhibitor [51]. Indeed, experiments with a small-molecule inhibitor of TPL-2 have recently suggested that T290 is autophosphorylated by TPL-2 itself after IL-1 β stimulation of IL-1R-expressing 293T cells [52]. TPL-2 must also be phosphorylated on S400 in its C-terminal tail to activate MEK following LPS stimulation of macrophages [53]. Different experimental systems have suggested that S400 is either autophosphorylated by TPL-2 (IL-1 β -stimulated IL-1R-293T cells) or transphosphorylated by an unknown kinase (LPS-stimulated RAW264.7 macrophages) [52, 53]. Interestingly, a recent study has shown that the extracellular nutrient arginine positively regulates the phosphorylation of TPL-2 on both T290 and S400, and consequently TPL-2 activation following LPS stimulation

[54]. Although the mechanism underlying this effect has not yet been worked out, these data imply that nutritional status may directly influence ERK activation in innate immune responses via regulation of TPL-2 phosphorylation.

In summary, current data indicate that activation of TPL-2 MEK kinase activity involves at least two regulatory steps: auto- and/or transphosphorylation on two residues (T290 and S400), and release from p105-mediated inhibition, which is triggered by IKK-induced p105 proteolysis by the proteasome. The IKK complex, therefore, directly regulates both NF- κ B and the ERK MAP kinase activation in innate immune responses via NF- κ B1 p105. However, although p105 inhibits TPL-2 phosphorylation of MEK, it does not directly regulate TPL-2 catalytic activity [53, 55]. Therefore, it is possible that TPL-2 phosphorylates substrates other than MEK when complexed with p105, and independently of regulation by the IKK complex.

Regulation of TPL-2 stability by ABIN-2

Tandem-affinity purification and peptide mass fingerprinting identified A20-binding inhibitor of NF- κ B 2 (ABIN-2) as an NF- κ B1 p105-interacting protein [56, 57]. Cotransfection experiments indicate that ABIN-2 can also interact directly with TPL-2, but preferentially forms a ternary complex with p105 and TPL-2. Consistently, a substantial fraction of endogenous ABIN-2 is associated with p105 and TPL-2 in primary macrophages [57]. ABIN-2 was originally identified in a two-hybrid screen, with the NF- κ B inhibitory protein A20 as bait, and overexpression experiments indicate that ABIN-2 may be a downstream effector of A20, mediating its NF- κ B inhibitory function [58]. ABIN-2 contains an UBAN (ubiquitin binding in ABIN and NEMO) ubiquitin-binding domain, which is essential for its ability to inhibit NF- κ B in overexpression experiments [59]. Binding analyses with recombinant ABIN-2 have indicated interaction with linear ubiquitin chains, and also weak binding to K63-linked ubiquitin chains [60].

Initial experiments to investigate the significance of ABIN-2 association with p105 and TPL-2 utilized siRNA knockdown in 293 cells. These experiments demonstrated that ABIN-2 is required to maintain TPL-2 protein stability [57], which was subsequently confirmed by analysis of various types of primary cells isolated from *Abin2*^{-/-} (also termed *Tnip2*^{-/-}) mice [61]. Consequently, LPS and TNF activation of ERK is reduced, but not absent, in *Abin2*^{-/-} macrophages. Since LPS activation of ERK in macrophages is completely dependent on TPL-2 [29], this implies that the small amount of TPL-2 pro-

tein present in *Abin2*^{-/-} macrophages is still activated in the absence of ABIN-2. Consistent with this hypothesis, retroviral expression of TPL-2 in *Abin2*^{-/-} macrophages substantially increases LPS- and TNF-induced ERK activation [61]. ABIN-2, therefore, does not seem to be important for activation of TPL-2. In addition, ABIN-2 is released from p105 and TPL-2 after LPS stimulation, and is not associated with the pool of TPL-2 that can activate MEK [57].

It is unlikely that the sole function of ABIN-2 is to stabilize TPL-2, since this does not require the conserved UBAN domain of ABIN-2 [61]. Retroviral expression of ABIN-2 deletion mutants has also demonstrated that ABIN-2 binding to A20 is not required for stabilization of TPL-2 in macrophages. The importance of ABIN-2 ubiquitin-binding activity and interaction with A20 in immune responses remains unclear. Interestingly, ABIN-2 has been reported to interact with SMRT (silencing mediator of retinoic acid and thyroid hormone receptors) [62], a core component of corepressor complexes that actively repress expression of a fraction of inflammatory response genes in macrophages [63]. TLR activation of these genes requires the active removal of SMRT, in addition to the recruitment of activators and coactivators. ABIN-2 has also been shown to interact with Baf60a, a subunit of the SWI/SNF chromatin-remodeling complex [64], which positively regulates a subset of TLR4-induced genes in macrophages [65]. Tandem-affinity purification has demonstrated that ABIN-2 complexes with Rel subunits [56], although it is not known whether these are direct interactions or are mediated via NF- κ B1 p105. Since ABIN-2 is released from p105 following LPS stimulation of macrophages [57] and can enter the nucleus [64], it is possible that ABIN-2 regulates gene expression by controlling the interaction of SMRT, Baf60a and/or Rel subunits with target genes.

Regulation of immune and inflammatory responses by TPL-2

Analyses of *Tpl2*^{-/-} mice have suggested critical functions for TPL-2 in immune responses, which are summarized in Table 1. Initial experiments investigated the role of TPL-2 in inflammatory responses. *Tpl2*^{-/-} mice were found to produce very low levels of TNF after intraperitoneal LPS injection and to be resistant to septic shock induced by LPS and D-galactosamine [29]. TPL-2 is also required for optimal TNF production by LPS-stimulated macrophages, the major cellular source of TNF during inflammatory responses, while LPS-induced TNF production by DC is partially dependent on TPL-2 [29, 37]. However, TPL-2 is not universally involved in

induction of TNF in macrophages, since curdlan stimulation of dectin-1, which activates ERK via Raf, induces TNF independently of TPL-2 expression [37, 66]. The requirement for TPL-2 in TNF-induced innate immune responses is, therefore, both cell- and stimulus specific.

Pharmacological inhibition of ERK activation with MEK inhibitors impairs LPS induction of TNF by macrophages, implying that TPL-2 regulates TNF production by controlling ERK activation [29, 67]. Initial experiments suggested that TPL-2/ERK might regulate TNF production at a post-transcriptional level by promoting the export of *Tnf* mRNA from the nucleus [29]. In apparent support of this hypothesis, a recent study using TPL-2 and MEK inhibitors showed that TPL-2/ERK controls, at the post-transcriptional level, the expression of the nuclear mRNA export receptor Tip-associated protein (TAP) [68]. However, pre-TNF protein levels in LPS-stimulated primary macrophages are minimally affected by pharmacological MEK inhibition or TPL-2 deficiency, indicating that defective intracellular TNF mRNA transport is unlikely to explain the failure of *Tpl2*^{-/-} macrophages to produce TNF [67]. Rather, TPL-2/ERK signaling is required for the appearance of pre-TNF at the cell surface and processing of pre-TNF to soluble TNF, which may both be controlled by ERK-mediated phosphorylation of TNF α -converting enzyme (TACE) on T735 [69]. TNF maturation, but not pre-TNF production, has also recently been shown to be blocked in LPS-stimulated macrophages isolated from a new *Tpl2* mutant mouse strain, called *Sluggish*, in which the TPL-2 kinase domain is partially deleted. This study confirms the importance of TPL-2 in the posttranslational control of TNF production [70].

TPL-2 also positively regulates mRNA and protein induction of IL-1 β and IL-10 following stimulation of macrophages and DC with LPS or CpG (TLR9 ligand) [36, 37]. MEK inhibitor experiments suggest that TPL-2 controls TLR-induction of IL-10 and IL-1 β via ERK [36, 61], while positive regulation of IL-1 β production by TPL-2 following stimulation with the dectin-1 ligand, curdlan, appears to be ERK independent [37]. TLR induction of IL-12 p70 and IFN (interferon)- β is inhibited by TPL-2/ERK signaling, and this occurs, at least in part, independently of any effects on IL-10 induction [36, 71]. TPL-2 signaling therefore has complex pro- and anti-inflammatory effects on the production of cytokines in innate immune responses. In addition, TPL-2 not only controls the production of cytokines but also the cellular response to TNF and IL-1 β , since TPL-2 expression is required for activation of ERK by both of these proinflammatory cytokines [32, 52, 72]. Indeed, the development of TNF-induced Crohn's-like inflammatory bowel

Table 1 Effects of TPL-2 deficiency on immune and inflammatory responses

Model	Phenotype	Expression of effectors	References
Septic shock (LPS/D-gal)	Resistant	Serum: TNF ↓, IL-1β ↓	[29, 37]
IBD	Delayed onset (<i>Tnf^{ΔARE/ΔRE}/Map3k8^{-/-}</i>) Spleen: T cells ↑ (CD4 ⁺ CD44 ^{lo} ↑, CD8 ⁺ and CD4 ⁺ CD44 ^{hi} ↓)		[73]
Zymosan injection	Inflammation ↓		[37, 104]
	<i>Intraperitoneal</i>	<i>Intraperitoneal</i> Serum: TNF ↓	
	Neutrophil recruitment ↓ Macrophage recruitment ↓		
	<i>Intraplantar</i>	<i>Intraplantar</i> TNF ↓, IL-1β ↓, IL-6 ↓ G-CSF ↓, GM-CSF ↓, NGF ↓ MCP-1 ↓, MIP-α ↔, MIP-1β ↓, KC ↓ PGE ₂ , LTB4 ↑ (5 h), PGE ₂ , LTB4 ↓ (24 h)	
	Neutrophil recruitment ↓ Hypernociception ↓		
Bronchoalveolar inflammation (OVA-induced)	Inflammation ↑		[77]
	Bronchoalveolar exudates: Lymphocytes ↑ Eosinophils ↑	Bronchoalveolar exudates: IL-4 ↑, IL-5 ↑ Eotaxin ↑ IgE ↑	
Caerulein-induced pancreatitis	Inflammation ↓		[85]
	Pancreas: Neutrophil activity/recruitment ↓	Pancreas: IL-6 ↓ MCP-1 ↓, MIP-2 ↓	
<i>L. monocytogenes</i> infection	Lethality ↑ Bacterial burdens in liver and spleen ↑	Serum: TNF ↔, IL-12 (p70) ↑, IL-27 ↑ (<i>In vitro</i> infected BMDM and splenocytes: IL-1β ↓)	[37]
<i>L. major</i> infection	Parasite load ↔	LN CD4 ⁺ T cells (Ag-restimulated): IFN-γ ↑, IL-4 ↓	[79]

Model	Phenotype	Expression of effectors	References
<i>T. gondii</i> infection	Susceptibility ↑ Lethal upon <i>Tpl2</i> ^{-/-} T cell → <i>Rag2</i> ^{-/-} % of infected PECs ↑ Spleen and PECs: NK and T cell numbers ↔	Serum: IFN-γ ↓, IL-12 ↑, IL-6 ↔ MCP-1 ↓ LN (Ag-restimulated): IFN-γ ↓	[77]
Ag-specific responses		Serum: <i>OVA</i> + <i>CFA</i> : IgG _{2a} ↑ <i>OVA</i> + <i>atum</i> : IgG ₁ ↓ IgE ↑ (Ag-specific and total) [77] IgE ↓ [79]	[77, 79]

Abbreviations and symbols used: ↑, increase; ↓, decrease; ↔, unaffected; Ag, antigen; IBD, inflammatory bowel disease; LN, lymph node; PECs, peritoneal exudate cells.

disease in *Tnf*^{ΔARE/ΔARE} mice, which constitutively over-produce TNF, is reduced in the absence of TPL-2 expression [73, 74]. TPL-2 is therefore important in driving TNF-induced inflammation.

It will be important in the future to determine how the complex effects of TPL-2 on the production and response to cytokines regulate immune responses to pathogens, in which multiple pattern recognition receptors are activated and a complex interplay of cytokines is required to mount an effective immune response. One recent study demonstrated that *Tpl2*^{-/-} mice have increased pathogen burdens compared to wild-type controls after infection with *Listeria monocytogenes*, an intracellular Gram-positive bacterium [37]. The increased susceptibility of *Tpl2*^{-/-} mice to listeriosis correlates with a substantial reduction of IL-1β production following infection. However, while IL-1β is required for optimal anti-listerial responses [75, 76], it remains to be established whether defective IL-1β induction explains the increased susceptibility of *Tpl2*^{-/-} mice to *L. monocytogenes* infection.

Tpl2^{-/-} mice also have an impaired immune response to the intracellular parasite *Toxoplasma gondii*. Surprisingly, transfer experiments with purified T cells suggest that this is due to a T-cell intrinsic defect, rather than an altered innate immune response [77]. Resistance to *T. gondii* critically depends on IFN-γ production [78], and *in vitro* experiments revealed that TPL-2 is required for optimal differentiation of naïve T cells to Th1 effector cells producing IFN-γ [77]. Interestingly, in an ovalbumin-induced model of asthma that is driven by Th2 effector cells, lung inflammation is exacerbated in *Tpl2*^{-/-} mice compared to wild-type controls [77]. Negative feedback regulation between Th subsets may explain this phenotype, whereby decreased Th1 differentiation in absence of TPL-2 results in increased Th2 polarization. It has been suggested that defective TCR-mediated activation of ERK in *Tpl2*^{-/-} CD4⁺ T cells results in reduced T-bet and Stat4 expression, and consequently in reduced Th1 differentiation [77]. However, the reduction in TCR-induced ERK phosphorylation in TPL-2-deficient cells is relatively modest and it is possible that TPL-2 has other signaling functions in Th1 differentiation.

In contrast to the above-mentioned studies suggesting a requirement for TPL-2 in Th1 differentiation, an independently generated *Tpl2*^{-/-} mouse strain has been reported to mount a Th1-skewed immune response following *Leishmania major* infection [79], possibly due to the increased production of IL-12 by TPL-2-deficient innate immune cells. These conflicting results may be explained by differences in how the two *Tpl2*^{-/-} mouse strains were generated, genetic background differences of the mice or by differential responses elicited by the two pathogens

used in the studies. If the latter explanation is correct, this would suggest that the net effect of TPL-2 deficiency on effector T-cell development may be dependent on the specific type of immune response involved. In addition, TPL-2 may not only directly influence the differentiation of the Th1 cell subset, as it has recently been reported that production of the Th17-promoting cytokine IL-23 is impaired in LPS-stimulated *Tpl2*^{-/-} macrophages [80]. This suggests that TPL-2 may positively regulate Th17 cell differentiation. It will be very interesting to investigate this possibility *in vivo* using *Tpl2*^{-/-} mice, given the importance of Th17 cells in autoimmune diseases [81].

TPL-2 expression is required for CD40 activation of ERK in B cells [35]. *In vitro* experiments suggest that IgE switching of purified splenic B cells induced by CD40 plus IL-4 requires TPL-2 activation of ERK [35]. However, IgE antibody production is not defective in *Tpl2*^{-/-} mice, possibly due to enhanced Th2 cell differentiation (see above), increasing the available concentration of IL-4, which promotes switching to IgE [77]. Analysis of B cells purified from TPL-2-deficient *Nfkb1*^{-/-} mice has also suggested that TLR4 utilizes TPL-2 to activate ERK in B cells [82]. This pathway is proposed to positively regulate B cell survival by promoting the degradation of the pro-apoptotic protein Bim [83], which is directly phosphorylated by ERK [84]. However, the importance of TLR4 activation of ERK via TPL-2 in B cell antibody responses is not known.

Several studies indicate that TPL-2 can also control inflammatory responses by signaling in non-hematopoietic cells. For example, TPL-2 positively regulates the development of pancreatic and lung inflammation during caerulein-induced acute pancreatitis [85]. By generating bone marrow chimeras, pancreatic inflammation was shown to be controlled by TPL-2 in non-myeloid cells. Analyses of pancreas homogenates demonstrate that caerulein activation of ERK, JNK and AP-1 is dependent on TPL-2 expression, as is the induction of MCP-1 (monocyte chemoattractant protein-1), MIP-2 (macrophage-inflammatory protein-2) and IL-6 [85]. Thus, TPL-2 may regulate pancreatic inflammation during pancreatitis by mediating proinflammatory signals and the generation of neutrophil chemoattracting factors. *In vitro* experiments also show that TPL-2 is involved in inflammatory cytokine activation of ERK and lipolysis in adipocytes, and is upregulated in adipose tissue in obese mice and humans [86]. TPL-2 therefore may play a role in adipose tissue dysfunction in obesity and type-2 diabetes.

TPL-2 kinase as an anti-inflammatory drug target

The proinflammatory cytokine TNF plays an impor-

tant role in the pathogenesis of rheumatoid arthritis (RA) and Crohn's disease, and biological agents that block its activity have been used to treat these diseases [87, 88]. However, only a fraction of RA and Crohn's sufferers respond well to anti-TNF antibodies. Consequently, there is still a need for more effective, less expensive and orally active drugs for RA and Crohn's treatment. One approach is to target the signaling pathways that regulate the production of TNF [89]. TPL-2 is critical for production of TNF during TLR-induced inflammatory responses, and is an attractive drug target since TPL-2 is not required for normal development or viability. Furthermore, TPL-2 only regulates MEK activation in response to inflammatory stimuli, since activation of MEK by growth factors and antigen receptors is mediated by Raf isoforms [90]. Amino acid sequence comparisons also demonstrate that the TPL-2 kinase domain has relatively low homology to other kinases and is also the only human kinase domain with a unique proline (P145) rather than a conserved glycine on the Gly-rich loop [48]. Therefore, it may be possible to identify inhibitors that are selective to TPL-2, which do not affect other kinases.

Using *in vitro* assays in which recombinant truncated TPL-2 (M30-TPL-2ΔC) phosphorylates the MEK activation loop [21], high-throughput screening by the pharmaceutical companies Abbott and Wyeth/Pfizer have identified different classes of small-molecule TPL-2 inhibitors (see George and Salmeron [91] for a comprehensive review). Several of these appear to be relatively specific and block LPS-induced ERK activation and TNF production in primary macrophages at low micromolar concentrations [92, 93]. In addition, three of Wyeth/Pfizer compounds have been reported to have efficacy *in vivo*, blocking TNF production in mice after intraperitoneal LPS injection [94].

Although there has been considerable progress toward the generation of specific TPL-2 inhibitors, more work is needed to develop compounds suitable for *in vivo* use, which should be of sufficient potency and have the appropriate physicochemical properties. TPL-2 remains a difficult drug target to develop further, since the lack of a crystal structure has prevented structure-based drug design. Indeed, it is unlikely that it will be technically possible to crystallize isolated M30-TPL-2ΔC, since this is associated with heat shock proteins and has a tendency to form insoluble aggregates, suggesting that it is not correctly folded [21]. Furthermore, full-length recombinant TPL-2 has an even greater tendency to aggregate. Analyses of *Nfkb1*^{-/-} and *Abin2*^{-/-} cells suggest that production of correctly folded and stable recombinant TPL-2 requires its association with p105 and ABIN-2 [34, 38, 61]. Coexpression of p105 and ABIN-2 may eventually

allow structural determination of TPL-2, which could be used in structure-based drug design. However, it is unclear whether drugs developed from the structure of complexed TPL-2 would also target the physiologically relevant form of TPL-2, which is released from p105 [41]. Nevertheless, the pharmaceutical industry is likely to remain interested in developing TPL-2 as a drug target, given its critical role in regulating TNF production in inflammation.

Conclusions and future research directions

Since its initial discovery in 1991, much progress has been made in our understanding of the regulation and functions of TPL-2 MAP3 kinase. The discovery by the Tschlis laboratory of the critical role played by TPL-2 in the induction of TNF in inflammatory responses using *Tpl2*^{-/-} mice was particularly important [29]. This result identified TPL-2 as a potential anti-inflammatory drug target, and continues to stimulate both academic and industrial research on this protein. However, TPL-2 also has anti-inflammatory functions, such as the negative regulation of IL-12 and IFN- β production in myeloid cells [36]. It will therefore be important to test *Tpl2*^{-/-} mice in a variety of autoimmune disease models to establish whether TPL-2 inhibition is likely to have an overall anti-inflammatory effect. It will also be important to test the effect of TPL-2 deficiency on immune responses against various pathogens to determine whether long-term pharmacological inhibition of TPL-2 is likely to significantly increase susceptibility to opportunistic infection.

Dysregulation of the ERK MAP kinase pathway is common in many human cancers, often arising because of mutations in the MEK kinase B-Raf [95]. Targeted inhibitors of BRAF and its downstream target MEK are already undergoing clinical testing [96]. Although *Tpl2* was originally identified as an oncogene, DNA sequence analyses have failed to identify *Tpl2* mutations in primary human cancer cells. However, activation of ERK signaling by TPL-2 could still contribute to the transformation of cells in which IKK is constitutively switched on, such as in diffuse large cell lymphoma [97], since IKK-induced p105 proteolysis facilitates TPL-2 activation. This may be an interesting question to investigate in the future, since specific inhibitors of TPL-2 are currently under development and could potentially be used to treat such tumors.

The role of p105 in regulating the MEK kinase activity of TPL-2 is well established [41, 46]. However, our understanding of how phosphorylation regulates TPL-2 activation is less clear. For example, it is not known why

S400 phosphorylation is required for TPL-2 activation of MEK after LPS stimulation in macrophages [53], especially since TPL-2^{S400A} has normal catalytic activity when expressed in Jurkat or 293T cells [52, 98]. In addition, the functional importance of several other known phosphorylation sites on TPL-2 has yet to be investigated [54]. The identity of the kinases that phosphorylate TPL-2 also remains unknown, as is the role of phosphatases in regulating TPL-2 signaling activity.

One key outstanding question is whether TPL-2 only controls MAP kinase signaling via phosphorylation of MAP2 kinases or whether TPL-2 can phosphorylate other classes of proteins and consequently regulate other signaling pathways. The function of the TPL-2-associated ubiquitin-binding protein, ABIN-2, in immunity is also completely unclear. Since ABIN-2 expression is required to maintain steady-state TPL-2 levels [61], progress in this area will require the generation of more sophisticated *Abin2*-mutant strains, which block ABIN-2 signaling function (e.g., ubiquitin-binding function) without affecting TPL-2 stability.

The consequences of linking NF- κ B and ERK activation via IKK-induced p105 proteolysis are also unknown. One interesting question is whether these two p105-dependent signaling pathways regulate the transcription of the same target genes in TLR-stimulated macrophages. If this is the case, at least for some TLR-induced genes, p105 proteolysis could potentially promote transcription via synergistic interactions between NF- κ B and AP-1 transcription factors on the promoters of target genes [99]. What is clear, however, is that care has to be taken in attributing the phenotypes of *Nemo*-, *Ikk2*- and *Nfkb1*-deficient mice solely to impaired NF- κ B activation, as TPL-2 signaling function will also be affected.

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