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Molecular and physiological roles of Pellino E3 ubiquitin ligases in immunity

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Summary: The sensing of foreign agents by the innate and adaptive immune system triggers complex signal transduction cascades that culminate in expression of gene patterns that facilitate host protection from the invading agent. Post-translational modification of intracellular signaling proteins in these pathways is a key regulatory mechanism with ubiquitination being one of the important processes that controls levels and activities of signaling molecules. E3 ubiquitin ligases are the determining enzymes in dictating the ubiquitination status of individual proteins. Among these hundred E3 ubiquitin ligases are a family of Pellino proteins that are emerging to be important players in immunity and beyond. Herein, we review the roles of the Pellino E3 ubiquitin ligases in innate and adaptive immunity. We discuss their early discovery and characterization and how this has been aided by the highly conserved nature of innate immune signaling across evolution. We describe the molecular roles of Pellino proteins in immune signaling with particular emphasis on their involvement in pathogen recognition receptor (PRR) signaling. The growing appreciation of the importance of Pellino proteins in a wide range of immune-mediated diseases are also evaluated.

Keywords: *Pellino E3 ubiquitin ligases, innate immunity, Toll-like receptors, NOD-like receptors, inflammation, ubiquitination*

Conserved innate immune signaling as the gateway to discovering Pellino

The primary sensing of invading pathogens by the host immune system is facilitated by pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs). Such PRRs include families of receptors like Toll-like receptors (TLRs) (1) and NOD-like receptors (NLRs) (2) that trigger signal transduction pathways to regulate gene expression patterns that are adapted toward protecting the host from the pathogen. Taking TLRs as the founding members of the PRR family and the paradigm for PRR signaling, these receptors promote activation of nuclear factor-κB (NF-κB) and interferon (IFN) regulatory factors (IRFs) to induce proinflammatory cytokines, like interleukin-1 (IL-1) and tumor necrosis factor (TNF), and anti-viral interferons (IFNs) (3) (Fig. 1). TLRs are transmembrane proteins with many

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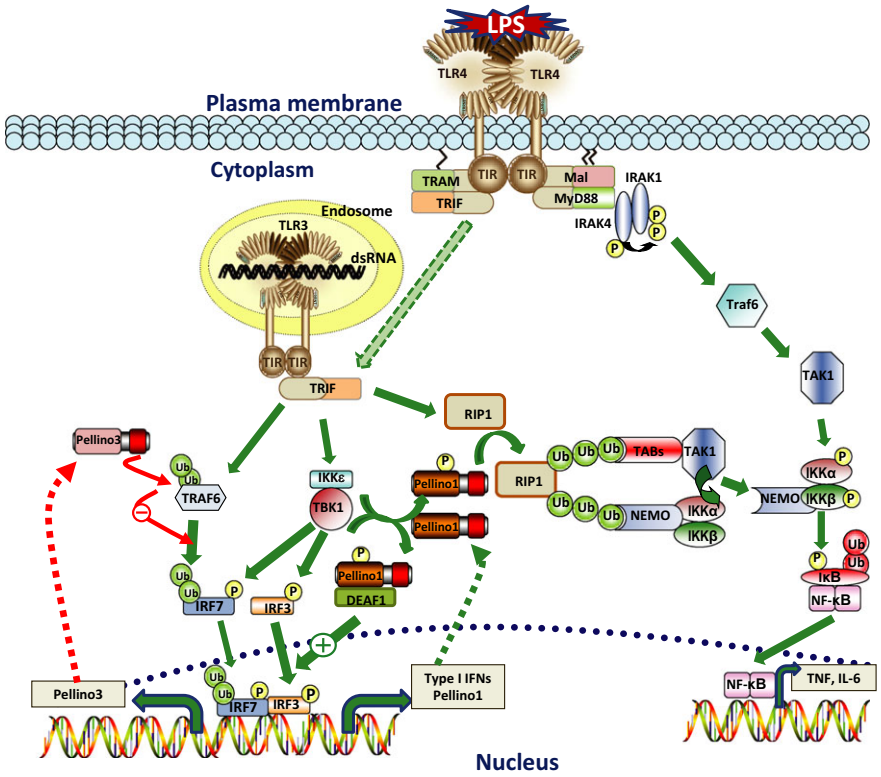

Fig. 1. Pellino1 and Pellino3 regulate Toll-like receptor (TLR) signaling. Lipopolysaccharide (LPS) stimulates TLR4 to interact with MyD88, via the bridging adapter Mal. This results in recruitment and phosphorylation of IRAKs that stimulate TRAF6 to sequentially activate TAK1 and the IKK complexes resulting in downstream activation of NF-κB and induction of the pro-inflammatory genes encoding TNF and interleukin-6 (IL-6). TLR4, via TRAM, and TLR3 recruit TRIF that then interacts with RIP1. Pellino1 catalyses K63-linked polyubiquitination of RIP1 that facilitates the recruitment of the TAK1 and IKK complexes via the ubiquitin-binding domains of the associated TGFβ-activated kinase 1 binding proteins (TABs) and NF-κB essential modulator (NEMO) respectively. This culminates in TAK1-mediated activation of NF-κB. TRIF also activates IKKε/TBK1 to phosphorylate and activate IRF3 and IRF7 resulting in induction of type I IFNs. IRF3 also induces Pellino1 that is subject to phosphorylation and activation by IKKε/TBK1. Pellino1 interacts with the deformed epidermal autoregulatory factor-1 (DEAF1) transcription factor and both of these proteins augments binding of IRF3 to the IFNβ promoter. TRIF can also trigger TRAF6-mediated K63-linked polyubiquitination of IRF7 that promotes its nuclear translocation and induction of Pellino3. The latter catalyzes K63-linked polyubiquitination of TRAF6 that inhibits the TRAF6/IRF7 interaction, impairs ubiquitination of IRF7 culminating in reduced nuclear translocation of IRF7 and repression of type I IFN expression.

members of the family, like TLR4, being located in the plasma membrane where they can recognize PAMPs such as lipopolysaccharide (LPS) on the microbial surface (4). Other TLRs, like TLR3, are located in endosomal membranes where they can detect nucleic acid from viruses that have infected the cell. All TLRs share with the IL-1 receptor (IL-1R) an intracellular Toll/IL-1R (TIR) homology domain and stimulation of TLRs by respective PAMPs facilitates the recruitment of specific TIR adapter proteins to the TIR domain of the activated TLR (1). All TLRs, with the exception of TLR3, recruit the TIR adapter myeloid differentiation primary response protein 88 (MyD88) (5). Most of these TLRs and IL-1R interact directly with MyD88, via homotypic TIR associations, but TLR4 requires the bridging adapter MyD88 adapter like (Mal) to recruit MyD88. The association of MyD88 with TLRs facilitates recruitment of members of the IL-1R-associated kinase (IRAK) family, such as IRAK-1 and IRAK-4, leading to their hyper-phosphorylation and interaction with the E3 ubiquitin ligase TRAF6 (6–9). The formation of K63 polyubiquitin chains by TRAF6 serves to bring TAK1 kinase into close proximity with its substrates IκB kinases (IKKs) (10, 11). Activation of the latter leads to phosphorylation and subsequent proteasomal degradation of the NF-κB inhibitory proteins IκB, allowing for nuclear translocation of NF-κB and its induction of a plethora of pro-inflammatory genes (12, 13). Most TLRs use this MyD88-dependent pathway to activate NF-κB, but TLR4 can additionally deploy another adapter protein, TIR domain-containing adapter protein inducing IFN-β (TRIF), via bridging TRIF-related adapter molecule (TRAM), to trigger a MyD88-independent pathway that also activates NF-κB (14). TLR3 interacts directly with TRIF that then activates RIP1 kinase to trigger downstream IKK-mediated activation of NF-κB (15, 16). TRIF can also deploy the IKK-related kinases IKKε (also known as IKKi) and TANK-binding kinase

1 (TBK1) to catalyze phosphorylation and activation of IRF3 and IRF7 leading to their nuclear translocation and induction of type I IFNs (3, 17).

These PRR signaling pathways are highly conserved across evolution with early studies on innate immunity in primitive organisms like *Drosophila* informing discovery on the components and organization of PRR pathways in mammalian systems (18). In an analogous manner to MyD88-dependent signaling by mammalian TLRs, *Drosophila* Toll utilizes adapter molecules, like dMyD88 and tube, to connect to the IRAK homologue Pelle (19, 20) and Pelle then triggers downstream activation of NF-κB family members Dorsal and Dif to induce the expression of antibacterial genes (21–23). Using Pelle as a bait in a yeast two hybrid screen, Pellino was first identified in *Drosophila* as a Pelle-interacting protein (24). A later report demonstrated that Pellino is required for induction of the antimicrobial peptide drosomycin and as a positive regulator of innate immunity in response to Gram-positive bacterial infection (25). Interestingly a more recent study has reported a directly contrasting role for *Drosophila* Pellino as a negative regulator of Toll signaling and inhibitor of antibacterial responses to Gram-positive bacteria (26). The authors demonstrate that upon stimulating Toll, Pellino is recruited to the plasma membrane-bound *Drosophila* MyD88 resulting in ubiquitination and degradation of *Drosophila* MyD88 (dMyD88) and suppression of downstream Toll signaling and repression of anti-bacterial peptides. It is presently unclear as to why these 2 studies demonstrate such directly opposing roles for Pellino in *Drosophila* Toll signaling.

The initial discovery of Pellino in *Drosophila* laid the foundation for the subsequent identification of three members of the mammalian Pellino family (Pellino1, 2, and 3) with Pellino3 being expressed in two alternative spliced forms (27–31). The Pellino-Pelle interaction is conserved in evolution as indicated by Pellino1 (28), Pellino2 (27, 32, 33), and Pellino3 (29) being capable of interacting with the Pelle orthologues IRAK-1 and IRAK-4. There is some confusion in relation to the requirement of IRAK kinase activity for mediating such interactions with some studies indicating a requirement (29, 34, 35) while we and others suggest that kinase activity is dispensable (32, 36, 37). The first studies also demonstrated that Pellino proteins can interact with other downstream TLR signaling molecules including TRAF-6 and TAK-1 initially suggesting that the primary Pellino function is to provide a scaffold framework for signaling complex assembly in TLR/IL-1R signaling (28, 29, 33, 38).

Pellino proteins contain RING-like and FHA domains

Early bioinformatic studies indicated that Pellino proteins contained a putative RING-like domain in their C-termini (30). A RING domain consists of a conserved pattern of cysteine and histidine residues that forms a structure that is capable of coordinating two zinc atoms and is a defining feature of the RING class of E3 ubiquitin ligases (39). Pellino proteins contain a conserved pattern of cysteine and histidine residues that resembles the canonical RING motif suggesting that they may be E3 ubiquitin ligases. This was supported when co-expression of Pellino proteins and IRAK-1 resulted in ubiquitination of the latter whereas Pellino forms with mutations in their RING-like domain were ineffective (34). We provided the first direct evidence to confirm that all three mammalian Pellino proteins have intrinsic E3 ligase catalytic activity *in vitro* (36). While co-expression of Pellino proteins and IRAK-1 in cells results in the ubiquitination of IRAK-1 by K63-linked polyubiquitin chains, in an *in vitro* setting Pellino1 can work with the E2 enzyme Ubc13-Uev1a to form K63-linked polyubiquitin chains, whereas in conjunction with UbcH4 or UbcH5a/UbcH5b E2 enzymes, the polyubiquitin chains have mainly K11 and K48 linkages (37). Furthermore, the K63-linked chains generated by Pellino1 *in vitro* are unanchored whereas the K11/K48 chains are anchored to IRAK or the Pellino protein itself. Whereas Pellino1 catalyzes the formation of unanchored K63-linked chains *in vitro*, the co-expression of Pellino1 and IRAK1 in cells leads to the formation of K63-linked chains that are attached to IRAK-1 (37). This difference may be due to Pellino proteins initially employing an E2 enzyme other than Ubc13-Uev1a to ligate the first ubiquitin to IRAK-1 in cells and this ubiquitin then seeds a growing K63-linked chain that is catalyzed by Pellino proteins in association with Ubc13-Uev1a. Alternatively, a different E3 ligase may add the first ubiquitin moiety.

While the RING-like domain is essential for mediating Pellino-induced ubiquitination of IRAK-1, it is dispensable for the Pellino-IRAK interactions (34, 35). Indeed truncation mutants of the Pellino proteins that lack the entire C-terminus RING-like domain are capable of binding to IRAK-1 (35). The x-ray crystal structure of this corresponding N-terminal fragment of Pellino2, that lacks its RING-like domain, has been resolved and uncovers a cryptic Forkhead-associated (FHA) domain that was not apparent from primary sequence analysis (35). The FHA domain is a recognized phosphothreonine-binding module (40, 41). The canonical FHA domain contains a number of highly

conserved residues that directly contact the phosphothreonine residue of the target protein and mutation of these corresponding residues in Pellino2 abrogates its ability to bind IRAK-1, suggesting that Pellino2 recognizes IRAK-1 via a functional FHA domain. Furthermore, the Pellino2 FHA domain can bind to phosphothreonine-containing peptides and enzymatic dephosphorylation of IRAK-1 precludes its binding to the Pellino2 FHA domain. While the target phosphothreonine residue(s) on IRAK-1 has yet to be identified the above findings strongly indicate that Pellino2 uses its FHA domain to recognize phosphorylated IRAK-1. In addition to Pellino2 containing a core FHA domain, the x-ray crystal structure also revealed that Pellino2 contains a 'wing' or appendage that is unique among FHA structures. The wing is tightly packed against the FHA core structure and may work with the FHA domain in recognizing target proteins. Based on amino acid similarity, apart from some loop regions, the core FHA domain and wing structures are conserved across the Pellino family (35) and the FHA-mediated recognition of phosphorylated target proteins is likely to be a common feature of all members of the family. However, as described below, members of the mammalian Pellino family do not appear to show functional redundancy with different Pellino proteins demonstrating preference for particular substrate proteins. A recent study has shown that whilst the FHA domain in Pellino proteins mediates interactions with target proteins via recognition of phospho-threonine residues, the affinity of the FHA domain for the target protein is also dependent on the local amino acid sequence context of the phosphothreonine residue, with the FHA domains from the different Pellino proteins displaying varying affinities for different surrounding sequences. This likely underlies the strong recognition of RIP1 but not TRAF6 by Pellino1 whereas Pellino3 demonstrates high affinity for TRAF6 (42).

Pellino proteins are modified by ubiquitination and sumoylation

While IRAKs are subject to ubiquitination by Pellino proteins the latter are substrates for the kinase activity of IRAKs. Both IRAK-1 and IRAK4 catalyze *in vitro* phosphorylation of Pellino proteins (32, 34, 37) resulting in increased E3 ligase activity (37) and auto-ubiquitination of Pellino1 on residues K169, K202, and K266 (43). In a cell setting, the consequence of IRAK-1-induced activation and ubiquitination of Pellino proteins may be Pellino degradation given that overexpression of a kinase-active form of IRAK results in polyubiquitination and decreased expression levels of Pellino proteins (36). Conversely, cells that lack IRAK-1 or expressing kinase-dead IRAK-1 demonstrate augmented expression levels of Pellino1 (44). If auto-ubiquitination of Pellino proteins leads to degradation the underlying mechanism remains to be defined. In a cell setting, Pellino proteins tend to catalyze ubiquitination of substrate proteins by K63-linked polyubiquitin chains whereas K48-linked chains normally provide the signature for proteasome-mediated degradation. However, a recent report has shown that ubiquitination of Pellino3 results in its lysosomal degradation by selective autophagy whereas blockade of the proteasome had no effect on Pellino3 degradation (45).

In addition to being subject to post-translational modification by ubiquitination, Pellino1 can also be modified by covalent attachment of the small ubiquitin-like modifier (SUMO) protein at residues K202, K266, K295, K299, and K303 (46). Such sumoylation is catalyzed by the SUMO-conjugating enzyme Ubc9 and it is interesting to note that some of the sumoylation sites in Pellino1 are common to the ubiquitination sites above suggesting competition between sumoylation and ubiquitination. The functional consequence of Ubc9-induced sumoylation of Pellino1 is currently unknown.

Phosphorylation of Pellino proteins

The IRAK-1 and IRAK-4 phosphorylation sites in Pellino1 have been identified and correspond to multiple serine (S)/threonine (T) residues with S76, S78, T80, S82, and T86 being located in the wing appendage outside the core FHA domain and T288 and S293 just prior to start of the RING-like domain (43). Interestingly, the phosphorylation of any one of the residues S76, T288, and S293, or by the combined phosphorylation of the residues S78, T80, and S82 is sufficient to reveal full Pellino1 E3 ligase activity. This has important consequences for any phosphatase-based mechanisms that may inactivate Pellino function since dephosphorylation of multiple sites would be required to terminate activity. Given such a challenge, it may not be surprising that Pellino degradation may be an important mechanism for negatively regulating Pellino function. Most of the IRAK phosphorylation sites in Pellino1 are conserved in Pellino2 and Pellino3, suggesting that the phosphorylation and activation of each member of the Pellino family by IRAKs is likely mediated by a common mechanism. However, some of these sites are also conserved in *Drosophila* Pellino and mutation of these

serine/threonine residues to alanine fail to affect the E3 ligase activity of *Drosophila* Pellino or its regulatory effects on Toll signaling (26).

More recently IKKε and TBK1 kinases, which mediate TRIF-dependent activation of IRF3, have also been shown to be capable of phosphorylating and activating Pellino1 (47). In this study, a yeast-two hybrid screen initially identified IKKε as an interacting protein with the FHA domain of Pellino1 and both IKKε and TBK1were subsequently shown to phosphorylate and activate the E3 ligase activity of Pellino1 *in vitro*. The major TBK1 phosphorylation sites on Pellino1 were also identified as S76, T80, T288, and S293. As discussed above, a number of these residues are key activation sites and provide a likely molecular basis to TBK-1/IKKε induced activation of Pellino1. While stimuli, such as LPS and Poly(I:C) that trigger the TRIF/TBK1/IKKε pathway, have been shown to stimulate the E3 ligase activity of Pellino1 in cells, it has not been technically possible to detect the phosphorylation sites on endogenous Pellino1 (47). Interestingly, in addition to activating Pellino1, the TRIF/TBK1/IKKε pathway can also induce expression of Pellino1 via the transcription factor IRF3, and this may explain the high levels of Pellino1 in rhinovirus-infected bronchial epithelial from COPD patients (48).

Since IRAK and TBK1/IKKε kinases are capable of phosphorylating Pellino1, a study explored the relative importance of these kinases in various pathways that can activate Pellino1 (44). Unexpectedly, the role of IRAK-1 appears to be restricted to mediating IL-1-induced activation of Pellino1, whereas TNF signaling and TLRs use TBK1/IKKε kinases to stimulate Pellino1. Given that a number of these TLRs can also strongly activate IRAK-1, the mechanism by which a given pathway selects a discrete kinase to phosphorylate and activate Pellino1 awaits clarification.

Molecular studies have thus demonstrated Pellino proteins to contain a FHA domain that enables their interaction with kinases such as IRAK-1, IRAK-4 and TBK1/IKKε resulting in Pellino phosphorylation, activation of their E3 ligase activity and ubiquitination of substrate proteins including some of their kinase partners such as IRAK-1. We now move to discuss the downstream functional and physiological consequences of these molecular roles of the Pellino family. The largely conserved nature of the FHA and RING-like domains across the Pellino family would suggest that Pellino proteins may share common functions but studies are revealing specialized physiological roles for individual members of the Pellino family.

Physiological roles of Pellino proteins in PRR signaling

Pellino1 mediates TRIF-dependent TLR signaling

Pellino1-deficient mice have been very valuable in implicating specific roles for Pellino1 in TLR3 and TLR4 biology (49). Pellino1-deficient mice are viable and develop normally but are less responsive than wildtype mice to the TLR3 and TLR4 ligands, Poly(I:C), and LPS, respectively, in terms of induction of pro-inflammatory genes like TNF and IL-6. Consequently, Pellino1 deficiency results in resistance to septic shock normally effected by Poly(I:C) and LPS. This is also consistent with reduced TLR3/TLR4-induced activation of IKKs and NF-κB in cells from Pellino1-deficient mice whereas the activation of these inflammatory pathways by other TLRs are generally unaffected suggesting a specificity for Pellino1 in mediating TLR3 and TLR4 signaling. Pellino1 was shown to mediate TRIF-dependent activation of NF-κB by interacting with the downstream kinase RIP1 and catalyzing its ubiquitination (49), thus triggering downstream activation of IKKs and NF-κB (15) (*Fig. 1*). This adds to our understanding of the sequence of intracellular signaling events in TLR3 and TLR4 pathways in that their stimulation results in the RIP homotypic interaction motif in TRIF interacting with RIP1 (16) followed by recruitment of Pellino1 (49). Since the latter is phosphorylated and activated by TBK1/IKKε, that are also triggered by TRIF (44), this results in Pellino1-mediated ubiquitination of RIP1 and the recruitment of TAK-1 and IKK complexes to the polyubiquitin chains (*Fig. 1*). TAK-1-mediated phosphorylation of IKKs culminates in downstream activation of NF-κB and a pathway by which Pellino1 can contribute to the expression of pro-inflammatory genes. This is also supported by a recent study demonstrating that mice expressing a Pellino1 transgene show increased levels of pro-inflammatory cytokines like TNF and IL-6 (50).

While TRIF utilizes RIP1 to activate the NF-κB pathway it can also recruit TBK1/IKKε kinases to activate IRF transcription factors and induce type I IFNs like IFNβ (14, 17, 51–53) (*Fig. 1*). However, fibroblasts from Pellino1-deficient mice retain full capacity for TLR3 and TLR4-induced activation of IRFs and expression of IFNβ suggesting that Pellino1 mediates TRIF-dependent activation of NF-κB and induction of pro-inflammatory genes but does not regulate the TRIF-IRF3-IFNβ signaling axis (49). Such selectivity of Pellino1 for the NF-κB arm also applies to human bronchial epithelial cells in which Pellino1 knockdown suppresses virus-induced expression of IL-6 and CXCL8 but not IFN-related genes (54). However, a knockin mouse expressing an E3

ligase dead form of Pellino1 has questioned the selective effect of Pellino1 on NF-κB (55). The inactive mutant form of Pellino1 showed similar expression patterns as wildtype Pellino although the expression levels of the mutant protein were lower. Unlike Pellino1-deficient mice, macrophages from the Pellino1-ligase dead knockin mice showed normal Poly(I:C)-induced ubiquitination of RIP1, activation of NF-κB and induction of pro-inflammatory genes (55). However, Poly(I:C)/LPS-induced expression of IFNβ was greatly reduced in macrophages and dendritic cells from knockin mice and this was associated with reduced recruitment of IRF3 to the IFNβ promoter. This suggests that the E3 ligase activity of Pellino 1 facilitates the recruitment of IRF3 to the IFNβ promoter with a recent report describing the transcription factor deformed epidermal autoregulatory factor 1 (DEAF1) as a Pellino1-interacting protein that associates with IRF3 and the IFNβ promoter to drive IFNβ expression (56) (*Fig. 1*).

Pellino1-deficient mice and knockin mice in which wildtype Pellino1 is replaced by a point mutant lacking E3 ubiquitin ligase activity offer contrasting conclusions on the roles of Pellino1 in TRIF-mediated signaling. The former indicates a critical role for Pellino1 in promoting ubiquitination of RIP1 and downstream inflammatory signaling, whereas findings from the knockin mice propose that the loss of E3 ligase activity in Pellino1 has no consequence for RIP1 ubiquitination but instead is key for specifically activating the IFNβ promoter. This may suggest that Pellino1 promotes ubiquitination of RIP1 in an indirect manner that is independent of its intrinsic E3 ligase activity.

Pellino3 negatively regulates TLR3 signaling

We have generated Pellino3-deficient mice and while they develop normally, they have been very valuable in revealing a novel regulatory role for Pellino3 in TLR3 biology (57). Cells from these mice respond normally to a range of TLR ligands with respect to induction of pro-inflammatory genes and type I IFNs but Pellino3 deficiency leads to augmentation of TLR-3 induced expression of type I IFNs (*Fig. 1*). The enhanced expression of IFNs confers on Pellino3-deficient mice a greater ability to clear encephalomyocarditis virus (EMCV). These findings indicate that Pellino3 negatively regulates TLR3-induced expression of type I IFNs and the underlying mechanism involves the targeting and inhibition of the transcription factor IRF7. In this model, TLR3 induces expression of Pellino3, via the TRIF/TBK1/IRF3 signaling axis, followed by interaction of Pellino3 with TRAF6 (*Fig. 1*). Pellino3 promotes polyubiquitination of TRAF6 and so precludes the interaction of TRAF6 with IRF7 and impairs polyubiquitination of the latter. Since ubiquitination of IRF7 is associated with its activation (58), Pellino3 acts to inhibit nuclear translocation of IRF7 to the IFNβ promoter and so decrease transcription of the *Ifnb* gene. This model thus highlights an important physiological role for Pellino3 as part of a negative feedback system in the TLR3 pathway in which activation of TLR3 drives TRIF-induced expression of Pellino3 that then targets IRF7 to repress ongoing TRIF-induced transcription of IFNβ. The negative regulation of IFNβ expression by Pellino3 is also employed by oxidized low-density lipoprotein (oxLDL) to inhibit LPS-induced expression of IFNβ (59, 60). However, the mechanism underlying these inhibitory effects of Pellino3 differ from its regulatory effects in the TLR3 pathway in that OxLDL, via the scavenger receptor-A1, activates Pellino3 to catalyze monoubiquitination of the protein TANK that normally acts as an adapter to connect TRAF3 to TBK1/IKKε and ultimately activation of IRF3 (60). Monoubiquitination of TANK precludes the association of TBK1 with TRAF3 and manifests oxLDL-mediated inhibition of IFNβ expression (60).

The regulatory effects of Pellino3 on IFNβ expression highlights opposing functions for members of the Pellino family in that the inhibitory roles of Pellino3 in TRIF signaling (57) contrast with the positive mediatory roles of Pellino1 in TRIF-dependent pathways (49, 55). However, given the various roles of the family members in this pathway and its importance in antiviral signaling we were intrigued to discover a viral Pellino homologue in an entomopoxvirus that, while lacking a RING-domain, contains a FHA domain and interferes with TLR-induced activation of NF-κB (61). The existence of a viral Pellino homologue implicates potential functional importance for Pellino proteins in antiviral immunity. Viruses tend to develop evasive strategies by targeting critical components of the antiviral response in the host. However, to date no homologues of Pellino have been found in viruses that show tropism for mammals.

Pellino2 and TLR4 signaling

Details on the role of Pellino2 in TLR signaling are relatively sparse. Knockdown of Pellino2 inhibits LPS-induced polyubiquitination of IRAK-1 (62) and activation of NF-κB (27, 63) and ERK and JNK pathways (62). It has also been shown that LPS promotes the interaction of Pellino2 with BCL10 to trigger downstream activation of NF-κB with

suppressor of cytokine signaling 3 (SOCS3) being capable of targeting this interaction to mediate IL-10-induced suppression of LPS signaling (63).

Pellino3 mediates Nod2 signaling

Much research focus in the Pellino field has explored the physiological roles of these proteins in TLR signaling. However, we have delineated a novel function for Pellino3 as a mediator in the pathway triggered by NOD2, a member of the NLR pathway (64) (*Fig. 2*). NOD2 is as an important player in regulating intestinal inflammation with loss of function mutations in NOD2 being associated with increased susceptibility to Crohn's disease (65–68). Nod2 senses bacterial infection by recognizing peptide derivatives of peptidoglycan (69, 70) and responds by recruiting and stimulating K63-linked ubiquitination of RIP2 kinase. In an analogous way to TRAF6 and RIP1 described earlier, the ubiquitination of RIP2 serves as a recruitment platform for TAK1 and IKK complexes facilitating NF-κB and MAPK activation and the induction of inflammatory genes (71–74). While a number of E3 ubiquitin ligases such as TRAF6 (73), cellular inhibitor of apoptosis proteins (cIAPs), and XIAP proteins (75–77) and ITCH (78) are proposed to mediate ubiquitination of RIP2, the functional importance of these effects remain to be defined (74, 76, 78, 79) and the identity of the functionally relevant E3 ligase for RIP2 is uncertain. We have recently used cells from Pellino3-deficient to demonstrate that Pellino3 is a key mediator of NOD2 signaling by acting as a direct E3 ligase for RIP2, promoting its K63-linked ubiquitination and triggering downstream activation of NF-κB and expression of pro-inflammatory proteins (64) (*Fig. 2*). We also propose that the Pellino3-induced ubiquitination of RIP2 may facilitate subsequent linear ubiquitination of the RIP2 signaling complex by XIAP. This would allow TAK1 to be recruited to the K63-linked chains and the NEMO component of the IKK complex to associate with linear chains thus facilitating TAK-1-induced activation of IKKs and downstream signaling (79, 80). This has intriguing similarity to a report proposing that IL-1 and TLR-induced activation of IKKs involves the modification of IRAK-1 and IRAK-4 by hybrid ubiquitin chains in which the IRAKs are initially modified by K63-linked chains to which linear chains are subsequently added (81). A paradigm may be arising in which Pellino proteins seed proteins for linear ubiquitination by initial attachment of K63 chains.

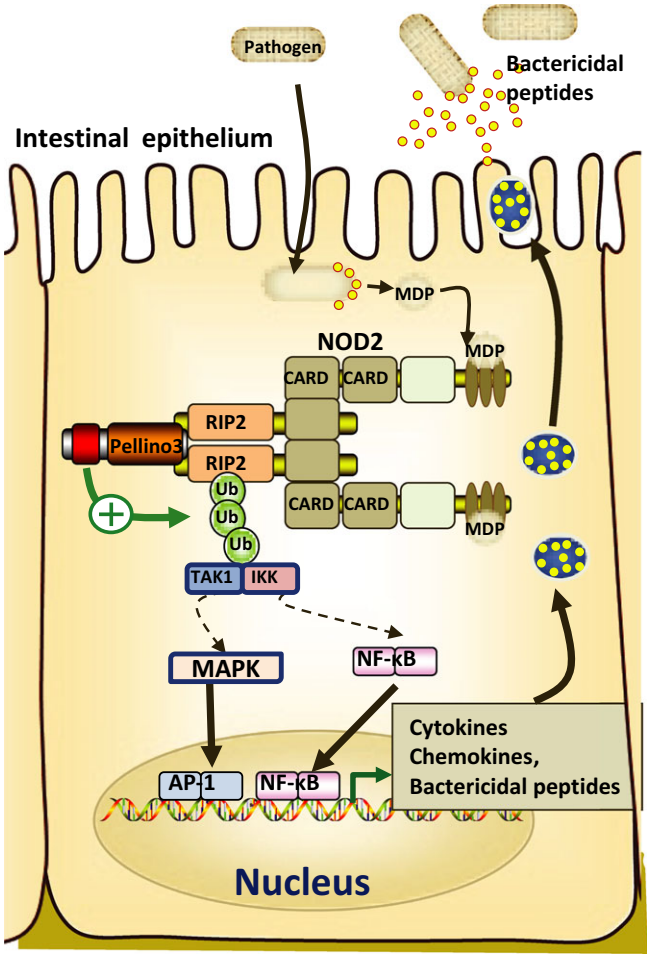

***Fig. 2.* Pellino3 ubiquitinates RIP2 and mediates NOD2 signaling.** The peptidoglycan-derived muramyl dipeptide (MDP) from bacterial pathogens triggers oligomerization of NOD2 that then utilizes its CARD domain to recruit the CARD-containing RIP2 kinase. Pellino3 is subsequently recruited to RIP2 and catalyses K63-kinked polyubiquitination of RIP2 allowing for co-recruitment of the TAK1 and IKK complexes. This facilitates TAK1-mediated activation of IKKs and downstream activation of NF-κB and activation of MAPK pathways that results in activation of AP-1. NF-κB co-operates with AP-1 to induce expression of cytokines, chemokines, and bactericidal peptides.

Given the importance of functional NOD2 in ensuring intestinal homeostasis, we also highlighted the physiological importance of Pellino3 in facilitating this homeostatic role of NOD2 by demonstrating more severe intestinal inflammation in Pellino3 knockout mice relative to wildtype counterparts when these mice were subject to various forms of experimental colitis (64). It was important to explore if this protective role of Pellino3 extended into a human context and the greatly decreased levels of Pellino3 that we observed in the colons of Crohn's disease patients certainly adds further credibility to Pellino3 deficiency being a significant contributory factor to intestinal pathophysiology.

Pellino proteins as regulators of IL-1 and TNF signaling

Given that the first Pellino protein was discovered in *Drosophila* Toll signaling and this pathway is highly conserved in

composition and organization, it not surprising that most research to date has focused on the potential roles of Pellino proteins in the related TLR pathways. However, we and others have also explored Pellino biology in the context of signaling pathways employed by some of the effector pro-inflammatory cytokines that are induced by TLRs. The IL-1 signal transduction pathway is especially pertinent given that IL-1R shares its TIR domain with TLRs and uses IRAK kinases to mediate MyD88-dependent signal transduction. TNF signaling was also of interest to our laboratory, since while not employing IRAK kinases, the TNF pathways are critically dependent on RIP kinases and it is clear from above that there is an emerging strong theme of an intimate relationship between the Pellino and RIP kinase families of proteins.

Are Pellino proteins involved in IL-1 signaling?

Early studies implicated a signaling role for Pellino1 in the IL-1 pathway by virtue of knockdown of Pellino1 suppressing IL-1-induced activation of NF-κB and induction of pro-inflammatory gene expression in HEK293 cells (28) and a murine intestinal epithelial cell line (82). Interestingly the latter study also reported that the anti-inflammatory cytokine TGF-β inhibits IL-1 signaling by inducing the inhibitory Smad6 protein to interact with Pellino1 and disrupt Pellino1 association with IRAK-1, IRAK-4, and TRAF-6. A more recent study has suggested that TGF-β also induces the related Smad7 protein to target Pellino1 and block the interaction of the latter with the IRAK-1/IRAK-4/TRAF-6 complex resulting in reduced IL-1 and LPS signaling (83). Both Smad6 and Samd7 are proposed to work in tandem, via their MH2 domains, to simultaneously target different regions of Pellino1 (residues 1–137 for Smad6 and residues 198–345 for Smad7) and optimally disrupt Pellino1 binding to its signaling complex. These findings suggest that Pellino1, via its interaction with the IRAK signaling complex, plays an important mediatory role in IL-1 signaling. However, IL-1 signaling is not affected in cells from IRAK-1 kinase-dead knockin mice that lack Pellino1 E3 ligase activity (44). Furthermore, Pellino1 does not regulate IL-1-induced polyubiquitination of IRAK-1 in HEK293 cells (62), and IL-1 signaling is fully intact in murine embryonic fibroblasts (MEFs) from Pellino1-knockout mice (49), suggesting that Pellino1 does not play a role in the IL-1 pathway.

Early studies in the field also demonstrated that knockdown of Pellino2 inhibits IL-1 induced activation of NF-κB (27, 63). Overexpression of Pellino2 in cells triggers activation of the ERK and JNK MAPK pathways (33) with Pellino2 knockdown attenuating IL-1 and LPS-induced activation of the MAPK pathways and decreasing the stability of transcripts encoding IL-6, IL-8, and TNF (62). Pellino2 knockdown also inhibits IL-1-induced polyubiquitination of IRAK-1 suggesting that Pellino2 may play a key role in promoting IRAK-1 ubiquitination and triggering downstream activation of the ERK and JNK pathways. The physiological role of Pellino2 in immunity awaits the generation of genetic models.

Overexpression of Pellino3 can activate ERK (29), JNK (29, 84), and p38 MAPK (38), while Pellino3 knockdown inhibits IL-1 and LPS-induced activation of p38 MAPK, the transcription factor cAMP-responsive-element-binding protein (CREB) and expression of the CREB-responsive gene IL-10 (38, 85). Similar approaches have suggested the shorter spliced form Pellino3b, negatively regulates IL-1-induced TAK-1-dependent NF-κB activation by ubiquitinating and stabilizing IRAK-1 with K63-linked chains, thus sequestering the TAK-1- complex in the membrane and preventing its activation of NF-κB in the cytosol (84). However, in the last few years we have generated Pellino3-deficient mice and have yet to detect any deficiency in IL-1 signaling.

It is clear from above that the role of Pellino proteins in IL-1 signaling is far from certain. This is somewhat surprising given that Pellino proteins were discovered as IRAK-interacting proteins, and IRAK kinases play important role in the IL-1 pathway. This suggests that Pellino proteins may have more functionally relevant substrate proteins such as TRAF6 and RIP kinases as described above. The critical role of the latter in TNF signaling prompted enthusiasm in our laboratory to probe the potential involvement of Pellino proteins in TNF pathways.

Pellino3 controls cell fate in TNF signaling

Early studies in the Pellino field had indicated a lack of role for Pellino1 and Pellino2 in TNF-induced activation of NFκB (27, 28), but we have recently demonstrated an important role for Pellino3 in controlling cell fate in response to TNF (86) (*Fig.* 3). TNF can activate NF-κB and induce pro-inflammatory gene expression but can also be a critical determining factor in dictating whether a cell lives or dies (79, 87). Stimulation of TNF receptor I (TNF-R1) leads to association of the latter with TNF-R1-associated death domain protein (TRADD) (88) and RIP1 (89) followed by recruitment of a number of E3 ubiquitin ligases including TRAF2, TRAF5, cIAP1, and cIAP2 thus forming a large multimeric complex termed Complex I (90–93) (*Fig.* 3). These E3 ligases catalyze polyubiquitination of RIP1 that is then

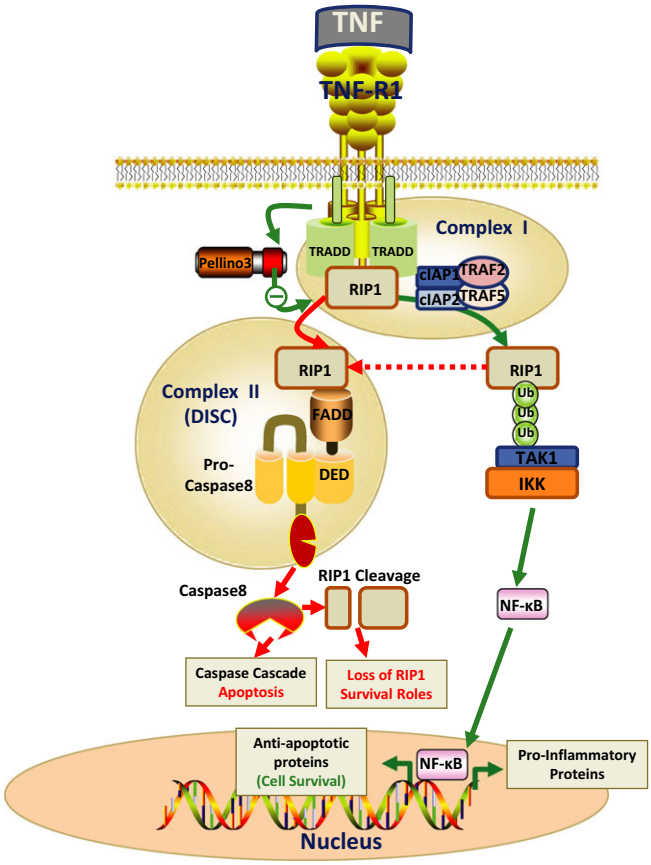

Fig. 3. Pellino3 protects cells from pro-apoptotic effects of TNF. Stimulation of TNF-RI with TNF leads to association with the adapter protein TRADD and RIP1 kinase. This is followed by recruitment of the E3 ubiquitin ligases TRAF2, TRAF5, cIAP1, and cIAP2 that catalyze K63-linked polyubiquitination of RIP1 as part of a multimeric complex termed Complex I. Ubiquitinated RIP1 deploys TAK1 and IKK complexes to activate NF-κB and induce pro-inflammatory gene expression. NF-κB also induces anti-apoptotic proteins that favor cell survival. Deubiquitination of RIP1 leads to its re-distribution to Complex II [also known as Death induced signaling complex (DISC)] with the death effector domain (DED)-containing Fas-associated death domain protein (FADD) and procaspase8. This results in auto-processing of caspase8 to generate catalytically activate caspase8 that triggers a downstream caspase cascade and ultimately apoptosis and cell death. Active Caspase8 can also cleave RIP1 to impair its cell survival roles. Pellino3 blocks the killing effects of TNF by interacting with RIP1 in a TNF-dependent manner to preclude the association of RIP1 with FADD and caspase8 thus reducing complex II formation and impairing the pro-apoptotic pathway. This also prevents caspase8-mediated cleavage of RIP1 to retain the pro-survival roles of the latter. TNF, tumor necrosis factor; NF-κB, nuclear factor-κB.

recognized by the ubiquitin-binding domains of proteins in the IKK and TAK1 complexes resulting in activation of NF-κB and pro-inflammatory gene expression (79, 94). While NF-κB can also induce anti-apoptotic genes to protect cells from death (95–97), de-ubiquitination of RIP1 represses activation of NF-κB, and the unmodified RIP1 associates with Fas-associated death domain (FADD) protein and pro-caspase8 to form Complex II [also known as death inducing signaling complex (DISC)] (98–100). Complex II promotes processing and activation of caspase8 triggering the caspase cascade and apoptosis (101). Caspase8 can also cleave RIP1 to further augment TNF-induced apoptosis. Interestingly, we have shown that loss or knockdown of Pellino3 expression sensitizes cells to TNF-induced apoptosis without regulating activation of the NF-κB pathway (102). Instead Pellino3 is cytoprotective by interacting, via its FHA domain, with RIP1 and blocking the interaction of the latter with FADD and caspase8 (*Fig.* 3). This prevents downstream caspase activation and apoptosis and also reduces caspase8-mediated cleavage of RIP1 to favor cell survival as an outcome to TNF signaling. Furthermore, TNF-induced hepatotoxicity and lethality is exacerbated in Pellino3-deficient mice (86), emphasizing the physiological importance of Pellino3 in regulating cell survival in response to TNF challenge. While the targeting of RIP1 is central to this regulation by Pellino3, intriguingly the effects are independent of its RING-like domain and, unlike the effects of Pellino1, are not mediated by affecting the ubiquitination status of RIP1. This indicates that Pellino proteins may also have functional roles that are independent of E3 ligase activity. This study in conjunction with the other emerging roles of the Pellino family serves to further highlight an expanding theme of Pellino proteins targeting kinase families, such as the IRAKs and RIPs, to regulate immune signaling.

Pellino proteins as regulators and mediators of disease

Given the emerging roles of Pellino proteins in innate immune signaling, coupled to the propensity of dysregulated immune responses to trigger disease, it is not surprising that various reports implicate Pellino proteins as regulators of pathophysiology. As already stated, we have demonstrated Pellino3 expression to be greatly diminished in colon samples from patients with Crohn's disease and given that we have also shown Pellino3-deficient mice to have aggravated pathology in colitis models, we propose a protective role for Pellino3 in inflammatory bowel disease (64). Elevated levels of Pellino1 and Pellino2 have been recorded in asthmatics (103), children with protracted bacterial bronchitis (104), and in transplant patients with acute kidney rejection (105). Furthermore, polymorphisms in the *PELI1* locus, that encodes Pellino1, associate with vasculitis in young children (106, 107) and with increased susceptibility to systemic lupus erythematosus in a Chinese population (108). While the latter studies are restricted to interpreting association of Pellino genes and proteins with disease, a number of disease

models have recently highlighted that Pellino proteins may play key roles in various pathogenic processes. These range from Pellino1 being shown to regulate autoimmunity and lymphomagenesis to a very recent study from our own group showing that Pellino3 plays a protective role in obesity-driven insulin resistance.

Pellino1 and autoimmunity

While Pellino1-deficient mice were initially observed to develop and grow normally, it was noticed that, with aging, these mice had enlarged lymph nodes and inflammatory cell infiltrates in tissues, strongly suggesting that absence of Pellino1 is associated with spontaneous autoimmunity (109). These mice also display higher frequency and number of memory T cells but lower frequency of naive cells. Costimulation of the T-cell receptor (TCR) and CD28 in their $CD4^+$ and $CD8^+$ T cells result in hyper-activation and enhanced proliferation compared to the same cells from wildtype mice. TCR/CD28 normally activates NF-κB subunits like c-Rel to induce CD28-responsive gene such as IL-2 that drives autocrine T-cell activation and proliferation whereas TCR activation in the absence of CD28 results in T-cell anergy. Interestingly, Pellino1 is induced in response to TCR/CD28 costimulation and promotes K48-linked polyubiquitination of c-Rel followed by c-Rel degradation (109). In the absence of Pellino1, TCR/CD28 promotes sustained activation of c-Rel that drives dysregulated activation and proliferation of T cells ultimately compromising self-tolerance (109, 110). Given this proposed role for Pellino1 in preventing autoimmunity it was somewhat surprising that a subsequent study described less severe experimental autoimmune encephalomyelitis (EAE), a murine model of multiple sclerosis, in Pellino1-deficient mice (111). However, the latter study described a microglia-specific role for Pellino1 in driving neuroinflammation. Pellino1 is unique in its family with its high level of expression in microglia. While the initial studies in macrophages and dendritic cells from Pellino1-deficient mice indicated Pellino1 to play a selective role in TRIF-mediated signaling (49), Pellino1 mediates MyD88 signaling in microglia, with loss of Pellino1 resulting in impaired pro-inflammatory gene induction by TLRs that use MyD88 as their exclusive adapter molecule (111). Intriguingly, Pellino1 does not regulate MyD88-induced activation of NFκB in microglia but instead mediates MyD88 signaling that leads to ERK, JNK, and p38 MAPK pathways. In microglia, MyD88 employs Pellino1 to mediate TRAF6-induced activation of cIAP1 and cIAP2 (112). The activated cIAP E3 ligases facilitate ubiquitination and degradation of TRAF3 (that normally tethers the MyD88 signaling complex to the membrane), thus allowing the liberated complex to sequentially activate TAK-1 and MAPKs in the cytosol and induce pro-inflammatory gene expression. In this pathway, Pellino1 seems to target cIAP2 since Pellino1-deficient microglia show impaired TRAF6-induced ubiquitination of cIAP2. However, it is not known if Pellino1 is the direct upstream E3 ligase for cIAP2. It should be noted that this defective MyD88 signaling, in the absence of Pellino1, applies specifically to microglia and not to peripheral immune cells. The latter express high levels of all Pellino family members (109), and thus the loss of Pellino1 may be masked by functional compensation by Pellino2 and/or Pellino3. These experimental models thus implicate Pellino1 as a mediator of neuroinflammation. This may have pathophysiological relevance since Pellino1 expression is increased in EAE and more pertinently in post mortem brain samples from patients with multiple sclerosis (111). Given neuroinflammation underlies a number of neurodegenerative disorders, it will be interesting to further explore the potential contribution of the Pellino proteins to various neuropathological states.

Pellino1 contributes to cardiac dysfunction

Recent reports have demonstrated Pellino1 to make an important contribution to cardiac dysfunction following myocardial infarction or mechanical stress (113, 114). Pellino1 expression was shown to be increased in cardiomyocytes and fibroblasts under hypoxic conditions in a murine model of myocardial infarction (113). Conditional deletion of Pellino1 in the myocardium or cardiac delivery of Pellino1 siRNA suppresses myocardial infarction-induced cardiac dysfunction, reduces scar size, and decreases pro-inflammatory gene expression and cardiac infiltration of inflammatory leukocytes. The protective effects of Pellino1 suppression are also paralleled with decreased ubiquitination of RIP1, and so the contribution of Pellino1 to cardiac damage may be due to its ability to promote ubiquitination of RIP1 and downstream pro-inflammatory signaling in TLR3 and TLR4 pathways (as described earlier). Similarly suppression of Pellino1 expression reduces mechanical stress-induced cardiac dysfunction, cardiac hypertrophy and cardiac fibrosis in rat hearts and these protective effects are associated with reduced expression of TGFβ (114).

Pellino1 promotes lymphomagenesis

The generation of Pellino1 transgenic mice, in which expression of the Pellino1 transgene is under the control of

the B-actin and CMV promoters, has revealed a potential role for Pellino1 in driving B-cell lymphoma formation (50). These mice display premature mortality with over half of the mice showing a wide range of tumors in various tissues. A particularly high frequency of lymphoid tumors in the thymus, spleen and lymph nodes are observed and these lymphoid tissues are characterized by increased numbers of proliferating and activated B cells. Interestingly tumors in the non-lymphoid tissue from the Pellino1 transgenic mice also demonstrate high numbers of proliferating B cells suggesting that overexpression of Pellino1 drives tumor formation that is strongly associated with B-cell activation and infiltration. Indeed the enhanced expression of Pellino1 was shown to drive lymphomagenesis. A mechanistic explanation was provided for these effects of Pellino1 by demonstrating that Pellino1 can interact with and promote K63-linked polyubiquitination and stabilization of the proto-oncogene BCL6. Furthermore, there is a strong correlation between the levels of Pellino1 and BCL6 in various B-cell lymphoma cell lines and this correlation extends to cells from patients with diffuse large B-cell lymphomas. This latter correlation may be of particular pathological relevance given that BCL6 can facilitate lymphomagenesis in diffuse large B-cell lymphomas (115) and high Pellino1 expression is associated with poor prognosis and survival outcome for patients with these lymphomas (50). The Pellino1-driven K63-linked ubiquitination of BCL6 presumably opposes the degradative K48-linked polyubiquitination that is normally catalyzed by the SKP1-CUL1-F-box protein (SCF) ubiquitin ligase complex and the stabilizing effect of Pellino1 on BCL6 may be especially manifested in diffuse large B-cell lymphomas given that the F-box protein FBXO11 of the SCF complex is inactivated in these lymphomas (116). Such findings highlight the potential for Pellino1 to be targeted therapeutically in relevant lymphomas. However, it should be noted that the above pro-tumorigenic role for Pellino1 was demonstrated in Pellino1 transgenic mice in which the Pellino1 transgene is randomly integrated into the genome and under the control of strong promoters. To further evaluate the suitability of Pellino1 as a therapeutic target in cancer, it would be valuable to assess the outcome of preclinical studies that characterize tumorigenesis in Pellino1-deficient mice.

Pellino3 protects against obesity-induced insulin resistance

It is well-accepted that obesity can drive low level inflammation resulting in damaging effects on metabolic health as clinically manifested by insulin resistance and diabetes (117). We observed reduced Pellino3 expression in human abdominal adipose tissue in obese subjects and in adipose tissue of mice fed on a high fat diet and this prompted us to probe the potential role of Pellino3 in regulating obesity-driven inflammation and insulin resistance (118). Intriguingly Pellino3-deficient mice demonstrate exacerbation of high fat diet-induced glucose tolerance, insulin resistance and hyperinsulinemia. We probed the mechanism underlying the enhanced insulin resistance and revealed that Pellino3 deficiency results in more inflamed adipose tissue as manifested by accumulation of adipose tissue macrophages, especially of the pro-inflammatory M1 subtype. Indeed we revealed an intrinsic role for Pellino3 in negatively regulating macrophage M1 polarization. This results in enhanced levels of pro-inflammatory proteins in the adipose tissue and serum of Pellino3-deficient mice on a high fat diet and these mice also display aggravated hepatic steatosis, a signature of obesity-driven inflammation. These findings clearly indicate that Pellino3 plays a role in controlling the extent of obesity-driven inflammation and in protecting against insulin resistance. We also delineate the mechanisms underlying these regulatory effects of Pellino3 on obesity-driven inflammation by showing that Pellino3 inhibits the transcription of the gene encoding IL-1β. This is of particular pathophysiological relevance since IL-1β is a critical mediator of insulin resistance and diabetes (119) and has become an important focus for therapeutic intervention in these conditions (120). The transcription of the *Il1b* gene is driven by the transcription factors NF-κB and hypoxia inducible factor-1α (HIF-1α) (121, 122) and we show that Pellino3 inhibits *Il1b* gene transcription by negatively regulating the stability of HIF-1α (118). Under normoxic conditions HIF-1α is modified by proline hydroxylation resulting in its K48-linked polyubiquitination and proteasomal degradation (123, 124) whereas in hypoxia, the low levels of oxygen precludes hydroxylation of HIF-1α resulting in its stabilization. We describe an additional mechanism that can further stabilize HIF-1α in that stimuli such as hypoxia, LPS and fatty acids, conditions that are commonly found in obese adipose tissue (125, 126), can trigger TRAF6 to catalyse K63-linked ubiquitination of HIF-1α and so preclude K48-linked ubiquitination resulting in enhanced stability (118) (Fig. 4). We also demonstrate that Pellino3 mediates ubiquitination of TRAF6 and so blocks the interaction of TRAF6 with HIF-1α, resulting in reduced K63-linked ubiquitination and destabilization of HIF-1α. Thus, the protective effects of

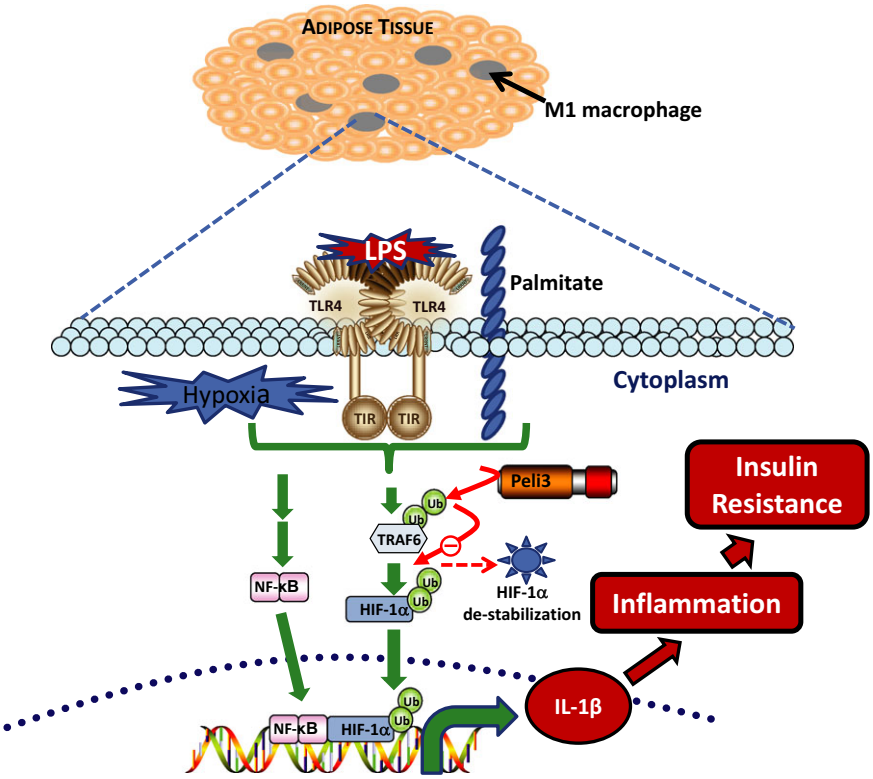

***Fig. 4.* Pellino3 protects against obesity-induced inflammation and insulin resistance.** M1 macrophages in obese adipose tissue are subject to various stimuli including lipopolysaccharide (LPS), palmitate, and hypoxia. These stimuli can trigger TRAF6 to activate NF-κB and to catalyze K63-linked ubiquitination and stabilization of HIF-1α. NF-κB and HIF-1α co-operate to induce transcription of the gene encoding IL-1β that drives inflammation-induced insulin resistance. Pellino3 acts to catalyze polyubiquitination of TRAF6 that inhibits the TRAF6/HIF-1α interaction, resulting in destabilization of HIF-1α. This impairs nuclear translocation of HIF-1α, repression of IL-1β expression and protection from inflammation-induced insulin resistance. IL-1, interleukin-1; NF-κB, nuclear factor-κB; HIF-1α, hypoxia inducible factor-1α.

Pellino3 in tempering obesity-driven inflammation and insulin resistance is underpinned by Pellino3 targeting TRAF6, leading to destabilization of HIF-1α and reduced expression of IL-1β.

A recent report has proposed that Pellino3 mediates the expression of pro-IL-1β, the precursor of IL-1β, and TNF in response to LPS (45). The authors demonstrate that Pellino3-specific shRNA cause partial inhibition of LPS-induced expression of IL-1β and TNF in macrophages. The proposed role of Pellino3 in mediating TNF and IL-1β expression in response to stimulation of macrophages with LPS differs from our conclusions from studies in macrophages from Pellino3-deficient mice (57, 118). We show no deficiency in LPS-induced expression levels of TNF in Pellino3-deficient macrophages (57) and augmented induction of IL-1β in response to co-stimulation of these macrophages with LPS and the fatty acid palmitate. (118). Whilst the basis to such differences are not immediately apparent, our studies to date on the physiological role of Pellino3 emphasize its anti-inflammatory function in preventing exaggerated inflammatory responses as highlighted by its protective roles in inflammatory bowel diseases and obesity-driven inflammation and insulin resistance.

Closing perspective

The highly conserved nature of innate immune signaling across evolution has proved highly valuable in using our understanding of the composition and organization of signaling pathways in primitive organisms to inform and aid discovery of equivalent systems and molecules in more advanced mammalian species. Such a relationship has proved very valuable in our journey to date through the field of Pellino biology. The first identification of a Pellino protein in *Drosophila* and its implied function in innate immunity provided a valuable reference to identify the three members of the mammalian Pellino family and informed the framework to explore their physiological roles. Whilst still early in this field of discovery and perhaps not receiving as much attention and profile as other related signaling molecules

that have been studied for longer, it is clear that Pellino proteins are emerging to be key players in immune signaling and an increased understanding of their biology can only aid our global appreciation of the workings of innate and adaptive immunity. It is also obvious that Pellino members have evolved far beyond their primary characterized role of targeting the IRAK homologue, Pelle. Whilst this interaction has been conserved in nature by virtue of IRAK-Pellino interactions, studies to date clearly emphasize that Pellino proteins have multiple targets beyond IRAKs. Indeed it is somewhat ironic that whilst the field has accrued detailed information on the nature of the molecular interactions between the IRAK and Pellino proteins, we have better understanding of the physiological or pathophysiological relevance of some of the other interactions of Pellino members with target proteins such as RIP kinases and TRAF6. Our increased awareness and identification of an increasing number of Pellino interactors is also helping to inform us about the diverse functions of these family members. In addition a single family member may target the same molecule to manifest various biological outcomes. This is indicated by our own studies that have revealed Pellino3 to ubiquitinate TRAF6 and so block TRAF6 interaction with IRF7 and repress type I IFN expression whilst Pellino3 can similarly target TRAF6 to preclude its binding to and activation of HIF-1α resulting in reduced expression of IL-1β and protection from the development of insulin resistance.

The ever expanding delineation of new roles for Pellino proteins is also highlighting that different members of the Pellino family fail to show functional redundancy and instead appear to have specialized roles. This appears surprising given the homology of the members and their common RING-like and FHA domains. Some of the specificity may be due to cell restricted expression of individual members such as predominant expression of Pellino1 in microglia and its role in mediating neuroinflammation. The Pellino proteins may also be subjected to differential regulation by varying kinases and indeed as discussed earlier different kinases phosphorylate and activate the Pellino proteins in the context of varying signaling pathways. Finally the most important determinant in dictating functional specificity for individual Pellino proteins may be facilitated by selective substrate recognition. The latter may seem unlikely given that the Pellino proteins share a FHA domain that can serve to recognize phosphorylated residues on target proteins to be ubiquitinated. However, the FHA domains from the Pellino members display varying affinities for different sequences that surround the phospho-threonine residues in target proteins (42) and this may facilitate that specificity of function for individual Pellino proteins.

While much of our detailed knowledge on Pellino biology focuses on the physiological roles of the family members, it is also clearly emerging that these proteins can contribute to and regulate diseases processes. Given this insight efforts will inevitably turn to designing strategies to target Pellino proteins for therapeutic application. A very recent study has provided the first indication that the specific targeting of Pellino proteins may be a fruitful avenue for new drug discovery. As described previously, TGF-β inhibits IL-1 and LPS signaling by inducing the inhibitory Smad6 and Smad7 proteins that subsequently interact with Pellino1 and disrupts Pellino1 signaling complexes (82, 83). Interestingly a synthetic membrane-tethered peptide corresponding to residues 422–441 of Smad6 has been recently shown to interact with Pellino1 and disrupt the TLR4-induced signaling complexes containing Pellino1 with RIP1 and IKKε/TBK1 (127). This peptide shows therapeutic potential in the treatment of experimental models of LPS- or polymicrobial-induced sepsis. This provides the first indication that Pellino proteins may be open for future exploitation as therapeutic targets. Such exciting promise heightens the need for continuing and expanding research on the molecular, physiological and pathophysiological roles of the Pellino family.

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