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Pellino 3b Negatively Regulates Interleukin-1-induced TAK1-dependent NFκB Activation*[S]

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Hui Xiao[‡], Wen Qian[‡], Kirk Staschke[§], Youcun Qian[‡], Grace Cui[‡], Li Deng[¶], Mariam Ehsani[§], Xiliang Wang[§], Yue-Wei Qian[§], Zhijian J. Chen[¶], Raymond Gilmour[§], Zhengfan Jiang[‖1], and Xiaoxia Li[‡2]

From the [‡]Department of Immunology, Cleveland Clinic Foundation, Cleveland, Ohio 44195, [§]Lilly Research Laboratories, Indianapolis, Indiana 46285, [¶]Howard Hughes Medical Institute, Department of Molecular Biology, University of Texas Southwestern Medical Center, Dallas, Texas 75390-9148, and [‖]Peking University, Beijing 00871, China

IL-1 receptor-associated kinase (IRAK) is phosphorylated, ubiquitinated, and degraded upon interleukin-1 (IL-1) stimulation. In this study, we showed that IRAK can be ubiquitinated through both Lys-48- and Lys-63-linked polyubiquitin chains upon IL-1 induction. Pellino 3b is the RING-like motif ubiquitin protein ligase that promotes the Lys-63-linked polyubiquitination on IRAK. Pellino 3b-mediated Lys-63-linked IRAK polyubiquitination competed with Lys-48-linked IRAK polyubiquitination for the same ubiquitination site, Lys-134 of IRAK, thereby blocking IL-1-induced IRAK degradation. Importantly, the negative impact of Pellino 3b on IL-1-induced IRAK degradation correlated with the inhibitory effect of Pellino 3b on the IL-1-induced TAK1-dependent pathway, suggesting that a positive role of IRAK degradation in IL-1 induced TAK1 activation. Taken together, our results suggest that Pellino 3b acts as a negative regulator for IL-1 signaling by regulating IRAK degradation through its ubiquitin protein ligase activity.

Interleukin-1 (IL-1),[3] a major pro-inflammatory cytokine, has a wide range of biological activities in inflammation. Genetic and biochemical studies revealed that IL-1R mediates a very complex pathway, involving a cascade of kinases organized by multiple adapter molecules into signaling complexes, leading to activation of the transcription factor NFκB. Based on studies by our group and others, we postulated a model for the IL-1 pathway (1–7). Upon IL-1 stimulation, the IL-1 receptor recruits adapter molecule MyD88 (8) and mediates the formation of complex I (IL-1R-MyD88-IRAK4-IRAK-TRAF6), where IRAK4 (IL-1 receptor-associated kinase 4 (9)) is activated, leading to hyperphosphorylation of IRAK (10), which creates an interface for its interaction with adapter Pellino 1 (11). The receptor proximal components are then released from the receptor to form an intermediate complex, followed by formation of complex II (IRAK-TRAF6-TAK1-TAB2-TAB3), leading to phosphorylation of TAK1 (transforming growth factor β-activated kinase, a MAP3K) and TAB2 (TAK1-binding protein 2) and TAB3 on the membrane (1, 2–7). Although the membrane-associated modified IRAK is ubiquitinated and degraded, complex III (TRAF6-TAK1-TAB2-TAB3) is then dissociated from complex II and translocated from the membrane to the cytosol, where TAK1 is activated, followed by the activation of IκB kinase (IKK) and NFκB (7).

Chen and co-workers (14, 15) showed that protein ubiquitination plays an important role in TRAF6-mediated TAK1 and IKK activation. The ubiquitin pathway generally involves three types of enzymes, ubiquitin-activating enzyme (E1 or Uba), ubiquitin-conjugating enzyme (E2 or Ubc), and ubiquitin protein ligase (E3 or Ubr) (16). The E3 ubiquitin protein ligases play a key role in recognition and selection of proteins targeted for ubiquitination. Many RING finger proteins have been shown to act as E3s, either by themselves or as part of a multisubunit E3 protein complex (17). TRAF6, a RING domain protein, has been shown to function as a ubiquitin protein ligase E3, and itself might be the target of ubiquitination, which leads to the activation of TAK1. Polyubiquitination of a target protein with the ubiquitin linked through Lys-48 is recognized by proteasome and ultimately degraded. However, polyubiquitination chains linked through Lys-63 of ubiquitin do not target the substrate for proteasome-mediated degradation, mediating protein-protein interaction and cell signaling instead. It has been reported that TRAF6-mediated Lys-63 polyubiquitination on itself plays an essential role in the activation of IKK and NFκB (18).

Recently genetic studies have provided further evidence for an essential role of TAK1 in IL-1 signaling. Two groups (19, 20) independently reported that TAK1 deficiency results in defects in IL-1 signaling. Intriguingly, whereas IL-1-induced JNK activation was completely abolished, NFκB activation was only partially impaired in TAK1-deficient cells, implicating an additional NFκB activation mechanism for the IL-1 pathway. We recently identified two parallel IL-1-mediated NFκB activation pathways as follows: TAK1-dependent and MEKK3-dependent (Fig. 1) (21). The TAK1-dependent pathway causes IKKα/β phosphorylation and IKKβ activation, leading to classical NFκB

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

[1] To whom correspondence may be addressed: Dept. of Cell Biology and Genetics, School of Life Sciences, Peking University, Beijing 100871, China. Tel.: 8610-62757923; E-mail: jiangzf@pku.edu.cn.

[2] To whom correspondence may be addressed: Dept. of Immunology, Cleveland Clinic Foundation, 9500 Euclid Ave. NE40, Cleveland, OH 44195. Tel.: 216-445-8706; E-mail: lix@ccf.org.

[3] The abbreviations used are: IL-1, interleukin-1; JNK, c-Jun N-terminal kinase; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin protein ligase; IKK, IκB kinase; siRNA, short interfering RNA; HA, hemagglutinin; TNF, tumor necrosis factor; IRAK, IL-1 receptor-associated kinase.

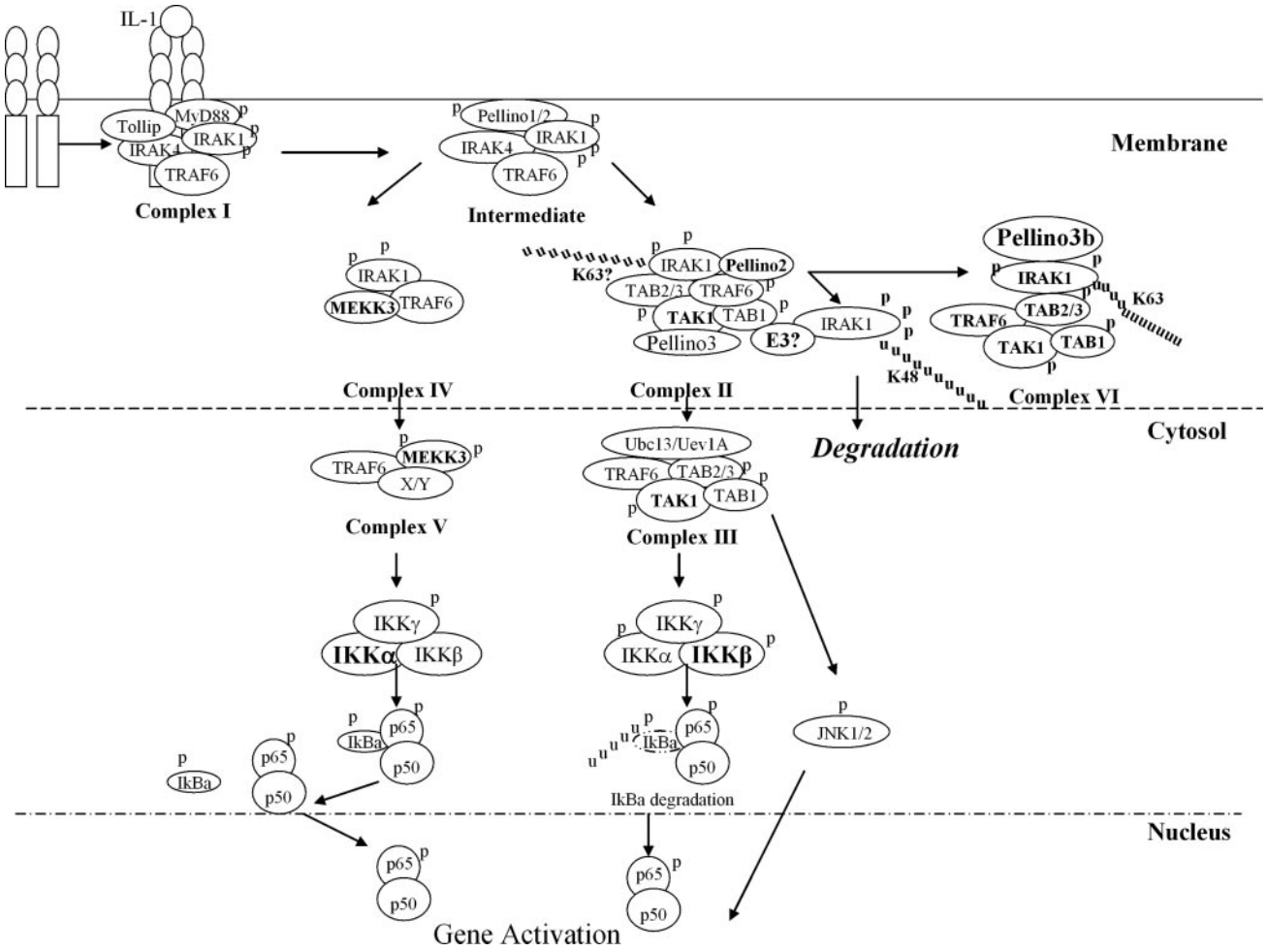

FIGURE 1. **Model of IL-1 signaling displaying the negative regulatory role of Pellino 3b.** Upon IL-1 stimulation, adapter molecules MyD88 and Tollip are first recruited to IL-1 receptor, which in turn recruits IRAK4, IRAK, and TRAF6, resulting in the formation of the receptor complex (*complex I*). During the formation of complex I, IRAK4 is activated, leading to the hyperphosphorylation of IRAK, which creates an interface for the interaction of Pellino 1/2 with the IRAK4-IRAK-TRAF6 complex (*Intermediate* complex). Pellino 2 might mediate Lys-63-linked IRAK ubiquitination, leading the interaction of intermediate complex Pellino 2-IRAK4-IRAK-TRAF6 with the membrane-bound pre-associated TAK1-TAB1-TAB2, resulting in the formation of *complex II* (TAK1 complex, IRAK-TRAF6-TAK1-TAB1–TAB2). An unknown E3 then mediates Lys-48-linked IRAK ubiquitination, leading to degradation of IRAK, resulting in the translocation of TRAF6-TAK1-TAB1–TAB2 (*complex III*) from the membrane to the cytosol where TAK1 is activated, eventually resulting in the activation of NFκB. Pellino 3b is up-regulated upon IL-1 stimulation and interacts with the TAK1 complex (*complex VI*), where Pellino 3b mediates efficient Lys-63-linked IRAK ubiquitination, competing with Lys-48-linked IRAK ubiquitination, thereby inhibiting IRAK degradation and subsequent release of the complex III from the membrane to the cytosol, attenuating TAK1-dependent NFκB activation.

activation through IκBα phosphorylation and degradation. The TAK1-independent MEKK3-dependent pathway induces IKKγ phosphorylation and IKKα activation, resulting in NFκB activation through IκBα phosphorylation and subsequent dissociation from NFκB but without IκBα degradation. These two pathways are regulated at the level of IRAK modification (21). Previous studies showed that IRAK is phosphorylated after it is recruited to the receptor, subsequently ubiquitinated, and eventually degraded upon IL-1 stimulation. A point mutation changing lysine 134 to arginine (K134R) in IRAK abolished IL-1-induced IRAK ubiquitination and degradation. The IRAK ubiquitination mutant is no longer degraded upon IL-1 stimulation and loses the ability to mediate the TAK1-dependent NFκB activation, while retaining the MEKK3-dependent signaling (21). At the moment, it is unknown which ubiquitin protein ligase E3(s) is responsible for the IL-1-induced ubiquitination of IRAK required for the activation of the TAK1-dependent NFκB activation pathway.

Pellino proteins have recently been reported as novel RING E3 ubiquitin ligases and implicated in IRAK ubiquitination. There are four Pellino family members, Pellino 1, 2, 3a, and splicing variant Pellino 3b, which are highly conserved from *Drosophila* to human. Although Pellino 1 and 2 share 82% amino acid identity, Pellino 3 has 70 and 71% amino acid identity with Pellino 1 and 2, respectively (11, 22–26). Although both Pellino 3a and 3b have a unique N terminus, containing 27 extra amino acids, Pellino 3b is missing an exon (exon 3, 24 amino acids) as compared with Pellino 3a. We and others have previously demonstrated that Pellino proteins form complexes with IRAK and TRAF6, implicating an important role of the Pellino proteins in IL-1 signaling. Although Pellino 1 and 2 have the ability to activate NFκB, Pellino 3a and 3b promote JNK activation but not NFκB. Recombinant forms of Pellinos are shown in an *in vitro* ubiquitination assay to be E3 ubiquitin ligases that catalyze Lys-63-linked polyubiquitination (27). Intriguingly, Ordureau *et al.* (28) recently reported that Pellino

1 protein can combine with UbcH3 to catalyze the formation of Lys-48-linked polyubiquitination *in vitro*, whereas Pellino 2 only induces the formation of Lys-63-poly(Ub)-IRAK1 in the cell culture model. If the Pellinos directly mediate IRAK polyubiquitination, then the critical questions are whether Pellinos mediate the formation of the polyubiquitin chain through Lys-63 or Lys-48 and what the function of Pellino mediated IRAK ubiquitination is.

In this study, we showed that IRAK can be ubiquitinated through both Lys-48- and Lys-63-linked polyubiquitin chains upon IL-1 induction, suggesting the complex role of IRAK ubiquitination on IL-1 signaling. In the search of E3 ligases for IL-1-induced IRAK ubiquitination, we found that Pellino 3b is the E3 ligase that promotes the Lys-63-linked polyubiquitination on IRAK. Mutation analysis revealed that the RING-like motif in Pellino 3b is required for its E3 ligase activity. Pellino 3b-mediated Lys-63-linked IRAK polyubiquitination competed with Lys-48-linked IRAK polyubiquitination for the same ubiquitination site (Lys-134) on IRAK, thereby blocking IL-1-induced IRAK degradation. Importantly, the negative impact of Pellino 3b on IL-1-induced IRAK degradation correlated with the inhibitory effect of Pellino 3b on IL-1-induced TAK1-dependent pathway, suggesting a positive role of IRAK degradation in IL-1-induced TAK1 activation. In support of this, proteasome inhibitor MG132 inhibited IL-1-induced TAK1-dependent signaling. Taken together, we conclude that Pellino 3b acts as a negative regulator for IL-1 signaling by regulating IRAK degradation through its E3 ligase activity.

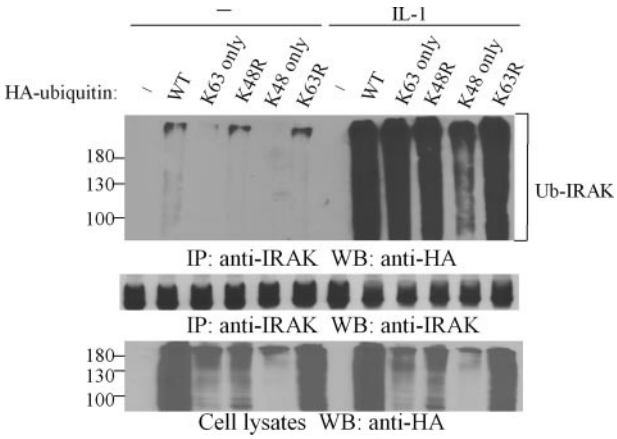

FIGURE 2. **IL-1-induced IRAK polyubiquitination is linked through both Lys-48 and Lys-63.** 293-IL-1R cells transfected with HA-tagged wild-type (*WT*) ubiquitin or ubiquitin mutants (including Lys-63 only, K48R, Lys-48 only, and K63R as described in the text) were stimulated with 1 ng/ml of IL-1 for 15 min or left untreated, followed by immunoprecipitation (*IP*) with anti-IRAK and Western analyses with anti-HA and anti-IRAK, respectively.

MATERIALS AND METHODS

Cell Lines and Reagents—C6 (HEK293/IL-1RI), I1A (HEK293/IRAK-deficient), and synoviocytes were grown on Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Oligonucleotides encoding either scrambled or Pellino 3 specific small hairpin RNAs were cloned into pSUPER to construct pSUPER-scrambled and pSUPER-pellino 3, respectively. 1 μg of pSUPER-scrambled or pSUPER-pellino 3 was transfected into C6 cells along with 0.1 μg of pBabo-puromycin by FuGENE 6 (Roche Applied Science). Two days after transfection, puromycin (1 μg/ml) containing Dulbecco's modified Eagle's medium was added to the cells to select puromycin-resistant clones. Following 10 days of puromycin selection, single clones were picked out and subjected to further analysis by anti-Pellino 3 blotting. cDNA encoding human Pellino 3b was cloned into pcDNA3.1 to create pcDNA3.1-FLAG-pellino 3b. pcDNA3.1 or pcDNA3.1-FLAG-pellino 3b (1 μg) was transfected into C6 cells along with pBabo-puromycin (0.1 μg) by FuGENE 6. Following puromycin selection for 10 days, single clones were picked out, and clones expressing FLAG-pellino 3b were confirmed by anti-FLAG blotting. IRAK (H-273, sc-7883), IRAK-agarose (F-4, sc-5288 AC), TRAF6 (H-274, sc-7221), IKKα/β (H-470, sc-7607), IκBα (FL, sc-847), IKKγ (FL-419, sc-4330), JNK1 (FL, sc-571), ubiquitin (FL-76, sc-9133, and P4D1, sc-8017), HA probe (Y-11, sc-805), and actin (C-11, sc-1615) were purchased from Santa Cruz Biotechnology, Inc. Anti-FLAGM2 antibodies (F7425 from rabbit; A2220 affinity gel from mouse), puromycin (P9620), and MG132 (C2211) were from Sigma. Phospho-IKKα (Ser-176)/β (Ser-180) (catalog number 2694), phospho-IKKγ (Ser-376) (catalog number 2689), phospho-stress-activated protein kinase/JNK (catalog number 9251), and phospho-IκBα (Ser 32)(catalog number 9241) were purchased from Cell Signaling Technology, Inc. Polyclonal anti-Pellino 3 and anti-TAK1 antibodies from rabbit were made by Lilly. Recombinant interleukin-1β was from R & D Systems.

Immunoprecipitation—Subconfluent C6 cells were stimulated with 1 ng/ml IL-1β for various times as indicated. Following washing with cold phosphate-buffered saline, cells were lysed in 0.5 ml of lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton, 1 mM EDTA, 5 mM NaF, 2 mM $NaVO_3$, 1 mM phenylmethylsulfonyl fluoride, 1× complete protease inhibitors). Cell lysates were cleared by centrifuging at 13,000 rpm for 10 min, and insoluble debris was discarded. For co-immunoprecipitation, cell lysates were incubated with 20 μl of anti-FLAG affinity gel at 4 °C overnight, and FLAG beads were washed with 1 ml of lysis buffer five times before dissolving in 40 μl of Laemmli buffer. When examining the ubiquitination of IRAK or Pellino 3b, immunoprecipitation was carried out under denaturing condition. Briefly, cell lysates containing 1% SDS were incubated for 10 min at 100 °C to disrupt the protein complexes. Denatured protein lysates were placed on ice for 5 min and then diluted 10 times with lysis buffer shown above. The cell lysates containing 0.1% SDS were immunoprecipitated with 20 μl of anti-FLAG affinity gel (A2220) or 30 μl of anti-IRAK-agarose (sc-5288 AC) at 4 °C overnight. Anti-FLAG and anti-IRAK beads were washed five times with lysis buffer containing 0.1% SDS and subsequently dissolved in 40 μl of Laemmli buffer.

In Vitro Ubiquitination Assay—His-tagged Pellino 1, Pellino 2, and Pellino 3b were expressed in *Escherichia coli* and purified by nickel-nitrilotriacetic acid-agarose from Qiagen according to the manufacturer's manual. The preparation of E1, Ubc13/Uev1A, Ubc5H, UbcH3, and ubiquitin proteins has been described previously (16). Recombinant IRAK, UbcH5, and UbcH3 were kindly provided by Dr. Philip Cohen (28). The *in*

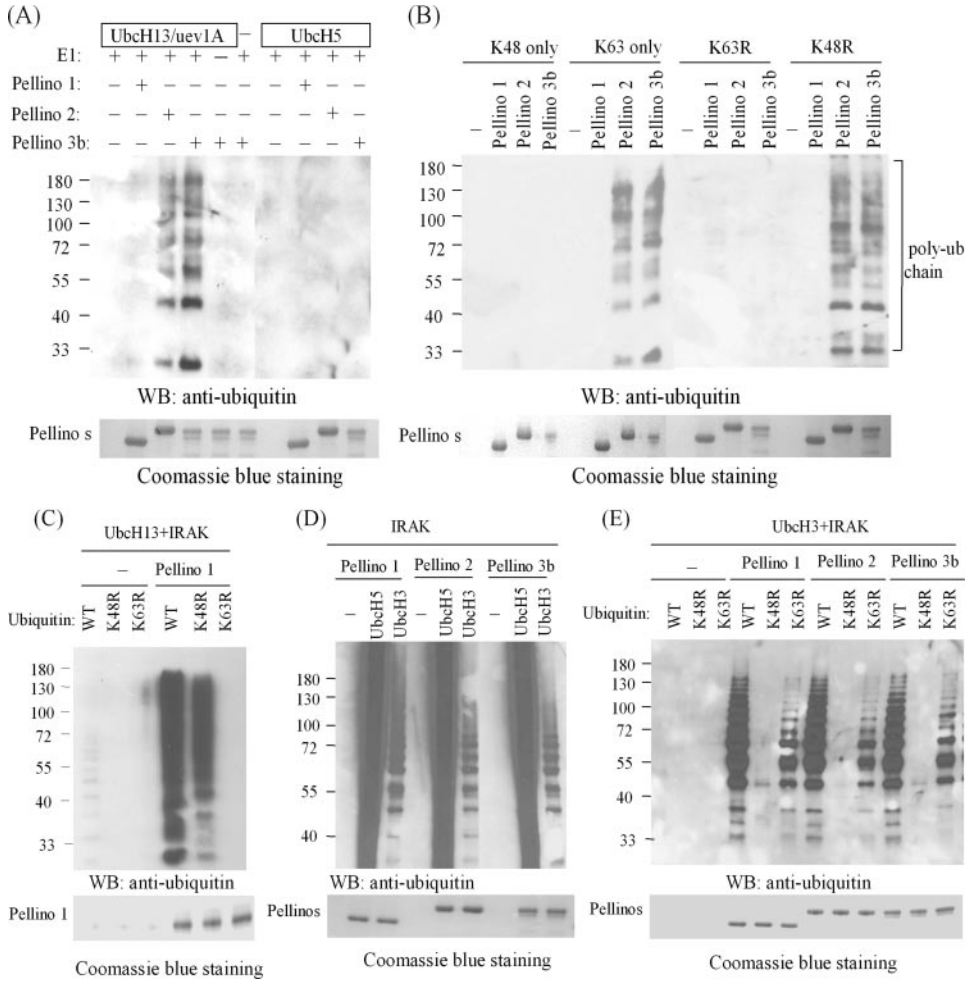

FIGURE 3. **Pellinos can mediate either Lys-63- or Lys-48-type polyubiquitination *in vitro*.** *A,* Pellino 3b and Pellino 2 act together with Ubc13/Uev1A to assemble polyubiquitin chain *in vitro*. Purified recombinant Pellino 1, Pellino 2, or Pellino 3b (1 μg) was incubated with 100 nM E1, 250 nM UbcH13/Uev1A or UbcH5, and 5 μg of ubiquitin at 30 °C for 1 h as described under "Materials and Methods." Following *in vitro* ubiquitination assay, 20 μl of Laemmli buffer was added into 10 μl of reaction. 15 μl of the reaction mixture was analyzed by Western blotting (*WB*) with anti-ubiquitin, and the other half was visualized by Coomassie Blue staining after resolving by 10% SDS-PAGE. *B,* Pellino 3b and Pellino 2 assemble Lys-63-linked polyubiquitin chain. Recombinant Pellino 1, Pellino 2, or Pellino 3b was incubated with E1, UbcH13/Uev1A, and various mutant ubiquitin at 30 °C for 1 h. *C,* phosphorylated Pellino 1 acts with UbcH13/Uev1A to assemble the Lys-63-linked poly(Ub) chain. Recombinant Pellino 1 was incubated with 0.5 μg of purified IRAK, 100 nM E1, 500 nM UbcH13/Uev1A, and various mutant ubiquitin at 30 °C for 1 h. *D,* phosphorylated Pellinos act together with UbcH5 or UbcH3 to assemble polyubiquitin chains *in vitro*. Purified recombinant Pellino 1, Pellino 2, or Pellino 3b (1 μg) was incubated with 0.5 μg of purified IRAK, 100 nM E1, 1 μM UbcH5 or UbcH3, and 5 μg of ubiquitin at 30 °C for 1 h. *E,* phosphorylated Pellinos act with UbcH3 to assemble Lys-48-linked polyubiquitin chain. Purified recombinant Pellino 1, Pellino 2, or Pellino 3b (1 μg) was incubated with 0.5 μg of purified IRAK, 100 nM E1, 1 μM UbcH3, and 5 μg of wild type (*WT*) or mutant ubiquitin at 30 °C for 1 h. Following *in vitro* ubiquitination assay, 20 μl of Laemmli buffer was added into 10 μl of reaction. 15 μl of the reaction mixture was analyzed by Western blotting with anti-ubiquitin, whereas the other half was visualized by Coomassie Blue staining after resolving by 10% SDS-PAGE.

vitro ubiquitination reaction was carried out in 1× reaction buffer (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 2 mM ATP, 2 mM MgCl, 10% glycerol). 1 μg of Pellino protein was incubated with 100 nM E1, 250 nM or 1 μM of E2, and 5 μg of ubiquitin at 30 °C for 1 h. The reaction was stopped by adding 20 μl of Laemmli buffer, and assembly of polyubiquitin chain was detected by anti-ubiquitin immunoblotting.

Immunoblotting—Whole cell lysates, immunoprecipitates, or *in vitro* ubiquitinated proteins were dissolved in Laemmli buffer and resolved by 10% SDS-PAGE. After electrophoresis, separated proteins were transferred onto polyvinylidene difluoride membrane (Millipore). For immunoblotting, the polyvinylidene difluoride membrane was blocked with 5% nonfat milk. Following incubation with specific primary antibody, horseradish peroxidase-conjugated secondary antibody was applied. The positive immune reactive signal was detected by ECL (Amersham Biosciences).

RESULTS

IL-1-induced IRAK Polyubiquitination Is Linked through Both Lys-48 and Lys-63—Upon IL-1 stimulation, IRAK is phosphorylated, polyubiquitinated, and eventually degraded (29). Although Lys-48-linked polyubiquitin chain has been exclusively related to protein degradation, Lys-63-linked polyubiquitin chain has been emerged as a type of polyubiquitin that promotes protein-protein association (4). To examine whether IL-1-induced polyubiquitination of IRAK is linked via Lys-63 or Lys-48, HA-tagged wild type and a series of mutants of ubiquitin were transfected into 293 cells. Cell lysates prepared from untreated or IL-1-treated transfected cells were immunoprecipitated with anti-IRAK, followed by Western analysis with anti-HA to detect HA-tagged ubiquitin. Following IL-1 induction, although we detected polyubiquitinated IRAK in cells transfected with wild-type ubiquitin, the ubiquitin mutants Lys-63 and Lys-48 (30) (all the lysines in ubiquitin are mutated to arginine except Lys-63 or Lys-48) were conjugated to IRAK as well. On the other hand, the ubiquitin mutants K63R and K48R (Lys-63 and Lys-48 are mutated to arginine) were also able to form a polyubiquitin chain on IRAK (Fig. 2). Taken together, these results suggest that IL-1 stimulation probably induces the assembly of both Lys-48- and Lys-63-linked polyubiquitin chains on IRAK.

Pellinos Mediate Both Lys-48 and Lys-63 Polyubiquitination in Vitro—Schauvliege *et al.* (26) recently reported that overexpression of Pellino 1 and 2 (to a lesser degree Pellino 3a) leads to IRAK ubiquitination. To test whether Pellino proteins can indeed function as E3 ubiquitin ligase and directly mediate polyubiquitination, we purified recombinant Pellino proteins and E1/E2 enzymes from *E. coli* and carried out *in vitro* ubiq-

uitination assay using ubiquitin as a substrate. Pellino 2 and 3b and to a much lesser extent Pellino 1 promoted the assembly of polyubiquitin chain when Ubc13/Uev1A was used as E2 (Fig. 3*A*), whereas Pellino 2 and 3b failed to promote polyubiquitination with UbcH5 as E2. To determine whether Pellinos mediate Lys-48 or Lys-63 polyubiquitination in combination with Ubc13/Uev1A, different ubiquitin mutants were used in the *in vitro* ubiquitination assay. These experiments showed that Pellino 2 and 3b can only form polyubiquitin chain with wild-type ubiquitin or ubiquitin mutants containing Lys-63, indicating that Pellino 3b- and Pellino 2-mediated polyubiquitin chain in combination with Ubc13/Uev1A is linked through Lys-63 (Fig. 3*B*).

Interestingly, Ordureau *et al.* (28) reported that phosphorylation mediated by IRAK can greatly increase the E3 ligase activity of Pellino 1 *in vitro*. Indeed, incubation of Pellino 1 with IRAK increased the ability of Pellino 1 in mediating Lys-63-type ubiquitination with UbcH13/Uev1A as E2 (Fig. 3*C*). Furthermore, in the presence of IRAK, all three Pellino proteins can combine with E2-conjugating enzyme UbcH5 or UbcH3 to catalyze polyubiquitination (Fig. 3*D*). Further analysis showed that Pellino proteins were able to mediate Lys-48-linked polyubiquitination with UbcH3 (Fig. 3*E*).

Pellinos Induce Formation of Lys-63-linked IRAK Polyubiquitination in Vivo—We and others (Schauvliege *et al.* (26)) have observed that that overexpression of Pellinos leads to IRAK ubiquitination. As shown in Fig. 4, *A* and *B*, co-expression of Pellinos with IRAK in 293 cells can lead to efficient IRAK ubiquitination. We then examined whether Pellinos mediate Lys-48 and/or Lys-63polyubiquitination on IRAK. Different ubiquitin mutants were co-transfected with Pellinos and IRAK into I1A cells (IRAK-deficient), followed by immunoprecipitation with IRAK and Western analysis with anti-HA to detect HA-tagged ubiquitin. We only detected IRAK polyubiquitination in cells co-transfected with Lys-63-containing ubiquitins (Fig. 4, *C* and *D*). The fact that a single mutation of Lys-63 in ubiquitin completely abrogated its ability to be assembled on IRAK by Pellinos strongly suggests that the Pellino-mediated IRAK polyubiquitination was linked through Lys-63, although IL-1 stimulation leads to both Lys-48- and Lys-63-linked IRAK polyubiquitination (Fig. 2). Taken together, although Pellinos were able to mediate either Lys-48- or Lys-63-linked polyubiquitination *in vitro* (depending on the specific E2s provided) (Fig. 3), all three Pellinos (Pellino 1/2/3b) mainly mediate Lys-63-linked IRAK polyubiquitination in cell culture model.

Pellino 3b Is a RING-like E3 Ubiquitin Ligase—In addition to its strong E3 ubiquitin ligase activity with Ubc13/Uev1A as E2 *in vitro*, Pellino 3b also exhibited the strongest activity in self-ubiquitination upon its overexpression in 293 cells as compared with the other Pellino family members (Fig. 5*A*). Interestingly, overexpressed Pellino 3b inhibited IL-1-induced NFκB activity measured by luciferase assay, whereas overexpressed Pellino 1 and 2 increased NFκB activity (Fig. 5*B*) (11), implicating different functions of these family members of Pellino. Because Pellino 3b displayed distinct function compared with Pellino 1 and 2, we decided to further investigate the E3 activity of Pellino 3b and its function in IL-1 signaling.

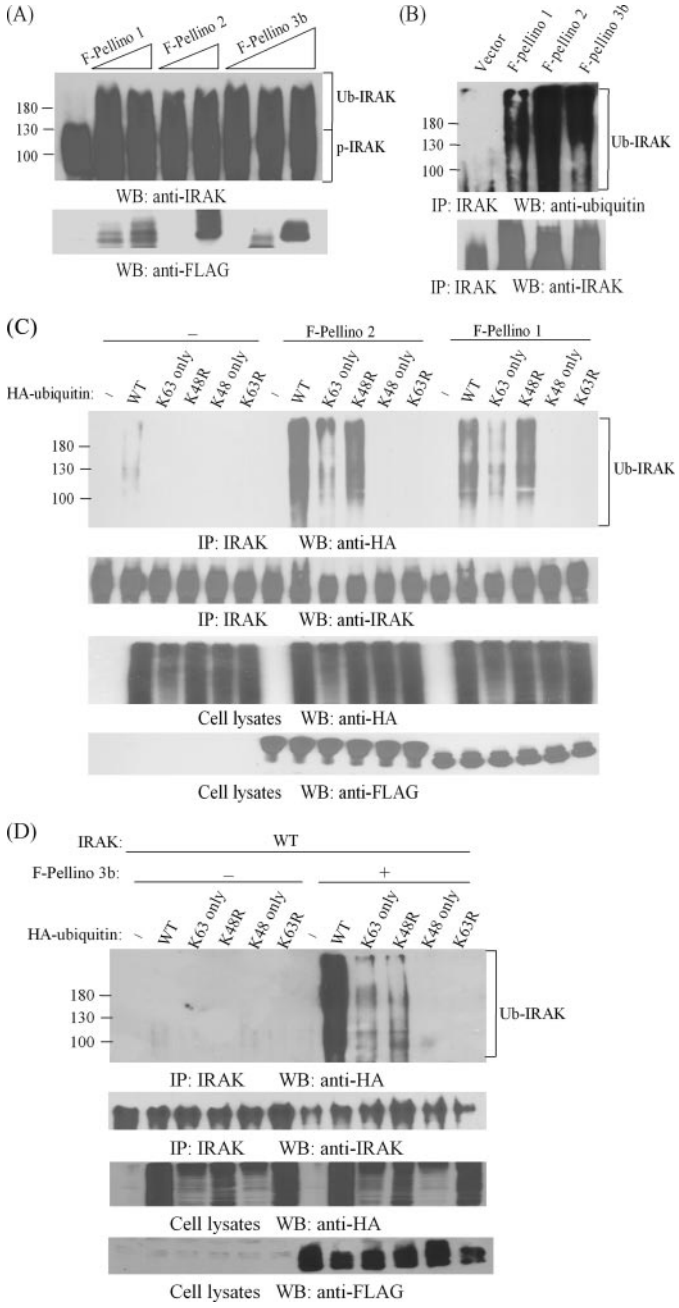

FIGURE 4. **Pellinos promote Lys-63-linked polyubiquitination of IRAK *in vivo*.** *A*, IRAK was co-transfected with FLAG-tagged Pellino 1, Pellino 2, or Pellino 3b into I1A cells (IRAK-deficient). Cell lysates from the transfected cells were analyzed by Western blotting (*WB*) with anti-IRAK and anti-FLAG (*A*) or immunoprecipitated (*IP*) by anti-IRAK-agarose and followed by anti-ubiquitin and anti-IRAK blotting (*B*). *C*, IRAK was co-transfected with HA-tagged wild-type or mutants of ubiquitin into I1A cells, with FLAG-pellino 1 or 2. Cell lysates were denatured and immunoprecipitated with anti-IRAK-agarose, followed by Western blotting with anti-HA and anti-IRAK. Cell lysates were directly analyzed by Western blotting with anti-FLAG or anti-HA. *D*, IRAK was co-transfected with HA-tagged wild-type or mutants of ubiquitin into I1A cells, with or without FLAG-pellino 3b. Cell lysates were denatured and immunoprecipitated with anti-IRAK-agarose, followed by Western blotting with anti-HA and anti-IRAK. Cell lysates were directly analyzed by Western blotting with anti-FLAG or anti-HA.

Pellino proteins have a RING-like motif (CHC2CHC2) at the C terminus. To examine whether that this RING-like motif is responsible for the E3 activity of Pellino proteins, we generated a set of mutants of Pellino 3b either by deleting the RING-like

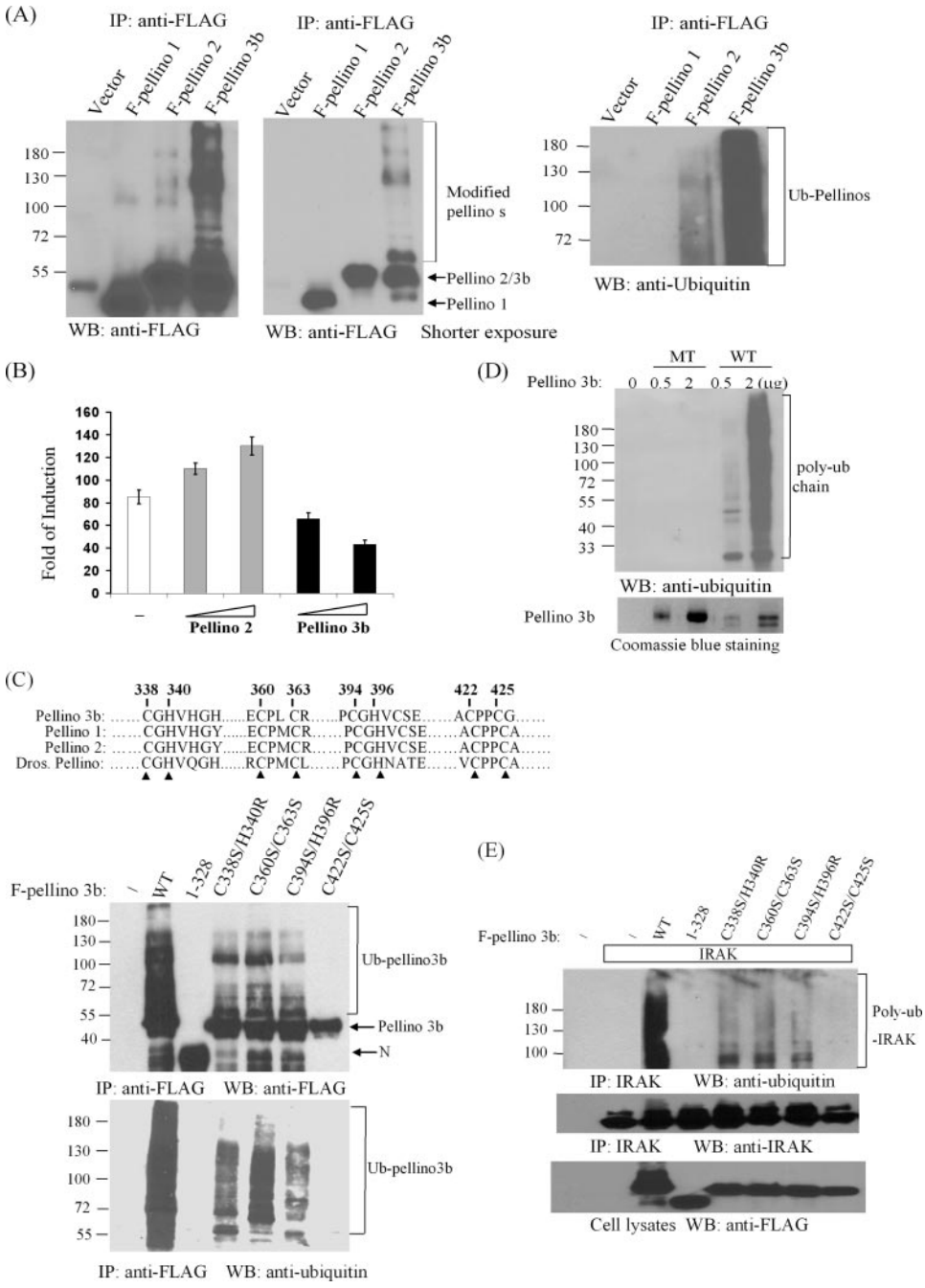

FIGURE 5. **The RING finger-like motif is required for the E3 ligase activity of Pellino 3b.** *A,* Pellino 3b and Pellino 2 promote self-ubiquitination *in vivo*. Cell lysates prepared for 293-IL-1R cells transfected with FLAG-tagged Pellino 1, Pellino 2, or Pellino 3b were immunoprecipitated (*IP*) with anti-FLAG followed by Western blotting (*WB*) with anti-ubiquitin and anti-FLAG, respectively. *B,* Pellino 2 and 3b differentially regulate NFκB activity. Various amounts (0–100 ng) of plasmids encoding Pellino 2 or Pellino 3b were co-transfected with Elam-luciferase reporter plasmid (*Renilla* luciferase reporter as control) into C6 cells. Following transfection for 20 h, cells were either left untreated or stimulated by IL-1 (1 ng/ml) for 6 h and then harvested for measuring luciferase activity. The relative luciferase units were counted by normalizing firefly luciferase activity against *Renilla* luciferase activity. The fold of induction was calculated for each sample as IL-1-induced relative luciferase units/nontreated relative luciferase units. *C,* RING-finger like domain is essential for the self-ubiquitination of Pellino 3b. Cell lysates prepared from 293-IL-1R cells transfected with wild-type (*WT*) Pellino 3b or Pellino 3b mutants were immunoprecipitated with anti-FLAG followed by Western analysis with anti-FLAG and ubiquitin. *D,* RING finger is essential for the E3 ligase activity of Pellino 3b. Purified Pellino 3b mutant (*MT*) protein (C422S/C425S) was incubated with E1, Ubc13/Uev1A, and ubiquitin to carry out *in vitro* ubiquitination assay as described above. *E,* IRAK was co-transfected with FLAG-tagged wild-type or mutants of Pellino 3b into I1A cells. Cell lysates from the transfected cells were denatured and immunoprecipitated with anti-IRAK-agarose, followed by Western analysis with anti-ubiquitin and anti-IRAK. Cell lysates were also directly analyzed by Western blotting with anti-FLAG.

motif (N) or mutating the individual conserved Cys (Cys to Ser) and His (His to Arg) in the RING-like motif. As shown in Fig. 5*C*, disruption or deletion of the RING motif impaired the ability of Pellino 3b to promote self-ubiquitination. Indeed, the RING-like motif is absolutely required for Pellino 3b to function as an E3 ligase *in vitro* (Fig. 5*D*). Mutations in the RING-like motif abolished the ability of Pellino 3b to ubiquitinate IRAK (Fig. 5*E*).

Pellino 3b-mediated Lys-63-linked Polyubiquitination Competes with Lys-48-linked Polyubiquitination on Lys-134 of IRAK—The fact that a point mutation changing lysine 134 to arginine (K134R) in IRAK abolished IL-1-induced IRAK ubiquitination and degradation suggests that Lys-134 is likely the ubiquitination site for the assembly of both Lys-48- and Lys-63-linked polyubiquitin chains on IRAK in response to IL-1 stimulation (21). Interestingly, we found that Pellino 3b-mediated IRAK polyubiquitination was greatly reduced when Pellino 3b was co-transfected with the K134R IRAK mutant, suggesting that Pellino 3b mediates polyubiquitination mainly on Lys-134 of IRAK (Fig. 6, *A* and *B*). Pellino 3b-mediated residual ubiquitination on K134R mutant might be due to a compensatory ubiquitination site in this IRAK mutant upon overexpression of Pellino 3b. Because Pellino 3b mediates Lys-63-linked IRAK polyubiquitination, it is unlikely for Pellino 3b to be responsible for IL-1-induced Lys-48-linked polyubiquitination and subsequent IRAK degradation. To examine the impact of Pellino 3b on IL-1-induced IRAK ubiquitination and degradation, we used Pellino 3 siRNA to knock down the expression of Pellino 3b. Interestingly, we found that IL-1-induced IRAK degradation was accelerated in clones where Pellino 3b expression was knocked down (Fig. 6*C*), indicating an important role of Pellino 3b in regulating IL-1-induced IRAK degradation. These results suggest that Pellino 3b probably contributes to IL-1-induced

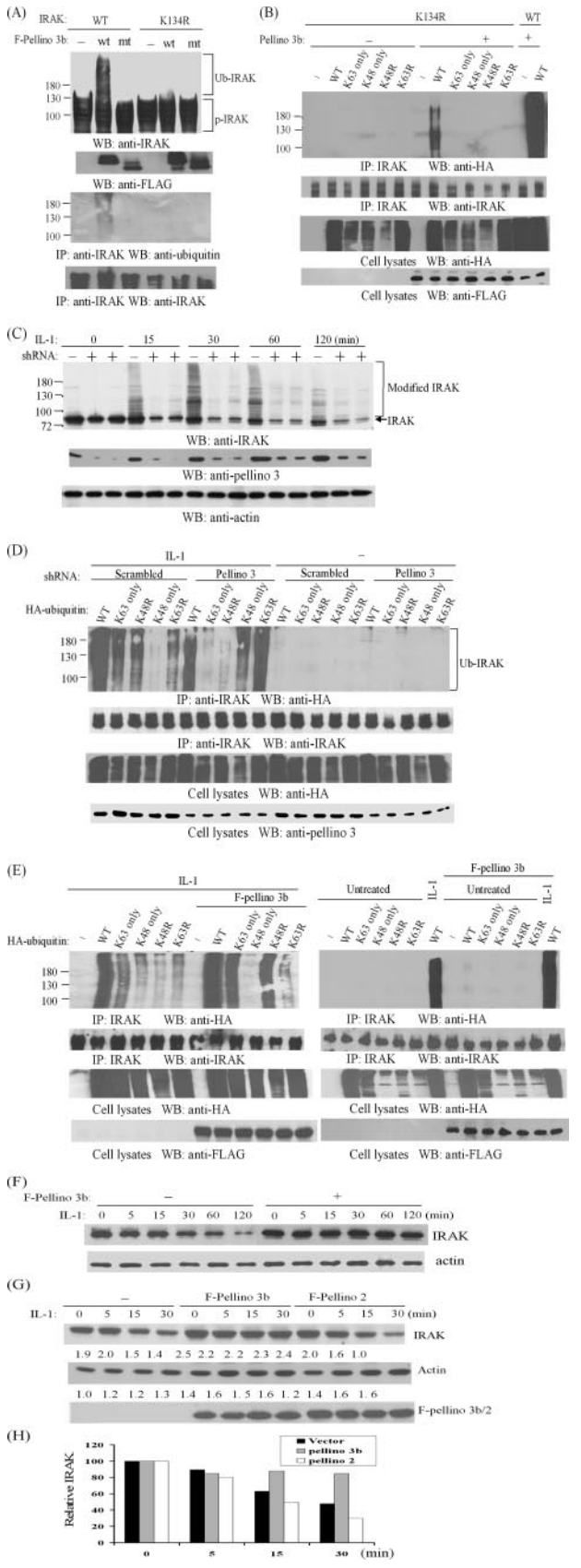

FIGURE 6. **Pellino 3b-mediated Lys-63-linked polyubiquitination inhibits IRAK degradation induced by IL-1.** *A,* Pellino 3b ubiquitinates IRAK on Lys-134. FLAG-tagged wild-type (*WT*) or mutants (*mt*) of Pellino 3b were co-transfected with wild-type or K134R mutant IRAK into I1A cells. Cell lysates were analyzed directly by Western blotting (*WB*) with anti-IRAK and anti-FLAG or immunoprecipitated (*IP*) by anti-IRAK and followed by anti-ubiquitin and anti-IRAK blotting. *B,* Lys-134 mutant IRAK plasmid was co-transfected with wild-type or mutants of HA-ubiquitin, with or without FLAG-pellino 3b, into I1A cells. Cell lysates were denatured and immunoprecipitated by anti-IRAK. Immunoprecipitates were probed with anti-HA and anti-IRAK. Cell lysates were also blotted with anti-HA and anti-FLAG. *C,* knockdown of Pellino 3 enhances IRAK degradation in IL-1 signaling. Control clone (−, stably transfected with pSUPER-scrambled into 293-IL-1R cells) and two Pellino 3 knockdown clones (+, stably transfected with pSUPER-pellino 3) were stimulated with 1 ng/ml IL-1 for indicated times. The cells lysates were analyzed by Western blotting with anti-IRAK, anti-Pellino 3, and anti-actin, respectively. *D,* wild-type or mutants of HA-ubiquitin plasmids were transfected into control or Pellino 3 knockdown cells. Two days after transfection, cells were either treated with IL-1 for 15 min or left untreated. Cell lysates were denatured and immunoprecipitated by anti-IRAK and immunoblotted with anti-HA and anti-IRAK. Cell lysates were also directly blotted with anti-HA and anti-Pellino 3. *E,* Pellino 3b-mediated Lys-63-linked polyubiquitination competitively inhibits its Lys-48-linked polyubiquitination of IRAK in IL-1 signaling. 293-IL-1R cells and 293-IL-1R cells stably transfected with FLAG-tagged Pellino 3b were transfected with HA-tagged wild-type or mutants of ubiquitin. The transfected cells were stimulated with 1 ng/ml IL-1 for 15 min or left untreated. Cell lysates were denatured and immunoprecipitated with anti-IRAK-agarose, followed by Western analysis with anti-HA and anti-IRAK. Cell lysates were analyzed by Western blotting with anti-FLAG and anti-HA. *F,* IL-1-induced IRAK degradation is abolished in FLAG-pellino 3b expressing clone. 293-IL-1R cells and 293-IL-1R cells stably transfected with FLAG-tagged pellino 3b were stimulated with 1 ng/ml of IL-1 for the indicated time. Cell lysates were analyzed by Western blotting with anti-IRAK and anti-actin. *G* and *H,* Pellino 2 and Pellino 3b exert different effects on IL-1-induced IRAK degradation. 293-IL-1R cells and 293-IL-1R cells stably transfected with FLAG-tagged Pellino 3b or Pellino 2 were stimulated with 1 ng/ml of IL-1 for the indicated time. Cell lysates were analyzed by Western blotting with anti-IRAK, anti-FLAG, and anti-actin, respectively. Anti-IRAK and anti-actin blots were quantified by densitometry, and the relative intensity (the weakest band of each blot was normalized as 1) is shown below the blot. Then the IRAK/actin ratio of each sample was calculated, and this is considered as the arbitrary IRAK protein amount presented in each sample. The arbitrary IRAK of the untreated samples of each cell line was set as 100%, and the relative arbitrary IRAK of the other samples (*versus* the arbitrary IRAK of the untreated sample from the same cell line) was calculated and plotted in *H*.

Lys-63-linked IRAK polyubiquitination, which might compete with IL-1-induced Lys-48-linked IRAK polyubiquitination, because both types of ubiquitination take place on the same ubiquitination site in IRAK (Lys-134 of IRAK). Such competition is abolished in the absence of Pellino 3b, which then leads to increased IL-1-induced Lys-48-linked ubiquitination resulting in increased IRAK degradation. In support of this notion, knocking down Pellino 3b expression reduced IL-1-induced Lys-63-linked IRAK polyubiquitination, but greatly increased IL-1-induced Lys-48-linked IRAK polyubiquitination (Fig. 6*D*). In addition, overexpression of Pellino 3b promoted IL-1-induced Lys-63-linked IRAK ubiquitination but inhibited IL-1-induced Lys-48-linked IRAK polyubiquitination and degradation (Fig. 6, *E* and *F*). Because Pellino 2 also mediates Lys-63-linked polyubiquitination on IRAK, we tested the impact of Pellino 2 on IL-1-mediated IRAK degradation. Interestingly, overexpression of Pellino 2, a positive regulator for IL-1 signaling, did not inhibit IL-1-induced IRAK degradation (Fig. 6, *G* and *H*). Instead, overexpression of Pellino 2 enhanced IRAK degradation (Fig. 6, *G* and *H*). These results indicate that Pellino 3b and Pellino 2 probably play differential roles in multiple steps of IRAK polyubiquitination and IRAK-mediated signaling.

Pellino 3b Negatively Regulates IL-1-induced TAK1-dependent Signaling Pathway—Pellino 1 and 2 have been implicated in IL-1 signaling and shown to have the ability to activate NFκB.

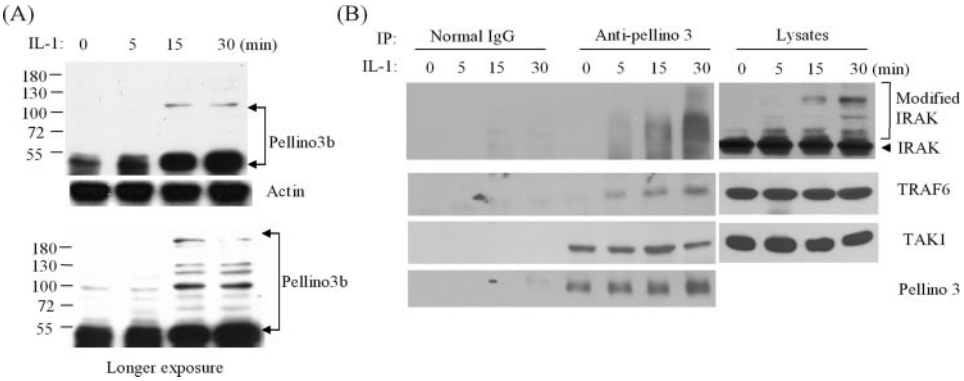

FIGURE 7. **Pellino 3b associates with TAK1-IRAK-TRAF6 (complex II) in IL-1 signaling.** *A,* IL-1 induces the expression and modification of Pellino 3 in 293-IL-1R cells. Cells lysated from 293-IL-1R cells untreated or stimulated with 1 ng/ml IL-1 for the indicated times were analyzed by Western blotting with anti-Pellino 3 and anti-actin. *B,* association of endogenous Pellino 3 and IRAK. Cell lysates from 293-IL-1R cells stimulated with IL-1 (1 ng/ml) were immunoprecipitated (*IP*) with anti-Pellino 3, and followed by immune blotting with anti-IRAK, anti-TRAF6, and anti-TAK1, respectively.

Windheim *et al.* (31) reported that Lys-63-linked IRAK polyubiquitination plays a critical role in IKK activation. We recently found that IL-1-induced TAK1 modification and IκBα degradation were reduced in Pellino 2 knockdown cells, suggesting the importance of Pellino 2 in TAK1-dependent NFκB activation pathway (data not shown). However, overexpression of Pellino 3a and 3b promotes JNK activation but not NFκB activation. Interestingly, IL-1 stimulation up-regulated the expression of endogenous Pellino 3 and induced slowly migrating modified forms of Pellino 3, suggesting a role of Pellino 3 in IL-1 signaling (Fig. 7*A*). Furthermore, IL-1 stimulation induced the interaction between endogenous Pellino 3 with IRAK and TRAF6, whereas Pellino 3 constitutively interacted with TAK1 (Fig. 7*B*).

We recently identified two parallel IL-1-mediated NFκB activation pathways, TAK1-dependent and MEKK3-dependent (Fig. 1). These two pathways are regulated at the level of IRAK modification. The K134R IRAK ubiquitination mutant is no longer degraded upon IL-1 stimulation and loses the ability to mediate the TAK1-dependent NFκB activation, while retaining the MEKK3-dependent signaling. Because Pellino 3b has significant impact on IL-1-induced IRAK ubiquitination and degradation, we hypothesized that Pellino 3b may play a major role in regulating the TAK1-dependent signaling pathway. Interestingly, we observed that IL-1 induced hyperphosphorylation of TAK1 and TAB1 in 293 cells transfected with Pellino 3 siRNA, whereas overexpression of Pellino 3b inhibited IL-1-induced TAK1 and TAB1 phosphorylation, suggesting a negative role of Pellino 3b in IL-1-induced TAK1 activation (Fig. 8, *A* and *B*). The TAK1-dependent pathway causes IKKα/β phosphorylation and IKKβ activation, leading to classical NFκB activation through IκBα phosphorylation and degradation. Although IL-1-induced IKKα/β phosphorylation, IκBα phosphorylation and degradation, and NFκB DNA binding activity were increased in the Pellino 3 knockdown cells as compared with the control cells, these signaling events were significantly inhibited in cells with overexpression of Pellino 3b (Fig. 8, *A–C*). In addition to its role in NFκB activation, TAK1 has been shown to be required for IL-1-induced JNK activation. In agreement with its negative regulatory role in the TAK1-dependent pathway, IL-1-induced JNK activation was also increased in Pellino 3b knockdown cells and decreased in 293 cells stably transfected with Pellino 3b (Fig. 8, *A* and *B*). Mutations in the RING motif impaired the ability of Pellino 3b to inhibit IL-1-induced signaling (Fig. 8*D*). It is important to note that a previous study reported that overexpression of Pellino 3 can lead to constitutive JNK activation (25). When we transiently transfected Pellino 3b into the 293 cells, a low level of JNK activation was observed (data not shown). However, in 293 cells stably transfected with Pellino 3b, constitutive JNK activation was not observed (Fig. 8*B*). Instead, Pellino 3b inhibited IL-1-induced JNK activation. On the other hand, Pellino 3b failed to inhibit TNFα-induced IκBα phosphorylation, IκBα degradation, or JNK activation (Fig. 8*E*), indicating the specific role of Pellino 3b in regulating IL-1 signaling. To further test the effect of Pellino 3b on IL-1 signaling, IL-1-induced IL-8 production was examined in media from Pellino 3b knockdown cells by enzyme-linked immunosorbent assay. As shown in Fig. 8*F*, IL-1-induced IL-8 production was increased by about 5-fold in Pellino 3b knockdown cells. Taken together, these results indicate that Pellino 3b negatively regulates the IL-1-induced TAK1-dependent pathway.

The K134R IRAK ubiquitination mutant is no longer degraded upon IL-1 stimulation and loses the ability to mediate the TAK1-dependent NFκB activation, suggesting that IL-1-induced ubiquitination and degradation of IRAK are required for the TAK1-dependent NFκB activation pathway. However, it is also possible that only the IL-1-induced IRAK ubiquitination is the necessary biochemical modification required for the activation of the TAK1-dependent pathway, and the IL-1-induced IRAK degradation is the consequence of activation. The fact that the negative regulatory impact of Pellino 3b on IL-1-induced IRAK degradation correlated with its inhibitory effect on IL-1-induced TAK1 activation suggests that IRAK degradation might be required for the activation of the TAK1-dependent pathway. In support of this, we found that the proteasome inhibitor MG-132 blocked IL-1-induced TAK1 activation, suggesting that IL-1-induced IRAK degradation is likely a necessary step in the activation of the TAK1-dependent pathway (Fig. 8*G*).

DISCUSSION

In this study, we report that Pellino 3b acts as a negative regulator for IL-1 signaling by regulating IRAK degradation through its E3 ligase activity. Our results showed that IRAK is ubiquitinated through both Lys-48- and Lys-63-linked polyubiquitination upon IL-1 induction. We recently found that a point mutation changing lysine 134 to arginine (K134R) in IRAK abolished IL-1-induced IRAK ubiquitination and degradation. These results suggest that Lys-134 is the site for both

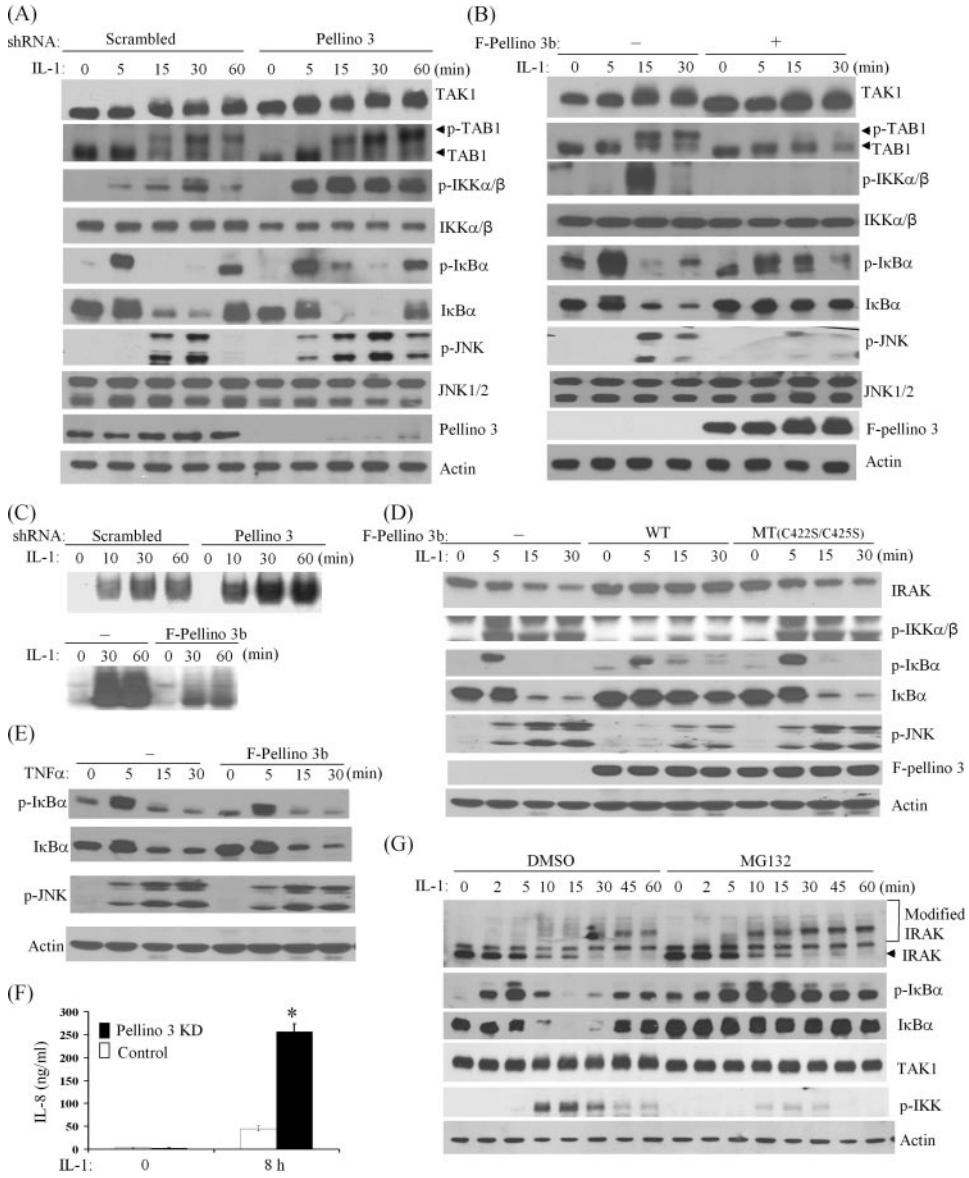

FIGURE 8. **Pellino 3b negatively regulates TAK1-dependent signaling induced by IL-1.** *A,* knockdown of Pellino 3 enhances IL-1-activated TAK1-IKK pathway. Pellino 3 knockdown and control cells were stimulated by 1 ng/ml IL-1 for the indicated time. Cell lysates were analyzed by Western blotting with anti-TAK1, anti-TAB1, anti-p-IKKα/β, anti-IKKα/β, anti-p-IκBα, anti-IκBα, anti-p-JNK, anti-JNK, anti-Pellino 3, and anti-actin. *B,* Pellino 3b inhibits IL-1-induced TAK1-IKK signaling. Control and FLAG-pellino 3b expressing cells were stimulated with 1 ng/ml IL-1 for the indicated time. Cell lysates were analyzed by Western blotting with anti-TAK1, anti-TAB1, anti-p-IKKα/β, anti-IKKα/β, anti-p-IκBα, anti-IκBα, anti-p-JNK, anti-JNK, anti-FLAG, and anti-actin. *C,* NFκB gel shift assay. Pellino 3 knockdown cells and Pellino 3b stably transfected cells, along with their control cells, were stimulated by IL-1 (1 ng/ml) for the indicated time periods. Cell lysates were incubated with ^{32}P-labeled NFκB gel shift oligonucleotides (sc-2511, Santa Cruz Biotechnology) and then resolved by 5% native polyacrylamide gel. *D,* E3 ligase activity is necessary for Pellino 3b to negatively regulate IL-1 signaling. The RING finger mutant (C422S/C425S) and wild-type (*WT*) Pellino 3b stably transfected cells were stimulated with 1 ng/ml IL-1 for the indicated time. Cell lysates were analyzed by Western blotting with anti-IRAK, anti-p-IKKα/β, anti-p-IκBα, anti-IκBα, anti-p-JNK, anti-FLAG, and anti-actin. *E,* Pellino 3b does not inhibit TNFα signaling. Control and FLAG-pellino 3b expressing cells were stimulated with 10 ng/ml TNFα for the indicated time. Cell lysates were analyzed by Western blotting with anti-p-IκBα, anti-IκBα, anti-p-JNK, and anti-actin. *F,* Pellino 3 negatively regulates IL-1-induced IL-8 production. Control and Pellino 3 knockdown cells were stimulated by 1 ng/ml IL-1 for 8 h or left untreated. Media were collected, and secreted IL-8 was measured by enzyme-linked immunosorbent assay. *G,* MG-132 inhibits IL-1-induced TAK1-IKK signaling. 293-IL-1R cells were untreated or treated with IL-1 with or without MG-132. Cell lysates were analyzed by Western blots with anti-IRAK, anti-TAK1, anti-p-IKKα/β, anti-IKKβ, anti-p-IκBα, anti-IκBα, and anti-actin.

Lys-48-linked and Lys-63-linked polyubiquitin chains on IRAK. We now found that Pellino 3b, a RING-like motif E3 ligase, played a critical role in IL-1-induced Lys-63-linked polyubiquitination on IRAK. Pellino 3b-mediated Lys-63-linked IRAK polyubiquitination competed with Lys-48-linked IRAK polyubiquitination for the same ubiquitination site, Lys-134 of IRAK, leading to inhibition of IL-1-induced IRAK degradation.

We recently identified two parallel IL-1-mediated NFκB activation pathways, TAK1-dependent and MEKK3-dependent (Fig. 1) (21). The TAK1-dependent pathway causes IKKα/β phosphorylation and IKKβ activation, leading to classical NFκB activation through IκBα phosphorylation and degradation. The TAK1-independent MEKK3-dependent pathway induces IKKγ phosphorylation and IKKα activation, resulting in NFκB activation through IκBα phosphorylation and subsequent dissociation from NFκB but without IκBα degradation. These two pathways are regulated at the level of IRAK modification (21). In this study, we showed that Pellino 3b efficiently inhibits IL-1-induced TAK1-dependent signaling events using siRNA or overexpression of Pellino 3b, including phosphorylation of TAK1/TAB1, phosphorylation of IKKα/β, and IκBα degradation (Fig. 8, *A* and *B*). The inhibition of Pellino 3b on the MEKK3-mediated pathway is probably less effective. Although TAK1-dependent signaling events (phosphorylation of TAK1/TAB1, phosphorylation of IKKα/β, and IκBα degradation) were severely abolished in cells with overexpression of Pellino 3b, significant levels of IL-1-induced phosphorylation of IκBα and NFκB DNA binding activity were still detected in the same cells, probably mediated by the MEKK3-dependent pathway (Fig. 8, *B* and *C*).

The fact Pellino 3b exhibited inhibitory effect on both IL-1-induced IRAK degradation and TAK1-dependent NFκB activation suggests an important role of IRAK degradation in IL-1-induced TAK1 activation. Consistent with this finding, the K134R IRAK ubiquitination mutant deficient in IL-1-induced IRAK degradation lost the ability to mediate IL-1-induced TAK1-dependent NFκB activation. Yamin and Miller (29) previously reported that proteasome inhibitor blocked

IL-1-induced IRAK degradation, resulting in accumulation of modified forms of IRAK. Consistent with Yamin and Miller (29), we found that MG132 treatment resulted in accumulation of modified forms of IRAK (Fig. 8*G*). Furthermore, the proteasome inhibitor MG-132 blocked IL-1-induced TAK1-dependent signaling, suggesting that IL-1-induced IRAK degradation might indeed be a necessary step in the activation of the TAK1-dependent pathway. Intriguingly, Windheim *et al.* (31) was unable to show the effectiveness of MG132 on IRAK degradation. Because of the complex modification pattern of IRAK, an extensive time course might be helpful to clearly observe IRAK degradation. The ubiquitinated proteins associated with IRAK might mask the effectiveness of MG132 treatment when IRAK is immunoprecipitated, followed by Western analysis with ubiquitin antibody. Such caution also needs to be taken for the analysis of IRAK ubiquitination mutants including K134R.

Although it was speculated that IRAK degradation may be one of the mechanisms to shut down IL-1 signaling to control inflammatory responses, as presented above, our recent studies suggest that IRAK degradation is also required for activation of the TAK1-dependent pathway. One possible role for IL-1-induced IRAK degradation in the activation of TAK1 is to release the TAK1 complex from the membrane-associated IRAK-TRAF6-TAK1-TABs complex. We have previously shown that TAK1-TABs are preassociated as a complex on the membrane before IL-1 stimulation (7). Upon IL-1 stimulation, the modified IRAK-TRAF6 complex dissociates from the receptor complex (complex I) and forms complex II with the preassociated TAK1-TABs on the membrane. The membrane-bound modified IRAK is eventually ubiquitinated and degraded, which is accompanied by the release of the TAK1 complex (TRAF6-TAK1-TABs) from the membrane to the cytosol, where TAK1 is activated (7). IL-1-induced Lys-48-linked IRAK ubiquitination and subsequent degradation might play a critical role in the release of TAK1 complex from the membrane to the cytosol. It is interesting that although the interaction of Pellino 3b with IRAK and TRAF6 is IL-1-dependent, Pellino 3b constitutively interacts with TAK1. It is possible that Pellino 3b is present in the membrane-associated TAK1 complex before IL-1 stimulation. IL-1 stimulation leads to the formation of IRAK-TRAF6-Pellino 3b-TAK1-TABs complex on the membrane, where Pellino 3b mediates Lys-63-linked IRAK polyubiquitination. Pellino 3b-mediated Lys-63-linked polyubiquitination may then compete with IL-1-induced Lys-48-linked IRAK ubiquitination, thereby blocking degradation and subsequent release of the TAK1 complex.

It is intriguing that Windheim *et al.* (31) reported that IL-1 triggers predominant formation of Lys-63-linked polyubiquitination of IRAK and is less abundant for Lys-48-linked polyubiquitination of IRAK. Because the degree of polyubiquitination chains formed on IRAK is proportional to the expression levels of HA-ubiquitin, the poor expression of K63R ubiquitin mutant might affect the interpretation of the results. In our study, we have now used not only K63R and K48R but also Lys-63-only and Lys-48-only ubiquitin mutants to definitively demonstrate the Lys-63- and Lys-48-linked polyubiquitin chain formation on IRAK in response to IL-1. One important question is then what is the E3 protein ligase responsible for IL-1-induced Lys-48-linked IRAK ubiquitination. TRAF6, a RING domain protein, has been shown to function as a ubiquitin protein ligase E3 in conjunction with E2 Ubc13/Uev1A to mediate Lys-63-linked polyubiquitination and lead to the activation of TAK1. We recently found that TRAF6 has the ability to mediate Lys-48-linked polyubiquitination when UbcH5 was used as E2 *in vitro*. However, IL-1 was still able to induce IRAK ubiquitination and degradation in TRAF6-deficient MEFs (data not shown), indicating that TRAF6 is not the E3 responsible for IL-1-induced K-48-linked IRAK ubiquitination. Recent studies showed that serine/threonine kinase Rip is ubiquitinated via Lys-63- and Lys-48-linked polyubiquitin chain by TRAF2 and A20, respectively, and competitively through the same ubiquitination site (12). A20 is unlikely to mediate Lys-48-linked IRAK ubiquitination, because A20 has been shown to play a negative regulatory role in TLR signaling by deubiquitinating TRAF6 (13), whereas our study suggests a positive role for Lys-48-linked IRAK ubiquitination and subsequent degradation in IL-1 signaling.

It seems unlikely for the other Pellino family members to mediate IL-1-induced Lys-48-linked IRAK ubiquitination, because the Pellino proteins only exhibited E3 activity for Lys-63-linked polyubiquitination *in vivo* (Fig. 4, *C* and *D*) (28). However, one possibility is that IL-1-induced phosphorylation of Pellinos (such as Pellino 2) by IRAK changes its specificity from Lys-63 to Lys-48 conjugations *in vivo*. Therefore, it would be premature to completely exclude the potential role of Pellinos as E3s required for IL-1-induced Lys-48-linked IRAK ubiquitination and subsequent degradation. In support of this, Pellinos were able to combine with UbcH3 to catalyze Lys-48-linked polyubiquitination *in vitro*. The timing of action, formation of specific signaling complexes, and relative expression levels of Pellinos *in vivo* might account for the differential mechanisms exerted by Pellino2 *versus* Pellino 3b in IL-1 signaling. Future studies are required to clarify the multiple steps of IRAK polyubiquitination and their specific roles in IRAK-mediated signaling.

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