Innate immune signaling in Drosophila is regulated by transforming growth factor β (TGFβ)-activated kinase (Tak1)-triggered ubiquitin editing

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Coordinated regulation of innate immune responses is necessary in all metazoans. In Drosophila the Imd pathway detects Gram-negative bacterial infections through recognition of diaminopimelic acid (DAP)-type peptidoglycan and activation of the NF-κB precursor Relish, which drives robust antimicrobial peptide gene expression. Imd is a receptor-proximal adaptor protein homologous to mammalian RIP1 that is regulated by proteolytic cleavage and Lys-63-polyubiquitination. However, the precise events and molecular mechanisms that control the post-translational modification of Imd remain unclear. Here, we demonstrate that Imd is rapidly Lys-63-polyubiquitinated at lysine residues 137 and 153 by the sequential action of two E2 enzymes, Ubc5 and Ubc13-Uev1a, in conjunction with the E3 ligase Diap2. Lys-63-ubiquitination activates the TGFβ-activated kinase (Tak1), which feeds back to phosphorylate Imd, triggering the removal of Lys-63 chains and the addition of Lys-48 polyubiquitin. This ubiquitin-editing process results in the proteasomal degradation of Imd, which we propose functions to restore homeostasis to the Drosophila immune response.

The Imd pathway is largely responsible for the robust antimicrobial peptide (AMP) induction observed after septic bacterial infection in Drosophila and thus is critically important for defense against invading pathogens (1, 2). This pathway is triggered by diaminopimelic acid (DAP)-type peptidoglycan (PGN) from bacterial cell walls. Immune-responsive cells recognize PGN through two peptidoglycan recognition proteins (PGRPs): the cell surface receptor PGRP-LC and the cytosolic receptor PGRP-LE (3). PGN recognition by these proteins (PGRPs) elicits the NF-κB response.

Once cleaved, Imd associates with the E3 ligase Diap2 and is rapidly ubiquitinated. This modification leads to the activation of the Drosophila homologs of the Tak1 and IKK (5) and ultimately to the activation of the NF-κB precursor Relish and induction of AMP genes expression.

Ubiquitination is a critical regulator of innate immune signaling, especially NF-κB pathways in mammals and insects. The number and topology of ubiquitin conjugations determine the fate of substrate proteins. For example, Lys-48-polyubiquitination targets proteins to proteasome for degradation (6), whereas Lys-63-polyubiquitin chains often function as scaffolds in signaling pathways, recruiting and activating downstream factors (7, 8). Ubiquitination requires the sequential action of ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s) and ubiquitin ligases (E3s). Whereas E3s are critical for substrate recognition, E2s play central role in determining chain topology (9, 10). In the Drosophila immune response, Lys-63-polyubiquitination of Imd plays a crucial role relaying signals to downstream kinases. In particular, we previously demonstrated that Imd polyubiquitination requires the E3 ligase Diap2 as well as the E2 ubiquitin-conjugating enzymes Ubc5 (Effete) and the Ubc13 (Bendless)-Uev1a complex (5).

Effete, also known as UbcD1, is a Drosophila member of the yeast Ubc4/5 family along with the human E2s in the Ubc2D (UbcH5) group (11–13). Bendless (UbcD3) is the Drosophila homolog of mammalian Ubc13/Ube2N, which dimerizes with ubiquitin enzyme variants (Uevs) to generate Lys-63 chains (12–14). However, the molecular mechanisms by which these two E2s function together in Imd polyubiquitination remain unclear.

The MAP3 kinase Tak1, complexed with the Drosophila Tab2 homolog (15), is known to function downstream of ubiquitinated Imd (5). Tab2 contains a conserved Lys-63-polyubiquitin binding domain (16, 17), suggesting activation of Tak1/Tab2 complex by association with Lys-63-polyubiquitinated Imd. Tak1 is required for activation of Drosophila IKK complex, which is essential for activation of NF-κB precursor Relish (18–20), the key transcription factor leading to induction of AMP genes.

In addition to ubiquitination, phosphorylation is another common type of post-translational modification observed in signaling pathways. Signal transduction often relies on cas-

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2 The abbreviations used are: AMP, antimicrobial peptide; PGN, peptidoglycan; PGRP, peptidoglycan recognition protein; IKK, IκB kinase; dsRNA, double-stranded RNA; ANOVA, analysis of variance; JNK, JNK kinase; qRT, quantitative real-time; Ub, ubiquitin; Uev, ubiquitin enzyme variant.
cades of kinase activation and phosphorylation. Previous research suggests that Imd is also phosphorylated upon immune stimulation (5). However, it is still unknown what kinases are responsible for Imd phosphorylation or what functional relevance this modification may have for immune signaling and defense.

Before this work it was demonstrated that Imd is polyubiquitinated and phosphorylated, yet no connection has been made between these types of post-translational modifications. Here we confirm that Imd is rapidly cleaved and Lys-63-polyubiquitinated upon immune stimulation and further demonstrate that this is followed by removal, or deubiquitination, of the Lys-63 chains and the addition of Lys-48-polyubiquitin. This ubiquitin editing strongly correlates with Imd phosphorylation and requires the Lys-63-activated MAP3K Tak1, creating a feedback loop that culminates in the proteasomal destruction of Imd.

**Results**

**Imd signaling regulated by Tak1-mediated ubiquitin-editing**

Previously, we have shown that PGN stimulation leads to the caspase-dependent cleavage and Lys-63-polyubiquitination of the adaptor protein Imd (5). Our earlier work suggests that Imd was Lys-63-polyubiquitinated but not Lys-48-conjugated. On the other hand, another report suggested that Imd is modified by both types of polyubiquitin chains (21). To examine the post-translational modifications of Imd more closely, a new assay was developed whereby a single Imd immunoprecipitation was assayed as above. *Dipt, diptericin. E. coli* were challenged with live *E. coli* for the indicated times. Endogenous Imd was assayed as above. All blots are representative of at least three independent experiments. In all cases, the open triangle marks the unmodified full-length Imd, the open arrow marks cleaved Imd, and the black arrow marks phosphorylated Imd.

**Imd is Lys-63- and Lys-48-polyubiquitinated as well as phosphorylated upon immune stimulation**

A. Specificity of ubiquitin chain antibodies. 100 ng of synthetic Lys-63, Lys-48, and Met-1 tetra-ubiquitin chains were each immunoblotted (IB) with antibody specific to total ubiquitin, Lys-63 ubiquitin, and Lys-48 ubiquitin. B. Two types of Imd ubiquitination induced by PGN stimulation. S2* cells were stimulated with PGN for the indicated times. Imd was immunoprecipitated (IP) from whole cell lysate, and immunoblotted with antibody specific to Lys-63, Lys-48, or total ubiquitin. C. Imd phosphorylation induced by PGN stimulation. Lysates from S2* cells stimulated with PGN were treated with lambda-phosphatase as indicated before analysis by immunoblot. D. Proteasomal degradation of Imd triggered by Lys-48 modification. S2* cells were treated with vehicle control (DMSO) or the proteasome inhibitor MG-132 before PGN stimulation for indicated times. Imd modifications were assayed as above. E. Imd modifications in adult flies. Control (w1118), Diap2Δ, and imd10191 adult flies were challenged with live *E. coli* for the indicated times.
polyubiquitination followed by intensive Lys-48 polyubiquitination peaking at 15 min, both of which progressively decreased and disappeared at later time points (Fig. 1B). The antibody detecting total ubiquitin showed a pattern that combined the Lys-63 and Lys-48 kinetics but slightly overrepresenting of Lys-63 modification, consistent with hypersensitive detection of synthetic Lys-63 Ub chains by this antibody (Fig. 1A). In addition to full-length (Fig. 1, open triangle) and cleaved Imd (open arrow), other inducible forms of Imd were observed (black arrows). λ-Phosphatase treatment combined with higher resolution SDS-PAGE demonstrated that these particular forms of Imd are phosphorylated (Fig. 1C). In these analyses, a fast migrating phospho-form is always observed (Fig. 1B and lower black arrow in Fig. 1C), which presumably represents phosphorylation of cleaved Imd. Often, a high molecular weight phospho-form is also observed, presumably due to phosphorylation of full-length Imd (upper black arrow in Fig. 1C).

Lys-48-polyubiquitination traffics conjugated proteins to the proteasome and/or autophagosomes for degradation. To determine whether Lys-48 conjugation is involved in the turnover of Imd through the proteasome pathway, S2* cells were treated with MG-132, a proteasome inhibitor, before stimulation with PGN (Fig. 1D). This treatment led to persistent Imd Lys-48-polyubiquitination but little change in the Lys-63 kinetics. Interestingly, the phosphorylated Imd also accumulated to higher levels in the MG-132-treated cells (bottom panel, Fig. 1D). These results indicate that Imd is degraded by proteasome after conjugation of Lys-48-polyubiquitin chains and suggest that phosphorylation might be linked to these ubiquitination/degradation events. On the other hand, blocking of autophagy by Atg1 RNAi did not cause any Imd accumulation (data not shown), suggesting that autophagy is not part of the degradation machinery for Imd.

To further analyze these patterns in vivo, adult animals were infected with Escherichia coli, and whole animal lysates were assayed by Imd immunoprecipitation and immunoblotting for post-translational modifications. Diap2 deficient (Diap2<sup>2c</sup>) and Imd null (imd<sup>10191</sup>) flies were included for comparison, relative to a control strain. Similar to S2* cells, Imd exhibited clear and transient infection-induced cleavage, ubiquitination, and phosphorylation in adult flies, whereas Diap2-deficient flies showed increased accumulation of cleaved Imd with a complete absence of ubiquitination or phosphorylation (Fig. 1E). The Lys-48- and Lys-63-specific antibodies did not have the sensitivity required for use in these whole animal assays (data not shown). Together, these cell-based and whole animal data show that Imd is multiply modified after immune activation with cleavage, two types of ubiquitination, phosphorylation, and ultimately proteasome-mediated degradation.

**Lys-137 and Lys-153 are the sites for Imd ubiquitin conjugation**

Next we sought to identify the ubiquitin conjugation sites on endogenous Imd with immunopurification and mass spectrometry. Two rounds of similar assays showed that lysine residues 137 and 153 were conjugated with ubiquitin, as indicated by diglycine tags after trypsin digestion and tandem MS (Fig. 2A, K<sup>+114</sup> in the spectrum).

To verify the result from mass spectrometry analysis, stable cell lines expressing Imd WT, K137R, K153R, or K137R/K153R were generated and transfected with dsRNA targeting the 3'-UTR of Imd to knock down the endogenous protein (RNAi efficiency is shown in Fig. 2B). PGN-stimulated Imd Lys-63- and Lys-48-polyubiquitination were completely absent when both lysine residues were mutated to arginine, whereas only Lys-63-polyubiquitination showed a considerable decrease when either Lys-137 or Lys-153 were singly mutated to arginine (Fig. 2C). Moreover, the signaling pathway activity, as measured by induction of the antimicrobial peptide gene Diptericin, showed ~40–50% reduction caused by either lysine mutation and 70% reduction by the double lysine mutation (Fig. 2D). By comparison, the control parental cell line showed 10% residual Diptericin induction in these knockdown conditions, indicative of the small amount of imd transcript that remained after RNAi treatment (Fig. 2B). These results demonstrate that ubiquitination at Lys-137 and Lys-153 is critical for Imd signaling in S2* cells.

To further explore the importance of Lys-137/Lys-153 in vivo, imd<sup>10191</sup> (null) flies expressing wild-type, K137R, K153R, or K137R/K153R imd were generated by PhiC31-mediated transgenesis. E. coli-triggered induction of Diptericin was significantly inhibited when both lysine residues are substituted with arginine (Fig. 2E), further highlighting the role of these two residues in Imd signaling in vivo.

**Imd ubiquitination requires Diap2 and multiple E2s**

Our previously published work showed that the E3 ligase Diap2 is required for Imd Lys-63-polyubiquitination (5). Now, using antibodies specific to Lys-63 and Lys-48 ubiquitin, we demonstrate that Imd Lys-63-polyubiquitination is prevented by knocking down Diap2, which leads to accumulation of cleaved Imd and a failure to generate Lys-48-conjugated Imd (Fig. 3A). Coincident with this lack of ubiquitination, Diptericin induction is also blocked with Diap2 knockdown (Fig. 3, B and C, for RNAi efficiency). The accumulation of cleaved Imd in this assay as well as in adult Diap2 mutant animals (Fig. 1E), strongly argues that Imd cleavage occurs before Lys-63-polyubiquitination.

To examine the detailed mechanism of Imd ubiquitination, we sought to more thoroughly characterize the role of two different E2 enzymes previously implicated: Ubc5 and Ubc13-Uev1a. RNAi targeting each of these E2s was optimized such that knockdown was at least 90% efficient, as measured by qRT-PCR (Fig. 4A). Each E2 was knocked down individually or in all possible combinations. Strikingly, total and Lys-63-polyubiquitination was completely abolished by Ubc5 depletion alone, whereas a low level (20–50%) of ubiquitination signal (both total and Lys-63) remained after knockdown of Ubc13 and/or Uev1a (Fig. 4B and intensity quantification in 4C). The induction of Diptericin showed a similar pattern (Fig. 4C). Knockdown of Ubc5 prevented Diptericin induction, whereas cells depleted of Ubc13 and/or Uev1a still induced a low level (5–15%). Similarly, Lys-48-polyubiquitination was also blocked.
in the absence of Ubc5 (Fig. 4D). On the other hand, the level of Lys-48-polyubiquitination observed with Ubc13 and/or Uev1a RNAi was mostly unchanged (Fig. 4D), suggesting that neither of these two E2s is involved in Imd Lys-48-polyubiquitination. Together, these data indicate that Imd Lys-63-polyubiquitination requires two E2s, which play distinct roles in the process, as discussed in more detail below.

**Tak1 is required for Imd phosphorylation and ubiquitin editing**

In addition to ubiquitination, Imd is phosphorylated upon immune stimulation, and the timing of this modification tracks with Lys-48-ubiquitination (Fig. 1B). Tak1, a Lys-63-polyubiquitin-activated MAP3K that functions downstream of Imd Lys-63-polyubiquitination, is implicated in Imd phosphorylation (25). To determine if Tak1 is required for Imd phosphorylation,
Imd signaling regulated by Tak1-mediated ubiquitin-editing

Tak1 directly phosphorylates Imd

To determine whether Tak1 is solely responsible for Imd phosphorylation, S2* cells were treated with dsRNA targeting two downstream kinases in the Imd-signaling pathway, IKKβ (encoded by *ird5*) or JNKK (encoded by *hep*). Unlike Tak1, depletion of neither IKKβ nor JNKK significantly affected the PGN-induced phosphorylation of Imd (Fig. 6, A and B and multiple blots quantified in C), indicating these kinases are not involved in phosphorylating Imd. However phosphorylation of full-length Imd was slightly (but not significantly) reduced in cells when JNKK was knocked, leaving open the possibility that JNKK contributes partially to phosphorylation of full-length, but not cleaved, Imd. To address whether Tak1 directly phosphorylates Imd, in vitro immunoprecipitation kinase assays were performed. FLAG-tagged Tak1 WT or kinase dead Tak1S176A was immunoprecipitated with anti-FLAG-agarose and used in a kinase reaction with recombinant SUMO-tagged Imd (or SUMO or MKK6 as controls) as substrates along with radiolabeled ATP (Fig. 6C). WT Tak1 but not Tak1S176A phosphorylated Imd and MKK6 but not SUMO. Furthermore, the addition of (5Z)-7-Oxozeaenol, a selective inhibitor of Tak1, to the in vitro kinase reaction inhibited Imd phosphorylation (Fig. 6D). Together these experiments support the conclusion that Tak1 directly phosphorylates Imd.

Discussion

In previous work we demonstrated that Imd is cleaved, Lys-63-polyubiquitinated, and phosphorylated upon immune stimulation (5). Although this earlier study did not find Lys-48-polyubiquitin chains, others have reported evidence of both Lys-63- and Lys-48-Imd modifications (21). However, the overall dynamics of, and interconnections between these Imd post-translational modifications remained unclear. Here we show that PGN stimulation of S2* cells leads to five different Imd modifications: proteolytic cleavage, Lys-63-polyubiquitination, phosphorylation, Lys-63-deubiquitination, and Lys-48-polyubiquitination occurring as early as 2 min after PGN stimulation. Although Lys-63 modification peaks early and then steadily declines, Lys-48-conjugation appears later, along with phosphorylation, and declines in a proteasome-dependent manner. These kinetics argue that Imd is sequentially conjugated with Lys-63 then Lys-48 ubiquitin, so-called ubiquitin editing, as has been reported for IRAK1 and RIP1 in mammalian innate immune signaling pathways (22).
In addition to ubiquitination, two slow-migrating species of Imd were detected and shown to be phosphorylated forms. Judging by their size, these two phospho-forms appear to be derived from either full-length Imd (upper) or cleaved Imd (lower) (Fig. 1C). Interestingly, we also observe persistence of phosphorylated Imd in the proteasome-inhibited samples (Fig. 1D), suggesting that Imd is both Lys-48-polyubiquitinated and phosphorylated before entering proteasome. Tak1 is required for these phosphorylation events as well as for ubiquitin editing, demonstrating a key role for this MAP3K in a negative feedback loop.

Conjugation of ubiquitin usually occurs on lysine side chains of target proteins, and mass spectrometry of immunopurified endogenously expressed Imd identified Lys-137 and Lys-153 as the sites of ubiquitin linkage. Note, our mass spectrometry analysis includes 50% coverage of Imd, and a cluster of four lysine residues at the very C terminus were not observed. Substitution of Lys-137 and Lys-153 residue with arginine prevented signal-induced ubiquitination of Imd in S2* cells and reduced expression of the AMP gene Diptericin in both cells and flies. These results demonstrate that both lysine residues are required for Lys-63-polyubiquitination and downstream signaling events. In S2* cells, mutation of single lysine residue led to a partial reduction of Lys-63-ubiquitination and a partial reduction of AMP gene induction. Surprisingly, a single lysine mutation did not correspondingly reduce Imd Lys-48-polyubiquitination, whereas the double lysine mutation completely blocked it. These results suggest that even the reduced signal, mediated by a single Lys-63-chain, is sufficient to trigger a robust feedback response with Lys-48-chain formation. On the other hand, a complete block in Lys-63-chains prevents Tak1 activation, which in turn fails to promote the ubiquitin editing of Imd. These findings are consistent with results observed with knockdown of Ubc5, Ubc13, and Uev1a. Ubc5 depletion prevented all Lys-63 ubiquitination and signaling (as measured by Diptericin induction), and subsequent Lys-48 modification was absent, whereas the Ubc13 and/or Uev1a knockdown showed residual Lys-63 chains and greatly reduced Diptericin expression but robust Lys-48-ubiquitination. These results also suggest that Lys-48-polyubiquitination may occur on lysine residues beyond Lys-137 and Lys-153, although more detailed mass spectrometric analyses is required to map these sites more thoroughly.

On the other hand, only double lysine mutation leads to significant reduction of AMP expression in adult flies, and this
Reduction is not as robust as in cultured cells. This pattern suggests that activation of the NF-κB protein Relish and its transcriptional targets do not solely rely on ubiquitination of Imd Lys-137/153. Other possible ubiquitination targets include the upstream caspase Dredd, which has been shown to be critical for signaling (26), or the E3 ligase Diap2. This redundancy may represent multiple parallel mechanisms that contribute to the NF-κB activation. Furthermore, tissue-specific immune differences may contribute to the discrepancy in Diptericin induction between macrophage-derived S2* cells and whole flies, in which the fat body rather than hemocytes is the major organ for inducible expression of AMPs.

Previous work has suggested that Diap2 is the E3 ligase for Imd ubiquitination (5). With the advantage of ubiquitin linkage-specific antibodies, data presented here show that Diap2 is required for Imd Lys-63-polyubiquitination and signaling, as measured by induction level of Diptericin. Moreover, the accumulation of cleaved but non-ubiquitinated Imd, in the Diap2 depleted cells and flies, provides further evidence that ubiquitination is downstream of Imd cleavage and highlights the role of Diap2 as the critical E3 in the Lys-63-modification of Imd. In addition, Imd is no longer Lys-48-modified when Diap2 is removed, suggesting that either Diap2 is also involved in Lys-48-conjugation or the failure of Lys-63-polyubiquitination leads to the loss of Lys-48-polyubiquitination. Given the role of Tak1 in this ubiquitin editing event, we favor the latter hypothesis and speculate another E3 is likely involved in the Lys-48 conjugation.

In addition to E3 ligases, E2 ubiquitin-conjugating enzymes are the other key factors in the ubiquitin conjugation reaction. We previously showed that Ubc5 and Ubc13-Uev1a are all involved in Imd ubiquitination (5). However, the mechanism by which these E2s collaborated was unclear. Results from in vitro reconstituted ubiquitination assays suggested a two-step reaction model for ubiquitin conjugation with different E2s (27–30). In particular, it was shown that some E2s, such as Ubc5, are effective at the initial ubiquitination of substrates but are ineffective at generating long chains, whereas other E2s, like Ubc13/Uev1a, are efficient at generating long ubiquitin chains.
but fail to conjugate substrate proteins. Thus, these two types of E2s can work together to generate long ubiquitin chains conjugated to target proteins. We propose that Imd undergoes ubiquitin chain initiation and elongation catalyzed by two separate E2s (Fig. 7). Once cleaved, Imd interacts with Diap2 through its baculovirus inhibitor repeat (BIR) repeats and is first modified by Ubc5-mediated substrate ubiquitination on lysines 137 and 153. Subsequently, the E2 complex of Ubc13-Uev1a pairs with an E3 (possibly Diap2 although another unidentified E3 is not excluded) and switches the reaction to the chain elongation mode, during which additional ubiquitin molecules are attached to the substrate-linked ubiquitin in a Lys-63-specific manner (31–35). In the absence of Ubc13/Uev1a, Ubc5 alone is still able to elongate the polyubiquitin chains but less efficiently and with unknown linkages.

Induction of Diptericin expression generally tracks with the Lys-63-polyubiquitination signal (but not the total Ub signal) observed in various E2 knockdown cells (Fig. 4C). The one exception is the samples in which Ubc13 and Uev1a are both knocked down and Ubc5 is still available. These samples display a similar Lys-63 intensity as the single Ubc13 or Uev1a RNAi lanes, but Diptericin induction is lower, close to background levels. Ubc5 alone is known to conjugate ubiquitin without linkage specificity, a random polyubiquitin chain that consists of all seven types of lysine linkages (29). Because the Lys-63 antibody recognizes Lys-63-linked diubiquitin (22), it is possible that Ubc5-catalyzed random ubiquitin chain elongation generates some Lys-63 diubiquitin linkages that are detected by this antibody but are unable to support signaling due to their altered topology and limited amounts of Lys-63-linkages. More detailed biochemical characterization of these Ubc5-catalyzed chains is required to confirm this hypothesis.

Lys-48 modification of Imd shows a subtle difference relative to the Lys-63 chains. Again, knockdown of Ubc5 causes com-
complete blocking of Lys-48 conjugation, but depletion of Ubc13/Uev1a has no effect. The failure of Lys-48 modification in the Ubc5-depleted cells may have two possible underlying causes. First, the complete lack of Lys-63 chains will fail to activate Tak1 and the subsequent ubiquitin editing feedback loop, whereas the Ubc13 and/or Uev1a RNAi display some residual Lys-63 activity and thus can trigger Tak1 and the feedback response. Alternatively, Ubc5 might be directly required for Imd Lys-48-polyubiquitination as shown in the degradation of proteins in multiple Drosophila pathways including eye development, maintenance of germ-line stem cells and apoptosis (36). These are not mutually exclusive possibilities.

Phosphorylation of Imd appears to be a major regulator of these ubiquitin-editing events. Knockdown of Tak1 prevents Imd phosphorylation in S2* cells and in adult flies. Moreover, immune-purified Tak1 can directly phosphorylate recombinant Imd in vitro, whereas neither JNKK nor IKK is required for phosphorylation of cleaved Imd, strongly arguing that Tak1 directly modifies Imd. RNAi depletion or drug inhibition of Tak1 prevents Lys-63-deubiquitination and the subsequent Lys-48-polyubiquitination/proteasome-mediated degradation, leading to accumulation of cleaved but unphosphorylated Imd, presumably an intermediate during chain editing. From these results, we infer that Tak1-mediated phosphorylation of Imd is required for ubiquitin editing. Future studies will reveal the underlying mechanisms by which phosphorylation triggers Lys-63-deubiquitination and Lys-48 chain conjugation. Nonetheless, these results are consistent with earlier reports of Imd regulation by Lys-63-deubiquitination and degradation (21).

Considering the results presented here together with earlier studies, we propose the following model of Imd signal activation and subsequent down-regulation (Fig. 8). One of the earliest events after PGN stimulation is the rapid cleavage of Imd by the caspase-8 homolog DREDD at Asp-30 (5). Cleaved Imd then interacts with BIR repeats of the E3 ligase Diap2 and is Lys-63-polyubiquitinated through the sequential action of Ubc5, for substrate conjugation, and Ubc13-Uev1a, for catalyzing long Lys-63 chains. These Lys-63-polyubiquitin chains are then likely to activate the Tak1/Tab2 kinase complex through the conserved Lys-63-binding motif in Tab2, which in turns signals through IKK complex to activate the NF-κB precursor Relish (20). Relish is central for the robust induction of AMP gene transcription. Meanwhile, Tak1 also mediates a retrograde signal that phosphorylates Imd and triggers ubiquitin editing and leads to the degradation of Imd through proteasome. This regulatory interaction between Tak1 and Imd represents a novel homeostatic loop whereby the Drosophila
immune response is rapidly activated but also quickly shut-
down. Future studies are necessary to determine function of
this feedback loop relative to other feedback mechanisms
reported for the Imd pathway (21, 37–45).

**Experimental procedures**

**Fly stocks**

The following fly strains were used in this work: w^{1118},
Diap2^{7c} (46), imd^{9191} (47), yw;wt and yw;Tak1^{1} (18).

**Antibodies**

Anti-Lys-63 (Apu3) and anti-Lys-48 (Apu2) ubiquitin antibi-
odies were purchased from EMD Millipore (Billerica, MA).
Total ubiquitin antibody (P4D1) was purchased from Santa
Cruz Biotechnology (Dallas, TX). Anti-FLAG M2 Affinity Gel
and anti-FLAG antibody were purchased from Sigma. Poly-
clonal Imd antibody was prepared by immunizing rabbits with
peptide (31 AAPVDDN41) according to the affinity-puri-
ﬁed package offered by New England Peptide (Gardner, MA).

**Peptidoglycan preparation**

A 5-liter culture of *E. coli* strain 1106 was collected and resus-
pended with 4% SDS solution before boiling at 100 °C for 30
min. Traces of SDS were removed by repeated centrifugation
and resuspending with sterilized water. Then the lysate was
sequentially treated with DNase, α-amylase, and Pronase.
These enzymes were removed by repeated centrifugation and
resuspending with sterilized water. At last the lysate was treated
with sonification and lyophilization and prepared as 1 mg/ml
stock.

**Immunoprecipitation and immunoblotting protein assay**

Cells or ﬂies were lysed in standard lysis buffer containing
10% glycerol, 1% Triton X-100, 150 mM NaCl, 24
mm β-glycerol phosphate, 2 mm EDTA, 1 mm 1,4 diithio-
reitol (DTT), 1 mm sodium orthovanadate, 5 mm N-ethylmaleimide,
1× protease inhibitor mixture. Total protein lysates was immu-
noprecipitated overnight at 4 °C followed by immunoblotting
protein assay.

**Proteasome inhibition assay**

S2* cells were treated with 20-hydroxyecdysone (1 μM) for
24 h before treatment of 10 μM MG-132 (Sigma) for 6 h fol-
lowed by PGN stimulation and protein assay.

**λ-Phosphatase assay**

S2* cells were stimulated with *E. coli* PGN for the indicated
times before lysis. The whole cell lysates (100 ng for each con-
dition) were treated without or with λ-phosphatase for 30 min
at 30 °C followed by immunoblotting with anti-Imd antibody.

**Protein puriﬁcation for mass spectrometry**

S2* cells were stimulated with PGN for 0 and 15 min and
lysed with the buffer described above. 20 mg of total protein
lysate was immunoprecipitated overnight at 4 °C with anti-Imd
antibody. Samples were washed twice with 5 ml of lysis buffer
and eluted with 200 μl of elution buffer (1 m citric acid, pH 2.2).
Elutes were separated by SDS-PAGE and stained with Coomassie
Blue. The corresponding bands were cut out for in-gel trypsin
digestion and analyzed by tandem mass spectrometry.

**In-gel digestion (MS)**

Gel slices were cut into 1 × 1-mm pieces and placed in 1.5-ml
Eppendorf tubes with 1 ml of water for 1 h. The water was
removed, and 50 μl of 250 mM ammonium bicarbonate was
added. For reduction, 5 μl of a 45 mm solution of DTT was
added, and the samples were incubated at 50 C for 30 min. The
samples were cooled to room temperature and then for alkyla-
tion, 5 μl of a 100 mm iodoacetamide solution was added and
allowed to react for 30 min. The gel slices were washed 2× with
1-ml water aliquots. The water was removed, and 1 ml of 50:50
(50 mm ammonium bicarbonate:acetonitrile) was placed in
each tube, and samples were incubated at room temperature for
1 h. The solution was then removed, and 200 μl of acetonitrile
was added to each tube at which point the gel slices turned
opaque white. The acetonitrile was removed, and gel slices were
further dried in a SpeedVac concentrator. Gel slices were rehy-
drated in 50 μl of 2 ng/μl trypsin (Sigma) in 0.01% Protease-
MAX Surfactant (Promega), 50 mm ammonium bicarbonate.
An additional aliquot of 50 mm ammonium bicarbonate was
added to fully submerge the gel slices. Samples were incubated
at 37 °C for 21 h. The supernatant of each sample was then
removed and placed in a separate 1.5-ml Eppendorf tube. Gel
slices were further dehydrated with 100 μl of 80:20 (aceto-
nitrile:1% formic acid). The extract was combined with the su-
pernatants of each sample. The samples were then dried down in a
SpeedVac concentrator (Savant Instruments, Inc.).

**LC/MS/MS on Orbitrap Velos**

Tryptic peptides were dissolved in 20 μl of 0.1% TFA, and a
3.5-μl aliquot was directly injected onto a custom packed trap
column (2 cm × 100 μm C18). Peptides were then eluted and
sprayed from a custom-packed emitter (75 μM × 25 cm C18)
with a linear gradient from 100% solvent A (0.1% formic acid in
5% acetonitrile) to 35% solvent B (0.1% formic acid in aceton-
itrile) in 90 min at a flow rate of 300 nL/min on a Proxeon Easy
nanoLC system directly coupled to a LTQ Orbitrap Velos mass
spectrometer (Thermo Scientific). Data-dependent acquisi-
tions were set up according to an experiment where full MS
scans from 350 to 2000 m/z were acquired in the Orbitrap at a
resolution of 60000 followed by 10 MS/MS scans acquired in the
LTQ ion trap instrument.

**Data analysis (MS)**

The raw data files were processed with Proteome Discoverer
v. 1.3 (Thermo Scientific) into peak lists and then searched
against the *Drosophila* Index of the UniProt database using the
Mascot Search engine version 2.3 (Matrixsciences, Ltd.). Parent
mass tolerances were set to 10 ppm, and fragment mass toler-
ances were set to 0.5 Da. The variable modifications of acetyl
(protein N-term), Gly-Gly on lysine, pyroglutamic for N-term
arginine, carbamidomethylation of cysteine, and oxidation of
methionine were used. Mascot search results were loaded into
the Scaffold software (Proteome Software, Inc.) to compare
sample results.
**Imd signaling regulated by Tak1-mediated ubiquitin-editing**

**RNAi**

DsRNA was produced using T7 RiboMAX Express Large Scale RNA Production System (Promega). S2* cells were split to 0.5 × 10⁶ cells/ml and incubated for ~24 h at 27 °C. 2 μg/ml dsRNA was delivered by calcium phosphate transfection, and cells were allowed to recover for 18 h at 27 °C. 1 μM 20-hydroxyecdysone was then added to the cells for 24 h. Finally, cells were treated with peptidoglycan (10 μg/ml) before harvesting for experiments.

**Imd lysine mutants assay**

Stable cell line expressing FLAG-Imd WT, K137R, K153R, or K137R/K153R were generated and then transfected with dsRNA targeting Imd 3'-UTR to knock down the endogenous Imd. Cells were allowed to recover for 18 h at 27 °C before treatment of 20-hydroxyecdysone (1 μM). Finally, cells were stimulated with peptidoglycan (10 μg/ml) and harvested for the experiments.

**E2 RNAi**

Each sample was transfected with 10 μg of dsRNA per target gene, and the total dsRNA used in each sample was equalized to 30 μg by adding GFP dsRNA. Cells were allowed to recover for 18 h at 27 °C before treatment of 20-hydroxyecdysone (1 μM). Finally, cells were stimulated with peptidoglycan (10 μg/ml) and harvested for the experiments.

**Transgenic Drosophila**

PhiC31 integrase-mediated transgenic rescue construct included the Imd coding region and about 3 kb of the surrounding gene region. This fragment was PCR-amplified and cloned into pattB vector. Lysine residues 137 and 153 were substituted with arginine individually or in combination, generating four donor plasmids: pattB-WT, -K137R, -K153R, and -K137R/K153R. To simplify the screening process, each plasmid contained a silent mutation between lysine 137 and 153, creating a SacII restriction digestion site. Confirmed transgenic flies were crossed with a double balancer and the null allele obtained from the Bloomington Drosophila Stock Center (National Institutes of Health Grant P40OD018537) was used in this study. We also thank Pascal Meier and Meike Broemer for providing the recombinant His6-SUMO-Imd10191 construct.

**RNA analysis**

Total RNA was extracted with TRIzol (Invitrogen), treated with DNase I, and purified by phenol/chloroform. Transcripts from the gene of interest were monitored by qRT-PCR (Bio-Rad). The gene expression level in each sample was normalized to RP49. Data from 3 biological independent experiments were collected, normalized with control samples (set as 1), and plotted as the mean ± S.D.

**Tak1 inhibitor assay**

S2* cells were treated with 20-hydroxyecdysone (1 μM) for 24 h before treatment of 2 μM (5Z)-7-oxozaenol (Sigma) for 3 h followed by PGN stimulation and protein assay.

**Kinase assay**

Recombinant proteins His6-SUMO-Imd(31–273), His6-SUMO and His6-MKK6-K28A were purified with nickel magnetic beads (EMD Millipore). Stable cell lines expressing FLAG-tagged Tak1 WT or S176A (kinase dead) from the Cu²⁺-inducible metallothionein promoter were treated with CuSO₄ (500 μM) for 6 h before lysing with lysis buffer as described above. 100–400 ng of total protein lysate from each cell line was immunoprecipitated with anti-FLAG antibody for 2 h at 4 °C. Kinases were then washed twice with 500 μl of lysis buffer and twice with 500 μl of kinase buffer (200 mM HEPES, 200 mM β-glycerol phosphate, 100 mM MgCl₂, 500 mM NaCl, 10 mM DTT, 1 mM sodium orthovanadate, 1 mM okadaic acid). Finally, kinases were incubated in kinase buffer containing 200 μM cold ATP, 5 μCi of [γ-³²P]ATP, and substrate (100 ng each) at 30 °C for 30 min. Samples were separated by SDS-PAGE, fixed with 10% methanol, 10% acidic acid, dried, and autoradiographed. For inhibitor studies, in vitro kinase assay were performed as described above, with the addition of (5Z)-7-Oxozaenol (or DMSO vehicle) to the reaction immediate before the addition of recombinant Imd substrate.

**Author contributions**—L. C. and N. S. conceived the work and wrote the manuscript. L. C. performed the majority of the experiments. S. M. and F. R. performed the other experiments. A. N. contributed to the manuscript preparation. N. P. provided an important initial observation. J. L. and S. S. performed the mass spectrometry analysis. N. S. supervised all aspects of the project.

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Imd signaling regulated by Tak1-mediated ubiquitin-editing

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