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Original Research

Ubiquitin C-Terminal Hydrolase L5 Plays an Essential Role in the Fly Innate Immune Defense against Bacterial Infection

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Abstract

Background: *Drosophila* ubiquitin carboxy-terminal hydrolase L5 (Uch-L5) functions as a critical component of the 26S proteasome to mediate degradation of polyubiquitinated proteins. It was recently shown to modulate tissue/organ development by targeting the Smoothened protein in the hedgehog pathway. However, whether it plays a role in controlling organismal immune response remains largely unknown. **Methods**: Reverse transcription plus quantitative polymerase chain reaction (RT-qPCR), dual-luciferase, and Western blot assays were used to explore the potential function of Uch-L5 in the innate immune regulation in cultured *Drosophila* S2 cells. Further genetic manipulations and bacterial infections were conducted to confirm the findings *in vivo*. **Results**: Silencing of Uch-L5 antagonizes the immune deficiency (IMD) but not the Toll innate immune signaling both *in vitro* and *in vivo*. Moreover, Uch-L5 positively contributes to the *Drosophila* innate immune response via its N-terminal Uch domain, which is the catalytical triad executing its deubiquitinase activity. **Conclusions**: Our studies shed light on a novel function of the deubiquitinase Uch-L5 in governing the anti-microbial defense in *Drosophila*.

Keywords: Uch-L5; IMD signaling; innate immune response; *Drosophila melanogaster*

1. Introduction

Innate immunity reacts quickly and efficiently upon recognition of extracellular pathogenic invasion. This constitutes the first line of defense of most organisms [1]. Pattern recognition receptors (PRRs) have been suggested to be mainly responsible for recognizing pathogenic stimuli, in order to trigger downstream signaling transductions, thereby inducing expressions of various immune effectors [2,3]. Several pioneering studies have demonstrated the pivotal roles of adaptor proteins and correlative modulators in the signal transductions from transmembrane and intracellular receptors to secreted effectors [reviewed in [1–6]]. Identification and characterization of novel adaptors/modulators has always been one of the hotspots in the basic research of innate immunity.

Drosophila melanogaster (fruit fly) is an excellent animal model for uncovering novel modulators in the innate immune signaling pathways, due to its powerful genetic approaches and worldwide resources of *Drosophila* mutants and transgenes. The fly innate immune response is mainly governed by two signaling pathways, namely the Toll and the immune deficiency (IMD) pathways [7–9], which are similar to the mammalian Myd88 (myeloid differentiation factor 88)-dependent TLR (Toll-like receptor) and TNFR (tumor necrosis factor receptor) signaling pathways, respectively. Upon infection by most fungi or Gram-positive bacteria, the Toll receptor-involved signaling is activated in order to trigger the induction of downstream anti-microbial peptides (AMPs), for instance drosomycin (Drs) and metchnikowin (Mtk) [10]. On the other hand, the IMD signaling pathway is mostly activated by Gram-negative bacteria and some types of Gram-positive bacteria, resulting in secretion of another set of AMPs like attacins (Att), cecropin (Cec), and diptericin (Dpt) for the host immune defense [11].

The IMD signaling pathway is normally activated by the binding of bacteria-derived DAP (meso-diaminopimelic acid)-type PGN (peptidoglycan) to the PGN recognition proteins PGRP-LC (located on the cell membrane) and/or PGRP-LE (intracellular) [12]. This binding further leads to the recruitment of the adaptor protein, Imd, to the receptor, together with Fadd (Fas-associated death domain) and the caspase Dredd (death related ced3/nedd2-like caspase) [13]. Dredd participates in the cleavage of Imd and the activation of the downstream kinase complex Tak1 (TGF-β activated kinase 1)/Tab2 (Tak1-associated binding protein 2) [14–16]. Activated Tak1/Tab2 phosphorylates and activates the IKK (IκB kinase) complex, consisting of Ird5 (immune response deficient 5) and Key (kenny), which in turn phosphorylate the NF-κB (nuclear factor κB) family protein Rel (relish), leading to its cleavage [17–20]. The

cleaved N-terminal active domain of Rel (Rel-68) translocates to the nucleus and activates the transcription of targeted effector genes [20].

Uncontrolled IMD signaling is detrimental to flies [21]. The maintenance of IMD homeostasis requires a series of regulators, among which are the ubiquitin-related enzymes including E3 ligases and Dubs (deubiquitinases). For instance, E3 ligases including Dnr1 (defense repressor 1) [22], Faf (fat facets) [23], Posh (plenty of SH3s) [24], and SkpA (Skp1-related A) [25] negatively contribute to IMD signaling through mediating the ubiquitin assembly of targeted substrates. Additionally, a Dub Usp36 (ubiquitin-specific protease 36) associates with Imd to antagonize its K63-linked ubiquitination, thereby down-regulating the IMD signals [26]. Some other Dubs such as Cyld (cylindromatosis) [27], Trabid (Traf-binding domain) [28,29], and Otu (ovarian tumor) [30] have been found to limit excessive activation of the IMD pathway under different physiological conditions. Recently, we noted that the ubiquitin carboxy-terminal hydrolase L5 (Uch-L5, also known as Uch37) functions as a typical Dub (belonging to the Uch sub-family of Dubs) to positively regulate Hh (hedgehog) signaling by restricting ubiquitination-mediated degradation of Smo (smoothened) [31]. However, little is known about the immunological role of Uch-L5 in the fly defense against microbial challenges.

In the present study, we focused mainly on exploring the potential role of Uch-L5 in regulating *Drosophila* innate immunity. We show that silencing of Uch-L5 results in markedly decreased IMD signaling, whereas ectopic expression of Uch-L5 behaves oppositely. The N-terminal Uch domain is both required and sufficient for Uch-L5 positively impacting on IMD signaling, suggesting that Uch-L5 executes its immune function relying on the Dub enzymatical activity. We further provide evidence displaying that Uch-L5 is essential for the immune defense of adult flies upon bacterial infections. Collectively, our results uncover a novel biological role of Uch-L5 in mediating the IMD innate immune reaction in *Drosophila*.

2. Materials and Methods

2.1 Drosophila Husbandry

Flies (*Drosophila melanogaster*) were raised at 25 °C with a 12 h of light/dark cycle and 65% humidity. Standard *Drosophila* medium (6.65% cornmeal, 7.15% dextrose, 5% yeast, 0.66% agar, 2.2% nipagin, and 3.4 mL/L propionic acid) was used to feed all flies. The w^{1118} strain was used as the *wild-type* control and all other flies were back-crossed with w^{1118} for isogenization (at least 5 generations). The detailed information of the flies used in this study are as follows: (1) key^{c02831} (#11044) and $Uch\text{-}L5^{j2B8}$ (#12078) were purchased from Bloomington *Drosophila* Stock Center; (2) *Uch-L5 RNAi #1* (#103481) was obtained from Vienna *Drosophila* Resource Center; (3) *Uch-L5 RNAi #2* (#04757.N) was acquired from Tsinghua Fly Center; and (4) *c564-gal4* was described previously [32].

2.2 dsRNA Synthesis and RNAi in S2 Cells

All dsRNAs were synthesized according to methods described previously [33]. In brief, DNA templates were amplified using specific primers (**Supplementary Table 1**). PCR products were then combined with 1/10 volume of 5 M NaCl and 2.5-fold volume of EtOH, followed by centrifugation for 10 min at high speed. The pellet was washed with 75% EtOH and dissolved in RNase/DNase-free water. The T7 *in vitro* transcription kit (Promega, Cat#P1300, Madison, Wisconson, USA) was used for dsRNA synthesis and purification according to the manufacture's protocol. Purified dsRNAs were diluted in RNase/DNase-free water to a final concentration of 1 μg/μL. The quality of dsRNAs was confirmed by agarose gel electrophoresis (**Supplementary Fig. 1A**). For gene silencing in S2 cells, dsRNA at the amount of 3 μg was added directly into S2 cells for 48 h.

2.3 S2 Cell Transfection and Luciferase Reporter Assay

S2 cells were cultured in a 27 °C incubator using insect medium (Gibco) with 10% FBS (Hyclone). The MycoAlert™ kit (Lonza, Cat#LT07-318, Basel, Halbkanton, Switzerland) was used for mycoplasma detection and validation of the authenticity of S2 cells. Lipofectamine™ 2000 (Thermo Fisher, Cat#11668019, Waltham, Massachusetts, USA) was used for plasmid transfection into S2 cells according to previous protocols [34]. The Att-Luc [35] and Drs-Luc [33] reporter systems were utilized to detect the relative activities of the IMD and Toll pathways, respectively. For the IMD pathway, we transfected S2 cells with the plasmid that express Imd, in order to induce the Att-Luc activity. As a positive control, we used the dsRNA targeting *kenny*, which is one of the pivotal genes of the IMD pathway. For the Toll pathway, the Myd88 expressing plasmid and the *pelle* dsRNA (positive control) were used. The methods for detecting the Firefly and Renilla luciferase activities were as follows: S2 cells were adequately lysed with 100 μL of passive lysis buffer (Promega, Cat#PR-E1941, Madison, Wisconson, USA). After centrifugation, 30 μL of the supernatant from each sample was added into a 96-well plate containing detection reagents. Firefly and Renilla luciferase activities were detected and finally the ratio of Firefly to Renilla was calculated. Results were analyzed based on data from 3 independent biological replications.

2.4 Reverse transcription plus quantitative polymerase chain reaction (RT-qPCR) Assay

S2 cells or fly samples were homogenized in Trizol Reagent (Invitrogen, Cat#15596026, Waltham, Massachusetts, USA). Chloroform solution (1/5 volume) was added into the sample, followed by intense votexing for 15 sec, and centrifugation at high speed for 15 min. The upper layer of the supernatant was transferred into a fresh tube and

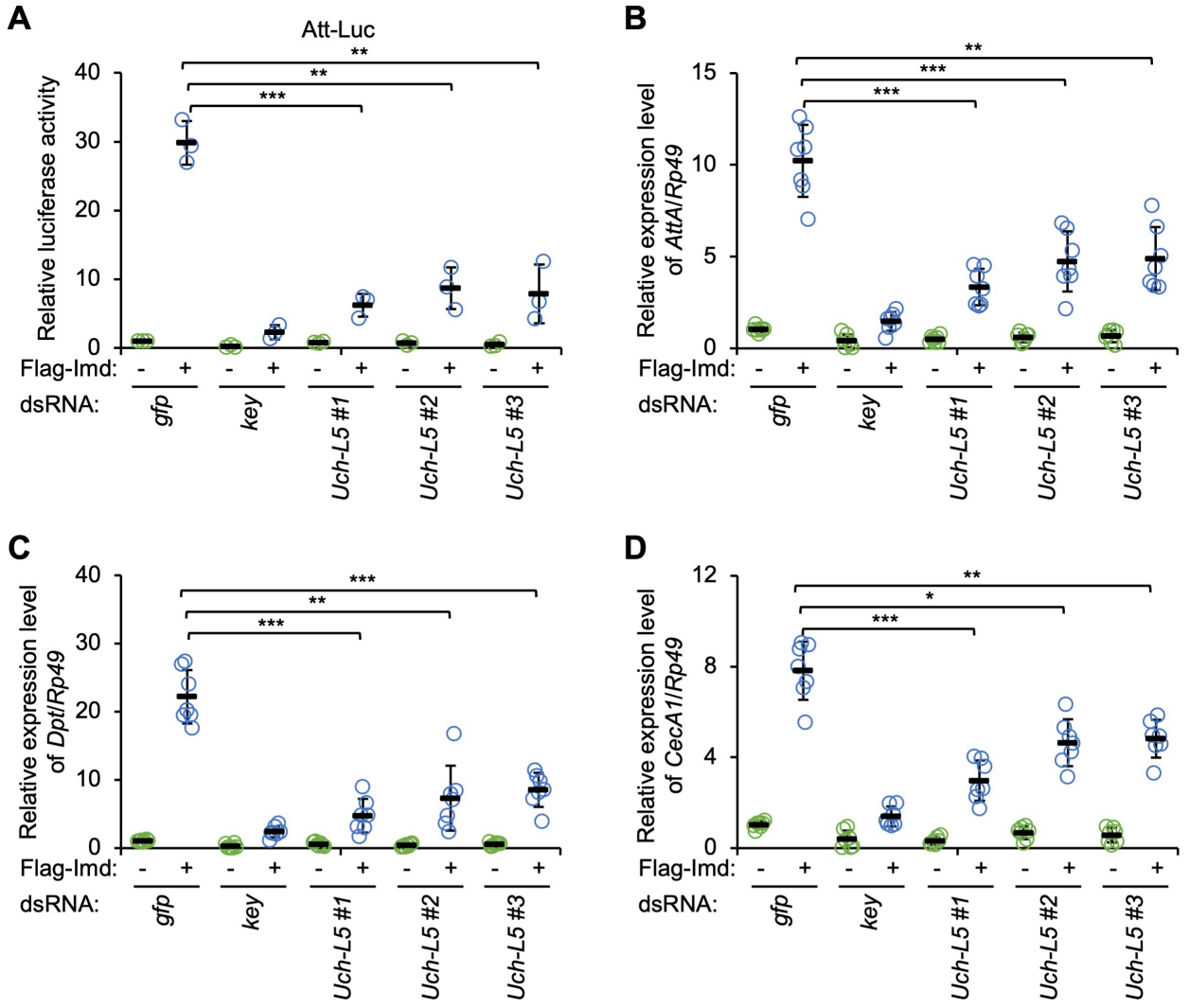

Fig. 1. Silencing of ubiquitin carboxy-terminal hydrolase L5 (Uch-L5) reduces immune deficiency (IMD) signaling in *Drosophila* S2 cells. (A) S2 cells were pretreated with indicated dsRNAs. 48 h later, cells were transfected with various combinations of expressing plasmids, followed by dual-luciferase assays. (B–D) S2 cells were pretreated with dsRNAs as in (A) for 48 h. Cells were then transfected with indicated expressing plasmids for 36 h and harvested for RT-qPCR assays to detect the mRNA levels of *AttA* (B), *Dpt* (C), and *CecA1* (D). In (A–D), each dot represents one independent replicate and data are shown as means plus standard errors. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

an equal volume of isopropanol was added. After centrifugation again at high speed for 10 min, the pellet was washed with 75% EtOH and diluted in RNase/DNase-free water. Samples were further incubated with DNase for 30 min, and the quality of RNA was assessed by examining the 260:280 ratio, and the pattern in the agarose gel electrophoresis assay. The first-strand cDNA synthesis kit (Transgen, Cat#AT341-01, Beijing, China) was used to reverse-transcribe RNA (1 μg) into cDNA. Quantitative PCR assays (in 3 technical repetitions) were performed using a SYBR Green Master Mix (Thermo Fisher, Cat#A46012, Waltham, Massachusetts, USA) in the Lightcycler 480 PCR platform (Roche, Cat#05015278001, Basel, Halbkanton, Switzerland). *Rp49* was used as the internal control. Results were analyzed based on data from 7 independent biological replications. The primers used in RT-qPCR experiments are shown in **Supplementary Table 1**.

2.5 Infection, Fly Survival, and Bacterial Burden Assay

Infection experiments were performed as previously described [36]. In brief, overnight bacterial cultures were harvested and diluted in sterile PBS at a concentration of $OD_{600} = 1$. The *Serratia marcescens* strain (#1.1215) was obtained from China General Microbiological Culture Collection Center (CGMCC) and the *Ecc15* was a kind gift from Dr. Dominique Ferrandon's laboratory (Institut de Biologie Moleculaire et Cellulaire, France). For injection, male flies were collected and anesthetizsed on the fly pad

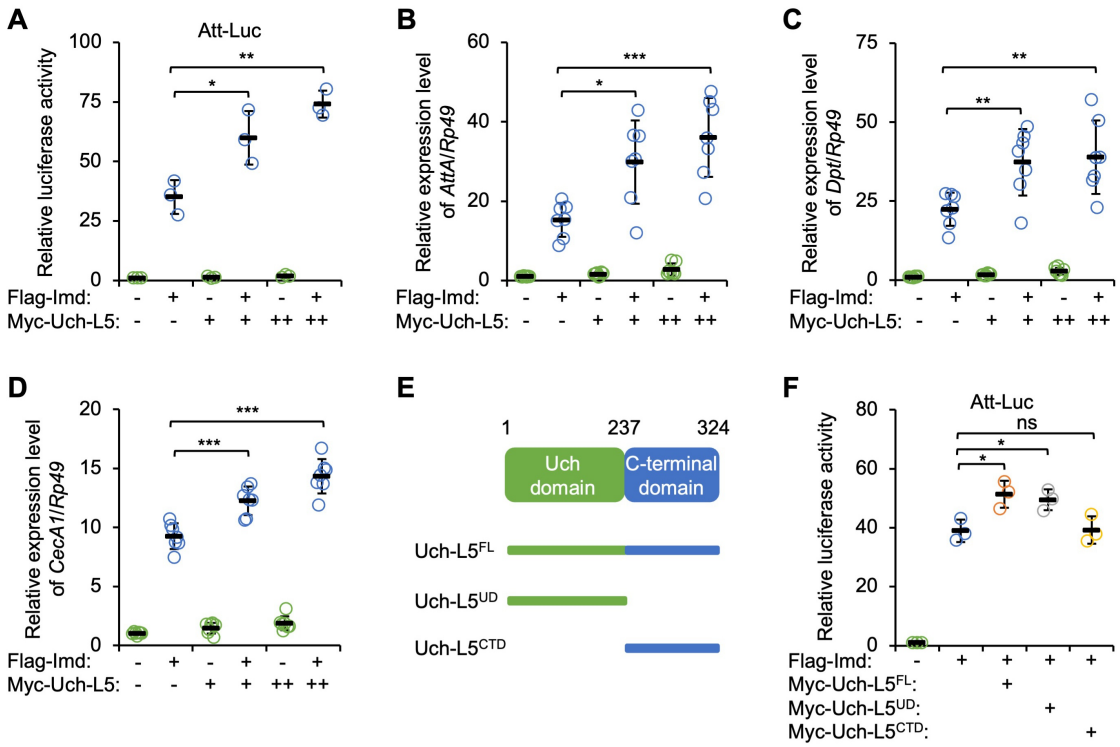

Fig. 2. Uch-L5 affects IMD signaling in a Uch domain-dependent manner. (A–D) S2 cells were transfected with expressing plasmids as indicated, followed by dual-luciferase assays (A) or RT-qPCR assays to examine the expression levels of *AttA* (B), *Dpt* (C), and *CecA1* (D). (E) Domain analysis of Uch-L5. (F) S2 cells were transfected with indicated combinations of expressing plasmids, followed by dual-luciferase assays. In (A–D,F), each dot represents one independent replicate and data are shown as means plus standard errors. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns $p > 0.05$.

with CO_2. Diluted bacteria (4.6 nL), or the same volume of PBS, was injected into each fly with a nanoliter injector (Nanoject III, Drummond, Cat#3-000-207, Broomall, Pennsylvania, USA). Flies that died within 2 h after bacterial infection were not included in further studies. For survival analysis, flies were transferred to fresh vials and counted for death every day. For bacterial burden experiments, 10 flies were collected, dipped in 75% EtOH, and volatilized with EtOH on the fly pad for several minutes. The flies were then homogenized in 200 µL sterile PBS, followed by serial dilutions. Finally, 100 µL of each dilution was inoculated on an LB agar plate at 30 °C for 12 h before we counted the numbers of the colonies.

2.6 Statistical Analysis

All statistical analyses were performed by using GraphPad Prism 9 (v. 9.4.0, Dotmatics, Boston, MA, USA). Data were shown as means plus standard errors. Statistical significance was determined by using the ANOVA or Mann-Whitney tests except for survival assays, in which the Log-Rank test (Kaplan-Meier method) was used. The p value of less than 0.05 was considered statistically significant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns (not significant) $p > 0.05$.

3. Results

3.1 Uch-L5 Positively Modulates IMD Signaling in Drosophila S2 Cells

We examined the potential role of Uch-L5, a previously described Dub modulating Hh signaling [31], in affecting the innate immune reaction in *Drosophila*. To do this, we first designed 3 types of dsRNAs that targeted different regions of the coding sequence of *Uch-L5* (referred to as *Uch-L5 dsRNAs #1*, *#2*, and *#3*, respectively, **Supplementary Fig. 1A**). Cultured *Drosophila* S2 cells were treated with these *Uch-L5 dsRNAs* (dsRNA that targeted *GFP* was used as the control), and the knockdown efficiency was monitored by RT-qPCR assays (**Supplementary Fig. 1B**). As illustrated in Fig. 1A, silencing of Uch-L5 resulted in drastic decreases (by ~75% to ~80%) of the Att-Luc activities upon Imd over-expression, indicating that Uch-L5 is a potentially positive regulator in the IMD signaling pathway in cultured S2 cells. Further, we performed RT-qPCR assays to detect the endogenous inductions of the AMPs that are downstream of the IMD pathway, including *AttA*, *Dpt*, and *CecA1*. We obtained consistent results: down-regulation of Uch-L5 significantly prevented the Imd-driven transcription of several AMP genes (Fig. 1B–D).

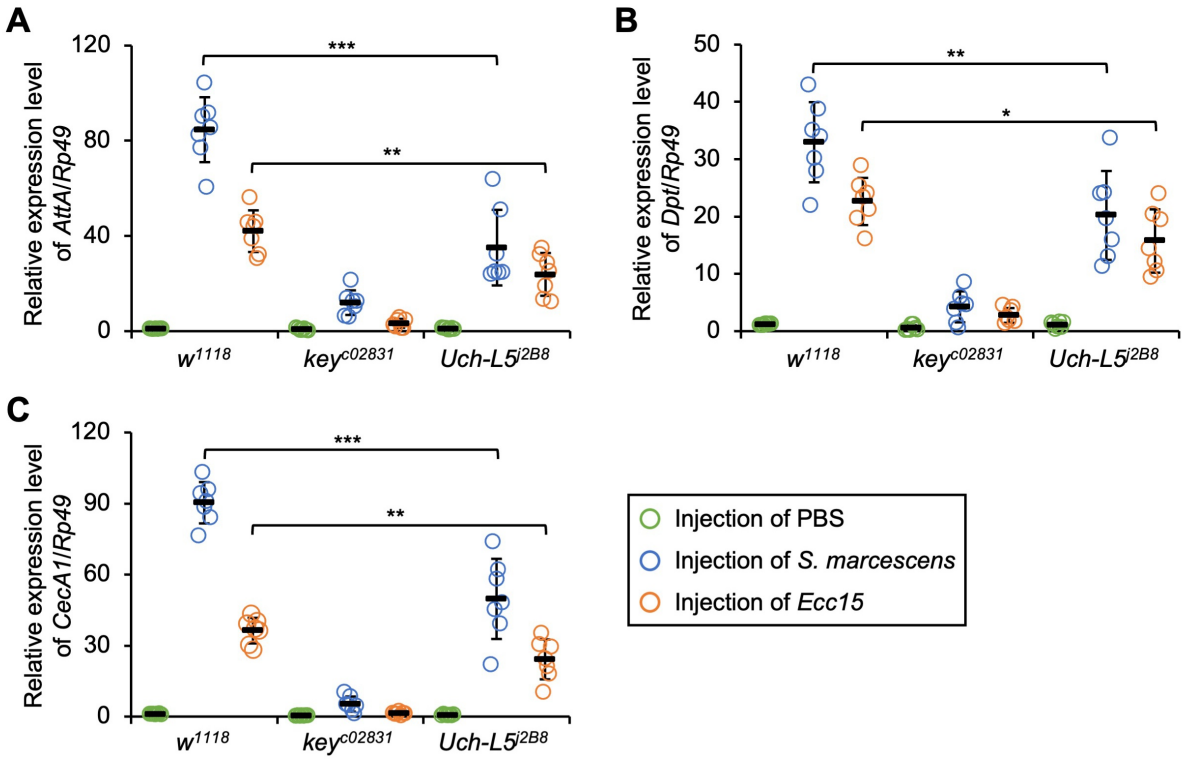

Fig. 3. Loss of *Uch-L5* antagonizes IMD downstream AMP expressions. (A–C) w^{1118} (*wild-type* control), key^{c02831}, and $Uch\text{-}L5^{i2B8}$ males (6- to 7-day) were injected with PBS, *S. marcescens*, or *Ecc15*. 6 h later, flies were harvested for RT-qPCR assays to detect the expression levels of *AttA* (A), *Dpt* (B), and *CecA1* (C). Each dot represents one independent replicate and data are shown as means plus standard errors. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.2 Uch-L5 is Dispensable for Affecting Toll Signaling

As mentioned in the Introduction, the *Drosophila* innate immune response is mostly governed by two signaling cascades, the Toll and the IMD pathways. We then conducted another luciferase reporter assay (Drs-Luc) in S2 cells, as previously reported, aiming to test whether Uch-L5 is also involved in affecting Toll signaling. However, we did not observe apparent alterations in the Myd88-driven Drs-Luc activities when Uch-L5 was knocked down, compared to that in the control (**Supplementary Fig. 2A**). Similar results were obtained when we looked at the transcript levels of *Drs* and *Mtk*, two well-known downstream AMP genes in the Toll pathway (**Supplementary Fig. 2B,C**). These data indicated that *Drosophila* Dub Uch-L5 is specifically involved in controlling the IMD other than the Toll innate immune signaling pathway in S2 cell cultures.

3.3 Uch-L5 Relies on the N-Terminal Uch Domain to Regulate IMD Signaling

We collected more evidence on the functional role of Uch-L5 in controlling IMD innate immunity by constructing a plasmid expressing Myc-tagged Uch-L5 in S2 cells. By again using the Att-Luc reporter system, we observed that ectopic expression of Myc-Uch-L5 enhanced IMD signaling in a dose-dependent manner (Fig. 2A). Accordingly, co-transfection of Uch-L5 expressing plasmid resulted in increased inductions (by ~32% to ~136%) of the IMD-downstream AMPs upon Imd over-expression (Fig. 2B–D), which indicated a positive role of Uch-L5 in modulating IMD signaling.

A recent study that explored the biochemical characteristic of Uch-L5 demonstrated that Uch-L5 harbors a typical Uch domain at its N-terminus, which is the catalytical region for its Dub enzymatical activity [31]. We therefore constructed two kinds of plasmids that expressed truncated forms of Uch-L5, including $Uch\text{-}L5^{UD}$ (Uch domain) and $Uch\text{-}L5^{CTD}$ (C-terminal domain) (Fig. 2E), and transfected them into S2 cells for Att-Luc reporter assay. As illustrated in Fig. 2F, over-expression of $Uch\text{-}L5^{UD}$, but not $Uch\text{-}L5^{CTD}$, mimicked the positive contribution of $Uch\text{-}L5^{FL}$ (full-length Uch-L5) to IMD signaling. Taken together, these data suggested that the N-terminal Uch domain is both required and sufficient for Uch-L5 to benifit *Drosophila* IMD signaling.

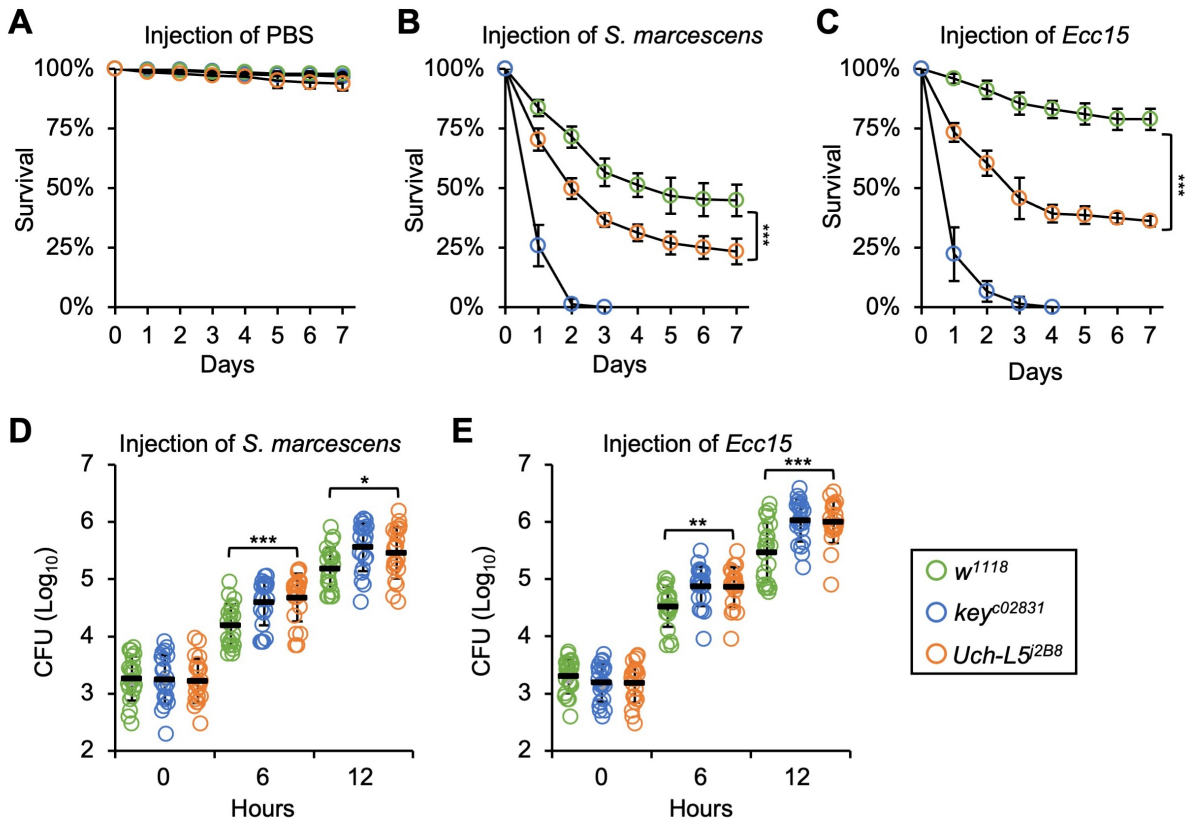

Fig. 4. *Uch-L5* plays a critical role in the fly anti-microbial defense. (A–C) w^{1118} (*wild-type* control), key^{c02831}, and $Uch\text{-}L5^{j2B8}$ males (6- to 7-day) were infected with PBS (A), *S. marcescens* (B), or *Ecc15* (C), followed by survival analyses. The numbers of flies in each figure are as follows. In (A), w^{1118}: 104, 103, 106; key^{c02831}: 105, 105, 102; $Uch\text{-}L5^{j2B8}$: 102, 103, 104. In (B), w^{1118}: 102, 104, 106; key^{c02831}: 104, 103, 105; $Uch\text{-}L5^{j2B8}$: 105, 103, 103. In (C), w^{1118}: 104, 107, 106; key^{c02831}: 106, 104, 103; $Uch\text{-}L5^{j2B8}$: 104, 103, 106. (D,E) w^{1118} (*wild-type* control), key^{c02831}, and $Uch\text{-}L5^{j2B8}$ males were infected with *S. marcescens* (D) or *Ecc15* (E), followed by bacterial burden assays at indicated time points (0, 6, and 12 h). Each dot represents one independent replicate and data are shown as means plus standard errors. For each replicate, 10 male flies were collected. In (B–E), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.4 Uch-L5 Mediates the IMD Innate Immune Defense in Adult Flies

We next sought to decipher the immune function of Uch-L5 *in vivo*. To this end, we collected w^{1118} flies (referred to as the *wild-type* control), key^{c02831} flies (*key* loss-of-function mutant as the positive control), and $Uch\text{-}L5^{j2B8}$ flies (*Uch-L5* loss-of-function mutant, isogenized with w^{1118}) for infection experiments using the Gram-negative bacteria *S. marcescens* (*Serratia marcescens*), to activate the host IMD innate immune defense. Six hours after infection, we detected decreases (by ~34% to ~56%) of *AttA*, *Dpt*, and *CecA1* in the *Uch-L5*-defective flies relative to those in the *wild-type* control (Fig. 3A–C). To confirm our results, we subjected these flies to infection with another type of Gram-negative bacteria, *Ecc15* (*Erwinia carotovora carotovora 15*). Similar reduction trends were observed when we compared the expression levels of AMPs in the *Uch-L5* and *key* loss-of-function mutants to those in the *wild-type* flies (Fig. 3A–C). In addition, we noted comparable survival rates of these flies (w^{1118}, key^{c02831}, and $Uch\text{-}L5^{j2B8}$) after injection of sterile PBS buffer (Fig. 4A). However, the *Uch-L5* loss-of-function mutant flies succumbed much faster than did the *wild-type* controls upon injection of either *S. marcescens* (Fig. 4B) or *Ecc15* (Fig. 4C), indicating that *Uch-L5* plays an essential role in the host defense against bacterial infections.

To test the involvement of *Uch-L5* in affecting the proliferation of injected pathogens (*S. marcescens* or *Ecc15*), we performed CFU (colony-forming-units) assays at different time points (0, 6, and 12 h) after injection. As shown in Fig. 4D,E, the burdens of *S. marcescens* and *Ecc15* in $Uch\text{-}L5^{j2B8}$ adults were significantly higher than those in the *wild-type* flies. In summary, our *in vivo* data strongly support the notion that *Uch-L5* plays a critical role in the host defense against bacterial pathogens in *Drosophila*.

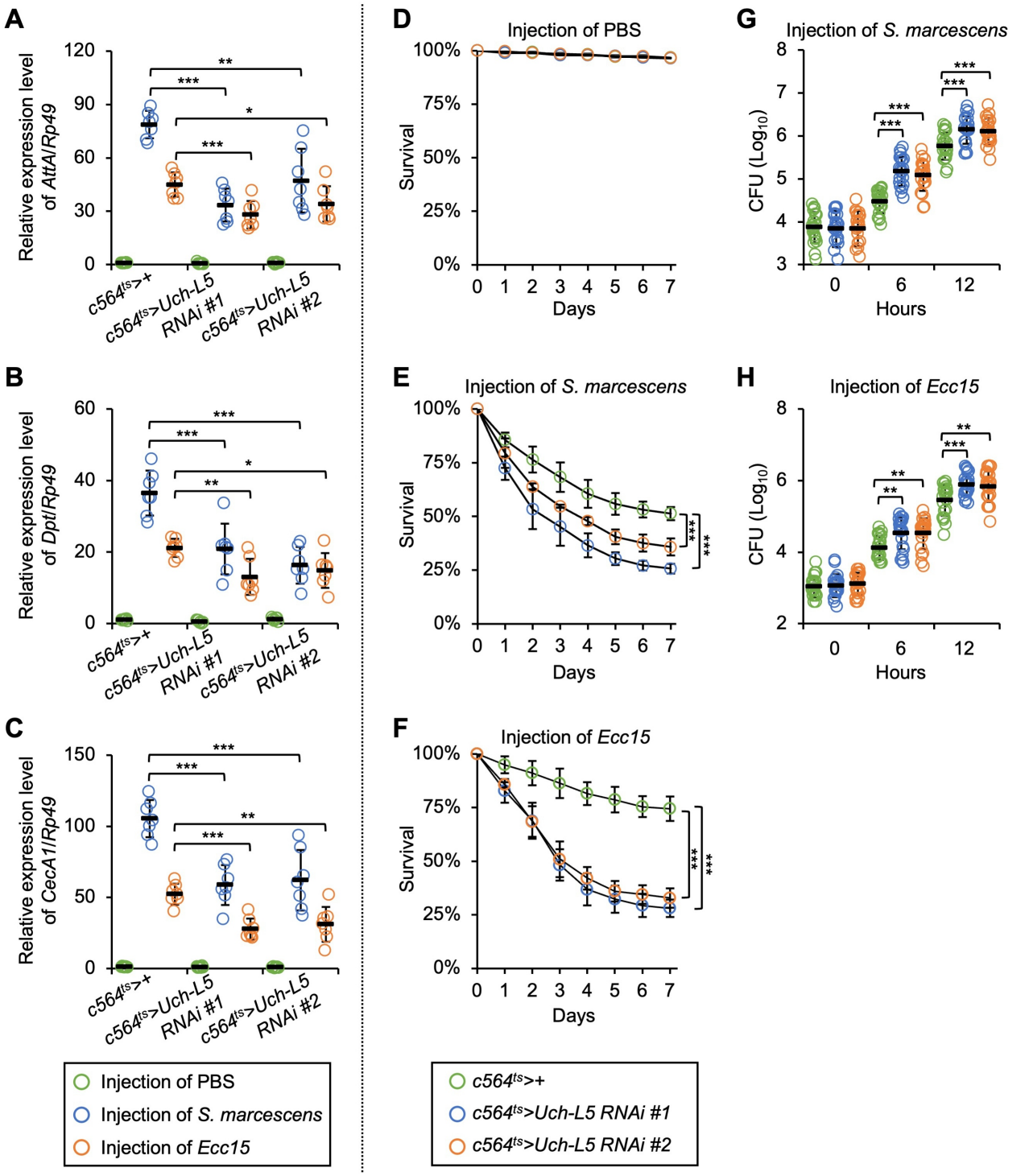

Fig. 5. Silencing of *Uch-L5* in fat body results in immune defects. (A–H) $c564^{ts}$>+ (*wild-type* control), $c564^{ts}$>*Uch-L5 RNAi #1*, and $c564^{ts}$>*Uch-L5 RNAi #2* males (6- to 7-day) were injected with PBS, *S. marcescens*, or *Ecc15*. Flies were collected for RT-qPCR assays to examine the inductions of *AttA* (A), *Dpt* (B), and *CecA1* (C) 6h after infection, or subjected to survival analyses (D–F), or bacterial burden assays (G,H). In (A–C, G,H), each dot represents one independent replicate and data are shown as means plus standard errors. In (D), the numbers of flies are as follows. $c564^{ts}$>+: 104, 105, 104; $c564^{ts}$>*Uch-L5 RNAi #1*: 103, 102, 102; $c564^{ts}$>*Uch-L5 RNAi #2*: 103, 106, 105. In (E), $c564^{ts}$>+: 102, 103, 106; $c564^{ts}$>*Uch-L5 RNAi #1*: 104, 105, 105; $c564^{ts}$>*Uch-L5 RNAi #2*: 103, 104, 102. In (F), $c564^{ts}$>+: 105, 104, 102; $c564^{ts}$>*Uch-L5 RNAi #1*: 106, 103, 104; $c564^{ts}$>*Uch-L5 RNAi #2*: 105, 105, 104. In (A–H), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.5 Silencing of Uch-L5 in Fat Body Leads to Immune Defects

Fat body is one of the main responsible immune tissues/organs during systemic infection in *Drosophila*. As the *Uch-L5* transcript is relatively abundant in the fat body cells, according to the high-throughput sequencing or array data in the FlyBase website (https://flybase.org/reports/FBgn0011327), we sought to investigate the functional role of *Uch-L5* in *Drosophila* fat body. Two different *Uch-L5 RNAi* (RNA interference) flies (*Uch-L5 RNAi #1* and *#2*, for detailed information, see Materials and Methods) were crossed with the fat body-specific driver *c564-gal4*. The *Tub-gal80^{ts}* strain was used in order to allow gene RNAi at adult stage (**Supplementary Fig. 3A**). The progenies, including *c564^{ts}>Uch-L5 RNAi #1* and *c564^{ts}>Uch-L5 RNAi #2*, were collected and subjected to *S. marcescens* or *Ecc15* infection (*c564^{ts}>+* was used as the *wild-type* control). Six hours after *S. marcescens* or *Ecc15* infection, the inductions of *AttA*, *Dpt*, and *CecA1* in *Uch-L5 RNAi* flies were prominently decreased compared to those in the control group (Fig. 5A–C). These results suggested that *Uch-L5* functions in fat body to modulate IMD signaling in response to bacterial challenge. Consistently, we observed that the *Uch-L5 RNAi* flies were less resistant to *S. marcescens* or *Ecc15* infection: they displayed higher mortality than did the *wild-type* control (Fig. 5D–F). Moreover, the bacterial burdens at various time points after infection were remarkably elevated by the loss of *Uch-L5* in fat body (Fig. 5G,H).

4. Discussion

Drosophila Uch-L5 belongs to the Uch sub-family of Dubs [31,37]. Dub-mediated cleavage of ubiquitin/poly-ubiquitin from ubiquitinated substrates has been widely studied and demonstrated to be involved in a broad range of cellular processes [reviewed in [38–40]]. However, our knowledge regarding the biological function of the fly Uch-L5 is incomplete. In the present study, we carried out a series of investigations using both *in vitro* and *in vivo* models. We showed that Uch-L5 behaves as a positive modulator in the fly IMD innate immune defense against bacterial stimuli. Our data shed light on a previously undescribed role of Uch-L5 in controlling organismal innate immunity.

How does Uch-L5 execute its essential role in regulating the IMD signaling pathway? A recent study by Zhou and colleagues illustrated that Uch-L5 depends on its N-terminal Uch domain to positively contribute to Hh signaling [31], highlighting the critical requirement of the Dub catalytical triad for Uch-L5 functioning. Indeed, when we utilized several truncated forms of Uch-L5 expressing plasmids and conducted Att-Luc reporter assays in cultured S2 cells, we observed that Uch-L5 without the Uch domain no longer promoted IMD signaling. It is of interest to note that the Dub enzymatical activity of *Drosophila* Uch-L5 is somehow autoinhibited by the CTD via oligomerization, which can be alleviated by the association of a co-factor such as Rpn13 [31,37]. However, we would like to conclude that this is not the case for the regulatory role of Uch-L5 in innate immune regulation, based on the following reasons: (1) ectopic expression of Uch-L5 without CTD showed a similar contribution to the IMD signaling as that of the Uch-L5^{FL} in S2 cells; and (2) co-expression of Rpn13 hardly affected the beneficial role of Uch-L5 in IMD signaling (**Supplementary Fig. 4A**). It seems that Uch-L5 relies on the same Uch domain to participate in different signaling cascades, but its CTD largely functions in distinct manners. Nevertheless, it would be worthwhile to generate corresponding *Uch-L5* transgenic flies to confirm these hypotheses *in vivo*.

Recently, numerous efforts have been made to decipher the critical role of Dubs in regulating IMD signaling. To date, only Imd, Tak1, and Dredd in the canonical IMD pathway, have been clearly demonstrated to be modified by ubiquitination/deubiquitination. Whether the other key factors also involve a ubiquitin-mediated modulation remains a mystery. Uch-L5 might therefore target a factor, or some of these factors, to enhance IMD signaling. The 48th lysine (K48)-linked ubiquitination has been suggested to primarily mediate protein recognition and degradation by the 26S proteasome, whereas the 63rd lysine (K63)-linked ubiquitination commonly governs signal transduction [41]. According to our current knowledge and the results of the present study, we speculate that the Dub Uch-L5 may inhibit the K48-linked ubiquitination of its substrate(s), thus improving its (their) stability. Future biochemical approaches, for instance co-immunoprecipitation and ubiquitination assays, will greatly help us further understand the molecular mechanism by which Uch-L5 benefits the IMD innate immune defense in *Drosophila*.

On the other hand, it is somehow lagged off that no Dubs have been shown to play a role in the Toll innate immune defense of *Drosophila*. This may be due to the fact that ubiquitination modification in Toll signaling is not as important as that in IMD signaling. Even though we failed to observe in the present study any involvement of Uch-L5 in controlling the Toll pathway, we cannot exclude the possibility that the Toll pathway is regulated by other Dubs. One strategy would be to explore Dubs that can physically associate with MyD88, a pivotal Toll downstream adaptor protein that undergoes ubiquitination [33].

5. Conclusions

We have demonstrated a critical regulatory role of the Dub Uch-L5 in *Drosophila* IMD innate immunity. The present study has provided insight into the understanding of the precise dynamic modulation of IMD signaling in response to bacterial infection.

Availability of Data and Materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Author Contributions

SJ and YH designed the research study. CZ, SZ, FK, YX, KS, YJ, JL, AQ, and XZ performed the research. CZ, SZ, FK, and YH analyzed the data. SJ and YH wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2811294.

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