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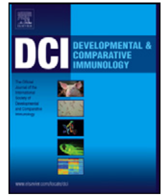

Dnr1-dependent regulation of the *Drosophila* immune deficiency signaling pathway

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ABSTRACT

Innate immunity is a critical metazoan defense strategy that rapidly detects and neutralizes invading microbes. As the signaling pathways that drive innate immune responses are evolutionarily conserved, there is considerable interest in the characterization of innate immune signaling in genetically tractable models, such as *Drosophila melanogaster*. *Drosophila* responds to detection of diamonopimelic-type microbial peptidoglycan through activation of the immune deficiency (Imd) pathway, a signaling pathway with numerous similarities to the mammalian pro-inflammatory TNF pathway. In this manuscript, we focus on a molecular and *in vivo* characterization of Dnr1, a putative regulator of Imd pathway activity. A previous cell culture RNAi screen indicated that Dnr1 may serve as a negative regulator of the Imd pathway. However, there are no *in vivo* data to validate this hypothesis and there are scant molecular data to identify the mechanism by which Dnr1 may inhibit the Imd pathway. In this manuscript, we present *in vivo* data that are consistent with a negative regulatory role for Dnr1 in the Imd pathway. Additionally, we provide molecular data to indicate that Dnr1 inhibits the Imd pathway at the level of the initiator caspase Dredd.

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1. Introduction

The innate immune response is a powerful, phylogenetically widespread first line of defense against microbial challenges [1]. In contrast to the elaborate adaptive immune responses of higher vertebrates, innate immune responses are extremely fast and rely exclusively on non-rearranging, germline-encoded gene products. Due to the high degree of evolutionary conservation of key regulators of innate immune signaling, genetically accessible organisms such as the fruitfly *Drosophila melanogaster* are potent tools for deciphering innate immune signaling pathways [2,3]. For example, identification of the Toll signaling pathway as a mediator of antimicrobial defenses in *Drosophila* led to the characterization of the critical role of Toll signaling in the activation of innate and adaptive defenses in higher vertebrates [4–7].

More recently, considerable efforts have been directed to the study of the *Drosophila* immune deficiency (Imd) signaling pathway, an innate immune response pathway that displays numerous parallels to the mammalian tumor necrosis factor (TNF) pathway (for a review of the Imd pathway see [3]). For example, both Imd and TNF pathways signal through conserved signal transduction molecules to engage caspase, c-Jun N-terminal kinase (JNK) and NF-κB modules that coordinate a rapid response to ongoing infections. In contrast to the TNF pathway, the transmembrane peptidoglycan recognition protein (PGRP-LC) detects diaminopimelic (DAP) acid-type peptidoglycan from Gram-negative bacteria, some Gram-positive bacteria and fungi and initiates the Imd pathway signaling cascade in response [8–12]. Imd pathway signaling proceeds through a series of signal transduction molecules and activates the JNK-responsive dAP-1 transcription factor and the NF-κB family member Relish (Rel). Cell culture studies indicate that the combined activities of dAP-1 and Rel mediate the bulk of the Imd pathway transcriptional response [13]. The dJNK/dAP-1 axis of the Imd pathway is activated in a rapid and transitory manner. Typically, JNK activation occurs within minutes and terminates within an hour, while AP-1 transcriptional activity terminates within 4 h [13,14]. In contrast, Rel activation occurs in a more prolonged manner. Rel is a p105 homolog with an N-terminal NF-κB domain and a C-terminal ankyrin domain that is presumed to tether Rel in the cytoplasm in the absence of PGRP-LC activation [15–17]. Infection initiates a signaling cascade that culminates in the proteolytic cleavage of Rel between the NF-κB and ankyrin domains [18,19]. The liberated NF-κB domain translocates to the

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Abbreviations: Imd, immune deficiency; PGRP-LC, peptidoglycan recognition protein; TNF, tumor necrosis factor; JNK, c-Jun N-terminal kinase; PGN, peptidoglycan; Rel, relish; Dnr1, defense repressor 1; *att*, attacin; dipt, diptericin; IAP, inhibitor of apoptosis; LPS, lipopolysaccharides.

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nucleus and drives a significant portion of the transcriptional component of the Imd pathway response. The transcriptional induction of several antimicrobial peptide genes such as *diptericin* (*dipt*) and *attacin* (*att*) are widely accepted signatures of Rel activation through the Imd pathway.

While the Rel protease requires definitive biochemical identification, several lines of evidence indicate that Rel cleavage depends on the activity of Dredd, a *Drosophila* initiator caspase with modest sequence similarities to mammalian caspase-8 [20]. *dredd* mutants display a significantly reduced ability to activate Imd signaling upon infection with Gram-negative bacteria and *dredd* mutants also display greatly enhanced lethality upon infection [21]. As Dredd forms a complex with Rel in *Drosophila* cell culture assays and Rel cleavage occurs at a caspase consensus cleavage site, it is widely assumed that Dredd is the protease responsible for Rel cleavage [19]. More recent data indicate that Dredd is also required to activate the JNK component of the Imd pathway, although the mechanism of Dredd-mediated JNK activation remains unclear [22].

Numerous genetic and molecular studies led to the identification of essential components of the Imd signal transduction pathway. In contrast, there are only a limited number of reports on negative regulators of Imd pathway signaling [23–28]. A recent report identified defense repressor 1 (Dnr1) as a putative negative regulator of Dredd activity in the Imd pathway [29]. Dnr1 is a conserved protein with an N-terminal FERM domain and a C-terminal RING domain. RING domains are established E3 ubiquitin ligases that target substrates for proteasomal degradation. The RING domain of Dnr1 shares striking similarities with the C-terminal RING domains frequently found on a family of caspase inhibitors known as inhibitor of apoptosis (IAP) proteins [30]. Depletion of Dnr1 from *Drosophila* S2 cells by RNAi led to the transcriptional activation of a Dredd/Rel responsive reporter construct (*dipt-lacZ*). Peptidoglycan-mediated activation of Dredd/Rel signaling also resulted in the transient stabilization of Dnr1 in S2 cell culture assays. These data led to the proposal that Dnr1 is a negative regulator of the Imd pathway and that Rel activation results in the stabilization of its own inhibitor (Dnr1), thereby contributing to termination of Imd pathway signaling. Consistent with a role for Dnr1 in the regulation of caspase activity, we recently demonstrated that overexpression of Dnr1 in S2 cells blocked pharmacological or genetic induction of apoptosis, while depletion of Dnr1 from S2 cells sensitized the cells to pro-apoptotic signals [31].

In this study, we present a comprehensive cell culture and *in vivo* characterization of interactions between Dnr1 and the Imd pathway. We demonstrate that Dnr1 forms a complex with Dredd in S2 cells, and that overexpression of Dnr1 significantly decreases Dredd proteins levels. Furthermore, we show that Dnr1-mediated depletion of Dredd requires a catalytically active RING domain. In addition we demonstrate that overexpression of Dnr1 in cells or in whole animals greatly decreases Imd pathway signaling and sensitizes adult *Drosophila* to infections with Gram-negative bacteria. In addition, we show that loss of Dnr1 from adult *Drosophila* results in a transitory up-regulation of Imd pathway-responsive transcripts. These data are consistent with a role for Dnr1 as a negative regulator of Dredd activity and suggest that a significant element of Dnr1-dependent regulation of the Imd pathway proceeds through the destruction of Dredd in a RING domain-dependent manner.

2. Materials and methods

2.1. S2 cell culture

S2 cells were maintained at 25 °C in HyQ TNM-FH medium (HyClone) supplemented with 10% heat-inactivated fetal bovine serum, 50 U penicillin/ml and 50 μg streptomycin/ml (all Gibco). For transient transfections, plasmid DNA (1 μg/10^6 cells) was incubated with DDAB (40 μl/10^6 cells) and serum-free cell culture medium (20 μl/10^6 cells) for 20 min at room temperature. The transfection mix was then added dropwise to S2 cells (10^6 cells per ml) and incubated overnight at 25 °C. Stable S2 cell lines were generated by transfecting cells with the respective plasmids and the pCoHygro hygromycin B resistance selection plasmid (Invitrogen) at a ratio of 19:1. Transfections were performed with 3×10^6 S2 cells in 3 ml cell culture medium and cells were passaged with cell culture medium containing hygromycin B (300 μg/ml, Sigma) for three weeks to select for stable transfected cell lines. For immunoprecipitation assays, S2 cells that stably express HADreddC408A were transiently transfected with mycDnr1 or mycDnr1C563Y expression plasmids and incubated for 24 h at 25 °C. Cells were then collected by centrifugation at $1000 \times g$ for 3 min and lysed in 1 ml lysis buffer (50 mM HEPES (pH 7.5), 10 mM EDTA (pH 8.0), 50 mM KCl, 50 mM NaCl, 1 mM $MgCl_2$, 0.1% NP40, protease inhibitors (Roche inhibitor cocktail tablets), phosphatase inhibitors (Sigma, phosphatase inhibitor cocktail)) for 10 min at 4 °C. Cellular debris was cleared by centrifugation at $21,000 \times g$ for 10 min at 4 °C and the supernatant was incubated with rabbit anti-myc antibodies (Sigma) overnight at 4 °C. The samples were incubated with Protein G Sepharose (Amersham Biosciences) for 1 h at 4 °C. Beads were pelleted at $300 \times g$ for 30 s and washed four times in lysis buffer. Beads were then resuspended in sample buffer, vortexed and boiled prior to Western blot analysis. For LPS treatments, S2 cells were incubated with 50 μg/ml LPS (*E. coli* 055:B5, Sigma) for the indicated periods. For MG132 experiments, S2 cells were transfected for 16 h prior to addition of MG132 and then incubated with the indicated concentration of MG132 for an additional 4 h.

2.2. Generation of expression constructs

The HADnr1 and HADnr1C563Y expression constructs have been described previously [29]. UAS-HADnr1 was generated by cloning full-length Dnr1 cDNA into pUAST as a Kpn I/Xba I fragment. All other expression constructs were generated using the gateway recombination system (Invitrogen). myc Dnr1 and mycDnr1C563Y were made by cloning the respective Dnr1 coding regions into pENTR/D-TOPO (Invitrogen) and recombining each

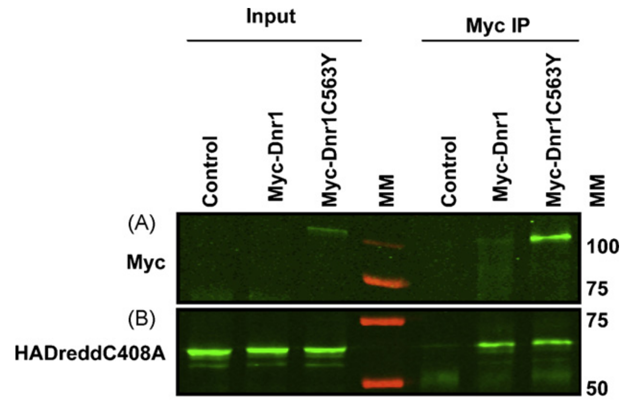

Fig. 1. Dnr1 forms a complex with Dredd. (A and B) Western blot analysis of lysates from S2 cells that constitutively express HADreddC408A probed with anti-myc (A) and anti-HA (B) antibodies. Cells were transfected with myc-tagged Dnr1 expression plasmids as indicated and immunoprecipitated with anti-myc antibodies. Whole cell input lysates are shown in lanes 1–3 and the corresponding immunoprecipitates are shown in lanes 5–7. Molecular mass markers are shown in lane 4. We detect a co-precipitation of HADreddC408A with both Dnr1 variants (lanes 6 and 7), while we only detect a weak precipitation of HADreddC408A in the absence of Dnr1 (lane 5).

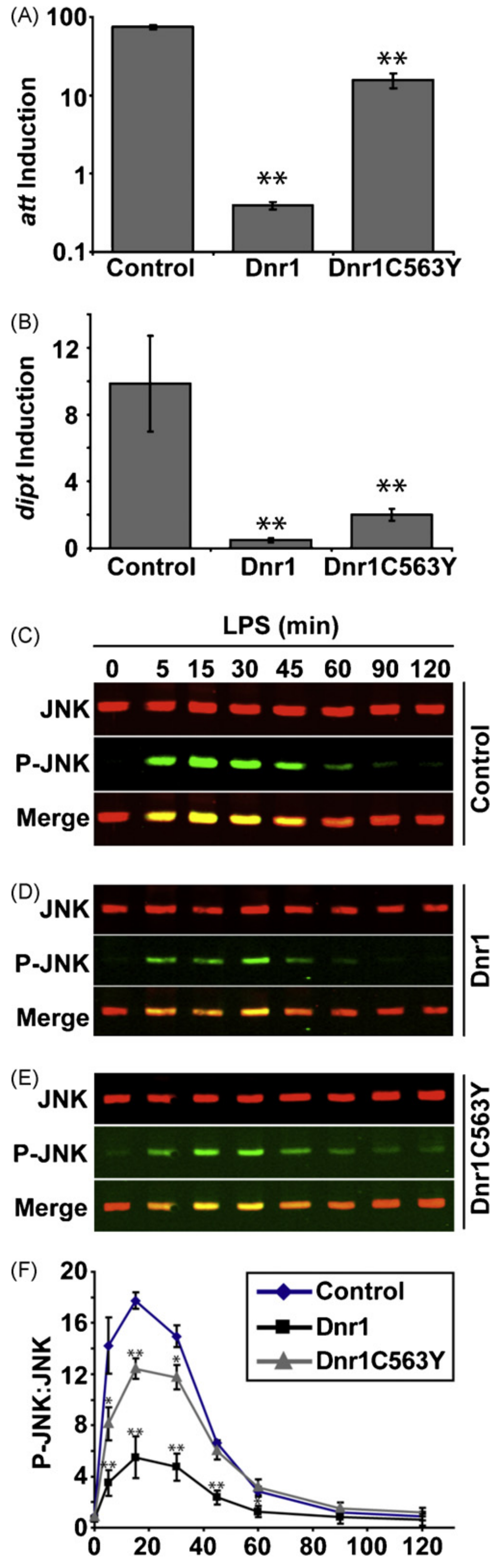

Fig. 2. Dnr1 blocks Imd pathway activity in S2 cells. (A and B) Quantitative real-time PCR assays to determine the relative peptidoglycan-mediated induction of *att* (A) and *dipt* (B) in control S2 cells (column 1), S2 cells that constitutively express Dnr1 (column 2) and S2 cells that constitutively express Dnr1C563Y (column 3). Each column shows the induction levels 8 h after peptidoglycan exposure for the respective cell lines relative to the 0 h value. The results shown are the average of three independent experiments and error bars indicate the standard error. Expression of either variant of Dnr1 significantly inhibits the peptidoglycan-mediated expression of *att* and *dipt* in comparison to control S2 cells (**$p < 0.01$). (C–E) Peptidoglycan-dependent phosphorylation of JNK in control S2 cells (C), S2 cells that constitutively express Dnr1 (D) and S2 cells that constitutively express Dnr1C563Y (E). Each cell line was treated with peptidoglycan for the indicated period and lysates probed for total JNK and phosphorylated JNK (p-JNK) by Western blot analysis. For C–E, total JNK is shown in the upper blot, p-JNK is shown in the center blot and the two panels are false colored and merged to form the lower blot, with JNK labeled in red and p-JNK labeled in green. A representative Western blot is shown for each cell line. (F) Quantification of the relative intensity of the p-JNK to JNK signal for each sample in C–E. The p-JNK to JNK signal at 0 min was assigned a value of one for each cell type and all other ratios are reported relative to that value. The results shown are the average of three independent experiments and error bars indicate the standard error.

clone with pAMW. DreddC408A has been described previously [20]. HADreddC408A was generated by cloning DreddC408A into pENTR/D-TOPO (Invitrogen) and recombining with pHHW. HADreddmyc was generated by cloning full-length Dredd cDNA into pENTR/D-TOPO (Invitrogen) and recombining with pAFHW. DreddΔPDmyc was generated by cloning DreddΔPD (an N-terminal truncation of Dredd that lacks amino acids 1–287) into pENTR/D-TOPO (Invitrogen) and recombining with pAFHW.

2.3. Western blotting and protein quantification

For Western blot analysis of S2 cells, 10^6 cells were lysed in lysis buffer, incubated at 4 °C for 10 min with occasional vortexing and resuspended in sample buffer. For Western blots of adult *Drosophila*, 10 flies were homogenized in 30 μl fly lysis buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 5 mM EDTA (pH 8.0), 0.2% NP40, protease inhibitors (Roche inhibitor cocktail tablets)) and suspended in sample buffer. All samples were boiled prior to separation by SDS-PAGE. Rabbit anti-myc, mouse anti-myc, mouse anti-actin and mouse anti-HA antibodies were purchased from Sigma. Rabbit anti-JNK was purchased from Santa Cruz Biotechnology and mouse anti-active JNK was purchased from cell signaling. Mouse anti-tubulin was a generous gift from Dr. Sarah Hughes. All secondary antibodies were purchased from Invitrogen. Protein quantification of Western blots was performed with an Aerius reader (Licor) following the manufacturer's recommendations.

2.4. Quantitative real-time PCR

For qRT-PCR assays, total RNA was purified from 10^6 S2 cells or 40 adult *Drosophila* using Trizol (Invitrogen) according to the manufacturer's instructions and incubated with DNase to eliminate residual DNA. cDNA was amplified from 5 μg RNA using SuperScript III and oligo dT primers (both Invitrogen), according to the manufacturer's instructions. qRT-PCR was performed in an Eppendorf realplex 2 PCR machine using SyBr green as a detection reagent. The following primers were used to detect the respective transcripts: *actin* forward 5′-TGCCTCATCGCCGACATAA-3′, *actin* reverse 5′-CACGTCACCAGGGCGTAAT-3′; *att* forward 5-AGTCA-CAACTGGCGGAC-3′, *att* reverse 5′TGTTGAATAAATTGGCATGG-3′; *dipt* forward 5′-ACCGCAGTACCCACTCAATC-3′, *dipt* reverse 5′-ACTTTCCAGCTCGGTTCTGA-3′; *drs* forward 5′-GTACTTGTTCGC-CCTCTTCG-3′, *drs* reverse 5′-ATTTAGCATCCTTCGCACCA-3′; *dnr1* forward 5′-GAGAAGGAGCATGTGCTGAG-3′, *dnr1* reverse 5′-ATG-TGCTCCATGGACTTCTG-3′. All samples were normalized to actin expression levels and quantified relative to a calibrator using the ΔΔCt method.

2.5. Drosophila husbandry

All *Drosophila* strains were cultured on standard cornmeal medium at 25 °C. UAS-HADnr1 transgenic lines were generated according to standard protocols in a w^{118} background. hsGAL4 flies were obtained from Dr. Sarah Hughes and UAS-Dnr1IR flies were

obtained from the National Institute of Genetics (Japan). For heat-pulse mediated inductions of transgenic constructs the appropriate strains were exposed to a 1 h heat pulse at 37 °C and returned to incubate at 25 °C. For injury experiments, flies were either pricked with a sharpened tungsten needle cleaned in ethanol (sterile injury) or with a sharpened tungsten needle immersed in a pellet of an overnight *E. coli* DH5α culture (septic injury). For kill curve analysis, 50 2–3 days old flies were infected with *E. coli* and their survival monitored on a daily basis.

3. Results

3.1. Dnr1 interacts with Dredd

As previous data suggest that Dnr1 impacts on the Imd signaling pathway through inhibition of Dredd, we performed a series of tests to determine if Dnr1 and Dredd form a complex in *Drosophila* cell culture assays. To this end, we generated a stable S2 cell line that constitutively expresses an HA-tagged Dredd variant where the active site cysteine was replaced with an alanine through site-directed mutagenesis (HADreddC408A). We chose proteolytically inactive Dredd for these experiments to circumvent the possibility of Dredd-mediated cleavage of Dnr1, or additional unknown components of a putative Dnr1–Dredd complex. We then transfected cells that express HADreddC408A with expression plasmids that drive constitutive expression of myc-tagged Dnr1 (mycDnr1), or a mutagenized Dnr1 variant where the essential RING domain active site cysteine was replaced with a tyrosine (mycDnr1C563Y). Similar to previous results [29,31], we always detected higher levels of HADnr1C563Y expression than HADnr1 in lysates from S2 cells (Fig. 1). We believe that this likely reflects the inability of the RING domain mutant to induce proteasomal destruction of Dnr1C563Y. We then precipitated lysates from both cell types with an anti-myc antibody and tested for co-precipitation of HADreddC408A. As a control, we precipitated lysates of S2 cells that express HADreddC408A alone with an anti-myc antibody. We reproducibly detected co-precipitation of HADreddC408A from lysates of cells expressing mycDnr1 or mycDnr1C563Y (Fig. 1, lanes 6 and 7). In contrast, we only detected a minor precipitation of HADreddC408A from control S2 cells that did not express HADnr1 (Fig. 1, lane 5). Thus, we conclude that Dredd specifically forms a complex with Dnr1. As HADreddC408A complexes with mycDnr1C563Y, we propose that the catalytic activities of Dredd and the RING domain of Dnr1 are not required for complex formation.

3.2. Dnr1 blocks Imd pathway activity in S2 cells

Dredd is an essential component of the Imd pathway with overlapping roles in activation of the NF-κB and JNK signaling modules. As Dnr1 forms a complex with Dredd and loss of Dnr1 triggers activation of an Imd pathway reporter construct, we asked if expression of Dnr1 blocks Imd pathway activity in S2 cells. Initially, we determined if Dnr1 affected the induction of two Imd-responsive antimicrobial peptides; *diptericin* (*dipt*) and *attacin* (*att*). To this end, we established S2 cell lines that constitutively

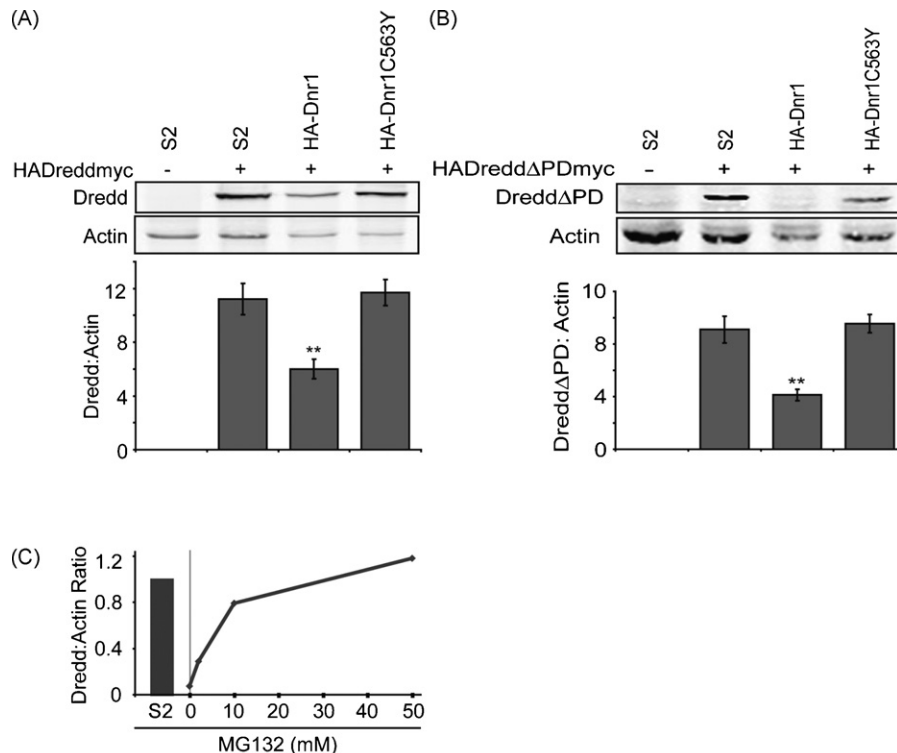

Fig. 3. Dnr1 depletes Dredd protein in a RING domain-dependent manner and independent of the prodomain of Dredd. (A) Anti-HA (upper blot) and anti-actin (lower blot) Western blot analysis of lysates from control S2 cells, S2 cells that constitutively express HADnr1 or S2 cells that constitutively express HADnr1C563Y. Cells were transfected with an HADreddmyc expression plasmid where indicated. A representative Western blot is shown. The ratio of the HA to actin signal was determined for each sample (lower panel). The results shown are the average of three independent experiments and error bars indicate the standard error. The HADreddmyc:actin ratio is significantly lower in HADnr1 expressing cells (**$p < 0.01$) than in control S2 cells, or S2 cells that express HADnr1C563Y. (B) A representative anti-HA (upper blot) and anti-actin (lower blot) Western blot analysis of lysates from control S2 cells, S2 cells that constitutively express HADnr1 or S2 cells that constitutively express HADnr1C563Y. Cells were transfected with an HADreddΔPDmyc expression plasmid as indicated. The ratio of the HA to actin signal was determined for each sample (lower panel). The results shown are the average of three independent experiments and error bars indicate the standard error. The HADreddΔPDmyc:actin ratio is significantly lower in HADnr1 expressing cells (**$p < 0.01$) than in control S2 cells, or S2 cells that express HADnr1C563Y. (C) Quantification of the HADreddmyc:actin ratio in control S2 cells (column) or S2 cells that express HADnr1. HADnr1 cells were treated with increasing concentrations of MG132 as indicated.

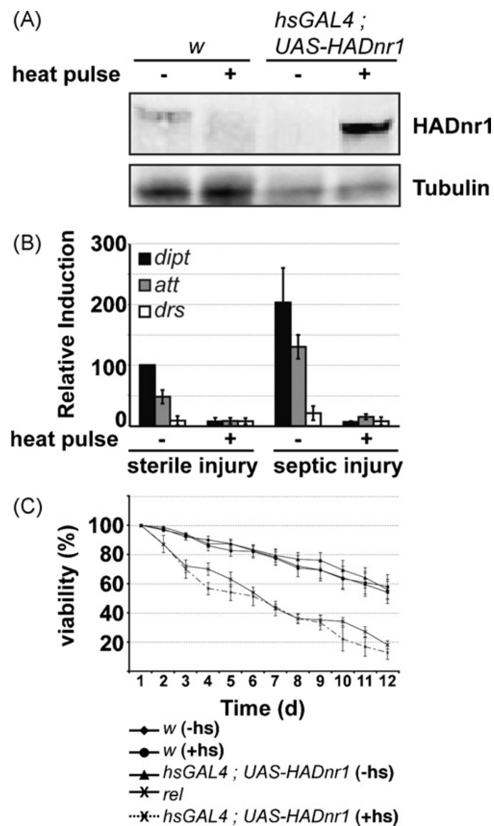

Fig. 4. Dnr1 blocks Imd pathway activity in adult *Drosophila*. (A) Western blot analysis of lysates from adult *w* or *hsGAL4;UAS-HADnr1* flies. Flies were treated with a heat pulse where indicated to induce GAL4 expression. Lysates were probed for HA (upper blot) and tubulin (lower blot) as a loading control. A transient heat pulse induces expression of HADnr1. (B) Quantitative real-time PCR analysis of the relative induction levels for three antimicrobial peptides (*dipt*, *att* and *drs*) in *hsGAL4;UAS-HADnr1* flies. Flies were treated with a heat pulse where indicated and exposed to a sterile or septic injury as shown. The expression levels for *dipt* in non-infected control *hsGAL4;UAS-HADnr1* flies that were not exposed to a heat pulse was assigned a value of one and all other expression values are reported relative to that value. Results are the average of three independent experiments and error bars indicate the standard errors. (C) The viability levels of *w* or *hsGAL4;UAS-HADnr1* flies after septic injury with a needle soaked in *E. coli*. Flies were treated with a heat pulse prior to infection where indicated. The viability of rel^{E38} flies after infection is shown as a control. Results are the average of three independent experiments and error bars indicate the standard errors.

express HADnr1, or HADnr1C563Y. We then incubated the cells with commercial preparations of lipopolysaccharides (LPS). Commercially available LPS are contaminated with minor amounts of peptidoglycan and stimulate Imd pathway signaling [10]. We used quantitative real-time PCR to compare induction of *dipt* and *att* in control S2 cells or S2 cells that express Dnr1 or Dnr1C563Y. In each case, overexpression of Dnr1 or Dnr1C563Y significantly blocked peptidoglycan-mediated induction of *att* and *dipt* (Fig. 2A and B). We observed a significantly greater inhibition of Imd pathway activity in cells that expressed Dnr1 than in cells that expressed Dnr1C563Y. For example, while we observed a near 10-fold decrease in peptidoglycan-mediated *att* induction in cells that constitutively expressed HADnr1C563Y compared to control S2 cells, we detected a 100-fold decrease in the peptidoglycan-mediated induction of *att* in cells that constitutively expressed HADnr1 compared to control S2 cells. Indeed, peptidoglycan essentially failed to induce expression of either *att* or *dipt* in cells that constitutively express Dnr1. Given that we always detect considerably higher levels of expression of HADnr1C563Y than of HADnr1, we conclude that the greater degree of inhibition observed with HADnr1 is not merely an expression artifact. Instead, we believe that our data show that the RING domain of Dnr1 makes significant contributions to the Dnr1-mediated inhibition of the Imd pathway.

To confirm that Dnr1 expression inhibits Imd pathway activity in S2 cells, we then examined the impact of Dnr1 expression on peptidoglycan-induced JNK activation. We monitored JNK activation by probing Western blots of lysates from LPS-treated cells with a monoclonal antibody that specifically detects the dual-phosphorylated, active form of JNK. Imd pathway signaling induces a transitory phosphorylation of JNK (Fig. 2C) and this phosphorylation requires Dredd activity [22]. We observed significantly lower degrees of JNK phosphorylation in lysates from peptidoglycan-treated S2 cells that express Dnr1 (Fig. 2D) or Dnr1C563Y (Fig. 2E). We quantified the ratio of p-JNK:JNK for each sample and confirmed that overexpression of Dnr1 or Dnr1C563Y significantly inhibits peptidoglycan-dependent JNK phosphorylation. Similar to our observation with *dipt* and *att* expression, we observed a stronger inhibition of JNK phosphorylation in cell that express Dnr1 than in cells that express Dnr1C563Y, even though the levels of Dnr1C563Y expression are considerably higher. Thus, we conclude that expression of wild-type Dnr1 blocks the Imd pathway in S2 cells, while expression of HADnr1C563Y partially blocks Imd pathway signaling. These data are in line with the hypothesis that Dnr1 acts as a negative regulator of Imd pathway activity. In addition, these data indicate that the RING domain of Dnr1 plays a critical role in Dnr1-mediated inhibition of the Imd pathway.

3.3. Dnr1 regulates Dredd protein levels in a RING domain-dependent manner

As Dnr1 forms a complex with Dredd and the RING domain of Dnr1 contributes to Dnr1-dependent inhibition of Imd pathway activity, we reasoned that Dnr1 blocks the Imd signaling pathway by regulating the levels of Dredd protein. To test this hypothesis, we transfected plasmids that drive constitutive

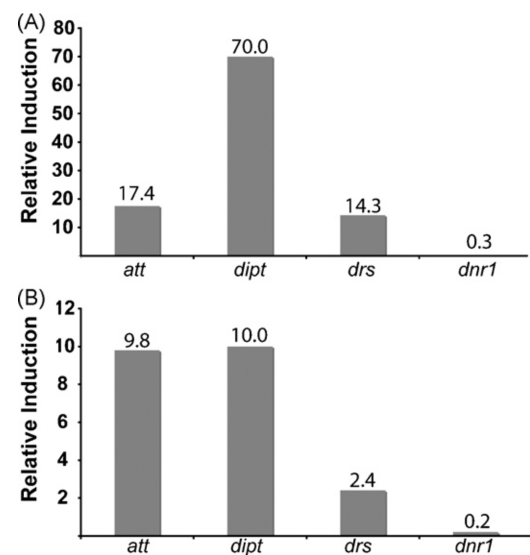

Fig. 5. Dnr1 depletion induces expression of antimicrobial peptides in adult *Drosophila*. (A and B) Quantitative real-time PCR measurements of the relative induction levels of three antimicrobial peptides (*att*, *dipt* and *drs*) and *dnr1* in uninfected *hsGAL4;UAS-Dnr1IR* flies 24 h after a heat pulse. Values are shown for two independent *UAS-Dnr1IR* (A and B, respectively) lines and are typical of three independent measurements for each line. The expression levels are reported relative to *hsGAL4;UAS-Dnr1IR* flies that were not exposed to a heat pulse. In both lines, a heat pulse induces a drop in *Dnr1* transcript levels and a corresponding increases in the expression levels of *att*, *dipt* and *drs*.

expression of N-terminally HA-tagged and C-terminally myc-tagged Dredd (HADreddmyc) into S2 cells or stable S2 cells that constitutively express HADnr1 or HADnr1C563Y. We then probed lysates from each cell type for levels of HADreddmyc relative to a control protein (actin). We detected a significant decrease in the relative expression levels of HADreddmyc in HADnr1-expressing cells in comparison to control S2 cells (Fig. 3A). In contrast, expression of HADnr1C563Y failed to decrease the levels of HADreddmyc in comparison to control S2 cells (Fig. 3A). Thus, we conclude that Dnr1 decreases the levels of Dredd in S2 cells in a manner that requires a catalytically active RING domain.

We previously detected a similar effect of Dnr1 on the pro-apoptotic caspase Dronc. These observations led us to speculate that Dnr1 recognizes a conserved feature of initiator caspases and targets them for proteasomal degradation. As initiator caspases are characterized by long prodomains, we hypothesized that Dnr1 specifically recognizes a common feature of the prodomain of initiator caspases. To test this hypothesis, we generated an N-terminal truncated variant of HADreddmyc that lacks the prodomain (HADreddΔPDmyc). We reasoned that the HADreddΔPDmyc construct would be insensitive to Dnr1-dependent depletion in S2 cells. We then transfected expression plasmids that drive constitutive expression of HADreddΔPDmyc into S2 cells or stable S2 lines that constitutively express Dnr1 or Dnr1C563Y and measured the levels of HADreddΔPDmyc in each cell type relative to a control protein (actin). Similar to our observations with full-length Dredd, overexpression of Dnr1C563Y failed to decrease the levels of HADreddΔPDmyc relative to actin in comparison to control S2 cells (Fig. 3B). Interestingly, Dnr1 significantly decreased the levels of HADreddΔPDmyc relative to actin (Fig. 3B).

As RING domains are known E3 ubiquitin ligases, we reasoned that Dnr1-dependent depletion of Dredd protein levels proceeded through ubiquitin-mediated proteolysis. To test this hypothesis, we transfected HADreddmyc expression plasmids in S2 cells that express Dnr1 and then added increasing doses of the proteasomal inhibitor MG132. As expected, increased proteasomal inhibition resulted in a sharp increase in HADreddmyc levels in S2 cell that express Dnr1. Combined, these data indicate that Dnr1 drives the proteasomal destruction of Dredd in a RING domain-dependent manner and that prodomain motifs of Dredd are not essential for Dnr1-dependent destruction of Dredd.

3.4. Dnr1 expression blocks Imd pathway activity in vivo

To probe interactions of Dnr1 with the Imd signaling pathway in a physiologically relevant *in vivo* setting, we generated a transgenic *Drosophila* line that inducibly expresses N-terminally HA-tagged Dnr1 under control of yeast UAS promoter sequences (*UAS-HADnr1*). We crossed *UAS-HADnr1* flies to a strain that expresses the yeast GAL4 transcriptional activator under the control of a heat-inducible promoter (*hsGAL4*) to generate a *UAS-HADnr1;hsGAL4* strain. We detected robust induction of HADnr1 upon exposure of *UAS-HADnr1;hsGAL4* to a brief heat pulse (Fig. 4A).

We then used quantitative real-time PCR to measure the consequences of Dnr1 induction on the Imd pathway response to challenges with *E. coli*. Consistent with numerous previous reports, piercing the cuticle of adult *Drosophila* with a sterile needle results in an injury response that is characterized by the induction of a series of antimicrobial peptides (sterile injury model, Fig. 4B). This antimicrobial response is heightened upon pricking the cuticle of adult *Drosophila* with a needle soaked in *E. coli* (septic injury model). Specifically, a septic injury with *E. coli* results in a strong induction of known Imd-responsive antimicrobial peptides such as *dipt* and *att* and a milder induction of Toll-responsive transcripts, such as *drosomycin* (*drs*) (Fig. 4B). Induction of Dnr1 prior to either a sterile or septic injury almost completely blocked expression of all Imd pathway sensitive transcripts upon injury (Fig. 4B). Based on these data, we conclude that expression of Dnr1 in adult *Drosophila* blocks activation of the Imd pathway in response to challenges with Gram-negative bacteria.

We then tested whether Dnr1-mediated inhibition of Imd pathway activity has physiological consequences for the host response to infection with *E. coli*. Wild-type flies survive septic injury with Gram-negative bacteria due to a robust Imd pathway response. We noticed that the survival rate of infected *UAS-HADnr1;hsGAL4* flies in the absence of a heat pulse was indistinguishable from the survival rate of control wild-type flies that were infected with or without a prior heat pulse (Fig. 4C). In contrast, *UAS-HADnr1;hsGAL4* flies that were treated with a heat pulse prior to infection displayed a significantly reduced viability upon septic injury with *E. coli* (Fig. 4C). Indeed, the viability of infected, heat pulse-treated *UAS-HADnr1;hsGAL4* flies was indistinguishable from infected rel^{E38} flies, a null allele of *relish* (Fig. 4C). Thus, similar to our cell culture data, we observed that induction of Dnr1 in flies prevents expression of Imd-responsive transcripts and greatly decreases the ability of adult flies to combat Gram-negative bacterial infection. We note that these data are in line with the hypothesis that Dnr1 acts as an inhibitor of Imd pathway activity.

3.5. Loss of Dnr1 induces transient expression of Imd-responsive transcripts

Previous data indicated that RNAi-mediated depletion of Dnr1 from S2 cells resulted in the induction of a Rel-responsive reporter construct (*dipt-lacZ*) in the absence of LPS [29]. We therefore, sought to determine whether loss of Dnr1 would result in induction of Rel-responsive gene products in a more physiologically relevant setting. To this end, we used quantitative real-time PCR to monitor the expression of *dipt* and *att* in adult flies that inducibly express dsRNA constructs that target Dnr1 (*UAS-Dnr1IR*). Such inducible RNAi lines are widely used for targeted depletion of known transcripts by RNAi under *in vivo* settings. We generated *UAS-Dnr1IR;hsGAL4* lines that allow the induction of *Dnr1* dsRNA upon exposure to a brief heat pulse.

A heat pulse alone does not activate the Imd signaling pathway [32]. Induction of the *Dnr1IR* construct in the absence of infection typically resulted in a three to 4-fold reduction of *Dnr1* transcript levels (Fig. 5A) and approximately a 10-fold increase in the levels of expression of *dipt* and *att* 24 h after induction. We note that we observed similar results with a second *UAS-Dnr1IR* strain (Fig. 5B). The spike in *dipt* and *att* expression levels was relatively short-lived for both strains; the expression levels for all three antimicrobial peptides had returned to basal levels within three days of induction of the heat pulse (data not shown). Thus, we conclude that loss of Dnr1 *in vivo* results in a temporary increase in the expression of Imd pathway-responsive transcripts such as *dipt* and *att*.

4. Discussion

Numerous molecular and genetic studies have made major contributions to the identification and characterization of positive components of the Imd signal transduction cascade. In contrast, there has been a relative lag in attempts to identify negative regulators of Imd signaling. A previous cell culture RNAi screen identified Dnr1 as a putative inhibitor of the Imd pathway in *Drosophila* [29]. Specifically, depletion of Dnr1 from S2 cells, led to ectopic activation of an Imd-responsive reporter construct (*dipt-*

lacZ). In addition, activation of the Imd pathway stabilized Dnr1 protein levels. Based on these data, it was proposed that Dnr1 is a negative regulator of the Imd pathway and that engagement of the Imd pathway stabilizes Dnr1 protein levels, thereby contributing to termination of the Imd pathway signal. However, to date there are no *in vivo* data to support a role for Dnr1 in Imd pathway regulation and no molecular data to identify the mechanism by which Dnr1 impinges on the Imd pathway.

In this manuscript, we present molecular, cell culture and *in vivo* data that are consistent with a role for Dnr1 as a negative regulator of the Imd pathway. We demonstrate that Dnr1 and Dredd form a complex in *Drosophila* S2 cells and that overexpression of Dnr1 in S2 cells significantly reduces Dredd protein levels. As a caveat, we point out that the lack of available anti-Dredd antibodies prevented us from analyzing interactions of Dnr1 with endogenous Dredd. Interestingly, a mutant variant of Dnr1 that lacks an essential RING domain catalytic residue retains the ability to bind Dredd, but fails to affect Dredd protein levels. These data indicate that the RING domain of Dnr1 is critical for the regulation of Dredd protein. In addition, we demonstrate that overexpression of Dnr1 in S2 cells or adult *Drosophila* blocks key features of Imd pathway activity, such as JNK phosphorylation or the transcriptional activation of antimicrobial peptide gene expression. Furthermore, we show that overexpression of Dnr1 significantly reduces the ability of adult *Drosophila* to combat Gram-negative bacterial challenges. Importantly, we also demonstrate that depletion of Dnr1 from two independent *Drosophila* strains results in a temporary induction of Imd pathway transcripts. Thus, whereas overexpression of Dnr1 is sufficient to block Imd pathway signaling, loss of Dnr1 induces a transient activation of the Imd pathway. We believe that these data are most consistent with a model for Dnr1 where Dnr1 functions as a negative regulator of Imd pathway signaling activity.

From a mechanistic perspective, we propose that Dnr1 inhibits the Imd pathway at the level of the initiator caspase Dredd. Specifically, we propose that Dnr1 binds and targets Dredd for proteasomal destruction. In support of this hypothesis, we note that the RING domain of Dnr1 bears strong similarities to the RING domains of members of the IAP family [30]. RING domain-bearing members of the IAP family inhibit caspase activity and target caspases for proteasomal destruction via ubiquitination [30,33,34]. In addition, IAPs undergo auto-ubiquitination and thereby initiate their own destruction. While Dnr1 lacks the caspase-binding BIR motifs typical of *bona fide* IAPs, our data indicate that Dnr1 binds Dredd in S2 cells. Furthermore, site-directed mutagenesis of the RING domain of Dnr1 greatly diminishes the ability of Dnr1 to decrease Dredd protein levels, inhibit Imd pathway signaling and regulate Dnr1 protein levels. We also note that our observations are in line with our previous report that Dnr1 partially blocks the pro-apoptotic caspases in S2 cells [31]. Thus, it appears reasonable to assume that Dnr1 performs "IAP-like" functions in the regulation of the Imd pathway. Based on our observations, we propose that Dnr1 forms a complex with Dredd and targets Dredd for destruction through the proteasome, thereby inhibiting the Imd pathway. Presently, we do not know whether Dnr1 binds Dredd directly, or whether additional proteins are involved in establishment of a Dredd–Dnr1 complex. Although Dnr1 lacks a BIR motif, we cannot exclude the existence of alternative caspase-interacting motifs on Dnr1.

We note that while the Dnr1C563Y variant is not as effective as Dnr1 at inhibiting Imd pathway activity, the C563Y mutant version retains a partial ability to prevent JNK phosphorylation and Rel transcriptional activity in cell culture assays. The diminished ability of Dnr1C563Y to block Imd signaling relative to Dnr1 is not a consequence of reduced expression of the C563Y variant, as we always detect greater levels of Dnr1C563Y expression than Dnr1. Instead, we believe that Dnr1C563Y retains an ability to inhibit Imd signaling through its interactions with Dredd. In this model, we propose that Dnr1C563Y binds and sequesters Dredd, thereby effectively decreasing the available pool of Dredd activity for Imd pathway signaling.

Similar to our observations on the impact of Dnr1 on pro-apoptotic signaling pathways, we only detected minor effects of Dnr1 loss on Imd pathway activity. While overexpression of Dnr1 completely blocks Dredd-dependent features of Imd pathway signaling, we only detected a modest, transitory activation of Imd signaling upon Dnr1 depletion in wild-type flies. We believe there are several likely explanations for these observations. From a homeostatic perspective, we consider that it is likely a major disadvantage to the fly to have the Imd pathway in a permanent "on" mode, as this is metabolically consuming and may result in hyperactivation of potentially deleterious signaling cascades or the accumulation of large amounts of damaging molecules. This phenomenon is well documented in higher eukaryotes, where hyperactivation of the NF-κB signaling pathway is directly linked to numerous cancers and other pathological conditions [35–37]. Indeed, a considerable amount of mammalian NF-κB responsive transcripts serve to diminish NF-κB activity, thereby acting as a negative feedback loop within the NF-κB pathway [38–40]. Given that the transcriptional response of *Drosophila* to Rel activation results in altered expression profiles of scores of individual transcripts [13,41,42], we consider it likely that a subset of these transcripts prevent unchecked Rel activity. In addition, we consider it likely that overlapping constitutive inhibitors of Imd pathway activity readily replace Dnr1 activity upon loss of Dnr1 function. Several candidate constitutive inhibitors have been described previously and we anticipate more may yet be found [23,24,26–28]. In summary, we propose that Dnr1 acts as a negative regulator of the Imd pathway as part of a broader collection of negative elements within the Imd pathway.

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