Ubiquitylation of the initiator caspase DREDD is required for innate immune signalling

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Caspases have been extensively studied as critical initiators and executioners of cell death pathways. However, caspases also take part in non-apoptotic signalling events such as the regulation of innate immunity and activation of nuclear factor- κ B (NF- κ B). How caspases are activated under these conditions and process a selective set of substrates to allow NF- κ B signalling without killing the cell remains largely unknown. Here, we show that stimulation of the *Drosophila* pattern recognition protein PGRP-LCx induces DIAP2-dependent polyubiquitylation of the initiator caspase DREDD. Signal-dependent ubiquitylation of DREDD is required for full processing of IMD, NF- κ B/Relish and expression of antimicrobial peptide genes in response to infection with Gram-negative bacteria. Our results identify a mechanism that positively controls NF- κ B signalling via ubiquitin-mediated activation of DREDD. The direct involvement of ubiquitylation in caspase activation represents a novel mechanism for non-apoptotic caspase-mediated signalling.

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Introduction

The conjugation and deconjugation of ubiquitin (Ub) to target proteins influence diverse biological processes that can contribute to tumour formation when deregulated (Hoeller and Dikic, 2009). The conjugation of Ub, thereby, can stimulate the assembly of transient signalling hubs in which the Ub adduct forms part of a new docking site for proteins with specialized binding motifs that help to build reversible, short-lived signalling centres (Grabbe and Dikic, 2009). Ub-mediated signalling is particularly important for activation of nuclear factor- κ B (NF- κ B) during innate immune responses (Skaug *et al*, 2009). Deregulated activation of NF- κ B is recognized as one of the major underlying causes for chronic, cancer-related inflammation that drives tumour development, and is seen in most tumour types including leukaemia, lymphomas and solid tumours (Karin and Greten, 2005; Grivennikov *et al*, 2010; Nathan and Ding, 2010). Hence, a better understanding of the processes that regulate NF- κ B and innate immunity is critically important.

Metazoans live in close contact with a multitude of microbes with which they establish complex reciprocal interactions. *Drosophila* relies on innate immunity responses to combat microbial challenges (Ferrandon *et al*, 2007; Lemaitre and Hoffmann, 2007). Depending on the invading microorganism, *Drosophila* activate either the Toll or immune deficiency (*imd*) pathway (Lemaitre *et al*, 1995, 1996). Infection with fungi or certain Gram-positive bacteria activates the Toll pathway, which initiates an intracellular signalling cascade that culminates in the nuclear translocation of the NF- κ B-like transcription factors Dif and Dorsal (Lemaitre *et al*, 1996; Manfrulli *et al*, 1999; Meng *et al*, 1999; Rutschmann *et al*, 2000a, 2002). On the other hand, the IMD pathway is activated by Gram-negative bacteria, which in turn stimulates activation of the NF- κ B-like transcription factor Relish (also known as IRD4) (Hedengren *et al*, 1999). Activation of both pathways induces the expression of a plethora of NF- κ B responsive antimicrobial peptide (AMP) genes, which are important for fending off invading microorganisms (Tzou *et al*, 2000).

The IMD signal transduction pathway shows striking similarities to the ones stimulated by members of the mammalian TNF-receptor super-family (Tanji and Ip, 2005). These pathways are Ub-dependent signal transduction cascades in which Ub ligases, Ub receptors and deubiquitylating enzymes (DUBs) build up, recognize and remove Ub signals, allowing the temporally controlled assembly of protein complexes that lead to the activation of kinases that regulate NF- κ B. Ub, thereby, functions as a binding surface for proteins with Ub-binding domains (UBD), also referred to as Ub receptors (Dikic *et al*, 2009). The conjugation of Ub requires a stepwise process that involves Ub-activating enzymes (E1), Ub-conjugating enzymes (E2) and Ub protein ligases (E3) (Dikic *et al*, 2009). E3s confer substrate specificity and enable

the formation of an isopeptide linkage between the carboxyl-terminus of Ub (glycine (G)76) and the amino group of a reactive side chain of the substrate. Ub can be conjugated either as a single moiety or as chains of variable length (Komander, 2009). Further complexity is provided by different linkage types, as Ub moieties can be conjugated to one another via different lysine (K) residues within Ub. At least eight different types of Ub chains exist. The different types of Ub chain linkages exert distinct functional outcomes (Hoeller and Dikic, 2009). This is predominantly due to the fact that the different chain types adopt distinct topologies, which in turn are recognized by specific Ub receptors (Komander, 2009).

Signalling through the IMD pathway is activated by the Gram-negative cell wall component diaminopymelic-type peptidoglycan (DAP-PGN). DAP-PGN is recognized by the pattern recognition protein PGRP-LE and the transmembrane receptor PGRP-LCx (Choe *et al*, 2002; Gottar *et al*, 2002; Ramet *et al*, 2002; Leulier *et al*, 2003; Kaneko *et al*, 2004, 2006). Binding of DAP-PGN to PGRP-LCx causes receptor oligomerization and triggers recruitment of the adaptor protein IMD (Choe *et al*, 2005), which carries a C-terminal death domain (DD) that is similar to the one of the mammalian adaptor protein RIP1 (Georgel *et al*, 2001). Through its DD, IMD subsequently recruits dFADD (also known as BG4), which in turn binds and presumably activates the *Drosophila* caspase-8 orthologue Death-related ced-3/Nedd2-like protein (DREDD; also known as DCP2) (Leulier *et al*, 2000, 2002; Naitza *et al*, 2002). Following activation, DREDD cleaves off the amino (N)-terminal portion of IMD, thereby exposing an evolutionarily conserved inhibitor of apoptosis (IAP)-binding motif (IBM) at the neo-N-terminus of IMD (Paquette *et al*, 2010). This IBM is recognized by the E3 ligase *Drosophila* IAP protein 2 (DIAP2), which brings it into position for IMD ubiquitylation. In conjunction with the E2s Effete (UEV1a) and UEV1a/Bendless (UEV1a/UBC13), DIAP2 targets IMD for K63-linked polyubiquitylation (Zhou *et al*, 2005; Paquette *et al*, 2010). According to the current model, the attached Ub chains function as scaffolds for the recruitment of the *Drosophila* MAP kinase kinase kinase dTAK1/TAB2 and the Relish kinase complex IRD5/Kenny (IKK β /IKK γ) (Ferrandon *et al*, 2007). Ub-dependent recruitment of dTAK1/TAB2 and IRD5/Kenny is thought to be mediated by their respective Ub receptors TAB2 and Kenny (Rutschmann *et al*, 2000b; Silverman *et al*, 2000; Lu *et al*, 2001; Vidal *et al*, 2001; Silverman *et al*, 2003; Kanayama *et al*, 2004; Kleino *et al*, 2005; Zhuang *et al*, 2006). Ub-dependent complex formation is presumed to result in activation of dTAK1, which in turn phosphorylates and activates IRD5/Kenny and MKK4/7 (Silverman *et al*, 2003; Geuking *et al*, 2009). While MKK4/7 promotes JNK activation, IRD5/Kenny phosphorylate Relish (Rutschmann *et al*, 2000b; Silverman *et al*, 2000; Lu *et al*, 2001; Vidal *et al*, 2001; Geuking *et al*, 2009), which activates its transcriptional activity (Erturk-Hasdemir *et al*, 2009). In addition to IRD5/Kenny-mediated phosphorylation of Relish, activation also requires DREDD-dependent proteolytic processing of Relish (Elrod-Erickson *et al*, 2000; Leulier *et al*, 2000; Stoven *et al*, 2000, 2003; Erturk-Hasdemir *et al*, 2009). Activated DREDD cleaves off an inhibitory C-terminal ankyrin repeat domain of Relish, thereby allowing the translocation of the N-terminal portion to the nucleus

where it induces expression of AMP genes (Silverman *et al*, 2000; Stoven *et al*, 2000, 2003; Erturk-Hasdemir *et al*, 2009). Loss-of-function mutations in most of the components of the IMD signalling cascade results in an immune deficiency phenotype, in which animals become acutely susceptible to infection by Gram-negative bacteria. Common to all these mutants is their failure to induce expression of antibacterial peptide genes and, therefore, to fend off bacterial infection (Ferrandon *et al*, 2007; Lemaitre and Hoffmann, 2007). While E3 ligases promote IMD signalling via ubiquitylation, pathway activation can be suppressed via Ub deconjugation by dUSP36 and dCYLD (Tsichritzis *et al*, 2007; Thevenon *et al*, 2009), reinforcing the importance of Ub in the regulation of innate immunity.

At present, DIAP2 is the sole E3 ligase implicated in Ub-mediated IMD signalling (Gesellchen *et al*, 2005; Kleino *et al*, 2005; Leulier *et al*, 2006; Huh *et al*, 2007). DIAP2 is a member of the evolutionarily conserved IAP protein family, whose members are best known for their ability to regulate caspases and apoptosis (Gyrd-Hansen and Meier, 2010). The defining feature of an IAP protein is the presence of the baculovirus IAP repeat (BIR) domain(s), a zinc-binding fold of ~70 amino-acid residues that mediates protein interactions. Most IAPs harbour additional domains such as the C-terminal RING finger domain that provides them with E3 Ub ligase activity by mediating the transfer of Ub from E2s to target substrate.

Although it is clear that DIAP2 is required for Rel/NF- κ B activation (Gesellchen *et al*, 2005; Kleino *et al*, 2005; Leulier *et al*, 2006; Huh *et al*, 2007), the precise mechanism through which DIAP2 mediates Ub-dependent activation of NF- κ B remains ill defined. At present, the only known targets for DIAP2-induced ubiquitylation are the adaptor protein IMD and DIAP2 itself (Paquette *et al*, 2010). To gain a better understanding of Ub-dependent NF- κ B activation, and to explore the possibility that DIAP2 mediates its effect through modulating caspases, we studied the molecular mechanism through which DIAP2 triggers Ub-dependent signalling. Here, we show that DIAP2 interacts with the initiator caspase DREDD and that DIAP2 targets this caspase for non-degradative ubiquitylation in a signal-dependent manner. Further, we find that impaired ubiquitylation of DREDD blocks IMD and Relish cleavage and renders mutant flies acutely sensitive to Gram-negative bacterial infection. Thus, the Ub chains on DREDD might function as anchor points for Ub receptors that help dimerization and full activation of DREDD. The mechanism of DREDD activation might parallel the one of caspase-8 in mammals, where the Ub receptor p62 reportedly promotes aggregation, activation and processing of CUL3-modified caspase-8 (Jin *et al*, 2009). Together, our data indicate that DIAP2-mediated ubiquitylation of DREDD is required for innate immune signalling.

Results

DIAP2 binds to DREDD and targets it for polyubiquitylation in a RING finger-dependent manner

To determine the molecular mechanism through which DIAP2 controls innate immunity, we first determined the contribution of individual domains of DIAP2 that are required

Figure 1 DIAP2 binds and ubiquitylates the initiator caspase DREDD. (A, B) DIAP2's RING finger and both BIR domains are required to resist *Ecc15* bacterial infection. *Diap2^{7c}* mutant animals were reconstituted with rescue constructs expressing the indicated DIAP2 proteins. To confirm the requirement of DIAP2 E3 ligase activity for the IMD pathway (A), DIAP2 constructs were ubiquitously expressed in transgenic flies under the control of *daughterless* (*Da*)-GAL4. Animals were pricked with a needle previously dipped into *Ecc15* and the survival rates monitored. To evaluate the contribution of individual BIR domain of DIAP2 (B), transgenic flies were generated using germ-line-specific phiC31 integration (Bischof *et al*, 2007). Transgenes were expressed using the fat-body-specific c564-Gal4 driver line. Animals were injected with 13.8 nl of *Ecc15* bacterial solution (optical density of 300) and the survival rates monitored. The experiments were performed using at least 20 flies for each genotype. Shown is a representative experiment of at least three repeats. C466Y represents a RING mutant of DIAP2, which lacks E3 ligase activity (Ribeiro *et al*, 2007), D163 and D263A disrupts the BIR pocket of BIR2 and BIR3, respectively. (C, D) DIAP2 binds to DREDD. Reciprocal binding assays with DIAP2 and DREDD. The indicated constructs were transfected into S2 cells and GST-tagged DIAP2 (C) and V5-tagged DREDD (D) were purified by Glutathione resin and α-V5 antibodies, respectively. The expression and the presence of the co-purified proteins were assessed by immunoblotting with the indicated antibodies. (E) DIAP2 ubiquitylates DREDD in a RING finger-dependent manner. HA-Ub was co-expressed with DREDD-V5 in S2 cells together with vector control, DIAP2^{WT} or the DIAP2 RING mutants DIAP2^{C466Y} and DIAP2^{F469A}. Cells were lysed under denaturing conditions and ubiquitylated proteins isolated using α-HA antibodies. The presence and ubiquitylation of DREDD and DIAP2 was assessed with the indicated antibodies. (F) DREDD is transiently ubiquitylated in a signal-dependent manner. S2 cells were transfected with DREDD-V5. Subsequently, cells were treated with 1 μg/ml DAP-PGN for the indicated time points before being harvested. DREDD was immunoprecipitated using α-V5 resin and ubiquitylated DREDD analysed using α-Ub antibodies. (G) DIAP2 promotes the conjugation of K63-linked Ub chains on DREDD. The indicated constructs were transfected into S2 cells, and HA-tagged DREDD and IMD were purified under denaturing conditions. The presence of the purified proteins and identity of the respective Ub chains were detected by immunoblotting using the indicated antibodies (see Materials and methods for details).

for IMD signalling. To this end, we used UAS-driven transgenes encoding specific DIAP2 mutants and assessed their ability to rescue the lethality of *Diap2* mutant flies following septic injury by *Erwinia carotovora carotovora* 15

(*Ecc15*), a Gram-negative bacteria. Transgenes were expressed ubiquitously under the control of *daughterless* (*Da*)-GAL4. While expression of wild-type (WT) DIAP2 rescued the lethality of the *Diap2* mutant flies (*Diap2^{7c}*) (Leulier *et al*, 2006) in

response to *Ecc15* septic injury, reconstitution with an E3-defective DIAP2 mutant (DIAP2^{RINGmut}) completely failed to protect *Diap2* mutant animals (Figure 1A), which is in agreement with an earlier report (Huh *et al*, 2007). Of note, DIAP2^{RINGmut}, which carries a mutation that removes the zinc-coordinating cysteine required for the proper folding of the RING domain, is as stable as WT DIAP2 (Ribeiro *et al*, 2007). To evaluate the contribution of individual BIR domain of DIAP2, we generated transgenic flies using germ-line-specific phiC31 integration into pre-defined landing sites, which allows accurate comparison between mutants (Bischof *et al*, 2007). DIAP2 transgenes encoding DIAP2 mutants with amino-acid (aa) substitutions in either the BIR2 (D163A) or BIR3 (D263A) domain (Ribeiro *et al*, 2007) efficiently rescued the sensitivity to *Ecc15* infection seen in *Diap2*^{2c} mutant flies. In contrast, double mutants carrying a substitution in BIR2 and BIR3 (D163A/D263A) failed to provide protection from *Ecc15* septic injury (Figure 1B). This is consistent with the observation that either BIR2 or BIR3 can mediate IMD binding (Paquette *et al*, 2010). Given that expression of DIAP2^{RINGmut} fails to rescue *Ecc15*-mediated lethality of *diap2*^{2c} mutant animals, this indicates that DIAP2-mediated ubiquitylation of IMD pathway components is indispensable for IMD signalling.

To identify components of the IMD pathway that are targeted by DIAP2, we first performed binding assays with DIAP2 and known members of this pathway. We co-expressed DIAP2 with tagged components of the IMD pathway in S2 cells and analysed their ability to interact with each other. The interaction between DIAP2 and PGRP-LCx was performed in the presence or absence of DAP-PGN to assess whether receptor activation is required for binding to DIAP2. PGRP-LCx failed to interact with DIAP2 under these conditions (data not shown). Cleaved IMD, which is truncated at aspartic acid residue 30 following pathway activation and exposes a *bona fide* IBM at the neo-amino-terminus of the C-terminal portion of IMD, readily bound to DIAP2 (data not shown; Paquette *et al*, 2010). While DIAP2 failed to associate with other IMD pathway components such as dFADD, IRD5, dTAB2 and dTAK1 (data not shown), DIAP2 tightly bound to DREDD (Figure 1C). DIAP2 also bound to the heterodimeric E2 UEV1A/Bendless that promotes the conjugation of K63-linked polyubiquitin chains, as previously reported (data not shown; Zhou *et al*, 2005; Paquette *et al*, 2010). Reciprocal pulldown experiments confirmed the interaction between DREDD and DIAP2 (Figure 1D).

To assess whether DIAP2 can target DREDD for ubiquitylation, we next expressed non-tagged DIAP2 in S2 cells together with HA-tagged Ub and V5-tagged DREDD. Ubiquitylated proteins were affinity purified under denaturing conditions using α -HA columns (see Materials and methods for details), and the presence of ubiquitylated proteins was assessed by immunoblotting the eluate. Under these conditions, DIAP2 efficiently ubiquitylated DREDD (Figure 1E) and IMD (data not shown; Paquette *et al*, 2010). Ubiquitylation of DREDD was dependent on DIAP2's E3 ligase activity, as the RING mutants DIAP2^{F469A} and DIAP2^{C466Y}, which abrogate the Ub-E3 activity of IAPs (Ribeiro *et al*, 2007; Ditzel *et al*, 2008), failed to ubiquitylate DREDD (Figure 1E, top panel, compare lane 3 with lanes 4 and 5). Together, these data indicate that DIAP2 can bind to DREDD and promote its polyubiquitylation.

Next, we determined whether ubiquitylation of DREDD occurred in a signal-dependent manner. For this purpose, we induced the IMD pathway in *Drosophila* S2 cells using bacterial-derived DAP-PGN. For this assay, we relied on endogenous DIAP2, but stably expressed DREDD-V5, because no antibody is currently available that detects endogenous DREDD. Interestingly, while ectopically expressed DREDD remained unmodified under these conditions, treatment with DAP-PGN induced prominent and transient ubiquitylation of DREDD (Figure 1F). DREDD ubiquitylation occurred as early as 1 min and was no longer detectable 40–60 min after DAP-PGN treatment. Consistent with the notion that DIAP2 can bind to the heterodimeric E2 UEV1 A/Bendless, we found that DIAP2 promoted the conjugation of K63-linked Ub chains on DREDD (Figure 1G). Likewise, DIAP2 also triggered K63-ubiquitylation of IMD. Given that DIAP2 binds and ubiquitylates DREDD, and that DIAP2 is the key E3 ligase of the IMD pathway (Gesellchen *et al*, 2005; Kleino *et al*, 2005; Leulier *et al*, 2006; Huh *et al*, 2007), these data suggest that activation of the IMD pathway results in transient, DIAP2-mediated ubiquitylation of DREDD.

DIAP2 binds to the DED1 of DREDD

DIAP2 is the first IAP found to interact with a death effector domain (DED)-containing initiator caspase. To better understand the nature of this interaction, we generated various deletion mutants of DREDD and DIAP2 (Figure 2A) and determined the domains that are required for their association. Similar to caspase-8, DREDD contains a long pro-domain with two DEDs in tandem (Chen *et al*, 1998). This is followed by the large (p20) and small (p10) caspase subunits. Co-immunoprecipitation assays revealed that DIAP2 readily bound to the prodomain of DREDD (DED1/2, aa 1–264) (Figure 2B). In contrast, DIAP2 failed to associate with the C-terminal caspase domains (p20/p10, aa 265–517) of DREDD. Further, the binding of DIAP2 to DREDD appeared to depend on the presence of the DED1 because DIAP2 only weakly bound to DREDD- Δ DED1 (aa 143–517) that lacks DED1. Consistently, DED1 in isolation co-purified with DIAP2 (Figure 2C), while the DED2 did not interact with DIAP2. This indicates that the first DED is necessary and sufficient for DREDD to bind to DIAP2. Intriguingly, we found that the adaptor protein dFADD also selectively bound to DED1 (Figure 2D), which is in contrast to an earlier report (Hu and Yang, 2000). Although dFADD and DIAP2 both bound to DED1, they did not compete with one another for the binding to DREDD (Figure 2E). This is evident because co-expression of dFADD did not abrogate the interaction of DIAP2 with DREDD. Together, our data indicate that DIAP2 and dFADD bind to the DED1 of DREDD, forming a trimeric complex.

To identify the region of DIAP2 that interacts with DREDD, we first examined the requirement of the BIR domains, since BIRs generally function as protein interaction motifs (Eckelman *et al*, 2008). We found that the BIR domain-containing amino-terminal portion of DIAP2 (DIAP2-BIR1/2/3, aa 1–338) bound to DREDD as efficiently as WT DIAP2. In contrast, the carboxy-terminal portion of DIAP2 (DIAP2^{281–498}), which lacks the BIR domains, failed to associate with DREDD (Figure 2F). Based on the presence of a deep peptide-binding groove, BIR domains can roughly be grouped into type-I and type-II domains (Gyrd-Hansen and

Meier, 2010). Type-I BIR domains lack a peptide-binding groove, or possess a shallow pocket only, whereas type-II BIRs carry a distinctive hydrophobic cleft through which they can bind to IBMs. According to this classification, DIAP2's BIR1 represents a type-I, whereas the BIR2 and BIR3 domains

are type-II domains. Co-immunoprecipitation assays indicated that DIAP2's BIR1 domain in isolation did not interact with DREDD (Figure 2G). Under the same conditions, DREDD readily associated with the BIR2 and BIR3 domains. To determine whether DREDD interacted with these BIRs

through their respective peptide-binding grooves, we examined the ability of DREDD to bind to DIAP2 proteins that carry substitution mutations of the Zn^{2+} -coordinating Cysteine residue in the BIR2 (C149G) and/or BIR3 (C249G) (Ribeiro *et al*, 2007). Intriguingly, single or double mutations in the BIR2 and/or BIR3 did not abrogate the binding of DIAP2 to DREDD (Figure 2H). This is in stark contrast to the interaction between DIAP2 and IMD, which was lost when the peptide-binding groove of both BIR2 and BIR3 were mutated (Figure 2I) (Paquette *et al*, 2010). The observation that DIAP2^{C149/C249} retained its ability to interact with DREDD indicates that the DREDD-binding surface of DIAP2 does not coincide with the one required for binding to the neo-amino-terminus of IMD. It also indicates that the interaction between DIAP2 and DREDD does not rely on an IBM-like motif in DREDD.

The loss-of-function DREDD allele D44 carries a mutation that disrupts DIAP2-mediated ubiquitylation

Loss-of-function mutations in DREDD occur throughout the primary sequence of DREDD (Leulier *et al*, 2000). The alleles *B118*, *D55* and *F64* generate either premature stop codons or frameshift changes in the DREDD prodomain. In contrast, the loss-of-function *dredd* allele *D44* carries a missense mutation (G120R) in the sequence encoding the DED1 (Figure 3A). Importantly, *D44* blocks *dipteracin* expression to the same degree as *B118*, *D55* and *F64* (Leulier *et al*, 2000). The strong phenotype associated with the G120R mutation of *D44* indicates that the DED1 is essential for DREDD's function in immunity. Given that DIAP2 and dFADD both interact with the DED1 of DREDD, we examined the functional consequence of the G120R mutation.

The 3D structure of DREDD's prodomain was modelled using the X-ray structure of the DEDs of the viral-FLIP MC159 (pdb 2bbz) as a template (Yang *et al*, 2005). The predicted tandem DEDs of DREDD adopt a dumbbell-shaped arrangement, with the two DEDs at two opposing ends (Figure 3B and C). According to the structural modelling, DED1 (residues 18–242) adopts a classical death fold with a six-helical bundle structure. Glycine 120, thereby, is positioned in a loop that connects helix $\alpha 5a$ with $\alpha 6a$ (Figure 3B). The small side chain of G120 faces outwards and does not contribute to the overall fold of the death motif. Consequently, substitution of G120 to R120 results in DREDD with a positively charged side chain protruding from DED1 (Figure 3C, right panel). Even though the

G120R is a loss-of-function mutation, DREDD^{D44} was fully competent in binding to dFADD and DIAP2 (Figure 3D and E). This is evident, because DREDD^{D44} bound to DIAP2 (Figure 3D) and dFADD (Figure 3E) like WT DREDD. The G120R mutation also did not affect auto-processing of DREDD following its overexpression, as evidenced by the conversion of full-length DREDD to processed DREDD (Figure 3D). Since initiator caspases are activated following dimerization-induced conformational changes, this suggests that the G120R mutation does not affect DREDD dimerization. Accordingly, DREDD^{D44} readily homo-dimerized with WT or mutant DREDD (Figure 3F and G). Further, upon overexpression, mutant DREDD cleaved Relish like its WT counterpart, in both *Drosophila* S2 cells (Figure 3H) and mammalian 293T cells (data not shown). While this overexpression experiment does not address the physiological mechanism of DREDD activation, it demonstrates that the G120R mutation neither influences the inherent catalytic activity of DREDD, for example by distorting the catalytically active centre, nor interferes with substrate entry. We also assessed the protein stability of DREDD^{D44} following cycloheximide treatment, and found that the protein half-life of DREDD^{D44} was comparable with the one of WT DREDD (Figure 3I).

Next, we examined whether the G120R mutation affects DIAP2-mediated ubiquitylation of DREDD. To this end, we performed ubiquitylation assays with DIAP2 and WT or mutant DREDD. Intriguingly, DREDD^{D44} was significantly less ubiquitylated by DIAP2 than WT DREDD (Figure 4A). This is unexpected, as DIAP2 binds to DREDD^{D44} as efficiently as to WT DREDD (Figure 3D). Furthermore, while treatment with DAP-PGN induced prominent and transient ubiquitylation of WT DREDD, DREDD^{D44} failed to be ubiquitylated in a signal-dependent manner (Figure 4B). Of note, the base-line ubiquitylation status of DREDD^{D44} was higher than WT DREDD, indicating that DREDD^{D44} can be modified under steady-state conditions. However, signal-dependent ubiquitylation of DREDD^{D44} did not occur upon treatment with DAP-PGN. This demonstrates that the G120R mutation affects the ability of DREDD to be ubiquitylated in a signal-dependent manner. Most likely, the protruding positively charged side chain of R120 (Figure 3C) prevents DIAP2-mediated ubiquitylation allosterically, possibly by repositioning DREDD in respect to DIAP2's E2s, which moves the Ub-acceptor lysines of DREDD out of reach for the E2, making them no longer available for Ub transfer.

Figure 2 DIAP2 binds to the DED1 of DREDD. (A) Schematic representation of the DIAP2 and DREDD constructs used in this study. (B) DIAP2 binds to the pro-domain of DREDD. Co-IP assays with DIAP2 and various fragments of DREDD. S2 cells were co-transfected with HA-tagged DIAP2 and V5-tagged DREDD. Expression and the presence of co-purified proteins were analysed by immunoblotting with the indicated antibodies. (C) DIAP2 associates with DED1 of DREDD. Co-IPs were performed with DIAP2 and individual DEDs or the prodomain of DREDD as in (B). (D) dFADD also binds to the DED1 of DREDD. Co-IPs were performed with dFADD and individual DEDs or the prodomain of DREDD as in (B). (E) Competition assay with DIAP2, dFADD and DREDD indicates that DIAP2 and dFADD can bind to DREDD simultaneously. Co-IPs were performed with dFADD, DIAP2 and DREDD. Expression and the presence of co-purified proteins were analysed as in (B). (F) The N-terminal portion of DIAP2 interacts with DREDD. Co-IPs were performed with DREDD and the indicated fragments of DIAP2. Expression and the presence of co-purified proteins were analysed as in (B). (G) The BIR2 and BIR3 domains mediate binding to DREDD. Co-IPs were performed with DREDD and individual BIR domains of DIAP2. Expression and the presence of co-purified proteins were analysed as in (B). An asterisk marks a cleavage product of BIR3. (H) The interaction between DIAP2 and DREDD does not require the hydrophobic pocket of the BIR2 and BIR3. Co-IPs were performed with DREDD and the indicated WT or mutant BIR domains. Expression and the presence of co-purified proteins were analysed as in (B). (I) In contrast, the binding of DIAP2 to cleaved IMD critically depends on the hydrophobic pockets of the BIR2 and BIR3. IMD corresponds to the DREDD-cleaved form of IMD (aa 31–273) and was expressed using the Ub-fusion technique (Varshavsky, 2000; Tenev *et al*, 2005). Co-IPs with DREDD and the indicated WT or mutant BIR domains were performed as in (H). BIR1/2/3 encompasses DIAP2's N-terminal segment that carries the BIR1, BIR2 and BIR3 domains.

Figure 3 The G120R loss-of-function mutation of DREDD disrupts DIAP2-mediated ubiquitylation. **(A)** Schematic diagram of the domain architecture of DREDD. Residue G120 maps to the DED1 of DREDD. **(B)** Ribbon representation of the prodomain structure of DREDD (residues 40–235). The 3D structure of DREDD's prodomain was modelled using the X-ray structure of the DEDs of the viral-FLIP MC159 (pdb 2bbz) as a template. The six-helical bundle structure of the death fold of DED1 is indicated. The position of G120 is highlighted in red. **(C)** Molecular surfaces of the same residues as shown in **(B)**. G120 and G120R of WT DREDD (left panel) and DREDD^{D44} (right panel) are highlighted in red. DED1 is indicated in green, while the DED2 is depicted in grey. **(D)** DREDD^{D44} binds to DIAP2 like WT DREDD. Co-IPs were performed with DIAP2 and DREDD^{WT} and DREDD^{D44}. Expression and the presence of co-purified proteins were analysed by immunoblotting with the indicated antibodies. Of note, DIAP2 only co-purifies full-length DREDD, but not processed DREDD (p20/p10) that lacks the pro-domain. **(E)** The G120R mutation does not affect the ability of DREDD to bind to dFADD. Co-IPs with dFADD and DREDD^{WT} or DREDD^{D44} were performed as in **(D)**. **(F, G)** DREDD^{D44} is not impaired in its ability to hetero- or homodimerize with WT and mutant DREDD^{D44}. Co-IPs with DREDD^{WT} and DREDD^{D44} were performed as in **(D)**. **(H)** The G120R mutation does not affect the inherent catalytic activity of DREDD, since DREDD^{D44} can cleave Relish similar to DREDD^{WT} upon overexpression. S2 cells were co-transfected with FLAG-tagged Relish and empty vector, V5-tagged WT, catalytically inactive (C408A) or G120R mutant DREDD. Relish cleavage was assessed by immunoblotting using α -FLAG antibodies. **(I)** The G120R mutation does not affect DREDD's protein stability. Cycloheximide (CHX) chase experiments to compare the protein stability of DREDD^{WT} and DREDD^{D44}. Equal loading was assessed with α -actin antibodies.

Impaired ubiquitylation of DREDD abrogates expression of AMP genes following septic injury

The observation that DREDD^{D44} fails to be ubiquitylated in a signal-dependent manner is consistent with a model in which

ubiquitylation of DREDD is required for innate immunity. To investigate the role of DREDD ubiquitylation in NF- κ B-mediated induction of AMPs, we first analysed the expression levels of *attacin D* and *diptericin* following septic injury with

Figure 4 The G120R mutation impairs DREDD ubiquitylation. (A) DIAP2 ubiquitylates DREDD^{D44} less efficiently than WT DREDD. HA-Ub was co-expressed with DREDD^{WT} and DREDD^{D44} in S2 cells together with vector control, DIAP2^{WT} or the DIAP2 RING mutant DIAP2^{F469A}. Cells were lysed under denaturing conditions and ubiquitylated proteins isolated using α-HA antibodies. The presence of DREDD and DIAP2 and the DREDD ubiquitylation was assessed with the indicated antibodies. (B) DREDD^{D44} fails to be ubiquitylated in a signal-dependent manner. S2 cells were co-transfected with HA-Ub and DREDD^{WT} or DREDD^{D44}. Subsequently, cells were treated with 1 μg/ml DAP-PGN for the indicated time points before being harvested under denaturing conditions. Ubiquitylated DREDD was purified and detected as described in (A). An asterisk marks a cross-reactive band.

the Gram-negative bacteria *Ecc15*. Of note, *attacin D* and *diptericin* are AMP genes that are controlled by the IMD pathway (Lemaitre *et al*, 1997). While WT flies readily induced expression of *attacin D* and *diptericin*, flies with mutations in *Relish* (*Relish*^{E20}) or *Tak1* (*Tak1*¹), failed to mount such an IMD response (Figure 5A) (Leulier *et al*, 2006; Huh *et al*, 2007). Likewise, homozygous *Dredd*^{D44} mutants similarly failed to induce Relish-mediated expression of *attacin D* and *diptericin*. AMP induction in *Dredd*^{D44} mutants was as impaired as in *Dredd*^{B118} animals that harbour a stop codon in the DREDD prodomain, which is consistent with an earlier report (Leulier *et al*, 2000). Given that DREDD^{D44} fails to be ubiquitylated in a signal-dependent manner, but otherwise behaves like WT DREDD with regards to its protein stability, catalytic potential and its ability to bind to DIAP2 and dFADD, these data suggest that signal-dependent ubiquitylation of DREDD is essential for Relish-mediated induction of AMP genes.

To investigate the consequence of DREDD ubiquitylation in protecting flies against bacterial infection, we determined the survival profile of *Dredd*^{D44} mutant flies that were infected with *Ecc15*. Consistent with the notion that *Dredd*^{D44} mutants fail to induce AMP genes, *Dredd*^{D44} flies were highly susceptible to injection with the Gram-negative bacteria *Ecc15* (Figure 5B) (Leulier *et al*, 2000). Likewise, *Dredd*^{B118}, *Dredd*^{L23}, *Diap2*^{7c}, *Relish*^{E20} and *Tak1*¹ mutant animals also died following infection by *Ecc15*. *Dredd*^{D44} and *Diap2*^{7c} mutant animals did not impair AMP production in response to infection with *Micrococcus luteus*, a Gram-positive bacteria known to activate the Toll pathway (data not shown). Taken together, these data indicate that loss of the Ub-E3 ligase for DREDD, or loss of signal-dependent ubiquitylation of DREDD, as seen in *Dredd*^{D44} flies, dramatically sensitizes animals to infection by Gram-negative bacteria.

To gain some mechanistic insights into why *Dredd*^{D44} mutants fail to induce AMP production, we performed a time course of immune stimulation and examined whether endogenous IMD and Relish were processed in *Dredd*^{D44} mutant animals in a signal-dependent manner. We previously have shown that IMD is ubiquitylated following DREDD-mediated cleavage (Paquette *et al*, 2010). Therefore, we used IMD ubiquitylation as a proxy to investigate DREDD activation. Intriguingly, we found that IMD failed to become ubiquitylated in *Dredd*^{D44} mutant flies (Figure 5C), which indicates that cleavage of IMD is impaired in these animals. Moreover, JNK activation (as measured by expression of *puckered*, see below) in response to immune stimulation is blocked in *Dredd*^{D44} mutant flies (Figure 5D). This is consistent with the observation that IMD ubiquitylation, which is required for activation of dTAK1, a JNK upstream kinase, is lost in *Dredd*^{D44} mutant animals. Consistent with the notion that ubiquitylation of DREDD is required for DREDD activation in response to septic injury with *Ecc15*, we found that *Dredd*^{D44} mutant flies also failed to cleave Relish (Figure 5E). While cleaved Relish was detected in extracts from WT flies within 45 min of infection with *Ecc15*, *Dredd*^{D44} mutant animals completely failed to induce Relish cleavage following immune stimulation. Likewise, Relish cleavage was similarly impaired in *Dredd*^{B118} mutants. In contrast, *Tak1*¹ mutants partially cleaved Relish as previously described (Delaney *et al*, 2006). This indicates that impaired ubiquitylation of DREDD, through point mutation of DREDD (G120R), prevents DREDD-mediated cleavage of IMD and Relish. The observation that inefficient ubiquitylation of DREDD prevents IMD and Relish cleavage indicates that signal-dependent ubiquitylation of DREDD is a key step in innate immune-mediated activation of NF-κB.

Figure 5 The G120R mutation impairs signal-dependent cleavage of Relish and Imd, and abrogates activation of AMP gene expression. (A) Quantitative RT-PCR analysis of *attacin D* and *dipteracin* induction in the indicated fly strains. *rp49* was used as the experimental expression standard. Shown are the relative expression ratios of *attacin D/rp49* and *dipteracin/rp49* 5 h post-infection. Values are normalized to non-pricked *Canton^S*. Np stands for non-pricked. Error bars indicate SEM from three independent experimental repeats using at least 20 flies per repeat. (B) *Dredd^{D44}* mutant flies die upon infection with *Ecc15*. Adult flies of the indicated genotypes were subjected to *Ecc15*-mediated septic injury and their survival rates were monitored at the indicated time points. Error bars indicate SEM from three independent experimental repeats using at least 20 flies per repeat. (C) Immunoblot analysis assessing ubiquitylation of IMD, which requires DREDD-mediated cleavage of IMD (Paquette *et al*, 2010). The indicated fly strains were exposed to septic injury and fly lysates were used to immunoprecipitate IMD. The presence and modification of IMD was subsequently analysed by immunoblotting with the indicated antibodies. DD1 and *w¹¹¹⁸* fly strains are WT strains that served as controls. (D) IMD-mediated activation of JNK in the indicated fly strains was measured by evaluating expression of *puckered*, a *bona fide* downstream target of the JNK signal transduction cascade. Values were normalized to mock injections. The asterisks indicate a *P*-value of *P* < 0.01, calculated on raw data before normalization. Ns designates non-significant differences. (E) Immunoblot analysis assessing cleavage of Relish in the indicated fly strains. While Relish cleavage occurred in a signal-dependent manner in *yw* flies, Relish failed to be cleaved in *Dredd^{B118}* and *Dredd^{D44}* following septic injury with *Ecc15*. In contrast, Relish was partially cleaved in *Tak1¹* mutant flies. Equal loading was assessed with α -actin antibodies. An asterisk indicates a cross-reactive band.

Discussion

Ub-dependent signalling is critically important for the ability of the *Drosophila* fat body to produce AMP genes in response to Gram-negative bacterial infection (Zhou *et al*, 2005; Paquette *et al*, 2010). Previous studies have implicated ubiquitylation of IMD in innate immune signalling (Paquette *et al*, 2010). We now show that one of the key steps in innate immune-mediated activation of NF- κ B appears to be ubiquitylation of DREDD. Several lines of evidence support the notion that DIAP2-mediated ubiquitylation of DREDD is critically important for activation of AMP genes. First, the E3 ligase DIAP2, which is indispensable for innate immune-mediated activation of NF- κ B and protection from Gram-negative bacterial infection, physically interacts with DREDD and targets it for non-degradative polyubiquitylation. DIAP2-mediated ubiquitylation of DREDD, thereby, relies on the presence of a functional RING finger of DIAP2, which strongly suggests that DIAP2 is directly ubiquitylating DREDD. Second, ubiquitylation of DREDD occurs in a

signal-dependent manner. Third, failure to stimulate DREDD ubiquitylation, as seen in *Dredd^{D44}*, is associated with a severe loss-of-function phenotype abrogating IMD cleavage and ubiquitylation as well as Relish maturation. DREDD activation may follow a similar principle as the one of caspase-8 in mammals, where the Ub receptor p62 promotes aggregation and activation of CUL3-modified caspase-8 (Jin *et al*, 2009).

While innate immune signalling induces prominent and transient ubiquitylation of WT DREDD, treatment with DAP-PGN failed to promote ubiquitylation of *Dredd^{D44}*. The G120R mutation maps to DED1 of DREDD, and although it impairs DIAP2-mediated ubiquitylation, this mutation does not affect the ability of DREDD to bind to itself, DIAP2, and dFADD. It also does neither destabilize *Dredd^{D44}* nor affect its inherent catalytic centre or substrate entry. Although *Dredd^{D44}* behaves like WT DREDD in most aspects, the G120R mutation abrogates its ability to be ubiquitylated in a signal-dependent manner. Substitution of G to R at position

120 generates DREDD with a positively charged side chain protruding from DED1. Most likely, the protruding positively charged side chain prevents DIAP2-mediated ubiquitylation allosterically, possibly by re-positioning DREDD in respect to DIAP2's E2, which moves the Ub-acceptor lysines (K) of DREDD out of reach for the E2, making them no longer available for Ub transfer. Recent structural studies indicate that Ub-acceptor K residues must be positioned within 50 angstrom (Å) of the active site cysteine of the E2 for Ub transfer (Duda *et al*, 2008). Ultimately, crystal structure analysis of DIAP2/E2 bound to WT and mutant DREDD will be required to validate this model.

Intriguingly, expression of *attacin D* and *dipteracin* is blocked to the same degree in *dredd^{D44}* and *Dredd^{B118}* animals, which lack DREDD expression due to a premature stop codon at position 127 (Leulier *et al*, 2000). The strong phenotype associated with the G120R mutation together with the observation that this mutation selectively affects signal-dependent ubiquitylation strongly suggests that ubiquitylation of DREDD is essential for DREDD's function in immunity. Consistently, we find that flies harbouring the G120R mutation fail to cleave IMD and Relish and induce expression of AMP genes in response to septic injury with Gram-negative bacteria. *Dredd^{D44}* mutant flies also exhibit significantly lower basal expression of *attacin D* and *dipteracin* under non-challenged conditions (data not shown), indicating that production of AMPs in response to commensal microbiota is also affected in these animals. Together, these data are consistent with a model whereby ubiquitylation of DREDD is required for signal-dependent activation of DREDD. This subsequently leads to processing of IMD, which exposes an IBM that associates with DIAP2 allowing DIAP2-mediated ubiquitylation of IMD. In addition, Ub-dependent activation of Relish is also required for the induction of AMPs.

The observation that impaired DREDD ubiquitylation correlates with loss of IMD and Relish cleavage may suggest that ubiquitylation of DREDD is required for DREDD activation under physiological conditions. This view is supported by a recent study indicating that the mammalian Ub receptor p62 promotes activation of ubiquitylated caspase-8 (Jin *et al*, 2009). While Ub-mediated clustering of DREDD might further support DREDD dimerization and activation, it is also possible that the Ub chains on DREDD and IMD might serve as docking sites for other proteins with UBD. In this respect, it is important to note that several downstream components, such as TAB2 and Kenny, carry putative UBDs. The Ub chains on DREDD and IMD may thereby bind and recruit the dTAK1/TAB2 and IKK (IRD5(IKKβ)/Kenny(IKKγ)) kinase complexes via the Ub receptors TAB2 and Kenny, respectively. Importantly, previous data indicate that IKK is required for signal-dependent processing of Relish (Silverman *et al*, 2000; Stoven *et al*, 2003; Erturk-Hasdemir *et al*, 2009). While IKK is necessary for DREDD-mediated cleavage of Relish, dTAK1 is surprisingly dispensable. This is evident because knockdown or genetic ablation of dTAK1 does not alter signal-dependent cleavage of Relish (Delaney *et al*, 2006; Erturk-Hasdemir *et al*, 2009). In contrast, loss of IKK completely blocks Relish cleavage in response to Gram-negative bacterial infection (Erturk-Hasdemir *et al*, 2009). Intriguingly, the kinase activity of IKK is not required for DREDD-mediated cleavage of Relish. Relish cleavage is rescued in *ird5^l* mutant flies reconstituted with either

WT or catalytically inactive mutant IRD5 (IRD5^{K50A}). This demonstrates that IKK not only functions as the kinase phosphorylating Relish but also fulfils a structural role that facilitates Relish cleavage. The observation that the kinase activity of IKK is dispensable for Relish cleavage is also supported by the observation that IKK-mediated phosphorylation of serines 528 and 529 of Relish is not required for its cleavage (Erturk-Hasdemir *et al*, 2009). While phosphorylation of Relish is dispensable for Relish cleavage, it is clearly required for the transcriptional activity of Relish. Taken together, these data also indicate that mere activation of DREDD is not sufficient for DREDD-mediated processing of Relish. In addition to DREDD activation, IKK protein, but not IKK-mediated phosphorylation of Relish, is also required. At present it remains unclear how IKK protein facilitates DREDD-mediated processing of Relish. However, given that IKK directly phosphorylates Relish (Silverman *et al*, 2000), IKK must be able to bind to Relish. In this respect, it is interesting to note that phosphorylation of human p105 by IKKβ requires direct binding of IKKβ to a motif in the PEST region of p105 (Heissmeyer *et al*, 2001; Salmeron *et al*, 2001). Hence, a similar motif might exist in Relish that enables its recruitment to IKK. Consistently, the C-terminal 107 residues of Relish are required for its cleavage (Stoven *et al*, 2003), suggesting that the IKK interaction motif is located within this stretch.

On the basis of the new findings, we propose a refined model for IMD signalling (Figure 6). As previously established, ligand binding induces recruitment of IMD, Dredd and dFADD to PGRP-LCx (step 1). Recruitment of DIAP2 targets DREDD for K63-linked ubiquitylation (step 2), which allows Ub-mediated aggregation and activation of DIAP2-modified DREDD (step 3). Active DREDD subsequently cleaves IMD (step 4), which exposes an IBM at the neo-amino-terminus of cleaved IMD. The IBM of IMD binds to the BIR2/3 of DIAP2. This provides DIAP2 with an additional contact point within the signalling complex, reinforcing complex stability and allowing DIAP2-mediated ubiquitylation of IMD, and quite possibly other components of the signalling complex (step 5). The Ub chains on IMD and DREDD may serve as scaffolds for the recruitment of dTAK1 and IKK (step 6). Since IKK can bind to Relish, as evidenced by its ability to phosphorylate Relish, IKK might also bring Relish into close proximity of ubiquitylated and active DREDD, allowing DREDD-mediated proteolysis of Relish. The proximity to the signalling complex will also allow phospho-mediated activation of Relish (step 7). Subsequently, modified Relish translocates to the nucleus (step 8) where it drives expression of AMP genes (step 9).

The direct involvement of ubiquitylation in caspase-mediated endoproteolysis of substrates represents a novel mechanism for Ub-dependent activation of NF-κB. Interestingly, a similar mechanism might also exist in mammals. For example, loss of caspase-8 function in human patients reportedly is connected with defective activation of lymphocytes (Chun *et al*, 2002), a process that is known to require NF-κB. Moreover, mammalian p62 can promote aggregation, activation and processing of ubiquitylated caspase-8 in a stimulus-dependent manner (Jin *et al*, 2009). As DREDD is the orthologue of mammalian caspase-8, it will be interesting to examine whether ubiquitylation of caspase-8 might convert caspase-8 from a pro-death molecule into an NF-κB-activating signalling component.

Figure 6 Model depicting Ub-dependent activation of NF-κB during IMD signalling. (1) Ligand binding induces recruitment of IMD, DREDD and dFADD to PGRP-LC. (2) Recruitment of DIAP2 targets DREDD for K63-linked ubiquitylation. (3) This allows Ub-mediated aggregation, activation and processing of DIAP2-modified DREDD. (4) Active DREDD subsequently cleaves IMD, which exposes an IBM at the neo-amino-terminus of cleaved IMD. The IBM of IMD subsequently binds to the BIR2/3 of DIAP2. (5) This provides DIAP2 with an additional contact point within the signalling complex, reinforcing complex stability and permitting DIAP2-mediated ubiquitylation of IMD, and quite possibly other components of the signalling complex. (6) The Ub chains on IMD and DREDD may serve as scaffolds for the recruitment of dTAK1 and IKK. Since IKK can bind to Relish, as evidenced by its ability to phosphorylate Relish, IKK might also bring Relish into close proximity of ubiquitylated and active DREDD, allowing DREDD-mediated proteolysis of Relish. (7) The proximity to the signalling complex also allows phospho-mediated activation of Relish. (8) Subsequently, modified Relish translocates to the nucleus where (9) it drives expression of AMP target genes.

Materials and methods

Constructs

DIAP2 constructs were cloned into pAc5, pMT or pMT-IZ without tag or with C-terminal GST, HA or V5 tags. HA or V5 C-terminal tagged DREDD constructs were cloned into pMT. dFADD was cloned into pMT vector with C-terminal HA or Myc-tag. IMD was cloned into pMT or pAc5 and expressed as full-length protein (for Figure 1G) or alternatively as Ub-fusion protein to generate IMD exposing an N-terminal IBM (used in Figure 2I), corresponding to the DREDD-cleaved form of IMD (aa 31–273) (Paquette *et al*, 2010). Relish and Ub were cloned into pAc5 with N-terminal FLAG, HA or HIS-tag, respectively. Point mutants were generated by site-directed mutagenesis (Stratagene) according to the manufacturer's instructions. All constructs were cloned by PCR and verified by DNA sequencing.

Fly stocks

The following fly strains were used: *Canton^S*, *w¹¹¹⁸*, *yw*, *DD1* (wild-type) (Jung *et al*, 2001), *Diap2^{7c}* (Leulier *et al*, 2006), *Dredd^{D44}*, *Dredd^{B118}*, *Dredd^{L23}* (Leulier *et al*, 2000), *Tak1¹* (Vidal *et al*, 2001), *Relish^{E20}* (Hedengren *et al*, 1999), *c564-GAL4* (fat-body-specific driver), and *Da-GAL4*. The UAS-*Diap2* constructs encoding WT or mutant *Diap2* were generated by cloning the *Diap2* open reading frame in *EcoRI/XhoI*-digested pUAST or FlyC31 pUASTattB vector. *w¹¹¹⁸* flies or the landing platform fly strain 24749 were used to generate UAS-*Diap2* transgenic flies. *Drosophila* stocks and crosses were maintained at 25°C, unless stated otherwise.

Bacterial strains, infection experiments and survival analysis

In all, 2- to 4-day-old adult flies were used for infection experiments. Microbial septic injuries were performed by pricking adult flies in the lateral part of the thorax with a thin needle previously dipped into a concentrated (optical density of 200) culture of *Erwinia carotovora carotovora* 15 (*Ecc15*) or *Escherichia coli*. *M. luteus* was used as Gram-positive bacteria. Following septic injury, flies were incubated at 29°C and examined at different time points to monitor survival. The infected flies were transferred to fresh vials daily. For RT-qPCR analysis, adult flies were injected using the Nanoject II (Drummond Scientific). In all, 13.8 nl of cultured *Ecc15* bacterial solution (optical density of 300) was injected and flies were incubated at 25°C. The experiments were performed in triplicates using 20–40 flies for each genotype.

Quantitative RT-PCR

For quantitative analysis of *attacin D*, *dipterin*, *puckered* and *rp49* mRNA expression, RNA was extracted from whole animals using the RNeasy Mini kit (QIAGEN). cDNAs were synthesized using Quantitect Reverse transcription kit (QIAGEN) and quantitative PCR was performed using MesaBlue qPCR mastermix Plus and SYBR green (Eurogentech). SYBR green analysis was performed on a Lightcycler (Roche Diagnostics). The amount of mRNA detected was normalized to control *rp49* mRNA values. We used normalized data to quantify the relative levels of a given mRNA according to cycling threshold analysis.

Tissue culture and treatments

Drosophila S2 cells were cultured as described previously (Tenev *et al*, 2005) and transfected with Effectene (QIAGEN) according to the manufacturer's instructions. Peptidoglycan (DAP-PGN) isolated from *E. coli* was used at a concentration of 1 µg/ml for the indicated time points. Cycloheximide (Sigma) was used at a concentration of 50 µg/ml.

Immunoprecipitation and immunoblot analysis

Immunoprecipitation assays were performed as described previously (Tenev *et al*, 2005). In brief, cells were lysed in a buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol and 1 mM EDTA, and extracts were spun at 14 000 r.p.m. for 10 min at 4°C. HA-, or V5-tagged proteins were purified using antibody-IgG-coupled agarose beads (GE Healthcare, Sigma Aldrich, respectively). After 2 h incubation at 4°C, beads were washed three times in 10 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Triton X-100 and 5% glycerol. The following antibodies were used for immunoblotting: α-V5 (Serotec), α-HA (Roche), α-DIAP2 (Leulier *et al*, 2006), α-Myc (Sigma), α-Relish (in house), α-IMD (in house), α-FLAG (Sigma), α-K63-Ub, α-K48-Ub (Millipore), α-Ub (Cell Signaling), α-GST (Amersham) and α-Actin (Santa Cruz). Signals were visualized by chemiluminescence (GE Healthcare) or Odyssey Technology (LI-COR Biosciences). For total cell lysates, cells were lysed directly in Laemmli sample buffer before immunoblot analysis.

Ubiquitylation assays

For immunoprecipitation of ubiquitylated proteins, S2 cells were harvested in 200 µl of boiling 1% SDS in PBS, and the resulting lysate was heated at 100°C for 5 min. Lysates were diluted with 1 ml of 0.5% BSA, 1% Triton X-100 in PBS. DNA was sheared by

sonication, and the material centrifuged at 14 000g for 10 min. HA-tagged ubiquitylated proteins were purified from the supernatant using α-HA agarose beads. Purified proteins were washed four times with 0.5% BSA, 1% Triton X-100 in PBS and the eluate analysed by immunoblotting. For Figure 1G, HA-tagged DREDD and IMD were purified under denaturing conditions and the presence of the respective Ub chains were detected using Ub-linkage specific antibodies.

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Conflict of interest

The authors declare that they have no conflict of interest.

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