

The *Drosophila* Inhibitor of Apoptosis (IAP) DIAP2 Is Dispensable for Cell Survival, Required for the Innate Immune Response to Gram-negative Bacterial Infection, and Can Be Negatively Regulated by the Reaper/Hid/Grim Family of IAP-binding Apoptosis Inducers^{*[S]}

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Jun R. Huh⁺¹, Ian Foe[‡], Israel Muro[‡], Chun Hong Chen[‡], Jae Hong Seol[§], Soon Ji Yoo[¶], Ming Guo^{||}, Jin Mo Park^{**}, and Bruce A. Hay[‡]

From the [‡]Division of Biology, MC 156-29, California Institute of Technology, Pasadena, California 91125, the [§]Department of Biochemistry, Seoul National University, Seoul, Korea, the [¶]Department of Biology, College of Sciences, Kyung Hee University, Seoul 130-701, Korea, the ^{||}Department of Neurology, Brain Research Institute, The David Geffen School of Medicine, UCLA, Los Angeles, California 90095, and ^{**}Cutaneous Biology Research Center, Massachusetts General Hospital, Harvard Medical School, Charlestown, Massachusetts 02129

Many inhibitor of apoptosis (IAP) family proteins inhibit apoptosis. IAPs contain N-terminal baculovirus IAP repeat domains and a C-terminal RING ubiquitin ligase domain. *Drosophila* IAP DIAP1 is essential for the survival of many cells, protecting them from apoptosis by inhibiting active caspases. Apoptosis initiates when proteins such as Reaper, Hid, and Grim bind a surface groove in DIAP1 baculovirus IAP repeat domains via an N-terminal IAP-binding motif. This evolutionarily conserved interaction disrupts DIAP1-caspase interactions, unleashing apoptosis-inducing caspase activity. A second *Drosophila* IAP, DIAP2, also binds Rpr and Hid and inhibits apoptosis in multiple contexts when overexpressed. However, due to a lack of mutants, little is known about the normal functions of DIAP2. We report the generation of *diap2* null mutants. These flies are viable and show no defects in developmental or stress-induced apoptosis. Instead, DIAP2 is required for the innate immune response to Gram-negative bacterial infection. DIAP2 promotes cytoplasmic cleavage and nuclear translocation of the NF- κ B homolog Relish, and this requires the DIAP2 RING domain. Increasing the genetic dose of *diap2* results in an increased immune response, whereas expression of Rpr or Hid results in down-regulation of DIAP2 protein levels. Together these observations suggest that DIAP2 can regulate immune signaling in a dose-dependent manner, and this can be regulated by IBM-containing proteins. Therefore, *diap2* may identify a point of convergence between apoptosis and immune signaling pathways.

The inhibitor of apoptosis (IAP)² family proteins contain one or more repeats of an ~70-amino acid motif known as a baculovirus IAP repeat (BIR), which mediates interactions with multiple death activators and plays an essential role in the ability of these proteins to inhibit cell death. IAPs also contain a C-terminal RING E3 ubiquitin ligase domain that can target bound proteins, as well as the IAP itself, for ubiquitination and in some cases degradation (1). The *Drosophila* genome encodes two BIR and RING domain-containing IAP family members, DIAP1 and DIAP2, and ectopic expression of either protein inhibits apoptosis (2–4). DIAP1 is required continuously in many cells to inhibit the apical caspase Dronc and effector caspases activated by Dronc, such as Drice (5, 6). Critical interactions between caspases and DIAP1 are mediated by a surface groove within each DIAP1 BIR domain and short IAP-binding motifs (IBM) present in Dronc or Drice (7–9). Apoptosis in the fly can be induced by expression of proteins such as Reaper, Hid, Grim, Sickie, and Jafrac2 (the RHG proteins). Each of these proteins contains an N-terminal IBM that mediates competitive binding to DIAP1 through the same BIR surface grooves that are required for DIAP1-caspase interactions (8, 10). RHG proteins can also promote ubiquitin-dependent degradation of DIAP1. Both activities have the effect of liberating active caspases, resulting in apoptosis (6). IAPs that inhibit apoptosis, as well as inhibitory RHG counterparts, are also found in mammals (1, 3).

Several observations have suggested that DIAP2 might also be an important apoptosis inhibitor. DIAP2 can bind Rpr and Hid (11, 12) and the caspases Drice (4) and Strica (13). Overexpression of DIAP2 can also inhibit Rpr- and Hid-dependent apoptosis, developmental apoptosis in the eye (2), as well as apoptosis associated with decreased levels of *diap1* (4). In addition, RNAi-mediated knockdown of DIAP2 in the S2 cell line

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[S] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. 1–4.

¹ To whom correspondence should be addressed: Division of Biology, MC 156-29, California Institute of Technology, 1200 East California Blvd., Pasadena, CA 91125. Tel.: 626-395-3399; Fax: 626-449-0756; E-mail: haybruce@caltech.edu.

² The abbreviations used are: IAP, inhibitor of apoptosis; BIR, baculovirus IAP repeat; IBM, IAP-binding motif; E3, ubiquitin-protein isopeptide ligase; RT, reverse transcription; RNAi, RNA interference; dsRNA, double-stranded RNA; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; IKK, I κ B kinase; GFP, green fluorescent protein.

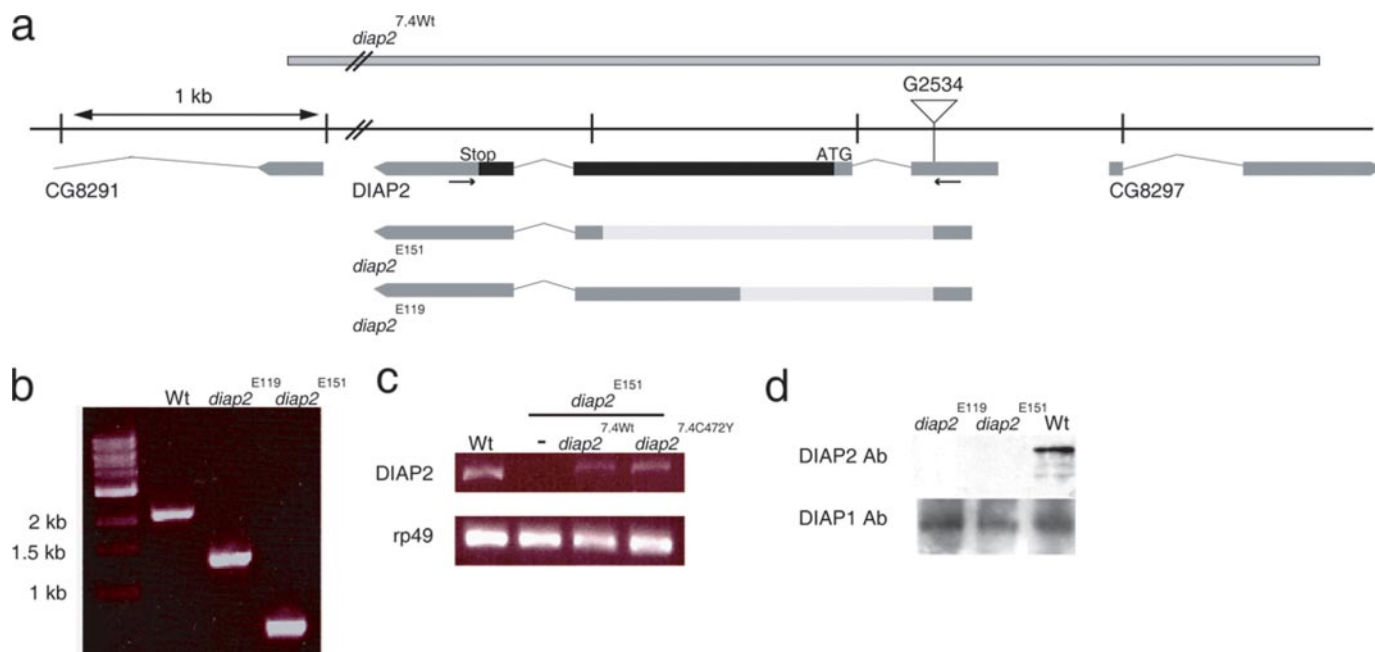


FIGURE 1. *diap2^{E119}* and *diap2^{E151}* are *diap2* null mutants. *a*, schematic of the *diap2* locus and surrounding region. Sequences deleted in *diap2^{E119}* and *diap2^{E151}* are indicated. *diap2^{E119}* removes 840 bp from the *diap2* locus, whereas *diap2^{E151}* removes 1,460 bp. Neither deletion affects neighboring genes. The 7.4-kb genomic fragment used to rescue the *diap2* mutant phenotypes is indicated. G2534 is the original P element inserted in the *diap2* 5'-untranslated region. Arrows indicate primer pairs used for genomic PCR. *b*, genomic PCR of homozygous *diap2* deletion mutants using the primer pairs indicated. *c*, results of RT-PCRs with primers specific for the DIAP open reading frame in different genetic backgrounds. Open reading frame sequences are expressed in wild type (Wt) flies and in *diap2^{E151}* flies carrying *diap2^{7.4Wt}* or the RING-mutated rescue *diap2^{7.4C472Y}* construct but not in *diap2^{E151}* alone. RP49 expression is used as a control. *d*, Western blot of adult flies probed with DIAP1 and DIAP2 antibodies. DIAP2, but not DIAP1, is absent in *diap2^{E119}* and *diap2^{E151}* flies.

has been reported to result in increased susceptibility to stress-induced apoptosis (14). RNAi of *diap2* in larvae and pupae has generated conflicting results. One group reported no effect of heat shock-induced expression of *diap2* dsRNA on developmental cell death or viability (15), and second group reported organismal lethality in response to ubiquitous *diap2* dsRNA expression (16). Finally, several RNAi-based studies in S2 cells have also provided evidence that DIAP2 is required for the innate immune response to Gram-negative bacteria infection (16, 17). However, these studies came to conflicting conclusions regarding the site of action for DIAP2 and involved primarily use of a single long time immortalized cell line.

Activation of apoptosis and the innate immune response both require tight control, and deregulation can lead to cancer, neurodegenerative diseases, immunodeficiency, or chronic inflammation (18, 19). Molecular links between apoptotic and immune signaling pathways may exist (20–23), and DIAP2 is an interesting candidate protein to act as a point of convergence. Questions regarding the role of DIAP2 as a cell death and/or immune regulator can best be approached through the characterization of deletion mutant animals. In contrast with previous work, we show here that *diap2* mutants are viable and do not show increased sensitivity to a number of different cell death stimuli. Instead, DIAP2 is required for immune deficiency signaling in response to Gram-negative bacteria infection. DIAP2 promotes but is not absolutely essential for Relish cleavage. Nonetheless, loss of DIAP2 is associated with a profound defect in Relish nuclear translocation and antimicrobial peptide (AMP) expression. Together, these results suggest that DIAP2 regulates Relish function at several points. Interestingly, DIAP2 levels could be dramatically reduced in cells expressing

the apoptosis inducers Rpr or Hid, suggesting an hypothesis in which DIAP2 acts as a point of convergence between immune and apoptotic signaling. Binding of death-inducing RHG proteins may act as a safety device to prevent unwanted chronic inflammation in the context to which massive cell death occurs during development or in response to environmental stress. Other IBM proteins may regulate DIAP2-dependent immune responses in other contexts.

EXPERIMENTAL PROCEDURES

Fly Stocks—All transformants were generated using standard techniques. *w¹¹¹⁸* flies were used as wild type. The P element line used to carry out imprecise excisions in the *diap2* region, G2534, was obtained from Genexel, Korea. Excisions were carried out using standard techniques. 300 independent excision lines were characterized at the *diap2* locus by genomic PCR using the primers indicated in Fig. 1. PCR fragments amplified from *diap2* mutant genomic DNA were fully sequenced to confirm deletion boundaries. Wild type rescue (*diap2^{E151}*; *diap2^{7.4Wt}*) and RING mutant rescue (*diap2^{E151}*; *diap2^{7.4C472Y}*) flies were generated by introducing a 7.4-kb genomic fragment that contains the DIAP2 open reading frame and nearby sequences into pCaSpeR4. Transformants containing these constructs were introduced into the *diap2^{E151}* genetic background. For the wild type 7.4-kb genomic fragment, three small fragments (3.2, 1.2, and 3 kb) were amplified from the BAC clone covering the DIAP2 genomic region and ligated into pCaSpeR4 with NotI, XbaI, HpaI, and XhoI, respectively. Primers 5'-AAGGAAAAAAGCGGCCGCAATAACCGAATCGG-AAAACG-3' and 5'-CGCTCTAGATTGTAAGAGGGAG-3' were used to generate the 3.2-kb fragment, and primers 5'-

CGCTCTAGATAAGCAACGCTCG-3' and 5'-CGCGTTAA-CCCACAGCCACCTGCAATC-3' were used to generate the 1.2-kb fragment, and primers 5'-CGCGTTAACAGGAAGTTAGCTAC-3' and 5'-CCGCTCGAGGGGCTCGAATTTGATTAGC-3' were used to generate the 3-kb fragment. For the 7.4-kb genomic fragment with the RING-mutation, changing Cys → Tyr at amino acid 472, alternative primer pairs (5'-CGCTCTAGATAAGCAACGCTCG-3' and 5'-CGCGTTAA-CCCACAGCCACCTACAATC-3') were used for the synthesis of the 1.2-kb fragment. GMR-Rpr, GMR-Hid, GMR-Dronc, GMR-Dream/Strica, GMR-DIAP2, UAS-Rpr, UAS-Hid, UAS-P35, *engrailed*-Gal4, *dipterocin*-GFP, UAS-dTak1, c564-Gal4, *hs-diap1*-rnai, *dredd*^{B118}, and *imd*¹ flies have been described previously (2, 15, 20, 21, 24–27). GMR-Drice flies, carrying both the GMR-Drice P20 and GMR-Drice P10 subunits, were generated by introducing Drice large (P20) or small (P10) subunit coding regions into pGMR separately. Transformants carrying both constructs were generated by recombination. Hs-Relish^{RHD} flies were generated by introducing the Relish^{RHD} coding region into pCaSpeR-hs-act vector (15, 28). UAS-*diap1*-miRNA and UAS-*diap2*-miRNA flies were generated by replacing 21-bp sequences within the mir-6 precursor backbone that are loaded into the RISC complex with sequences complementary to *diap1* or *diap2*.³

Fly Immune Challenge—All *Drosophila* stocks were raised at 25 °C. One- to 3-day-old adult male flies were pricked with a fine glass needle dipped in an overnight live culture of *Enterobacter cloacae* or *Staphylococcus aureus*, resuspended at 20× concentration in sterile S2 cell medium.

Irradiation-induced Stress—Forty wild type or *diap2*^{E151} 3rd instar larvae were placed in 1.5-ml tubes and X-irradiated with 2000 or 4000 rads. Subsequently, they were moved to fresh vials and scored for hatching efficiency. Each experiment was repeated three times. Error bars on figures indicate the standard deviation.

Oxidative Stress—One- to 3-day-old adult males were put in vials (20 flies to a vial, total five vials), with eight filter paper disks soaked with 500 μl of 5% sucrose and 20 mM methyl viologen (paraquat). Each experiment was repeated twice.

Salt Stress Tolerance—Solid medium that contained 2.5% sucrose, 1% agar, 0.15% methyl paraben, 0.003% methylene blue, and different concentrations of NaCl (0.4 or 0.8 M) was prepared in vials. Fifty young adult males of each genotype were introduced into these vials (25 flies per vial) and scored for survival over the next 4 days. Each experiment was repeated three times.

Heat Shock Induction—Wild type and *diap2*^{E151} mutant 3rd instar larvae carrying an *hs-diap1*-dsRNA construct were transferred to 1.5-ml microcentrifuge tubes and subjected to 10, 20, or 30 min of heat treatment in a 37 °C water bath. Each experiment was repeated in triplicate. For the induction of Relish^{RHD}, adult wild type or *diap2* mutant male flies carrying Hs-Relish^{RHD} were subjected to a 30-min heat shock at 37 °C and allowed to recover for 6 h at 25 °C before total RNA was isolated to quantify levels of antimicrobial peptide gene induction.

RNA Analysis—Total RNA was isolated from whole flies using the TRIzol reagent (Invitrogen). For real time PCR analysis, cDNAs were synthesized with the Superscript III reverse transcriptase system (Invitrogen). An amount of cDNA equivalent to 0.3 μg of total RNA was subjected to 40 cycles of PCR amplification for 15 s at 95 °C and a 1-min incubation at 60 °C. The output was monitored using SYBR Green core reagents and the ABI Prism 7900 system (Applied Biosystems). All results were normalized to the levels of *rp49* mRNA. Individual primer sequences are available upon request.

Protein Analysis—For immunoblot analysis, 20 adult flies were homogenized in lysis buffer (20 mM HEPES-KOH (pH 7.6), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, and 1× protease inhibitor (Roche Applied Science)) and then subjected to SDS-PAGE. After transfer to a nitrocellulose membrane, proteins were probed with mouse antibodies raised against DIAP2 (1:50), DIAP1 (25) (1:100), the C-terminal domain of Relish (29) (1:5; a generous gift from Svenja Stöven and Dan Hultmark, Umeå University, Umeå, Sweden), and tubulin (1:1,000; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City). DIAP2 antibodies were generated in mouse using a GST-DIAP2 fusion protein as the immunogen. Conditions for immunohistochemistry and confocal microscopy were described previously (25). Antibodies were used at the following concentrations: mouse anti-P35 (25) (1:100), mouse anti-DIAP2 (1:50), and rat anti-Relish (30) (1:1000, a generous gift from Young-Joon Kim, Yonsei University, Seoul, Korea).

Caspase Activity Assay—Fifteen wild type and *diap2*^{E151} mutant adult flies were homogenized in 100 μl of lysis buffer. 30 μg of each extract was added into 100 μl of caspase activity buffer (70.4 mM HEPES (pH 7.5), 140.8 mM NaCl, 1.4 mM EDTA, 0.14% CHAPS, 14.1% sucrose, 3.5 mM dithiothreitol, 5.6% glycerol, and 0.7% Triton X-100) in the presence of 100 μM Ac-DEVD-AFC (EMD Biosciences). Caspase activity was measured at 27 °C using a fluorometric plate reader (Fmax; Molecular Devices) in the kinetic mode with excitation and emission wavelengths of 405 and 510 nm, respectively. Assays were performed in triplicate.

Gene Switch System—Flies carrying the adult fat body gene switch driver S₁₀₆ in different genetic backgrounds were placed in empty vials with eight 3MM filter paper disks overnight (20 h) (31). To induce gene expression, disks were wetted with 800 μl of 500 μM RU486 (Sigma) in 2% sucrose solution. Following immune challenge, flies were allowed to recover in normal fly food vials for 4 h prior to RNA preparation.

RESULTS

Generation of *diap2* Deletion mutants; *diap2* Is Dispensable for Development—To examine DIAP2 (CG8293) functions *in vivo*, we generated two *diap2* deletion mutants, *diap2*^{E151} and *diap2*^{E119}, via imprecise excision of the P element G2534, which is located 100 bp upstream of the *diap2* start codon (Fig. 1a). *diap2*^{E119} deletes ~840 bp and *diap2*^{E151} ~1,460 bp downstream of the original P element insertion site, without affecting neighboring genes (Fig. 1b). RT-PCR and Western blot analysis failed to detect full-length *diap2* mRNA or protein in either mutant (Fig. 1, c and d, and Fig. 5a). Truncated *diap2* tran-

³ C. Chen and B. Hay, manuscript in preparation.

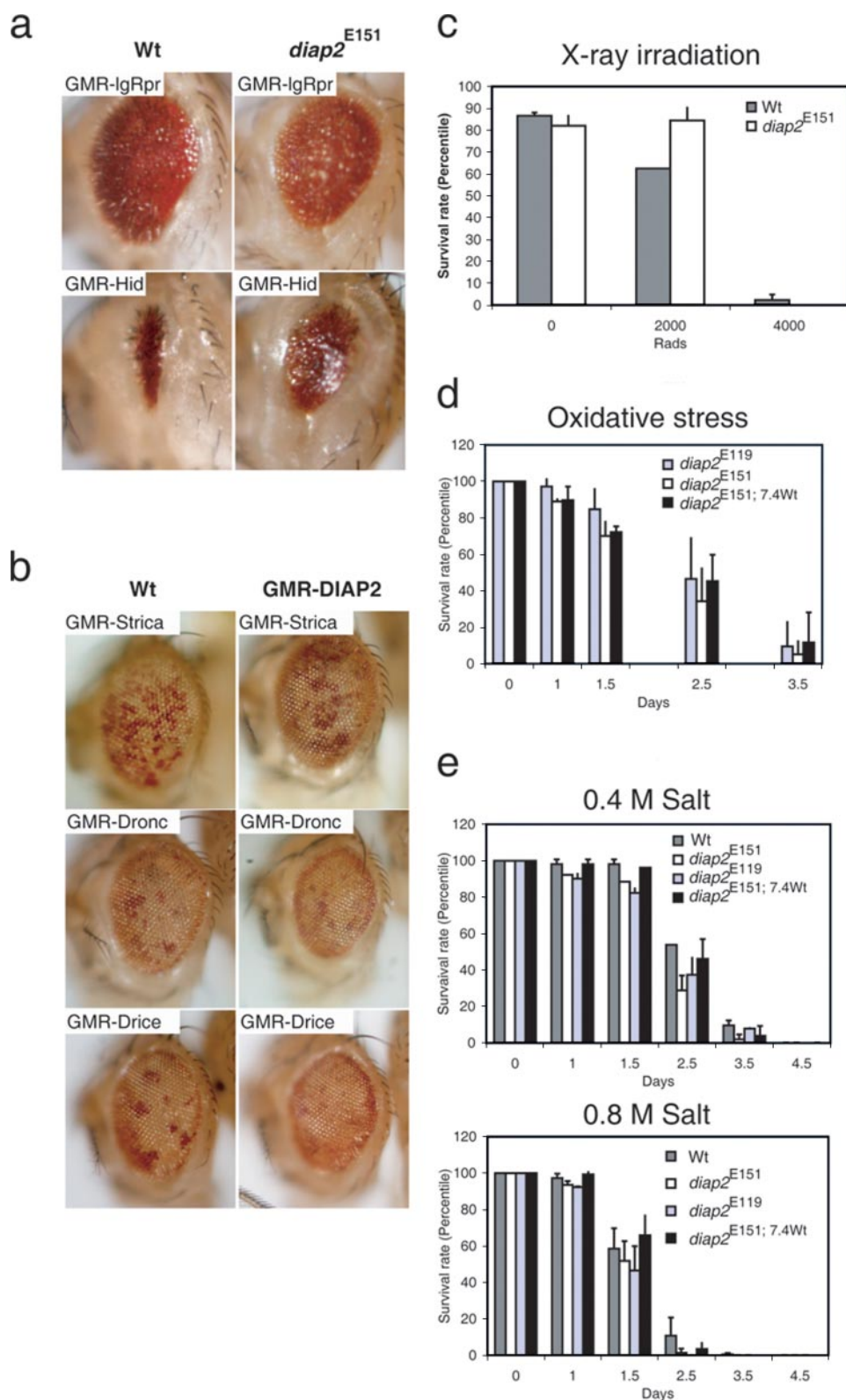


FIGURE 2. DIAP2 fails to inhibit multiple caspases and does not act as a significant inhibitor of death induced by expression of Rpr-, Hid-, X-irradiation, the free radical generator paraquat, or salt stress. *a*, removal of *diap2* fails to enhance cell death induced by Rpr or Hid overexpression. Wt, wild type. Adult eyes of various genotypes are shown: GMR-IgRpr/+; $E151/E151$; GMR-IgRpr/+; GMR-Hid/+; and $E151/E151$; GMR-Hid/+. *b*, DIAP2 overexpression in the fly eyes does not suppress cell death induced by expression of Strica, Dronc, or Drice. Genotypes are as follows: GMR-Strica/+ and GMR-Strica/GMR-DIAP2 (top row), GMR-Dronc/+ and GMR-Dronc/GMR-DIAP2 (middle row), GMR-DriceP20, GMR-DriceP10/+ and GMR-DriceP20, GMR-DriceP10/GMR-DIAP2 (bottom row). Loss of *diap2* does not confer sensitivity to x-ray induced stress (*c*), the free radical generator paraquat (*d*), or high salt (*e*).

scripts are detectable in *diap2*^{E151} and *diap2*^{E119} animals. However, these transcripts are unlikely to generate functional proteins because multiple in-frame start and stop codons precede the remaining C-terminal *diap2* coding sequences. Together these results suggest that *diap2*^{E151} and *diap2*^{E119} represent null alleles. Introduction of a 7.4-kb genomic fragment encoding wild type *diap2* (*diap2*^{7.4Wt}) or a point mutant of *diap2* with a mutation in the RING domain (*diap2*^{7.4C472Y}) into the *diap2*^{E151} mutant background resulted in expression of a full-length *diap2* transcript (Fig. 1, *a* and *c*) and protein (Fig. 5*a*). In contrast to mutations in *diap1* (alleles of *th*), which are lethal early in embryogenesis, animals homozygous for *diap2*^{E151} and *diap2*^{E119} are viable, healthy, and fertile. Therefore, *diap2* is dispensable for most, if not all, developmental cell death.

DIAP2 Does Not Inhibit Multiple Caspases, and Its Removal Does Not Sensitize Flies to Multiple Apoptotic Stimuli—In accordance with previous observations, overexpression of DIAP2 in the developing eye suppressed cell death induced by RHG protein overexpression (supplemental Fig. 1*a*) (2). However, removing *diap2* had little if any effect on RHG overexpression-induced apoptosis in the eye (Fig. 2*a*). Expression of *diap2* also failed to suppress death induced by expression of Dronc, Drice, Strica (Fig. 2*b*), and Dcp-1 (data not shown), indicating that DIAP2 does not inhibit the activity of multiple caspases, despite its structural and sequence similarity to DIAP1. This failure is particularly noteworthy in the case of Dronc and Drice, which are the central mediators of RHG-dependent cell death in the fly eye (32–36). These observations call into question the idea that DIAP2 normally functions as a cell death inhibitor. To explore this issue further, we exposed animals lacking DIAP2 to several stresses that lead ultimately to cell and organismal death as follows: X-irradiation, the free radical

Drosophila IAP2 Mutant Analysis

generator paraquat; and high salt, an activator of the p38 MAPK pathway (24, 37). If the physiological role of *diap2* is to prevent cell death, removal of *diap2* should render animals more sensitive to these stresses. However, compared with wild type or *diap2*^{E151} flies carrying the *diap2*^{7.4Wt} rescue construct, *diap2*^{E151} mutant flies did not exhibit increased sensitivity to any of these stresses (Fig. 2, c–e). Finally, we tested the hypothesis that *diap2* functions as a minor apoptosis inhibitor whose activity is only required in the absence of a major cell death inhibitor such as *diap1*. We transiently knocked down *diap1* levels in the third instar larvae using RNAi, in the presence or absence of *diap2*, and scored for larval survival (see “Materials and Methods”). Decreasing *diap1* in the presence of *diap2* resulted in almost complete lethality (<10% survival), whereas in the absence of *diap2* many more larvae survived (>35% survival) (supplemental Fig. 2a). Therefore, although our results do not exclude the possibility that *diap2* plays an anti-apoptotic role in some context, they strongly suggest that *diap2* normally plays little, if any role, in the regulation of the canonical caspase-dependent apoptosis pathway.

DIAP2 Is Required to Defend against Gram-negative Bacterial Infection—Bacterial infection, and the response to bacterial infection, constitutes a form of organismal and tissue stress that channels limited resources into a range of new tasks (19). Most notably, infection results in the activation of a multipronged immune response that involves phagocytosis, a melanin-producing phenol oxidase reaction, and a humoral response that leads to the expression of anti-microbial peptides and activation of the JNK signaling pathway (38–40). Two pathways, the Toll and IMD, mediate induction of the humoral response. In the Toll pathway, infection by Gram-positive bacteria or fungi triggers activation of the Toll receptor, which leads ultimately to the expression of AMPs such as drosomycin (38, 41, 42). The IMD pathway is activated in response to infection by Gram-negative bacteria when receptors such as PGRP-LC/ird7 bind Gram-negative peptidoglycan (26, 39, 43). PGRP-LC recruits IMD, which activates two downstream pathways. In one branch, IMD binds to dFADD (44), which recruits the caspase DREDD (45, 46). DREDD promotes (directly or indirectly) the cleavage of the REL/NF- κ B family member Relish in the cytoplasm (27, 47, 48). Once liberated from REL-49, the C-terminal κ B-like inhibitory domain, the N-terminal REL-68 fragment, which contains the DNA binding Rel homology domain, translocates to the nucleus where it drives the expression of AMPs such as dipterin, attacin, cecropin, and defensin (49). IMD also activates, through unknown mechanisms, the MAPK kinase kinase dTAK1 (50–52). dTAK1 is required to activate (perhaps through direct phosphorylation) the *Drosophila* signalosome equivalent (50, 52), which consists of homologs of the kinase IKK β (ird5), and an accessory subunit IKK γ (kenny) (53–55). The activated IKK complex phosphorylates Relish (48, 53), and this phosphorylation is required for Dredd-dependent cleavage of Relish (48).

When exposed to the Gram-positive bacteria *S. aureus*, wild type, *diap2*^{E151}, and *diap2*^{E119} flies died at similar rates (Fig. 3a). This suggests that *diap2* does not function as a general inhibitor of organismal death in response to bacterial infection or as an essential component of the Toll pathway. In contrast,

when exposed to the Gram-negative bacteria *E. cloacae* animals lacking *diap2* were much more sensitive than wild type flies. In fact, *diap2*^{E151} and *diap2*^{E119} adults died at a rate similar to that of animals lacking IMD (Fig. 3b), which is essential for the Relish-dependent production of Gram-negative-specific AMPs. Importantly, these phenotypes were completely suppressed in the presence of the wild type *diap2*^{7.4Wt} rescue transgene, demonstrating that they are due to loss of *diap2*. *diap2*^{E151} and *diap2*^{7.4Wt} flies actually showed greater resistance to infection than wild type flies (Fig. 3b), although they had decreased longevity in the absence of immune stimulation.⁴ The basis for this increased resistance is unknown, but it is interesting to speculate that it might reflect the fact that endogenous *diap2* levels are set so as to optimize an evolutionary compromise between immune resistance and longevity, a feature not retained with our transgene insertion. Finally, survival was not rescued in the presence of the *diap2* rescue transgene containing a point mutation in the RING domain, *diap2*^{7.AC472Y}, even though expression levels of wild type and mutant transgene mRNA (Fig. 1c) and protein (Fig. 3c and Fig. 5a) were similar.

To determine whether *diap2* regulates the production of AMPs, we infected wandering third instar larvae carrying a dipterin-GFP reporter construct (27) with *E. cloacae*. Wild type animals, as well as *diap2*^{E151} animals carrying a copy of the *diap2*^{7.4Wt} rescue transgene, showed strong GFP expression in response to infection (Fig. 3d). In contrast, GFP was undetectable in *diap2*^{E151} and *diap2*^{E119} animals, suggesting that *diap2* mutants are unable to mount an effective immune response. We also examined AMP expression using quantitative RT-PCR. When challenged with *E. cloacae*, wild type adult flies exhibited robust induction of attacin and dipterin. In contrast, the increases in attacin and dipterin were dramatically suppressed in *diap2*^{E151} and *diap2*^{E119} flies, even after 12 h (Fig. 3e). Importantly, these phenotypes were significantly suppressed in the presence of *diap2*^{7.4Wt} but not *diap2*^{7.AC472Y} (Fig. 4a). We note that the levels of the *diap2*^{7.4Wt} transgene were lower than those of endogenous *diap2* (Fig. 1c) and that dipterin expression levels were rescued to a somewhat lesser extent than those of attacin (Fig. 4a). This may simply indicate that the attacin promoter contains cis elements that buffer changes in the concentration of processed Relish whereas the dipterin promoter drives transcription in a manner directly proportional to the level of Relish expression, a question that requires further exploration. In any case, animals lacking *diap2* also failed to express two other Gram-negative-specific AMPs, cecropin and defensin, in response to bacterial challenge, suggesting a general failure to induce IMD-dependent antimicrobial peptide expression (Fig. 4b). In contrast to these observations with *diap2* mutants, when the number of copies of wild type *diap2* was increased from two (the endogenous chromosomal copies) to six (+ four copies of *diap2*^{7.4Wt}), the levels of antimicrobial peptide expression in response to immune challenge underwent a modest increase (supplemental Fig. 2d). This observation is potentially interesting because it suggests that regulation of *diap2* levels may serve to control the intensity and

⁴ J. R. Huh, unpublished observations.

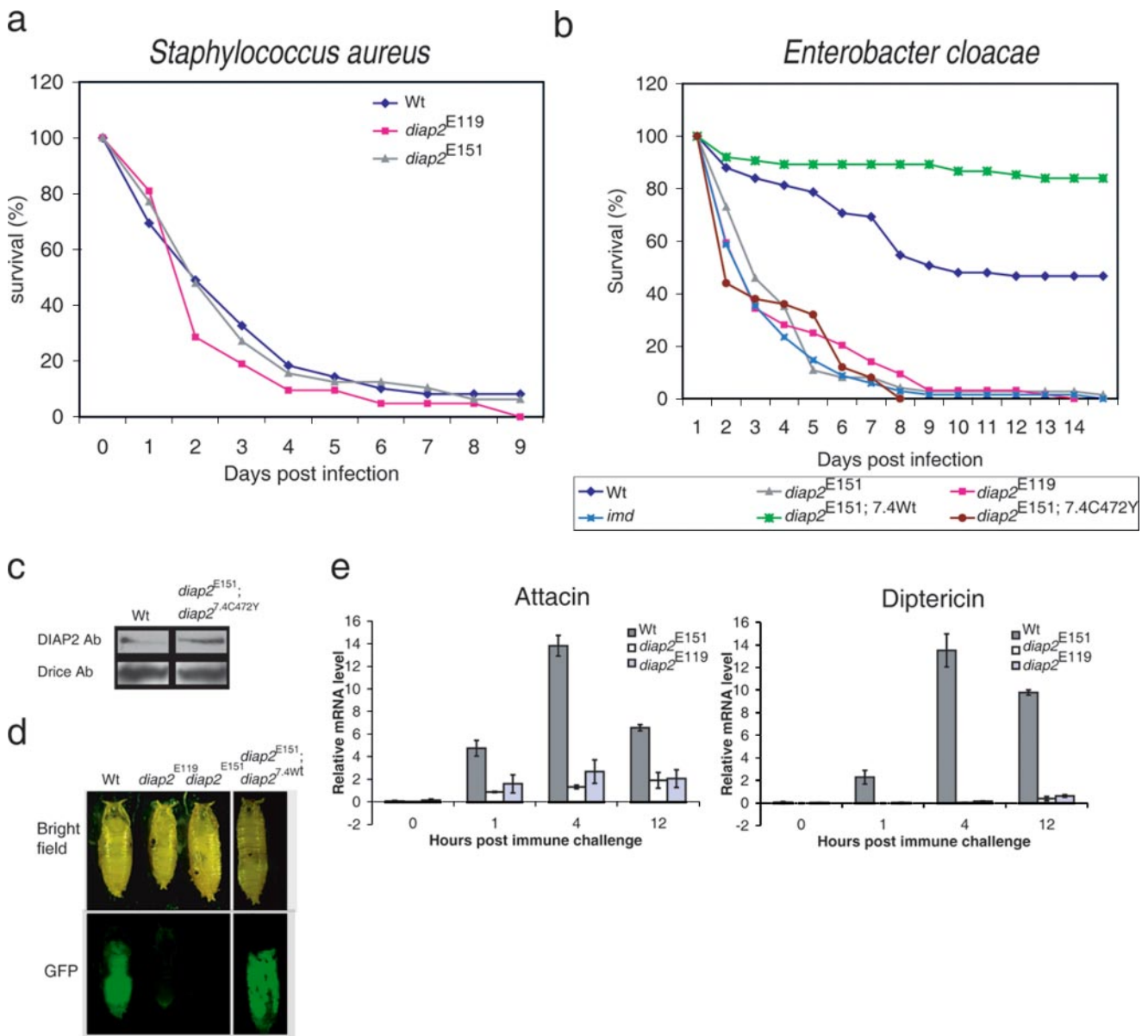


FIGURE 3. DIAP2 is required for survival and antimicrobial peptide induction following infection by Gram-negative bacteria. Fifty adult males of the indicated genotypes were immune challenged with *S. aureus* (a) or *E. cloacae* (b), and survival was monitored on a daily basis. Surviving flies were counted up to 9 or 15 days after infection, respectively. The results shown are representative of two (a) or three (b) independent experiments. *Wt*, wild type. *c*, Western blot of adult flies probed with DIAP2 and Drice antibodies. Similar level of DIAP2 are expressed in wild type flies and *diap2*^{E151} flies carrying the *diap2*^{7.4C472Y} rescue construct. *d*, Gram-negative bacterial infection induces the expression of a *dipterucin*-GFP reporter gene in the fat body of wild type and *diap2*^{E151}; *diap2*^{7.4Wt} flies but not in *diap2*^{E151} or *diap2*^{E119} mutants. *e*, real time PCR analysis of attacin and dipterucin transcript levels at different time points in wild type (gray bars), *diap2*^{E151} (white bars), or *diap2*^{E119} (light gray bars) adult flies after immune challenges with *E. cloacae*. Attacin and dipterucin expression are strongly suppressed in flies lacking *diap2*. Error bars represent the standard deviation of three independent experiments.

kinetics of the immune response. To localize the site of *diap2* action, we used micro-RNA-based RNAi⁵ to reduce *diap2* levels specifically in the adult fat body, the insect analog of the mammalian liver, and the major site of AMP synthesis. As shown in Fig. 4c, this treatment phenocopied the immune defects observed in *diap2*^{E151} and *diap2*^{E119} animals, thereby identifying the fat body as a major site of the *diap2* function.

The lack of AMP expression in *diap2* mutants could be due to death, degeneration, or altered cell fate in the fat body. Several observations suggest this is unlikely. First, apoptotic effec-

tor caspase activity (cleavage of the substrate DEVD) was not increased in *diap2* mutants before or after immune challenge (supplemental Fig. 2b). Second, fat body morphology in *diap2*^{E151} larvae (Fig. 5b) or adults (not shown) was indistinguishable from that of comparably staged wild type animals. Third, the expression of drosomycin, which is induced in the fat body in response to fungal or Gram-positive bacterial infections, was not significantly diminished in *diap2* mutants (Fig. 4d, upper panel). Finally, Gram-negative bacterial infection results in the TAK-dependent induction of the JNK pathway immediate-early response genes *puckered* and CG13482 in the fat body (28, 51). However, the levels of *puckered* (Fig. 4d, lower

⁵ C. Chen and B. Hay, manuscript in preparation.

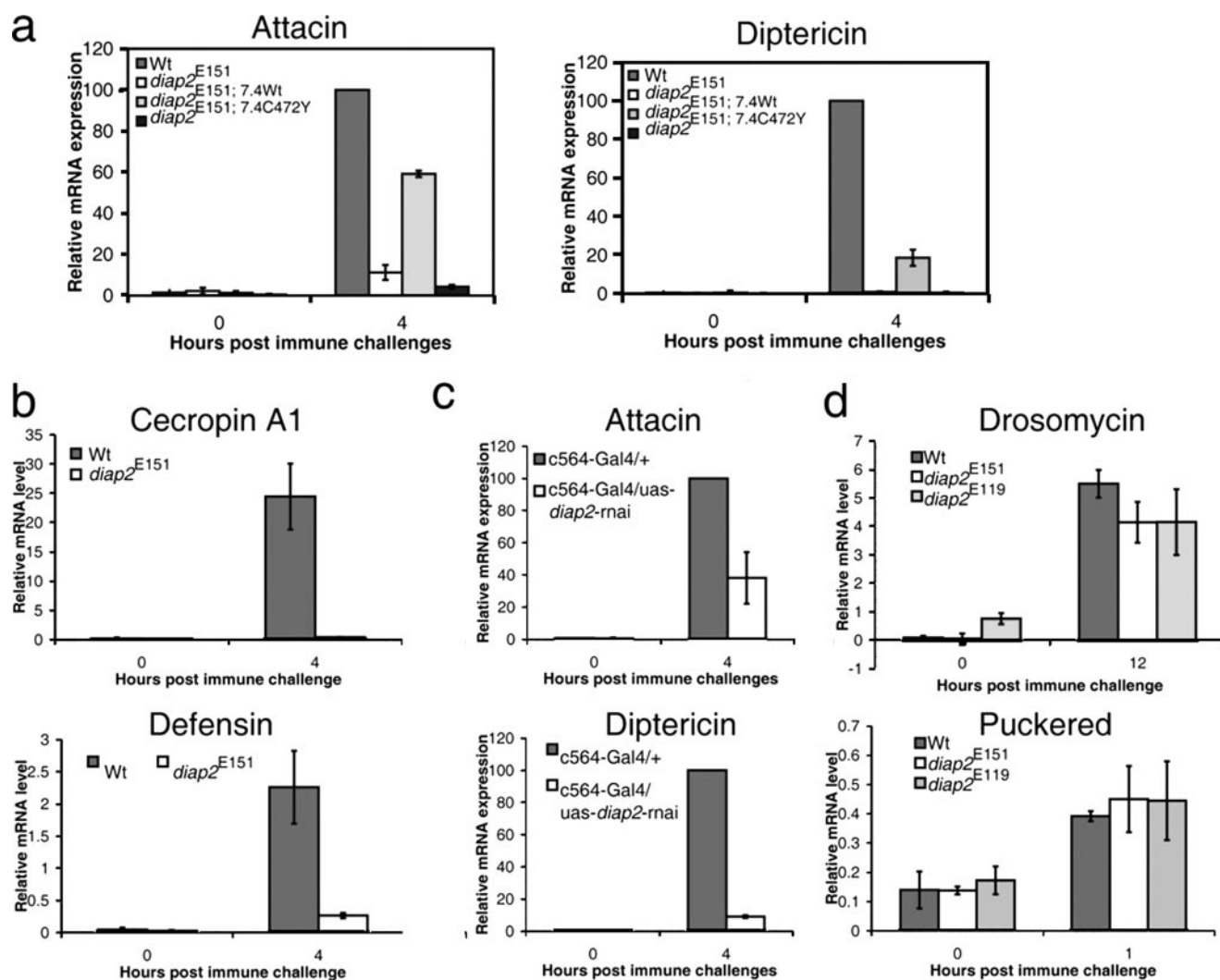


FIGURE 4. Expression of IMD-dependent antimicrobial peptides but not drosomycin, Toll pathway-dependent antimicrobial peptide, or *puckered*, a JNK target gene, are dramatically reduced in animals lacking *diap2*. *a*, attacin and dipteracin expression in response to immune challenge are strongly suppressed in *diap2^{E151}* flies, and these responses are restored in the presence of *diap2^{7.4Wt}*, but not *diap2^{7.4C472Y}*. Real time PCR analysis of attacin and dipteracin transcripts in wild type (gray bars), *diap2^{E151}* (white bars), *diap2^{E151}; diap2^{7.4Wt}* (light gray bars), or *diap2^{E151}; diap2^{7.4C472Y}* (black bars) adult flies following immune challenge. Error bars represent standard deviation of three independent measurements. *b*, real time PCR analysis of cecropin A1 and defensin transcript levels in wild type (gray bars) or *diap2^{E151}* (white bars) adult flies after infections with *E. cloacae*. Data represent mean \pm S.D. of three experiments. *c*, down-regulation of *diap2* in the fat body using micro-RNA-dependent RNAi suppresses attacin and dipteracin expression. The c564-Gal4 line expresses the transcriptional activator Gal4 in the adult fat body. This was used in conjunction with UAS-*diap2*-miRNA to target the *diap2* mRNA for degradation. Real time PCR analysis of attacin and dipteracin transcript levels in adult flies carrying c564-Gal4/+; dipt-GFP/+ (gray bars) or c564-Gal4/uas-*diap2*-miRNA; dipt-GFP/+ (white bars) after immune challenges with *E. cloacae*. Data represent mean \pm S.D. of three experiments. *d*, real time PCR of drosomycin and Puckered transcript levels in wild type (gray bars), *diap2^{E151}* (white bars), or *diap2^{E119}* (light gray bars) adult flies after immune challenge with *E. cloacae*. Data represent mean \pm S.D. of three experiments.

panel) and CG13482 (supplemental Fig. 2c) expression induced by immune challenge were similar in wild type, *diap2^{E151}*, and *diap2^{E119}* flies. Altogether, these observations argue that the immune defects in *diap2* mutants are not due to nonspecific effects on the fat body, such as increased cell death, and that DIAP2 is not required for the Toll-dependent immune response or Tak1-dependent JNK activation (28, 30, 51, 52, 56). Instead they suggest that the primary function of *diap2* is as an essential component of the humoral response to Gram-negative bacterial infection.

DIAP2, Including Its RING Domain, Is Required for Relish Function in the IMD Pathway—Activation of the IMD-dependent immune response ultimately leads to Dredd-dependent cleavage of the NF- κ B-like transcription factor Relish (27, 47,

48). The resulting cleaved N-terminal product, Relish^{RHD} (Rel homology domain), translocates into the nucleus and functions as a transcription activator for the induction of various AMP genes (49). Since *diap2^{E151}* animals lack IMD-dependent immune responses but have normal Toll-dependent signaling, we examined the fate of Relish in response to immune activation using an antibody that recognizes the C-terminal half of Relish (29). In unchallenged wild type animals, Relish was found mostly in the precursor form, although small amounts of processed Relish could also be observed (Fig. 5a, left panel). Relish processing was greatly increased post-challenge in wild type flies but not in flies lacking Dredd or Imd, which are required for challenge-dependent Relish cleavage. Flies lacking *diap2* showed a low level of challenge-independent Relish processing,

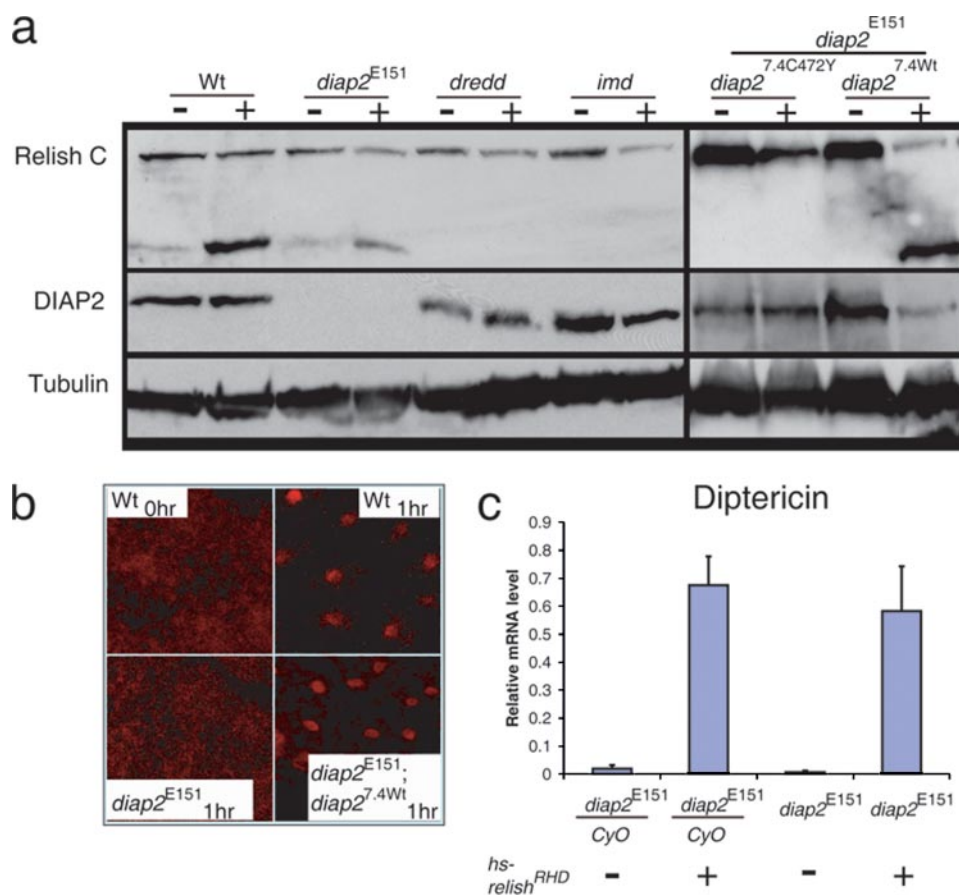


FIGURE 5. DIAP2 promotes cleavage and nuclear translocation of the NF- κ B transcription factor Relish in response to immune challenge. *a*, immunoblots of protein extracts (protein amount equivalent to three adult male flies) from untreated (–) and immune-challenged (heat-killed *Escherichia coli*) (+) flies of various genotypes, probed sequentially with anti-Relish C-terminal, anti-DIAP2, and anti-tubulin antibodies. The left and right panels are from different gels but are from the same experiment. In wild type and *diap2^{E151}, diap2^{7.4Wt}* flies, a 49-kDa Relish C-terminal fragment is present 1 h after immune challenge as a result of endoproteolytic processing. Cleavage of Relish is not observed in *dredd*, *imd*, and *diap2^{E151}* flies carrying *diap2^{7.4C472Y}*. As with wild type flies, *diap2^{E151}* flies show low levels of the C-terminal Relish cleavage product prior to immune challenge. However, in contrast to wild type flies, these levels increase modestly 1 h post-challenge. *b*, immunostaining of endogenous Relish in the 3rd instar larval fat bodies of various genotypes. Wild type fat body before (upper left) and 1 h after (upper right) immune challenge with *E. cloacae*. Relish is not localized to fat body nuclei in immune-challenged *diap2^{E151}* flies (lower left), but nuclear localization is restored in *diap2^{E151}* flies carrying *diap2^{7.4Wt}*. Lower right, *c*, heat shock-induced expression of Relish^{RHD} results in immune challenge-independent expression of diptericin in *diap2^{E151}* heterozygotes and homozygotes.

similar to that observed in unchallenged wild type flies. However, they showed only a modest increase in Relish cleavage in response to immune challenge. Importantly, high levels of challenge-dependent Relish cleavage were restored in the presence of the *diap2* wild type rescue transgene (*diap2^{7.4Wt}*) but not in the presence of the Ring mutant form (*diap2^{7.4C472Y}*). Together, these observations indicate that *diap2* is required to bring about normal levels of Relish cleavage and that this requires DIAP2 RING function. However, they also indicate that some challenge-independent, as well as challenge-dependent Relish cleavage (or stabilization of the C-terminal Relish cleavage product), occurs in the absence of *diap2*. Nuclear translocation of Relish^{RHD}, which occurs rapidly following immune stimulation in wild type animals, was undetectable in *diap2^{E151}* animals (Fig. 5*b*). As with Relish cleavage, the defect in Relish nuclear translocation was eliminated in the presence of the *diap2* genomic rescue transgene (Fig. 5, *a* and *b*).

of cleaved Relish (Relish^{RHD}), in the *diap2^{E151}* background, was sufficient to activate AMP expression. Indeed, heat shock-driven expression of Relish^{RHD} resulted in levels of diptericin and attacin expression in *diap2^{E151}* homozygous animals similar to those observed in *diap2^{E151}* heterozygotes (Fig. 5*c* and data not shown). This result indicates that DIAP2 is not absolutely required for Relish function post-cleavage. However, we note that the levels of cleaved Relish induced in the fat body in these experiments may be higher than in wild type and that cleaved Relish may normally be modified and/or function as a part of a complex, nuclear translocation, and/or function of which may require interaction with DIAP2. Given these uncertainties, and the observation that while loss of *diap2* reduces Relish cleavage it causes a more profound defect in AMP production, it remains possible that DIAP2 also has important roles downstream of Relish cleavage, as recently suggested by others (16). In either case, our observations demonstrate that

The *diap2^{7.4C472Y}* rescue transgene contains a point mutation in the RING domain. Mutation of the corresponding residue in DIAP1 results in a loss of DIAP1 E3 ligase activity (25). As noted above, *diap2^{E151}* and *diap2^{7.4C472Y}* flies were as sensitive to *E. cloacae* infection as *diap2^{E151}* flies and failed to induce attacin and diptericin expression in response to infection (Figs. 3*b* and 4*a*). As shown in Fig. 5*a*, Relish processing was largely blocked in these animals. These results suggest the DIAP2 RING, perhaps functioning as an E3 ligase, is required for Relish processing or other aspects of Relish function. As a further test of this hypothesis, we introduced the *diap2^{7.4C472Y}* genomic rescue fragment, which expresses wild type levels of *diap2^{CA72Y}* (Fig. 3*C*), into a wild type background. If the RING domain normally functions to promote ubiquitination of a substrate protein that is tethered to DIAP2 through interactions involving other sequences such as the BIRs, then RING mutated versions of DIAP2 might be expected to function as dominant negatives. Consistent with this hypothesis, the presence of *diap2^{7.4C472Y}* in a wild type background resulted in a 50% suppression of diptericin expression in response to Gram-negative bacterial infection (supplemental Fig. 2*d*). To determine whether *diap2* is required downstream of Relish cleavage, we asked if ectopic expression of

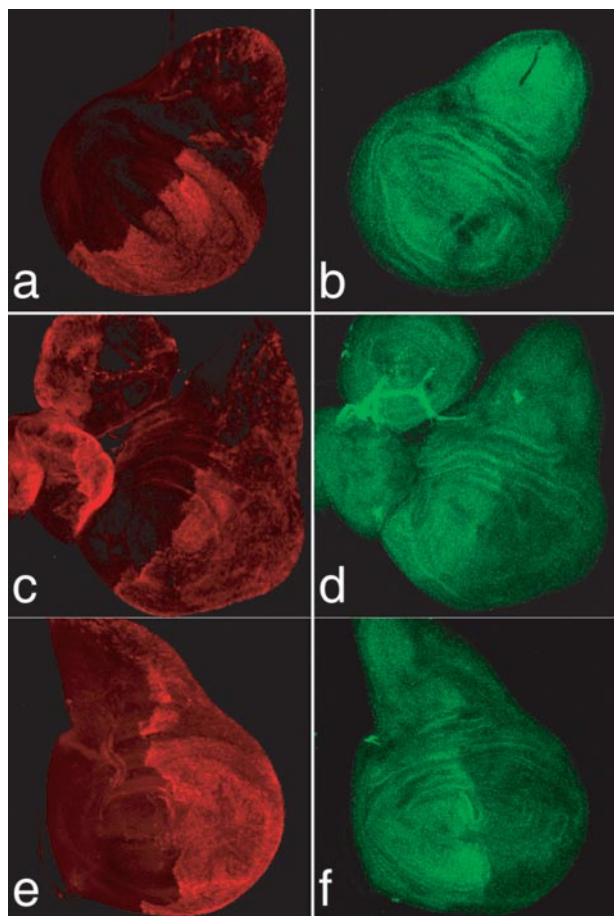


FIGURE 6. Expression of Hid or Rpr leads to loss of DIAP2 in *Drosophila* wing discs. Confocal images of wing discs from third instar larvae of various genotypes. Posterior is to the right. *a*, wing disc from third instar larvae of genotype UAS::p35; engrailed Gal4 (en::Gal4), stained with anti-P35, in red. *b*, wing disc in *a* stained with anti-DIAP2 in green. *c*, UAS::Rpr, UAS::p35; en::Gal4 wing disc stained with anti-P35, in red. *d*, wing disc in *c* stained with anti-DIAP2, in green. *e*, wing disc of genotype UAS::Hid, UAS::p35; en::Gal4 stained with anti-P35, in red. *f*, wing disc in *e* stained with anti-DIAP2, in green.

DIAP2 has an important role in promoting normal levels of Relish cleavage in response to immune challenge.

The DIAP2 BIR Serves as a Potential Point of Cross-talk between Apoptotic and Immune Signaling—IAP BIR domains contain a surface groove that is bound by proteins containing a short peptide motif, the IBM (supplemental Fig. 4) (6). RHG proteins bind DIAP1 BIRs via such IBMs and in doing so compete off bound proteins and promote DIAP1 degradation. The BIR domains of DIAP2 are very similar to those of DIAP1 (2), and RHG proteins can bind DIAP2 BIR domains through interactions that require their IBMs (4, 11, 12, 57). To explore the possibility that IBM-containing proteins can also regulate DIAP2 function, we expressed Rpr or Hid in the posterior compartment of a simple easy to visualize tissue, the wing imaginal disc, using the UAS-GAL4 system (see “Materials and Methods”) and examined DIAP2 levels. The baculovirus caspase inhibitor p35 was coexpressed along with these molecules to block cell death. Expression of p35 alone had no effect on DIAP2 expression, which was uniform throughout the wing disc (Fig. 6, *a* and *b*). In contrast, coexpression of Rpr or Hid with P35 resulted in a dramatic reduction of DIAP2 in the pos-

terior wing compartments (Fig. 6, *c–f*), similar to that seen for DIAP1 (25, 58). These results suggest that DIAP2, like DIAP1, is down-regulated in cells undergoing apoptosis.

Why might apoptosing cells choose to down-regulate DIAP2? One possibility is that this helps to prevent inappropriate immune activation in response to the presence of proinflammatory molecules released by dying cells. To explore this possibility, we created cells in which apoptosis effectors were activated at modest levels in the fat body (thereby inducing low levels of death) but in which this activation occurred independent of expression of RHG proteins (thus preventing down-regulation of *diap2* that would occur in response to their expression). We did this by targeting *diap1* mRNA for degradation specifically in the adult fat body, using a drug-inducible, fat body Gal4 driver (31), and a UAS-*diap1*-targeted micro-RNA (UAS-*diap1*-miRNA) that is known to promote the loss of *diap1* when expressed in other tissues (supplemental Fig. 1, *d* and *e*). As illustrated in supplemental Fig. 2*e*, a 20-h induction of UAS-*diap1*-miRNA had no negative effect on expression of the Gram-positive immune response gene drosomycin, indicating that the fat body was still largely intact. In contrast, with dipteracin, a Gram-negative response gene, a modest 20% increase in expression was seen. Most importantly, however, this increase in dipteracin was suppressed by coexpression of a micro-RNA targeting *diap2* (UAS-*diap2*-miRNA) that is known to promote the loss of *diap2* when expressed in other tissues (supplemental Fig. 1, *b* and *c*). No significant change in drosomycin expression was observed. Together, these observations suggest a model in which activation of apoptosis signaling pathways (which often involves decreasing *diap1* levels) can lead (through unknown mechanisms) to hyperactivation of immune responses unless *diap2* is also removed.

DISCUSSION

DIAP1 and DIAP2 both inhibit apoptosis when overexpressed, and DIAP1 is essential for the survival of many cells. Here we show that endogenous *diap2* does not function as a major regulator of apoptosis. DIAP2 expression does not inhibit the activity of multiple caspases, including the key apoptosis activators Drice and Dronc. In addition, animals in which *diap2* is deleted are viable, healthy, fertile, and as resistant as wild type animals to a number of stresses. The fact that DIAP2 can inhibit apoptosis when overexpressed probably reflects, at least in part, its ability to sequester and/or degrade apoptosis inducers that also target DIAP1, such as the RHG proteins. In this study, we show that DIAP2, perhaps functioning as a E3 ubiquitin ligase, is required for the IMD-mediated humoral response to Gram-negative bacterial infection, with DIAP2 being required for normal levels of Relish cleavage and nuclear translocation. DIAP2 may also function at other steps in the IMD pathway, although it is not absolutely required once Relish has undergone cleavage, at least when the cleaved version of Relish is expressed directly (see below). Finally, we observed modest but significant increases in the immune response when the endogenous DIAP2 genetic dosage was increased and a strong down-regulation of DIAP2 levels in the presence of the apoptosis inducers Rpr and Hid. As discussed further below, these observations suggest models in which IMD signaling is

sensitive to the levels of DIAP2, and IBM-containing proteins regulate DIAP2 function during apoptosis and perhaps other contexts, through interactions with its BIR domains.

Our observations, derived from *in vivo* studies of deletion mutants, stand in contrast to several recent reports, in which down-regulation of *diap2* was brought about by using long dsRNA to induce RNAi-dependent degradation of *diap2* mRNA. RNAi of *diap2* was reported to sensitize S2 cells to several cell death activators (14) and to result in pupal lethality when expressed ubiquitously (16). Differences between these results and our own are most simply explained as a result of RNAi off-targeting of unknown transcripts required for cell health and organismal viability in the earlier works. The ~500-bp dsRNAs used in the above-mentioned studies have the potential to be diced into many different 21-bp fragments for loading into the RISC complex and subsequent use as guide sequences. A number of recent reports have shown that off-targeting is a frequent occurrence (59–61) and can result in the production of toxic phenotypes (62). The basis for this has been made clear through experiments demonstrating that a single RISC-bound guide sequence can target many mRNA sequences, which need be only partially complementary to the guide strand (59, 63, 64).

Two groups, again using long dsRNA to target *diap2* in S2 cells, have also recently reported that *diap2* is required for the IMD response. One group concluded that *diap2* acted upstream of or at the level of TAK1 (17). This conclusion was based, in part, on the observation that reduction of *diap2* resulted in decreased expression of the dTAK1- and JNK-dependent early response gene *puckered* following immune challenge. In contrast, we found that expression of *puckered*, and a second early response gene, CG13482, were induced normally in animal mutants for *diap2* (Fig. 4d, lower panel, and supplemental Fig. 2c), making this site of action unlikely (see also below). How can these different observations be reconciled? One possibility is that differences in the systems are important. We measured immune responses in the context of the entire organism. In doing this our observations reflect the summed action of (and potentially cross-talk between) multiple cell types and signaling pathways. In contrast, cell line-based studies are, by their nature, much more focused. Thus, it seems not unreasonable to postulate that while Tak-dependent activation of JNK and/or JNK target genes may be sensitive to levels of *diap2* in one cell type, a different pathway may dominate in the intact organism. Alternatively, the effects of RNAi-mediated knockdown of *diap2* on *puckered* expression in S2 cells may again reflect off-targeting of unknown transcripts by long dsRNA.

In contrast to Gesellchen *et al.* (17), Kleino *et al.* (16) proposed DIAP2 functions in the IMD pathway downstream of Relish cleavage because they saw no effect of *diap2* RNAi on Relish cleavage in S2 cells. This difference from our observations, in which loss of *diap2* resulted in a significant decrease in Relish cleavage, may indicate differences in systems. However, we think it more likely to reflect the difficulty in generating null mutant phenotypes for *diap2* using RNAi. These authors did find that expression of pre-cleaved Relish was able to activate the immune response (and thus presumably able to translocate

into the nucleus) in cells treated with *diap2* dsRNA. Importantly, we observed a similar ability of pre-cleaved Relish to activate transcription of AMP in the complete absence of *diap2* in flies.

What do these observations tell us about the site(s) of DIAP2 action? Our observations demonstrate that DIAP2 plays a role in bringing about Relish cleavage, which is required for Relish nuclear translocation and function (29). Does DIAP2 function at other steps as well? The fact that loss of DIAP2 had a greater effect on nuclear translocation of endogenous Relish (Fig. 5b) and AMP production (Figs. 3 and 4) than it did on Relish cleavage (Fig. 5) is consistent with the hypothesis that it may. Expression of pre-cleaved Relish bypassed the requirement for DIAP2 in AMP production (Fig. 5c) (16), suggesting that DIAP2 is not absolutely required for Relish nuclear translocation or function. In addition, we have not observed nuclear translocation of DIAP2 in response to immune challenge.⁶ However, these observations do not exclude the possibility that the levels of pre-cleaved Relish generated in the fat body by heat shock are so high that Relish enters the nucleus through unphysiological pathways. For example, perhaps overexpression of pre-cleaved Relish titrates out an inhibitor of Relish nuclear translocation/function that is normally removed by DIAP2. It is also worth considering that pre-cleaved Relish expressed from a transgene may not be the same as processed Relish generated in response to IMD signaling. For example, endogenous Relish is phosphorylated by the IKK complex in response to IMD signaling (48, 52) and cleaved by Dredd. Processed endogenous Relish (but perhaps not transgene-expressed pre-cleaved Relish) may exist as a part of a complex and/or interact with factors that inhibit nuclear import unless DIAP2 promotes their removal. These hypotheses can best be explored through the identification of proteins that bind endogenous cleaved Relish and DIAP2. Finally, what is the significance of DIAP2-independent cleavage of Relish? Perhaps DIAP2 is not absolutely essential, mechanistically, for Relish cleavage (for example, if it acts as an inhibitor of an inhibitor of cleavage, some Relish may escape the inhibitor and thus be cleaved). Alternatively, other related E3s such as DIAP1 may be able to partially substitute for DIAP2 function (at least when DIAP2 is completely missing), an issue that requires further exploration. Regardless, our observation that loss of DIAP2 prevents a significant fraction of Relish cleavage and that DIAP2 is required for Relish-dependent AMP expression demonstrates that DIAP2 is a critical regulator of Relish function.

How does DIAP2 regulate Relish cleavage? It is unlikely that DIAP2 functions as an inhibitor of the caspase Dredd since DIAP2 does not inhibit other tested caspases, and Dredd, like DIAP2, is required for activation of the IMD pathway. However, it is worth noting that several mammalian IAPs that inhibit apoptosis when overexpressed, cIAP1 and cIAP2, bind active caspases even though they do not function as caspase inhibitors (65). Binding of DIAP2 to Drice and Strica has been reported (4). Binding between DIAP2 and Dredd has not been reported (it is not clear it has been searched for). Thus, it

⁶ J. R. Huh and B. A. Hay, unpublished data.

Drosophila IAP2 Mutant Analysis

remains possible that interactions between Dredd and DIAP2, perhaps involving ubiquitination by DIAP2, positively regulate Dredd activation, activity, or substrate targeting. Roles for E3 ligase activity of DIAP2 in the IMD pathway activation (upstream and/or downstream of Relish cleavage) are suggested by several observations. Expression of a RING domain point mutant version of *diap2*, *diap2*^{7.4C472Y}, in the *diap2*^{E151} mutant background failed to rescue Relish cleavage and IMD signaling (Figs. 3–5). In contrast, expression of this same mutant protein in a wild type background suppressed the IMD response, suggesting dominant negative activity (supplemental Fig. 2*d*). In mammals, ubiquitination exerts degradation-dependent effects on immunity through removal of I κ B and processing of NF- κ B. In contrast, activation of Tak1 and the IKK complex require ubiquitination in degradation-independent roles (66, 67). As discussed in the Introduction, *Drosophila* counterparts of Tak1 and the IKK complex are also essential for Relish processing. However, several observations suggest that *diap2* does not act at the level of dTAK1. First, as noted above, expression of puckered and CG13482, immediate transcriptional targets of the Tak1-JNK signaling module (30), are not affected in *diap2* mutant flies (Fig. 4*d* and supplemental Fig. 2*c*). Second, ectopic expression or down-regulation of *diap2* in the fly eye fails to influence Tak1-dependent cell killing, which is JNK-dependent (20) (supplemental Fig. 3). Recent observations suggest that activation of the IKK complex requires the *Drosophila* ubiquitin-carrier proteins Bendless (Ubc13) and dUEV1a, along with a yet to be identified E3 ubiquitin ligase (68). Both Ubc13 (MAALTP) and dUEV1a (MANTSS) contain potential IBM motifs at their N termini. It will be interesting to determine whether DIAP2 binds one or both these proteins and participates in this process.

Careful control over the intensity and timing of an immune response is important, because hyperactivation of the immune system induces fitness costs with respect to other aspects of the organism life cycle such as fertility and longevity. Hyperactivation can also lead to the induction of tissue inflammation and cell death (18, 19). In the context of these considerations, it is interesting that modest increases in the genetic dose of *diap2* resulted in an enhanced immune response, whereas expression of cell death activators such as Rpr or Hid brought about DIAP2 removal. Rpr and Hid, as well as other members of the RHG family, are induced or released from a sequestering environment in response to stresses that can lead to cell death and/or tissue damage. We speculate that in binding DIAP2 as well as DIAP1, these proteins accomplish two goals at once. They induce cell death through inhibition of DIAP1 and prevent activation of an immune response, which requires cleavage of Relish by the caspase Dredd, a process likely to be stimulated by loss of DIAP1 (a caspase inhibitor) and/or the activation of other caspases downstream of this loss. In other words, RHG protein binding to DIAP2 BIR domains may function in some contexts as a safety lock, down-regulating DIAP2 function, thereby preventing an inappropriate immune response in contexts in which there are high levels of cell death (perhaps during metamorphosis or larval fat body histolysis) or cell-damaging stress (Fig. 7*a*). This model is speculative, but it is testable and provides an explanation (perhaps partial) for the observation

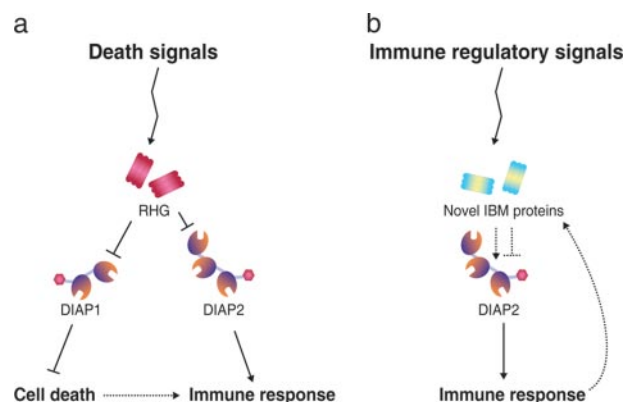


FIGURE 7. **Speculative models for DIAP2 regulation by IBM proteins.** *a*, cells undergoing apoptosis often express combinations of RHG proteins such as Rpr, Hid, and Grim, thereby promoting removal of DIAP1 and caspase activation. DIAP1 inhibits (by one of several different mechanisms) all tested *Drosophila* caspases. Therefore, if DIAP1 is normally required to limit Dredd activity (directly or indirectly), decreasing DIAP1 levels should lead (all other things being equal) to hyperactivation of an immune response. The fact that DIAP2 is also targeted by RHG proteins and is required for the IMD response may serve to limit this possibility. *b*, in an alternative scenario, activation of the IMD pathway may lead to the creation (through caspase cleavage) or expression of novel IBM proteins that regulate the stability of DIAP2 or its ability to interact with immune effectors. Such proteins could include a negative feedback loop. Indeed, several genes with potential IBM motifs (CG9646 and CG12113) were previously identified as suppressors of the IMD pathway in S2 cells (72).

that unlike necrotic cell death, which often leads to inflammation, apoptotic cell death is characterized by a lack of inflammation, and sometimes even immunosuppression (69, 70). Evidence consistent with such a model comes from our observation that activation of apoptosis effectors directly (by decreasing DIAP1), rather than through expression of RHG proteins (which would also down-regulate DIAP2), resulted in an increased immune response (supplemental Fig. 2*e*).

Related, alternative models can also be considered. For example, caspase cleavage exposes IBM-like motifs in many proteins (71). If this occurs in response to Dredd activation in the insect fat body, it could serve as a form of negative feedback, creating proteins that bind DIAP2 BIRs, displacing bound proteins required (we speculate) for the immune response, and/or promoting changes in DIAP2 localization or stability. Target genes activated by the immune response might play a similar role (Fig. 7*b*). RNAi in S2 cells of several transcripts encoding proteins with N-terminal sequences similar to those of known IAP-binding proteins or peptides (supplemental Fig. 4) results in increased immune activation (72), suggesting these as candidate negative regulators of DIAP2. IBM domain proteins might also play positive roles in immune regulation. In the context of this possibility, it is interesting to note that binding of the mammalian IBM protein Smac/Diablo promotes XIAP stabilization rather than degradation (73, 74). The hypothesis that IBM proteins regulate DIAP2 activity, in its capacity as an essential component of the innate immune system, is speculative but is testable. Given that IBM domain proteins function as evolutionarily conserved regulators of cell death by displacing IAP-bound proteins and promoting IAP degradation and that DIAP2 is able to bind proteins with these same motifs, it would be surprising if similar regulatory mechanisms were not utilized in the immune system.

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Addendum—A recent report also described the characterization of *Drosophila* IAP2 mutant flies (75). These authors placed DIAP2 function downstream or in parallel to Relish because *diap2* mutant phenotypes were not rescued by ectopic expression of full-length Relish. However, since DIAP2 plays a significant role in promoting Relish cleavage (our study), a failure of full-length Relish (which requires cleavage) to rescue immune responsiveness in *diap2* mutants is not surprising.

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