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The deubiquitinating enzyme DUBAI stabilizes DIAP1 to suppress *Drosophila* apoptosis

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Deubiquitinating enzymes (DUBs) counteract ubiquitin ligases to modulate the ubiquitination and stability of target signaling molecules. In *Drosophila*, the ubiquitin–proteasome system has a key role in the regulation of apoptosis, most notably, by controlling the abundance of the central apoptotic regulator DIAP1. Although the mechanism underlying DIAP1 ubiquitination has been extensively studied, the precise role of DUB(s) in controlling DIAP1 activity has not been fully investigated. Here we report the identification of a DIAP1-directed DUB using two complementary approaches. First, a panel of putative *Drosophila* DUBs was expressed in S2 cells to determine whether DIAP1 could be stabilized, despite treatment with death-inducing stimuli that would induce DIAP1 degradation. In addition, RNAi fly lines were used to detect modifiers of DIAP1 antagonist-induced cell death in the developing eye. Together, these approaches identified a previously uncharacterized protein encoded by *CG8830*, which we named DeUBiquitinating-Apoptotic-Inhibitor (DUBAI), as a novel DUB capable of preserving DIAP1 to dampen *Drosophila* apoptosis. DUBAI interacts with DIAP1 in S2 cells, and the putative active site of its DUB domain (C367) is required to rescue DIAP1 levels following apoptotic stimuli. DUBAI, therefore, represents a novel locus of apoptotic regulation in *Drosophila*, antagonizing cell death signals that would otherwise result in DIAP1 degradation.
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Programmed cell death, or apoptosis, is a genetically determined process by which a cell self-destructs for the benefit of the whole organism.[1] The apoptotic program is initiated by a number of signaling pathways following cell stress or developmental cues, ultimately leading to the activation of a family of cysteine proteases called caspases. Caspase pathways function as proteolytic cascades, wherein initiator caspases activated by dimerization cleave and activate executioner caspases, which in turn cleave multiple substrates to package the dying cell for destruction.[1–3]

For vertebrates, the core intrinsic apoptotic pathway is regulated by the adaptor protein Apaf-1, which is stimulated by cytochrome *c* released from the mitochondria to form the apoptosome, the activating protein complex essential for caspase 9 dimerization and activation.[4] In contrast, the analogous pathway in *Drosophila* does not appear to rely on mitochondrially released cytochrome *c*.[5–7] Instead, the *Drosophila* Apaf-1 homolog, DARK, appears to constitutively activate Dronc, the primary apical caspase in fly apoptosis.[8–10] Initiation of apoptosis is therefore kept in check by a family of caspase inhibitors known as the inhibitor of apoptosis proteins (IAPs).[2,11] These proteins antagonize active caspases by suppressing their enzymatic activity and inducing their degradation.[11,12]

The primary *Drosophila* IAP is DIAP1, whose loss of function is sufficient for inducing caspase-dependent cell death.[8–10] DIAP1 is an E3 ubiquitin ligase with an intrinsically active RING domain, which is required for DIAP1 to mediate its anti-apoptotic function.[13–15] In the absence of an apoptotic cue, DIAP1 binds to and post-translationally modifies a number of *Drosophila* caspases with ubiquitin and the ubiquitin-like protein NEDD8, thereby suppressing caspases and subsequent cell death through degradative and non-degradative mechanisms.[12,14,16] Apoptotic stimuli dampen DIAP1's anti-apoptotic activity, typically by transcriptionally inducing a family of IAP antagonists, including reaper (rpr), hid and grim (RHG).[2,17–19] These proteins bind particular baculoviral IAP repeat domains on DIAP1 to prevent DIAP1 from interacting with caspases.[20,21] Moreover, IAP antagonists reduce DIAP1 levels by globally suppressing protein translation[22,23] and by further decreasing the half-life of this fast-turnover protein through a mechanism involving UbcD1 (an E2 ubiquitin-conjugating enzyme) and DIAP1 autoubiquitination.[22,24–26] Notably, DIAP1 can also be ubiquitinated by an N-end rule ligase after caspase cleavage.[27]

Both the addition (by E3) and the removal (by deubiquitinating enzyme (DUB)) of ubiquitin can shape the stability and/or the activity of signaling proteins. Accumulating evidence shows that DUBs tune various cellular pathways, including those

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Abbreviations: DIAP1, *Drosophila* inhibitor of apoptosis protein 1; Dronc, *Drosophila* Nedd-2-like caspase; DUB, deubiquitinating enzyme; DUBAI, DeUBiquitinating-Apoptotic-Inhibitor; ETP, etoposide; IAP, inhibitor of apoptosis protein; Josephins, Machado–Joseph disease protein domain proteases; OTU, ovarian tumor protease; rpr, reaper; RHG, reaper, hid, grim; USP, ubiquitin-specific protease; WCL, whole-cell lysate; wt, wild type
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governing cell survival and death.[28–30] To date, nearly 100 human proteins have been predicted to possess deubiquitinating activity. On the basis of their domain structure and peptide similarity, DUBs are subclassified into six families: ubiquitin-specific proteases (USPs), ubiquitin C-terminal hydrolases, ovarian tumor proteases (OTUs), Machado–Joseph disease protein domain proteases (Josephins), JAMM/MPN domain-associated metallopeptidases and monocyte chemotactic protein-induced protein.[29,30] These enzymes help to maintain the free ubiquitin pool in cells by processing ubiquitin precursors translated as either linear polyubiquitin peptides or ubiquitin/ribosomal fusion proteins, and by recycling ubiquitin from proteins committed to proteasomal or lysosomal destruction.[30] Importantly, as mentioned above, they also modulate protein half-life by trimming ubiquitin from target proteins.

DUBs actively engage in the regulation of many key apoptotic regulators, including caspases, Bcl-2 family proteins and IAPs.[28,31] Although IAP ubiquitination has been studied extensively in *Drosophila* and in higher organisms, little is known about their deubiquitination.[26,32] Recently, human OTUB1 and USP19 have been suggested to inhibit TNFα-induced apoptosis via the stabilization of c-IAPs.[33,34] Interestingly, a catalytically inactive USP19 (lacking DUB activity) is still capable of suppressing the ubiquitination and degradation of c-IAPs *in vivo*, suggesting that DUBs may also in some way control protein stability through a mechanism independent of their enzymatic activities.[33] In *Drosophila*, genetic deletion of *scny* (*CG5505*, also known as e*mperor's thumb*), a fly homolog of human USP36, induces cell death associated with decreased DIAP1 levels; however, additional studies are required to determine whether scny controls DIAP1 directly or functions via other apoptotic regulators.[35]

Here we report the identification of the protein encoded by *CG8830*, an uncharacterized DUB, as a novel DIAP1-directed DUB; we have named this protein DeUBiquitinating-Apoptotic-Inhibitor (DUBAI). When overexpressed, DUBAI stabilized DIAP1 in *Drosophila* S2 cells exposed to UV or etoposide (ETP), two apoptosis inducers known to accelerate DIAP1 ubiquitination and degradation.[27,36] Consistent with these data in cultured cells, in DUBAI hypomorphic flies or those where DUBAI was knocked down by RNAi, RHG-induced cell death was enhanced in the developing eye, revealing its inhibitory role in *Drosophila* apoptosis. Moreover, DUBAI bound to DIAP1 *in vivo* and the catalytic residue predicted to be critical for DUB activity was essential for prolonging DIAP1's half-life on apoptotic stimuli. These data indicate that DUBAI is a novel IAP-directed DUB and a previously unrecognized factor controlling the fly apoptotic circuit.

Results

***Drosophila* S2 cell screen identifies DUBs that maintain DIAP1 levels during apoptosis.** As DIAP1's ubiquitination and degradation represent a critical control point in *Drosophila* apoptosis, we hypothesized that a DUB might control *Drosophila* cell death through stabilization of DIAP1. To evaluate this possibility, we developed a screen for DUBs able to maintain DIAP1 levels following apoptotic stimulation in S2 cells. Supporting information in Supplementary Table S1 summarizes the gene names, human homologs and sources of cDNA for each *Drosophila* DUB assayed. Several of these were well-characterized DUBs, such as Ubpy, CYLD and scny; others have a putative DUB domain based on peptide homology. When coexpressed with DIAP1 in S2 cells, scny downregulated DIAP1 levels even in the absence of an apoptotic stimulus, consistent with the reported death-inducing ability of this scny isoform in larvae.[35] In contrast, DUBAI (*CG8830*) and Ubpy (*CG5798*) were able to rescue DIAP1 from ETP-induced degradation (Figure 1). Similar results were seen in UV-treated cells (Supplementary Figure S1), indicating the preservation of DIAP1 by these two DUBs is not specific for ETP-induced apoptosis.

Targeted genetic RNAi analysis shows DUBAI is a negative regulator of rpr-induced eye ablation. Our initial screen identified DUBAI and Ubpy, both members of the

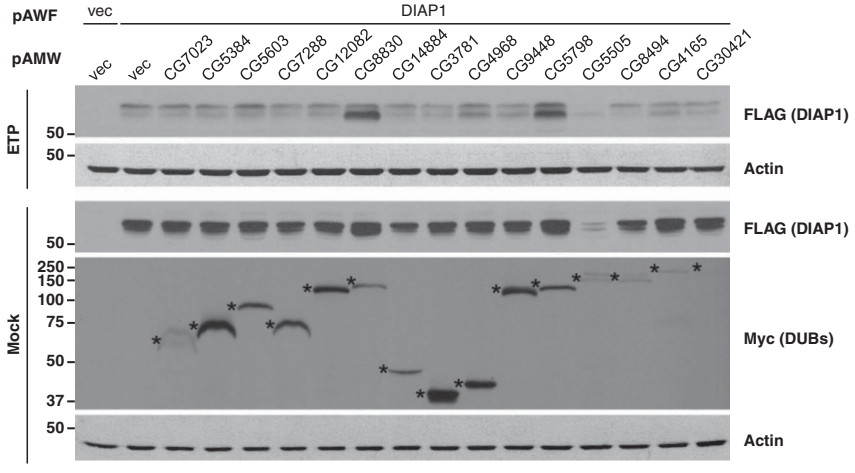


Figure 1 Identification of DUBs controlling DIAP1 levels in apoptotic cells. DUBAI/*CG8830* and Ubpy/*CG5798* prevent ETP-induced loss of DIAP1 in S2 cells. Anti-FLAG immunoblotting was performed to detect FLAG-tagged DIAP1 levels in ETP-treated and -untreated cells following expression of a panel of Myc-tagged DUBs designated by their CG number. Asterisks indicate the unmodified species of DUBs detected by Myc antibody immunoblotting. Actin levels are shown to indicate equal loading

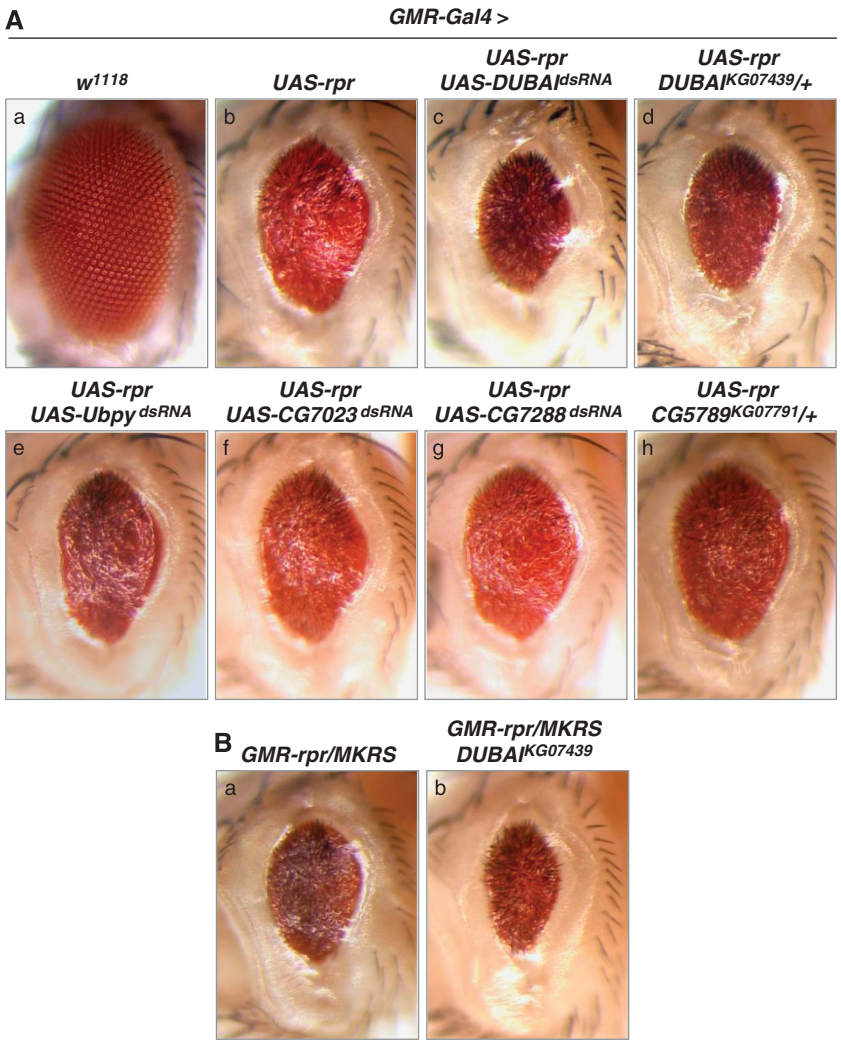
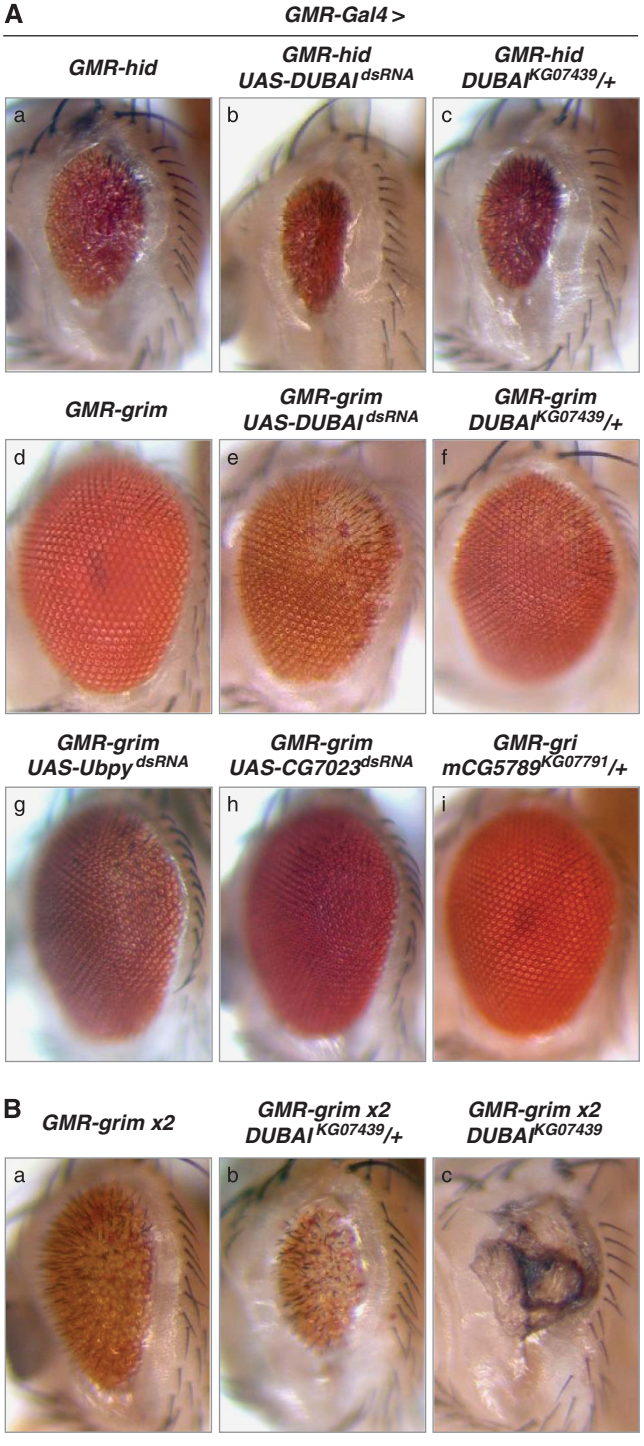


Figure 2 Loss of DUBAI, but not other DUBs, enhances rpr-induced eye ablation. Light micrographs of *Drosophila* adult eyes and their genotypes are shown. All experiments shown were carried out at 25 °C. (**A**) Eye apoptosis induced by *GMR-Gal4*-driven expression of *UAS-rpr* (**A**b) was significantly aggravated by *DUBAI* RNAi (**A**c) or a transposon that suppresses DUBAI expression genetically (*DUBAI*^{KG07439}) (**A**d), but not by dsRNA against other DUBs (**A**e–g and Supplementary Table S2) or *CG5789*^{KG07791} (**A**h). The eye of a wt fly bearing one copy of *GMR-Gal4* is shown as a control (**A**a). (**B**) Eye apoptosis induced by *GMR-rpr* (**B**a) is exacerbated by genetic reduction of *DUBAI* (**B**b). Genotypes: (**A**a) *GMR-Gal4/+; +*; (**A**b) *GMR-Gal4/+; UAS-rpr/+*; (**A**c) *GMR-Gal4/+; UAS-rpr/UAS-DUBAI*^{dsRNA}; (**A**d) *GMR-Gal4/ DUBAI*^{KG07439}*; UAS-rpr/+*; (**A**e) *GMR-Gal4/+; UAS-rpr/UAS-Ubpy*^{dsRNA}; (**A**f) *GMR-Gal4/+; UAS-rpr/UAS-CG7023*^{dsRNA}; (**A**g) *GMR-Gal4/+; UAS-rpr/UAS-CG7288*^{dsRNA}; (**A**h) *GMR-Gal4/+; UAS-rpr/ CG5789*^{KG07791}; (**B**a) *+; GMR-rpr/MKRS*; (**B**b) *DUBAI*^{KG07439}*; GMR-rpr/MKRS*

USP family of DUBs, as positive regulators of DIAP1 levels during apoptosis in cultured S2 cells. Therefore, in an alternative approach to both identify DIAP1-directed DUB(s) and validate the two genes isolated in the S2 cell screen, we performed a loss-of-function screen in flies using RNAi lines against a battery of DUBs in the USP family. Supplementary Table S2 summarizes the source of RNAi lines tested and their effects on eye phenotype when coexpressed with IAP antagonists. In support of the hypothesis that DUBAI stabilizes DIAP1 to inhibit apoptosis, *DUBAI* double-stranded (ds)RNA, but not others, significantly exacerbated eye ablation induced by *UAS-rpr* (Figure 2A and Supplementary Table S2). Similar results were observed with *DUBAI*^{KG07439}, where the expression of *DUBAI* is genetically suppressed by a transposon, when *GMR-rpr* was used as an alternative death inducer (Figures 2Ad and B), ruling out the potential position effects of UAS constructs and indicating that DUBAI negatively regulates rpr-induced cell death in the developing fly eye. By contrast, dsRNA designed against *Ubpy*, *CG7023* or *CG7288* had no or very limited effects on the eye phenotype induced by rpr (Figure 2A and Supplementary Table S2). Similarly, genetic disruption of an unrelated gene (*CG5789*) by the same transposon did not enhance the rpr phenotype (Figure 2Ah). These data suggest that *Ubpy* has an ancillary role in the control of *Drosophila* apoptosis in the eye (as opposed to in S2 cells). Notably, downregulation of *CG12082* also enhanced rpr-induced eye cell death, resulting in embryonic lethality (Supplementary Table S2), whereas ectopic expression of this DUB did not maintain DIAP1 levels during ETP- or UV-induced apoptosis in S2 cells (Figure 1 and Supplementary Figure S1). As USP5 (a human ortholog of CG12082) has been shown to generate ubiquitin monomers from unanchored polyubiquitin chains,[37] we speculate that the enhancement of the rpr phenotype

induced by *CG12082* RNAi may result from alterations in the free ubiquitin pool rather than a DIAP1-specific mechanism. Collectively, these data prompted us to focus on the further characterization of DUBAI, a candidate DUB that scored positively in both screens, as a protein able to suppress DIAP1 degradation in cultured S2 cells and whole flies.



DUBAI is able to suppress apoptosis induced by various IAP antagonists. Although all IAP antagonists are capable of inhibiting DIAP1, their molecular modes of action and regulation are somewhat distinct.[22,36] Similar to the results shown in Figure 2 for rpr-induced cell death, downregulation of DUBAI by dsRNA (Figure 3Ab) or one copy of the mutant *DUBAI*^{KG07439} allele (Figure 3Ac) further reduced eye size in the background of *GMR-hid* expression when compared with flies expressing *GMR-hid* alone (Figure 3Aa). Although expressing one copy of *GMR-grim* alone gave rise to no obvious eye pathology (Figure 3Ad), coexpression of dsRNA targeted against *DUBAI* (Figure 3Ae) resulted in significant loss of pigment cells. Similarly, flies carrying a mutant *DUBAI*^{KG07439} allele (Figure 3Af) exhibited enhanced grim-induced eye cell death, which was not seen when the same transposon was inserted to *CG5789* (Figure 3Ai). In contrast, coexpression of other DUB dsRNAs caused no or only marginal effects on the same genetic background (Figures 3Ag and h). A more severe eye phenotype, induced by the expression of two copies of *GMR-grim*, was sensitive to DUBAI levels, as expression of two copies of *GMR-grim* in *DUBAI*^{KG07439} homozygous flies (Figure 3Bc, which therefore have a lowered DUBAI levels) resulted in exacerbated eye ablation compared with flies carrying only one or no mutated *DUBAI* allele (Figures 3Ba and b).

Eye abnormality induced by loss of DUBAI at the restrictive temperature is ameliorated by DIAP1 overexpression or caspase inhibitor p35. To further understand the functional relationship between DUBAI and DIAP1, we examined their genetic interactions in the fly eye. Interestingly, overexpression of DUBAI caused a severe developmental defect in the eye (Supplementary Figures S2B and E), which could be rectified by *DUBAI* RNAi (Supplementary Figures S2B and D), but not by overexpression of the viral caspase inhibitor p35 (Supplementary Figures S2B and C) or DIAP1 (Supplementary Figures S2E and F). Coexpression of *UAS-Dronc* further aggravated the small eye phenotype induced by *UAS-DUBAI* (Supplementary Figures S2E and G). These data suggest that overexpressed DUBAI may activate non-apoptotic pathways in a gain-of-function setting. In contrast,

Figure 3 DUBAI suppresses eye abnormalities induced by IAP antagonists. Light micrographs of *Drosophila* adult eyes and their genotypes are shown. (**A**) Downregulation of *DUBAI* enhanced eye ablation induced by *GMR-hid* or *GMR-grim*. Eye phenotypes resulting from expression of hid or grim under the control of GMR promoter (**A**a and d), were exacerbated in flies expressing *DUBAI* RNAi (**A**b and e) or having one allele of *DUBAI*^{KG07439} (**A**c and f). Downregulation of Ubpy or CG7023 or genetic disruption of *CG5789* did not significantly enhance grim-induced eye ablation (**A**g–i). (**B**) DUBAI levels modulate eye phenotypes of *GMR-grim* homozygous flies. Replacement of one or both wt alleles with *DUBAI*^{KG07439} incrementally reduced DUBAI expression and worsened the eye ablation phenotype. Data shown in (**A**a–c) were carried out at 18 °C; others were performed at 23 °C. Genotypes: (**A**a) *GMR-Gal4/+; GMR-hid/+*; (**A**b) *GMR-Gal4/+; GMR-hid/UAS-DUBAI*^{dsRNA}; (**A**c) *GMR-Gal4/DUBAI*^{KG07439}*; GMR-hid/+*; (**A**d) *GMR-Gal4/+; GMR-grim/+*; (**A**e) *GMR-Gal4/+; GMR-grim/UAS-DUBAI*^{dsRNA}; (**A**f) *GMR-Gal4/DUBAI*^{KG07439}*; GMR-grim/+*; (**A**g) *GMR-Gal4/+; GMR-grim/UAS-Ubpy*^{dsRNA}; (**A**h) *GMR-Gal4/+; GMR-grim/UAS-CG7023*^{dsRNA}; (**A**i) *GMR-Gal4/+; GMR-hid/CG5789*^{KG0779,1}; (**B**a) *+; GMR-grim*; (**B**b) *DUBAI*^{KG07439}*/+; GMR-grim*; (**B**c) *DUBAI*^{KG07439}*; GMR-grim*

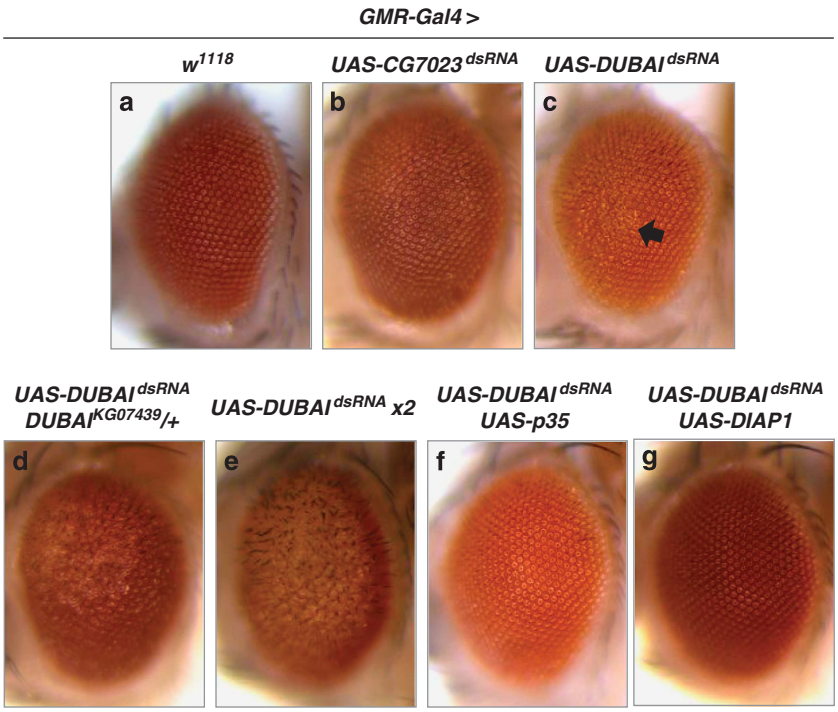


Figure 4 Loss of DUBAI causes eye ablation associated with caspase activation and DIAP1 deficiency. Light micrographs of *Drosophila* adult eyes and their genotypes are shown. (**a**) *GMR-Gal4* alone. (**b**) Downregulation of CG7023 in the fly eyes caused no clear abnormality. (**c**–**e**) The eye phenotype induced by *DUBAI* reduction correlated with DUBAI levels. *GMR-Gal4*-driven expression of one allele of *UAS-DUBAI*^{dsRNA} resulted in a rough eye phenotype (**c**, indicated by an arrow), which was enhanced in flies carrying one allele of *DUBAI*^{KG07439} (**d**). Expression of two alleles of *UAS-DUBAI*^{dsRNA} caused severe depigmentation in the eyes (**e**). (**f** and **g**) The eye abnormalities caused by the loss of DUBAI were suppressed by overexpression of p35, a viral caspase inhibitor (**f**), or by replenishment of DIAP1 with a UAS construct (**g**). All experiments were carried out at 29 °C. Genotypes: (**a**) *GMR-Gal4/+ ; +* ; (**b**) *GMR-Gal4/+ ; UAS-CG7023*^{dsRNA}*/+* ; (**c**) *GMR-Gal4/+ ; UAS-DUBAI*^{dsRNA}*/+* ; (**d**) *GMR-Gal4/DUBAI*^{KG07439}*; UAS-DUBAI*^{dsRNA}*/+* ; (**e**) *GMR-Gal4/+ ; UAS-DUBAI*^{dsRNA}; (**f**) *GMR-Gal4/+ ; UAS-DUBAI*^{dsRNA}*/UAS-p35*; (**g**) *GMR-Gal4/UAS-DIAP1; UAS-DUBAI*^{dsRNA}*/+*

downregulation of DUBAI in the fly eye by RNAi resulted in eye abnormalities associated with caspase activation. Specifically, GMR-driven expression of one allele of *DUBAI*^{dsRNA}, but not *CG7023*^{dsRNA}, caused a rough eye phenotype at 29 °C (Figures 4b and c), which was further enhanced in flies carrying one *DUBAI*^{KG07439} allele or flies expressing two copies of *UAS-DUBAI*^{dsRNA} alleles (Figures 4d and e), indicating that the eye phenotype was correlated with DUBAI levels. Importantly, this defect induced by reduced DUBAI levels could be rescued by p35 expression or overexpression of DIAP1 (Figures 4f and g). Systemic downregulation of DUBAI strongly affected fly viability, as *tubulin-Gal4*-driven coexpression of *DUBAI*^{dsRNA} with *Dicer2* resulted in pupal death at 29 °C. Flies homozygous for *DUBAI*^{KG07439} were similarly affected at 29 °C, whereas they exhibited no clear difference from the wild-type (wt) flies at the permissive temperatures (data not shown). These data are consistent with a model in which downregulation of DUBAI causes a reduction in DIAP1 levels, permitting subsequent caspase-dependent cell death.

DUB activity is indispensible for DUBAI to control DIAP1 levels. Consistent with a role for DUBAI in regulating DIAP1, we found these two proteins form a stable complex in S2 cells. As shown in Figure 5a, DIAP1 coprecipitated with both wt and C367A DUBAI (in which the putative catalytic Cys was changed to Ala), but not Ubpy, indicating the ability of DUBAI to interact with DIAP1 is likely independent of its enzymatic activity. We then tested whether the predicted catalytic Cys was required for DUBAI to maintain DIAP1 levels in apoptotic cells. As shown in Figure 5b, wt DUBAI maintained DIAP1 levels in ETP-treated S2 cells throughout the course of the experiment. However, ETP readily triggered DIAP1 degradation in cells transfected with control empty vector or the catalytically inactive mutant, indicating that DUBAI-mediated stabilization of DIAP1 likely relies on DUB activity.

Discussion

In *Drosophila*, ubiquitination of apoptotic regulators has a central role in orchestrating the death signaling circuit. The primary *Drosophila* IAP, DIAP1, functions as an E3 ligase to control caspase ubiquitination and thereby prevent the onset of the death program in healthy cells. Moreover, DIAP1 itself is suppressed by both auto- and transubiquitination that is stimulated by the apoptotic induction of IAP antagonists. Although much attention has been directed to DIAP1 ubiquitination, examination of its deubiquitination has been limited. Here we have shown that *CG8830*, an uncharacterized gene in *Drosophila*, encodes a novel DUB, DUBAI, which suppresses RHG-induced apoptosis by stabilizing DIAP1.

To examine how DUBs might impact *Drosophila* apoptosis, particularly by modulating DIAP1 stability, we took two

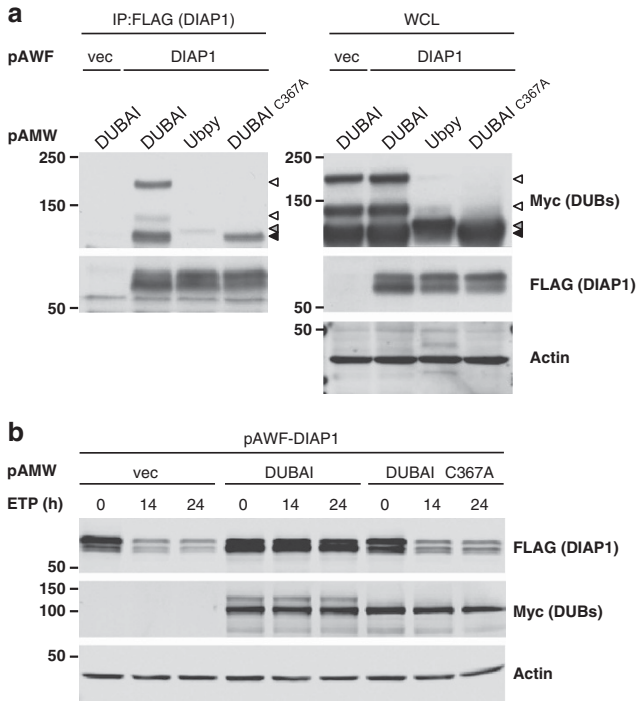


Figure 5 DUBAI mutated within the presumptive DUB catalytic domain cannot protect from apoptotic degradation of DIAP1. (**a**) Both wt and catalytically mutant (C367A) DUBAI/*CG8830*, but not Ubpy/*CG5798*, co-immunoprecipitated with DIAP1. FLAG-tagged DIAP1 was immunoprecipitated with FLAG beads from S2 cell lysate expressing indicated constructs. Co-immunoprecipitants were analyzed by western blotting with Myc antibody to detect DUBs that interact with DIAP1 (left panel). Expression levels of transfected constructs in the whole-cell lysate (WCL) were detected by the indicated antibodies. Actin antibody immunoblotting is shown to control for equal loading (right panel). Arrows in black and gray indicate unmodified DUBAI and Ubpy, respectively. Note that there appear to be post-translational modifications of DUBAI dependent on an intact catalytic domain as modified DUBAI species are detected in the wt, but not C367A mutant lanes (empty arrows). (**b**) Mutation at DUBAI's catalytic site in the putative DUB domain abrogated its protective effects on DIAP1. S2 cells expressing FLAG-tagged DIAP1 with empty vector, wt or catalytically mutant DUBAI were treated with ETP. Lysates were collected at the indicated time points and analyzed for DIAP1 and DUBAI levels by FLAG and Myc antibody, respectively. Actin levels are shown to indicate equal loading

complementary approaches. The gain-of-function screen carried out in cultured cells identified DUBAI and Ubpy as potential DIAP1-directed DUBs, as overexpression of these two proteins prevented ETP- and UV-induced DIAP1 degradation. A parallel loss-of-function assay with RNAi lines revealed that a decrease in DUBAI significantly enhanced rpr-induced eye cell death in the fly, whereas Ubpy deficiency had only a marginal effect. Although human USP19 has been implicated in the regulation of c-IAPs' stability,[33] knockdown of its fly homolog, CG30421, did not further modulate the rpr phenotype. Notably, DUBAI, but not Ubpy, formed a stable complex with DIAP1, further supporting DUBAI's dominant role in the control of DIAP1 stability and *Drosophila* apoptosis *in vivo*. For these reasons, we focused on the characterization of DUBAI. That said, we have not completely ruled out the involvement of genes that were expressed at only very low levels in our gain-of-function screen, nor the possibility that Ubpy contributes to DIAP1's stability through DUBAI or via a redundant pathway involving unidentified regulators. Downregulation of both Ubpy and DUBAI induces eye ablation similar to that caused by *DUBAI* dsRNA alone in flies expressing *GMR-grim*, implying that DUBAI might be the proximal regulator of DIAP1 in a regulatory circuit shared with Ubpy (data not shown). However, additional studies will be necessary to clarify their functional interactions.

To further dissect DUBAI's role in the apoptotic signaling repertoire, we examined DUBAI's involvement in cell death induced by various IAP antagonists. Interestingly, downregulation of DUBAI not only enhanced eye abnormalities induced by rpr, but also those caused by hid and grim. Therefore, the aggravated cell death caused by *DUBAI* RNAi must result from a loss of an anti-apoptotic regulator that works downstream of all these IAP antagonists, consistent with the model that compromised DUBAI activity causes a reduction in DIAP1 levels in the fly. Intriguingly, expression of $DUBAI^{dsRNA}$ at 29 °C (optimal temperature for the *GMR-Gal4* system[38]) caused a rough eye phenotype that could be completely rescued by blocking caspases with p35 or by replenishing DIAP1 levels by coexpression of a *UAS-DIAP1* construct, supporting the notion that DUBAI prolongs DIAP1's stability to halt caspase activation. Notably, many DUBs have been shown to form complexes with E3 ubiquitin ligases to control both ligase stability and the stability of their shared substrates.[39,40] Co-immunoprecipitation of DUBAI and DIAP1 is consistent with this paradigm.

It has been reported that genetic deletion of *scny* causes cell death associated with decreased DIAP1 levels in flies carrying two mutant alleles, but not in the heterozygote.[35] Therefore, the activity of residual protein might explain why scny did not emerge in our loss-of-function screen using RNAi lines.[41] Notably, scny deficiency was reported to enhance rpr- and grim-, but not hid-induced apoptosis, whereas overexpression of a long or a short *scny* isoform confers anti- or pro-apoptotic effects, respectively.[35] This suggests that scny function could be controlled by the balance of different isforms. In contrast, the two known *DUBAI* transcript variants translate into a peptide of identical sequence, whereas knockdown of DUBAI clearly exacerbated eye ablation induced by all IAP antagonists employed, including hid. Further analysis is required to understand the possible relationship between these DUBs.

In order to more fully understand how DUBAI modulates the ubiquitination status of DIAP1, we have tried to purify recombinant DUBAI from bacterial and baculoviral expression systems. Although the yield of catalytically inactive DUBAI was moderate in all systems tested, we failed to produce wt DUBAI despite many attempts. This prevented us from performing the *in vitro* assays necessary to demonstrate direct deubiquitination of DIAP1 by DUBAI. Nevertheless, given the physical interaction between DUBAI and DIAP1, the ability of a point mutation in DUBAI's putative catalytic site to prevent protection from ETP-induced DIAP1 degradation and the strong genetic interactions between DUBAI (*CG8830*) and DIAP1 (*th*), the most parsimonious explanation is that DUBAI directly controls DIAP1 by modulating its ubiquitination and stability.

According to the NCBI HomoleGene and BLAST, the closest human homologs of DUBAI are USP35 and USP38, respectively, although they have at most 35% sequence identity with DUBAI. Notably, amplification of the locus 11q14, where USP35 located, has been associated with a poor

prognosis in breast cancer patients,[42] whereas a genome-wide association study has linked the USP38-containing locus (4q31) to asthma susceptibility in the Japanese adult population.[43] However, the direct substrates and biological roles for these two human DUBs have yet to be elucidated.

DIAP1's central role in the fly death signaling circuit is well established, as is it's control by ubiquitination. We have shown here that DUBAI, a novel DUB encoded by *CG8830*, can control DIAP1 levels on apoptotic stimulation, thereby modulating the fly cell's balance between life and death. Whether regulation of DUBs will be as intricate as that of E3 ligases remains to be determined, but DUBAI may be a critical locus of apoptotic regulation in the fly, as it can effectively counteract the activity of the central cell death inducers, RHG, by modulating the levels of DIAP1.

Materials and Methods

DNA constructs and antibodies. cDNAs for DIAP1 and all DUBs were cloned into pENTR-3C (Invitrogen, Carlsbad, CA, USA). Fly DUBs (or their human homologs) known to control cell death were prioritized, together with other putative DUBs selected based on the availability of cDNA and RNAi fly lines. DIAP1 was recombined into pAWF, which contains a C-terminal FLAG tag and an actin promoter (DGRC gateway collection) using LR recombinase (Invitrogen). All DUBs were recombined into pAMW, which contains an N-terminal Myc tag and an actin promoter. HA and Myc antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). FLAG antibody was obtained from Sigma (Dallas, TX, USA).

Cell culture and transfections. *Drosophila* cells were grown in Schneider's media with 10% heat-inactivated FBS at 25 °C. Cell lysates for all experiments were made in lysis buffer (50 mM Tris, pH7.5, 150 mM NaCl, 1% NP40) supplemented with complete protease inhibitor cocktail (Roche, Indianapolis, IN, USA). For the gain-of-function assays, S2 cells were split into six-well plates. Four hundred nanograms pAWF-*DIAP1* and 200 ng pAMW-*DUB* were transfected with 4.8 μl Enhancer and 15 μl Effectene (Qiagen, Hilden, Germany) according to manufacturer's protocols. Cells from one well were split into three different plates the next day for DMSO, ETP (50 μM) or UV (100 mJ/cm^2) treatment. Cell lysates were collected before cell death become evident (18–24 h post treatment) and analyzed for the expression levels of DIAP1 and different DUBs with FLAG and Myc antibody, respectively. Co-immunoprecipitation assays were performed as previously described.[44] In brief, lysates from S2 cells transfected with pAWF-*DIAP1* and pAMW-*DUB* were incubated with FLAG beads at 4 °C for 1.5 h. Precipitates and whole-cell lysates were then analyzed by immunoblotting with indicated antibodies.

Transgenic flies. Sources of *Drosophila* stocks are available in (Supplementary Tables S2 and S3). *UAS-DUBAI* transgenic flies were generated according to standard protocols. The integration sites in different lines were mapped to individual chromosomes. Genotypes of progeny from each cross are shown in the figure legends. Expression of UAS constructs in the developing fly eye was achieved by *GMR-Gal4* drivers at temperatures specified. Micrographs of the eyes of age-matched female progeny are shown.

Conflict of Interest

The authors declare no conflict of interest.

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Author contributions

MJT and SK developed the concept. C-SY designed and performed all experiments. MJT cloned fly DUBs and initiated the gain-of-function screen. SAS collected and analyzed genetic interaction data. ACR and CDF carried out co-immunoprecipitation assays. SRH provided reagents. C-SY, MJT and SK interpreted the data and prepared the manuscript.

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