

Drosophila BRUCE inhibits apoptosis through non-lysine ubiquitination of the IAP-antagonist REAPER

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Active caspases execute apoptosis to eliminate superfluous or harmful cells in animals. In *Drosophila*, living cells prevent uncontrolled caspase activation through an inhibitor of apoptosis protein (IAP) family member, dIAP1, and apoptosis is preceded by the expression of IAP-antagonists, such as Reaper, Hid and Grim. Strong genetic modifiers of this pathway include another IAP family gene encoding an E2 ubiquitin conjugating enzyme domain, *dBruce*. Although the genetic effects of *dBruce* mutants are well documented, molecular targets of its encoded protein have remained elusive. Here, we report that *dBruce* targets Reaper for ubiquitination through an unconventional mechanism. Specifically, we show that *dBruce* physically interacts with Reaper, dependent upon Reaper's IAP-binding (IBM) and GH3 motifs. Consistently, Reaper levels were elevated in a *dBruce* $-/-$ background. Unexpectedly, we found that *dBruce* also affects the levels of a mutant form of Reaper without any internal lysine residues, which normally serve as conventional ubiquitin acceptor sites. Furthermore, we were able to biochemically detect ubiquitin conjugation on lysine-deficient Reaper proteins, and knockdown of *dBruce* significantly reduced the extent of this ubiquitination. Our results indicate that *dBruce* inhibits apoptosis by promoting IAP-antagonist ubiquitination on unconventional acceptor sites.

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Metazoans use a specific form of cell death known as apoptosis to eliminate damaged and potentially dangerous cells.¹ Indicative of its important role in tissue homeostasis, defective apoptosis is associated with a wide variety of diseases that includes cancer.²

Developmental programming or damage response pathways can trigger apoptosis by inducing pro-apoptotic gene expression. In *Drosophila*, four specific genes *grim*, *reaper*, *sickle* and *hid* are transcriptionally induced before apoptosis, and are required for most apoptosis that occurs in this organism.^{3,4} The encoded proteins are now widely referred to as IAP-antagonists, as they directly inhibit *Drosophila* IAP1 (dIAP1), a member of the inhibitor of apoptosis protein (IAP) family that contains signature Baculovirus IAP repeat (BIR) domains.⁵ Consistently, gain- or loss-of-function alleles of dIAP1 have been isolated from independent genetic screens as strong modifiers of IAP-antagonist-induced cell death.^{6,7} Once IAP-antagonists bind, dIAP1 itself undergoes ubiquitin-mediated degradation.^{8–11} In living cells, dIAP1 inhibits pro-apoptotic proteins, such as Dronc (initiator caspase), drICE and DCP-1 (effector caspases), as well as IAP-antagonists by tagging these targets with ubiquitin or ubiquitin-like proteins.^{12–16} Similarly in humans, IAP family members act as strong suppressors of apoptosis and a number of IAP-antagonists help sensitize these cells to various death stimuli.^{17–19} This pathway has drawn particular interest in recent years as abnormally high IAP levels confer apoptotic resistance to a number of cancer cell lines,²⁰ and synthetic

IAP-antagonist compounds show promise as anti-cancer drugs.²¹

Previous genetic screens had identified another IAP family gene, *dBruce*, as a major anti-apoptotic gene in *Drosophila*. *dBruce* encodes a giant (4876 a.a. protein) ubiquitin conjugating enzyme with a BIR domain, and its gain- or loss-of-function conditions modify Grim- and Reaper-induced cell death to a degree similar to dIAP1 alleles.^{22,23} Intriguingly, *dBruce* alleles do not affect cell death caused by another IAP-antagonist, Hid.²⁴ Unlike *dIAP1* mutants, most somatic cells can survive without *dBruce*. However, developing spermatids of *dBruce* $-/-$ flies prematurely activate caspases and undergo apoptosis.^{23,24} In spite of the strong genetic evidence linking *dBruce* in apoptosis regulation, ubiquitination targets of *dBruce* have not been identified. The presence of a ubiquitin conjugating (UBC) domain suggests that *dBruce* ubiquitinates pro-apoptotic proteins, such as Reaper and Grim. However, the difficulty of establishing biochemical links between *dBruce* and IAP-antagonists has led Vernooy *et al.*²² to suggest that *dBruce* regulates these proteins indirectly. Challenging that view, here we show the evidence that *dBruce* directly ubiquitinates Reaper at unconventional acceptor sites. Specifically, *dBruce* physically interacts with Reaper and the absence of *dBruce* leads to higher Reaper protein levels. Overexpression of the full-length *dBruce* strongly suppressed a Reaper-induced cell death phenotype, whereas this phenotype was only weakly suppressed by a truncated *dBruce* line lacking the UBC domain. Unexpectedly, the level

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Abbreviations: dIAP1, *Drosophila* Inhibitor of Apoptosis 1; IBM, IAP Binding Motif; GH3, Grim Helix 3; BIR, Baculovirus IAP Repeat; UBC, Ubiquitin Conjugating (domain); Dark, *Drosophila* Apaf1-related killer

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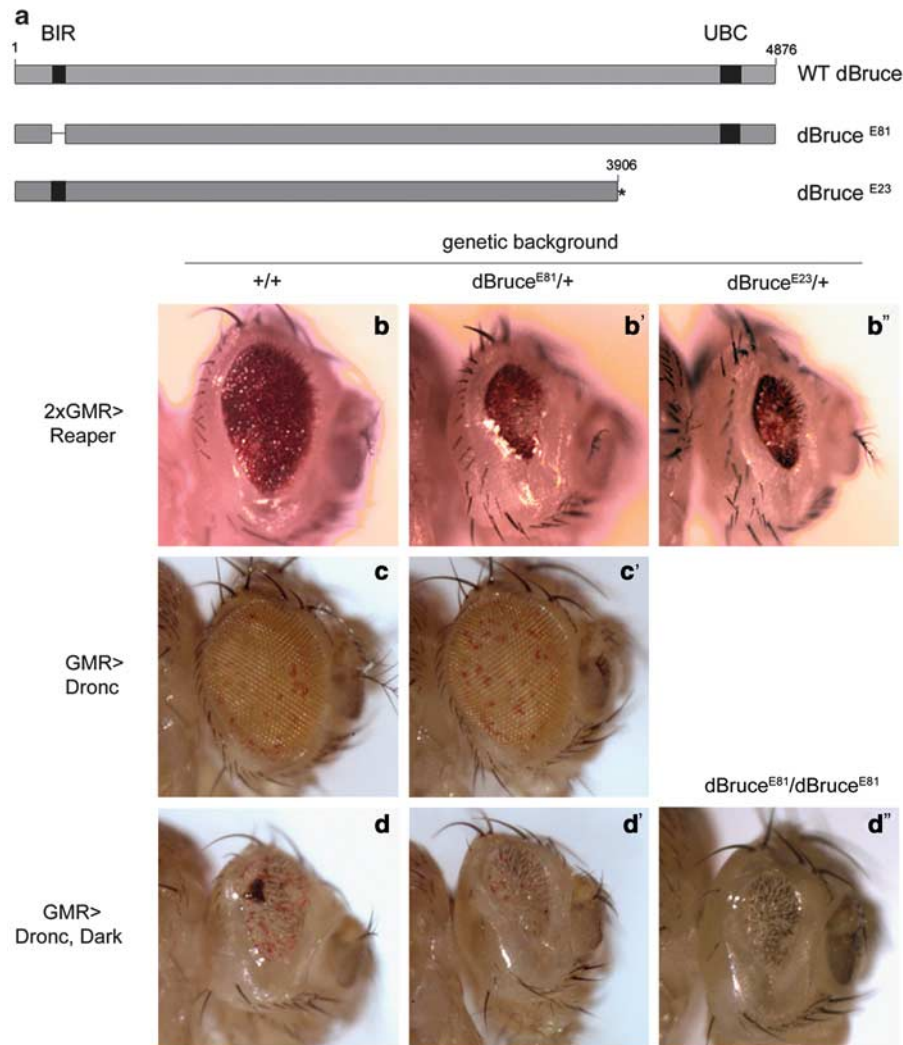


Figure 1 *dBruce* loss-of-function enhances Reaper-induced cell death in the eye, but not that caused by Reaper-independent apoptosome activation. (a) A schematic diagram of the *dBruce* primary structure, containing a BIR domain near the N-terminus, and a UBC domain near the C-terminus. *dBruce*^{E81} allele contains an in-frame deletion that eliminates the BIR domain,²⁵ whereas the *dBruce*^{E23} allele contains a premature stop codon that precedes the UBC domain. (b) Partial eye ablation caused by *reaper* overexpression during eye development (b), is enhanced in both, the *dBruce*^{E81}/+ background (b'), and the *dBruce*^{E23}/+ background (b''). On the other hand, *dBruce*^{E81}/+ condition does not impact the phenotype caused by expression of the apical caspase Dronc in the eye field (c and c'). Here, the uneven distribution of eye pigments is due to eye pigment cell death. The combined expression of the apoptosome components, Dronc and Dark causes a partial eye ablation (d), which is not affected in a *dBruce*^{E81}/+ background (d'). Additionally, no enhancement of the small eye phenotype owing to Dark and Dronc co-expression is observed in *dBruce*^{E81}/*dBruce*^{E81} homozygous condition (d and d''). Genotypes are: (b) *GMR-reaper*, *CyO*/+; +/+ , (b') *GMR-reaper*, *CyO*/+; *dBruce*^{E81}/+ , (b'') *GMR-reaper*, *CyO*/+; *dBruce*^{E23}/+ , (c) *GMR-G4*/+; *uas-Dronc*/+ , (c') *GMR-G4*/+; *uas-Dronc/dBruce*^{E81} , (d) *GMR-G4/CyO*; *uas-Dronc*, *uas-Dark*/+ , (d') *GMR-G4/CyO*; *uas-Dronc*, *uas-Dark/dBruce*^{E81} , (d'') *GMR-G4/CyO*; *uas-Dronc*, *uas-Dark*, *dBruce*^{E81}/*dBruce*^{E81}

of a mutant form of Reaper that lacks all internal lysines, thereby deficient of conventional ubiquitin acceptor sites, is also elevated in *dBruce* mutants. Furthermore, this form of Reaper is ubiquitinated and the extent of ubiquitination is reduced in *dBruce* knocked down cells, indicating that *dBruce* tags Reaper with ubiquitins on non-lysine acceptor sites.

Results

Reaper-induced cell death is modified by loss or gain of *dBruce* function. *dBruce* encodes a large protein with a BIR domain near the N-terminus, and a UBC domain near

the C-terminus (Figure 1a). To investigate the function of this gene, we primarily used two loss-of-function alleles: the *E81* allele has an in frame deletion that takes out the BIR domain,²³ and the *E23* allele has a stop codon that would not translate the UBC domain (Figure 1a and Materials and Methods). As previously reported with other loss-of-function alleles,²⁴ these two alleles behaved similarly in dominantly enhancing the *reaper* overexpression phenotype in the fly eye (Figures 1b–b''). To test the specificity of *dBruce* function, we performed genetic interaction assays between the *dBruce* alleles and downstream caspases of *Drosophila*. Unlike the *reaper*-induced phenotype, *dBruce*^{E81}/+ condition did not affect the pigment cell death phenotype

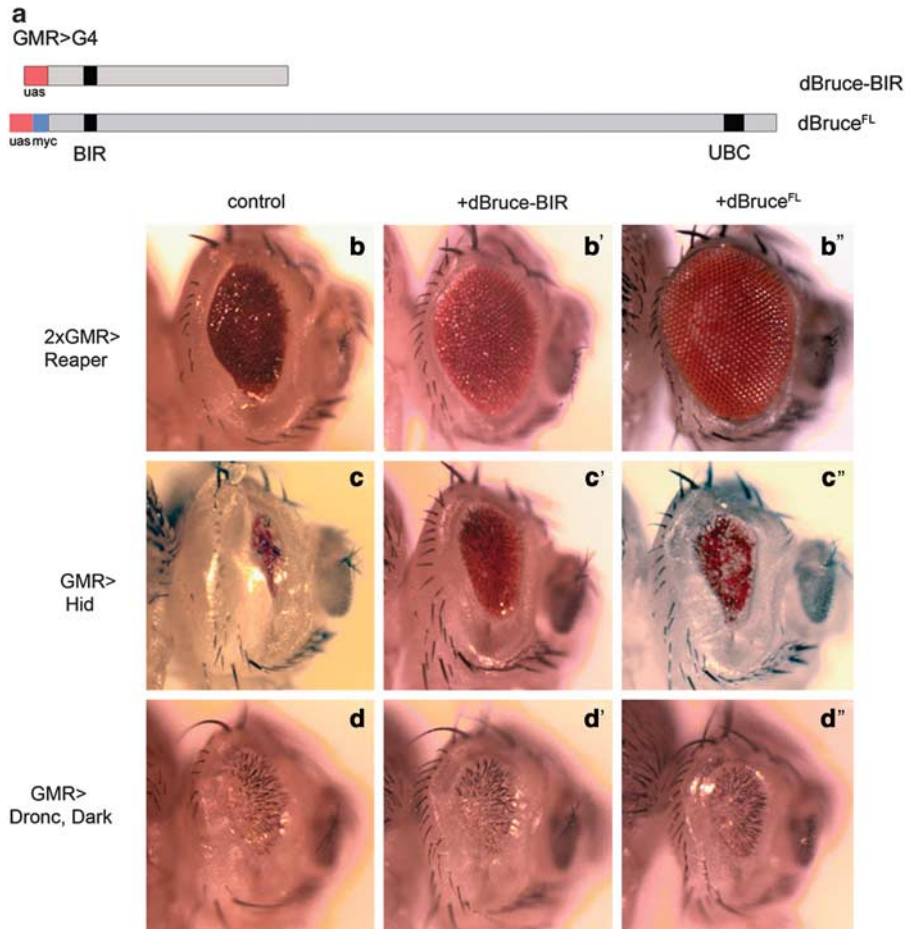


Figure 2 Overexpression studies reveal the importance of both the N-terminal and C-terminal sequences of *dBruce*. (a) A scheme showing the *dBruce* constructs used to rescue cell-death induced by Reaper, Hid, or Dronc and Dark together. The *dBruce-BIR* construct²⁵ contains the first 1622 a.a. residues of *dBruce*, which includes the BIR but not the UBC motif. The full-length *dBruce* contains the complete sequence. (b) Reaper expression in the eye causes partial eye ablation, and this is partially rescued by the *dBruce-BIR* construct (b'). Notably, full suppression is obtained only when the full-length *dBruce* is co-expressed (b''). The small eye phenotype induced by Hid overexpression (c) is also mildly suppressed by the *dBruce-BIR* construct (c'). In contrast to Reaper, however, the full-length *dBruce* did not fully rescue the Hid-induced eye phenotype (c''). Co-expression of the apoptosome components Dronc and Dark also led to a partial eye ablation (d), which was neither modified by the co-expression of *dBruce-BIR* (d') nor the full-length *dBruce* (d''). Genotypes: (b) *GMR-G4, ey-flp/+; GMR-Rpr,CyO/Sco; +/+*, (b') *GMR-G4, ey-flp/+; GMR-Rpr,CyO/uas-dBruce-BIR; +/TM6B*, (b'') *GMR-G4, ey-flp/+; GMR-Rpr,CyO/uas-dBruce^{FL}; +/TM6B*, (c) *GMR-G4, ey-flp/+; GMR-hid10/CyO; sb/TM6B*, (c') *GMR-G4, ey-flp/+; GMR-hid10/uas-dBruce-BIR; sb/TM6B*, (c'') *GMR-G4, ey-flp/+; GMR-hid10/uas-dBruce^{FL}; sb/TM6B*, (d) *GMR-G4/CyO; uas-Dronc, uas-Dark/TM6B*, (d') *GMR-G4/uas-dBruce-BIR; uas-Dronc, uas-Dark/TM6B* and (d'') *GMR-G4/uas-dBruce^{FL}; uas-Dronc, uas-Dark/TM6B*

caused by the overexpression of the initiator caspase Dronc (Figures 1c–c'). The results were further validated in a genetic condition where caspases were activated by the co-expression of Dronc and its adaptor, *Drosophila* apaf1-related killer (Dark).²⁵ The combined expression of Dronc and Dark in the developing eye imaginal discs generates a potent eye ablation phenotype owing to the assembly of the apoptosome, an oligomeric complex of Dark that activates Dronc for apoptosis,²⁶ providing a way to activate caspases independent of the IAP-antagonists. This phenotype was neither affected by *dBruce^{EB1}/+* nor by *dBruce^{EB1}-/-* conditions (Figures 1d–d''). These findings suggest that *dBruce* regulates apoptotic events upstream of the apoptosome.

To examine the effects of *dBruce* overexpression, we employed two *dBruce* transgenes, one that can express a truncated form of *dBruce* that retains the BIR domain

(*dBruce-BIR*),²⁶ and the other encoding the full-length *dBruce* (*dBruce^{FL}*) (Figure 2a and Materials and Methods). Co-expression of *dBruce-BIR* only moderately suppressed the *reaper* overexpression phenotype, whereas the *dBruce^{FL}* completely rescued that phenotype under otherwise similar conditions (Figures 2b–b''). *dBruce-BIR* also weakly suppressed the eye ablation phenotype caused by *hid* overexpression (Figures 2c and c'). Unlike the *reaper*-inflicted eye phenotype, however, *dBruce^{FL}* did not suppress the *hid* phenotype any better than that by *dBruce-BIR* (Figure 2b''). In addition, no suppression was observed when either *dBruce-BIR* or *dBruce^{FL}* was co-expressed with the apoptosome components, Dronc and Apaf1 (Figures 2d–d''). This is in agreement with the idea that *dBruce* does not regulate caspases directly, and point to IAP-antagonists as likely targets of *dBruce*. Although the anti-apoptotic function can be partly attributed to the *dBruce* BIR domain, the sequences

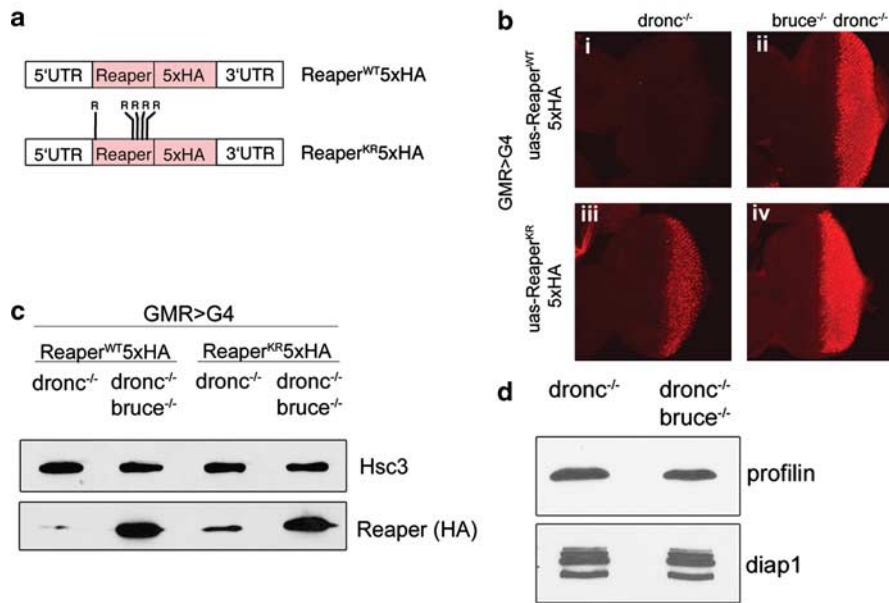


Figure 3 *dBruce* loss-of-function condition enhances Reaper protein levels, even in the absence of lysine residues. **(a)** A graphic scheme of the Reaper transgenes used in this study. The Reaper coding sequence was cloned in frame with a 5 × HA tag to generate a fusion protein, flanked by the 5′ and 3′UTR regions of the Reaper transcription unit. The transgenes were targeted for insertion in a predetermined genomic locus (51C). Reaper-KR has all internal lysine residues (at positions 20, 52, 56, 59 and 62) mutated to arginine. **(b)** Reaper protein levels as assessed by anti-HA immunolabeling (red). All experiments were performed in a *dronc*¹²⁹ *-/-* background to allow the survival of Reaper expressing cells. The levels of wild-type Reaper (Reaper-WT) tagged with 5 × HA are visualized in (i) and (ii). Reaper-KR levels similarly visualized with anti-HA are shown in (iii) and (iv). In the *dBruce*^{E81} *-/-* background, higher levels of both Reaper-WT and Reaper-KR are detected (ii) and (iv) as compared with the *dBruce*⁺ background (i) and (iii). Genotypes are: (i) *GMR-G4/uas-reaper*^{WT}5 × HA; *dronc*¹²⁹/*dronc*¹²⁹ (ii) *GMR-G4/uas-reaper*^{WT}5 × HA; *dronc*¹²⁹, *dBruce*^{E81}/*dronc*¹²⁹, *dBruce*^{E81} (iii) *GMR-G4/uas-reaper*^{KR}5 × HA; *dronc*¹²⁹/*dronc*¹²⁹ and (iv) *GMR-G4/uas-reaper*^{KR}5 × HA; *dronc*¹²⁹, *dBruce*^{E81}/*dronc*¹²⁹, *dBruce*^{E81}. **(c)** Confirmation of results in **(b)** through western blot. Extracts were prepared from ~30 eye imaginal discs of the specific genotypes as in **(b)**. Reaper levels as assessed through anti-HA blot (lower panel) can be compared with a control of anti-Hsc3 blot (upper panel). **(d)** Endogenous diAP1 levels are not altered in the *dBruce*^{-/-} background. Anti-diAP1 western blot (lower panel) can be compared with anti-Profilin controls (upper panel). Genotypes: *dronc*¹²⁹/*dronc*¹²⁹ (left lane) and *dronc*¹²⁹, *dBruce*^{E81}/*dronc*¹²⁹, *dBruce*^{E81} (right lane)

outside of the BIR domain (possibly the UBC domain) are also required for *dBruce*'s full anti-apoptotic function.

***dBruce* regulates the levels of wild-type and lysine-deficient mutant Reaper.** Although Vernooij *et al.*²² had previously speculated that Reaper is not a direct target of *dBruce*, the specificity of the genetic interactions prompted us to revisit this issue. As a first step, we analyzed the effect of *dBruce* mutations on the levels of Reaper protein expressed in the developing eye imaginal discs. To visualize ectopically expressed Reaper proteins, we generated a transgenic UAS-Reaper line with five tandem repeats of the HA epitope (5 × HA) added to its C-terminus (Figure 3a). In addition, we generated a similar UAS-Reaper line, in which all lysine residues were replaced with arginines (henceforth referred to as Reaper-KR), thereby eliminating conventional ubiquitin acceptor sites within this protein (Figure 3a). The HA epitope does not introduce any lysines. To ensure equivalent levels of transcription, both transgenes were targeted to the same genomic locus through the phiC31 integrase system (see Materials and Methods). Reaper protein expression analysis was performed in the *dronc* mutant background,²⁷ which prevents caspase activation and cell death that otherwise complicates the assessment of Reaper protein levels (Supplementary Figure S1). Immunostaining against HA

under these conditions showed faint signals from the wild-type Reaper protein (Reaper-WT), whereas a significantly higher signal was detected for Reaper-KR (Figure 3b, compare (i) and (iii)). These results indicate that Reaper protein levels are affected by internal lysine residues, most likely due to ubiquitin-mediated protein degradation.

To test whether *dBruce* affects Reaper levels, we examined the levels of HA-tagged Reaper expression in a *dBruce* mutant background. Consistent with the idea that *dBruce* promotes Reaper degradation, we found a significant increase in Reaper-WT levels in the *dBruce*^{E81} *-/-* condition (Figure 3b, compare (i) and (ii)). Unexpectedly, we found that Reaper-KR levels also increase under this condition, although to a lesser degree than Reaper-WT (Figure 3b, compare (iii) and (iv)). To rule out the possibility of an immunostaining artifact or epitope masking, we confirmed these results by western blot analysis of Reaper-HA levels from eye imaginal disc extracts (Figure 3c). In validation of our immunostaining results, we found that extracts from *dBruce*^{-/-} tissues have more than 10 times as much wild-type Reaper (*P* = 0.0417), and ~3 times as much Reaper-KR levels (*P* = 0.0497), as *dBruce*⁺ tissues (Figures 3c and 4e).

Reaper and *dBruce* physically interact through Reaper's N-terminal and GH3 motifs. The observation that Reaper-KR levels increase in *dBruce*^{-/-} tissues raised

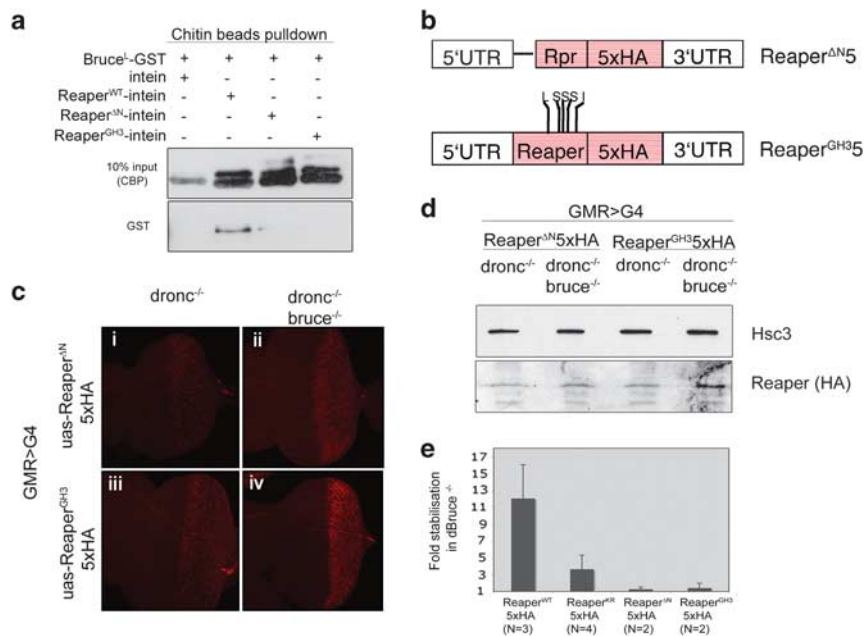


Figure 4 dBruce and Reaper physically interact. (a) A portion of dBruce containing the BIR domain fused with GST (Bruce^L-GST, residues 1–481) purified and eluted from glutathione beads was incubated with purified Reaper-CBD bound to chitin beads (or CBD alone). Physical interaction was detected between dBruce and Reaper^{WT}-CBD. Mutations in Reaper's N-terminus (Reaper^{ΔN}) or Reaper's GH3 domain (Reaper^{GH3}) abolish this interaction in otherwise identical conditions. (b) A schematic diagram of the Reaper mutant transgenes targeted to the 51C locus. Reaper^{ΔN} has a deletion encompassing the IBM motif (residues 2–8), while Reaper^{GH3} has mutations in the GH3 motif as follows: ³²W to L, ³⁴F to S, ³⁵L to S, ³⁶A to S and ³⁶V to I. (c) The *in vivo* levels of Reaper^{ΔN} ((i) and (ii)) and Reaper^{GH3} ((iii) and (iv)) proteins ectopically expressed in eye imaginal discs as assessed through anti-HA immunolabeling (red). The levels can be compared between *dBruce*^{+/+} ((i) and (iii)) and *dBruce*^{-/-} ((ii) and (iv)) conditions. Genotypes: (i) *GMR-G4/uas-reaper^{ΔN}5 × HA; dronc¹²⁹/dronc¹²⁹*, (ii) *GMR-G4/uas-reaper^{ΔN}5 × HA; dronc¹²⁹, dBruce^{E81}/dronc¹²⁹, dBruce^{E81}* (iii) *GMR-G4/uas-reaper^{GH3}5 × HA; dronc¹²⁹/dronc¹²⁹* and (iv) *GMR-G4/uas-reaper^{GH3}5 × HA; dronc¹²⁹, dBruce^{E81}/dronc¹²⁹, dBruce^{E81}*. (d) Confirmation of results in (c) through western blot analysis. Extracts from around 30 eye imaginal discs for each genotype were probed through anti-HA antibody (lower panel) and compared with anti-Hsc3 controls (upper panel). (e) Quantification of western blot results shown in (c). y axis shows estimated Reaper protein levels in *dBruce*^{-/-} imaginal disc extracts divided by those in control *dronc*^{-/-} extracts. N indicates the number of independent experiments performed, while the error bar shows standard deviation

two possibilities: in an 'indirect model' as proposed by Vernooij *et al.*,²² dBruce would affect an unknown intermediate protein whose function, in turn, affects Reaper levels. Among others, we considered dIAP1 as a potential mediator of dBruce's 'indirect effects' on Reaper, as previous studies have shown that dIAP1 acts as a ubiquitin ligase that can promote the degradation of Reaper.¹³ However, dIAP1 levels did not change in *dBruce* loss-of-function conditions (Figure 3d).

By contrast, a 'direct model' can be considered in which dBruce promotes ubiquitination of Reaper directly, but utilizes non-lysine residues as ubiquitin acceptor sites. In the latter case, mutating all internal lysine residues in the Reaper sequence would not be sufficient to prevent dBruce ubiquitination of Reaper. In a first step to examine the 'direct model', we tested possible physical interactions between dBruce and Reaper in an *in vitro* pull down assay using chitin binding domains (see Materials and Methods). Specifically, we found that recombinant Bruce-GST (residues 1–481 that include the BIR domain) associated with Reaper-WT in this assay (Figure 4a). We also performed binding assays with Reaper mutants that either lack the N-terminal IAP binding motif (IBM), or have mutations in the central helical domain (GH3). IBM is a motif that can bind to a related BIR domain present in dIAP1²⁸ and the GH3 has self-associating properties that promote the formation of multimeric IAP-antagonist

complexes.²⁹ Neither of these mutant proteins interacted with dBruce *in vitro* (Figure 4a). This result indicates that the BIR domain of dBruce has direct affinity for Reaper *in vitro*, and that this interaction is specific. These *in vitro* results are also consistent with the *dBruce*-BIR overexpression results *in vivo* (Figure 2), where *reaper*- and *hid*-induced cell death was moderately suppressed.

To test if dBruce regulates Reaper through the IBM and GH3 motifs *in vivo*, we generated transgenic constructs of these Reaper mutants with 5 × HA tags at their C-terminus (Figure 4b), and targeted them for insertion into a predetermined genomic locus (51C). Consistent with the idea that dBruce does not bind to these Reaper mutants *in vivo*, we observed no significant alteration of these Reaper protein levels in the presence or absence of dBruce (Figures 4c and d). We have quantified these results after western blot, and found that the stabilization fold is 1.25 for Reaper ΔN and 1.4 for Reaper-GH3, and the differences observed are not statistically significant ($P = 0.4096$ and $P = 0.5024$, respectively; see Figure 4e).

Reaper-KR is ubiquitinated *in vitro*, and the abundance of the ubiquitinated species is dependent on dBruce. Our finding that dBruce regulates Reaper levels prompted us to biochemically examine the extent of Reaper ubiquitination in cultured *Drosophila* S2R+ cell extracts. To

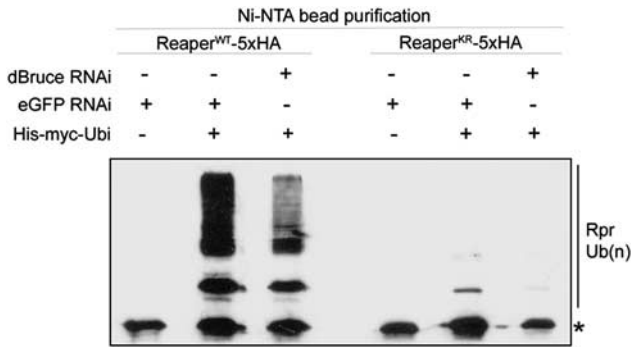


Figure 5 dBruce dependent ubiquitination of wild-type and lysine-deficient Reaper proteins. In order to purify ubiquitinated Reaper proteins from cultured cells, S2R⁺ cells were transfected with Reaper-5 × HA and His-tagged ubiquitin expression plasmids. Cells were treated with a proteasome inhibitor before his-ubiquitin-tagged proteins were purified from these extracts using Ni-NTA resin. The Reaper protein was detected through western blot with anti-HA antibody. The asterisk indicates the band corresponding to unmodified Reaper protein that was retained through unspecific matrix interaction. Higher molecular weight bands of Reaper-WT-5 × HA were detected only when His-ubiquitin was co-transfected (lanes 2, 3, 5 and 6), indicative of ubiquitin conjugation. His-ubiquitin dependent higher molecular weight Reaper-KR was also detected, although at a lower extent (lane 5). Knockdown of *dBruce* by RNAi decreases the abundance of the ubiquitinated species for both Reaper-WT and in Reaper-KR, supporting the idea that dBruce ubiquitinates Reaper on non-lysine residues

keep cells alive after Reaper transfection, we generated an inducible S2R⁺ cell line that expresses the baculovirus caspase inhibitor p35.³⁰ We transfected into these cells 5 × HA tagged Reaper with or without His-tagged ubiquitin. Purification of the ubiquitin-tagged proteins with Ni-NTA beads from these extracts, and subsequent detection of Reaper with anti-HA antibody revealed a ladder of higher molecular weight bands, corresponding to ubiquitinated Reaper species (Figure 5). These higher molecular weight species appeared only when His-ubiquitin was co-transfected (for example, compare between lanes 1 and 2 in Figure 5), indicating that they emerge as a result of ubiquitin conjugation. Consistent with the idea that Reaper is extensively ubiquitinated, wild-type Reaper exhibited a very prominent ubiquitination profile (Figure 5, compare lanes 1 and 2). Interestingly, Reaper-KR showed a more modest but unequivocal ubiquitination pattern (Figure 5, compare lanes 4 and 5). These results confirmed our hypothesis that Reaper must contain non-lysine ubiquitin acceptor sites.

There are a number of known ubiquitin-acceptor sites other than lysines. These include cysteines, serines, threonines and the N-terminal amino group.^{31–34} To examine the roles of these unconventional acceptor sites, we made Reaper-HA transgenes that have combinations of these residues mutated, on top of the five lysine mutations. These mutants made proteins that still appeared to have residual ubiquitin conjugation (Supplementary Figure S2). This result suggests that either another combination of residues might be responsible for accepting the ubiquitin moiety, or ubiquitination occurs at the N-terminal amino group. Testing the latter idea is impossible, as manipulating this N-terminus also abolishes Reaper's ability to bind BIR domains of its ubiquitinating enzymes.²⁸

To determine whether dBruce is responsible for the emergence of non-lysine conjugated ubiquitin chains on Reaper, we performed the Reaper ubiquitination analysis after knockdown of *dBruce* function. We found that dBruce RNAi treatment results in a decrease in the abundance of the ubiquitinated species, both with Reaper-WT and Reaper-KR (Figure 5, compare lanes 2 and 3, 5 and 6, respectively). The fact that these bands do not completely disappear may be owing to an incomplete knockdown of dBruce, or alternatively, owing to other ubiquitination enzymes that target Reaper. Taken together, these results support the idea that dBruce directly regulates Reaper protein levels by ubiquitinating it at lysine and non-lysine residues.

Discussion

As it was first identified, *dBruce* has attracted significant scientific interest as one of the strongest modifiers of apoptosis in *Drosophila*. Surprisingly, little is known about the molecular function and substrates of dBruce. Here, we established Reaper as a direct target of dBruce in flies. Our structure/function analysis of Reaper revealed the requirement of both the N-terminal IAP-binding domain and the central helical domain (GH3) for directly binding to dBruce. The IAP-Binding Motif (IBM) binds to the BIR domain of dIAP1,²⁸ and its requirement suggests that Reaper binds to a similar BIR motif present in dBruce. The GH3 motif has been recently characterized as a multimer-forming interface between IAP-antagonists,²⁹ which leads us to suspect that dBruce specifically recognizes active Reaper multimers, but not monomers. A similar GH3 motif is present in Grim, which genetically interacts with dBruce.²⁴ On the other hand, *dBruce* does not show strong genetic interactions with Hid, which lacks a GH3 motif. In mammals, the dBruce homolog BRUCE (otherwise known as APOLLON) can bind and ubiquitinate a mammalian IAP-antagonist, Smac.^{35,36} Noteworthy is the fact that Smac also forms dimers *in vitro*.³⁷ There are a number of other IAP-antagonists in mammals, and it remains to be seen whether their susceptibility to BRUCE correlates with their ability to form multimeric complexes.

Intriguingly, our data indicate that dBruce ubiquitinates Reaper on non-lysine ubiquitin acceptor sites. This is in contrast to dIAP1 and X-linked IAP, which are thought to ubiquitinate Reaper and caspases strictly on lysine side chains.^{13,15} Our results nicely explain a previously published observation that dBruce overexpression can somehow suppress apoptosis initiated by lysine-deficient Reaper.²² But unlike the conclusions drawn by those authors, we conclude that the inhibition is direct.

In our study, we did not find any evidence that *dBruce* regulates caspases directly. Specifically, *dBruce* does not affect the cell killing activity of the initiator caspase Dronc. The result is consistent with the observation that *dBruce* does not strongly affect Hid function, which also triggers apoptosis through the activation of initiator and effector caspases.²² These results have implications on the potential mechanisms of premature caspase activation that occurs in *dBruce* ^{-/-} spermatids. A simple model would suggest that, as in somatic tissues, dBruce primarily targets Grim and Reaper in differentiating spermatids, and the deregulation of these

IAP-antagonists lead to premature caspase activation. Alternatively, developing spermatids may have additional dBruce co-factors that allow dBruce to target caspases. In somatic tissues, it appears that *dBruce* regulates apoptosis primarily by acting as an IAP-antagonist inhibitor. As synthetic IAP-antagonists are actively being developed as anti-cancer compounds, the endogenous BRUCE levels may contribute to a particular cell's sensitivity or resistance to these compounds.

Materials and Methods

Generation of the p35 inducible cell line (S2R + p35). *XhoI/NotI* sites were used to subclone the full-length p35 cDNA³⁰ into the pMK33-HS vector, which contains a methallothionein promoter. This plasmid was transfected into S2R + cells, and hygromycin resistance was used to isolate a stable insertion line.

Cell culture and cell transfection. Cells were transfected using effectene (Qiagen, Valencia, CA, USA). To knock down *dBruce*, 15.6×10^6 S2R + p35 cells in 75 cm² T-flasks were transfected with 19.5 μ g dsRNA, 3.9 μ g of actin-Gal4 and 3.9 μ g *uas-dicer2*, 7 days before transfection of Reaper plasmids. To induce Reaper expression, cells were re-plated in six-well plates, and transfected with 1 μ g actin-Gal4 and 1 μ g of *uas-Reaper* plasmids. In order to visualize ubiquitinated proteins, 1 μ g His-Myc-Ubiquitin plasmid or empty vector was co-transfected. CuSO₄ was added to a final concentration of 0.5 mM to induce p35 expression to prevent Reaper from killing transfected cells. 3 days after transfection, cells were treated with 50 μ M MG132 for 6 h, then collected and resuspended in 150 μ l lysis buffer (1% SDS, 10 mM EDTA pH 8.0, 50 mM Tris-HCl pH 8.0 and 1 mM PMSF) supplemented with 5 mM NEM and protease inhibitors. Crude extract was sonicated and diluted 1:10 in IP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.0, 150 mM NaCl and 1 mM PMSF) supplemented with 5 mM NEM and protease inhibitors (Roche, Indianapolis, IN, USA). To purify proteins conjugated with His-tagged ubiquitin, each extract was incubated with 20 μ l Ni-NTA His-Bind Superflow beads (Novagen, San Diego, CA, USA) for 2 h at 4 °C and washed three times in PBS + 20 mM imidazole. SDS loading buffer (containing DTT that releases Ni²⁺ bound proteins from beads) was added to each sample before boiling for 5 min at 95 °C and loading onto a 15% SDS page gel. The nitrocellulose membranes were autoclaved for 30 minutes after transfer. To visualize Reaper through western blots, rat monoclonal anti-HA (Roche, clone 3F10) was used at 1:500 dilution.

Cloning. Generation of transgenes for fly transformation: to make a full-length *dBruce* transgene, a 19.7-kb genomic DNA encompassing the locus was cloned from the CH321-36F16 BAC clone (Pacman Resources) using the recombinering technology.³⁸ The construct was designed to have the coding sequence fused in frame with a myc-epitope into a pre-engineered UAS-myc-pACMAN vector. Reaper coding sequences with in frame 5 \times HA tags were cloned into *BamHI* sites engineered in between Reaper's 5' UTR and 3' UTR sequences. Using *EcoRI* sites, the engineered cDNAs were moved to the pUAST attB vector and inserted into the 51C locus using a PhiC31 integrase strain.³⁹ Generation of Reaper-CBD fusions and Bruce^L-GST: to generate recombinant protein fused to the chitin binding domain (CBD), coding sequences of wild-type or mutant Reaper were subcloned into the Nde1 site of the pTYB2 plasmid (N.E.B.), which encodes Reaper fused to the N-terminal to intein-CBD. Using the same approach, mutant Reaper lacking the N-terminal eight amino acids (Δ N), or with mutations in the GH3 domain²⁹ were subcloned into pTYB2. Bruce^L-GST was made by subcloning a PCR product that encodes from the original start codon to amino acid 481, into pGEX 4t-3. These recombinant proteins were expressed in BL21 cells according to standard protocols. His-Myc-Ubiquitin: a plasmid encoding N-terminal 6 \times His-Myc fused to mammalian ubiquitin³² was obtained from Michele Pagano, and *EcoRI/KpnI* sites were used to move the 6 \times His-Myc-ubiquitin into the pUAST vector.

Eye imaginal disc western blot quantification. About 30 pairs of eye imaginal discs were dissected for each genotype, and extracts were made in eye disc lysis buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, 150 mM NaCl and 1% SDS). Crude extracts were centrifuged 10 minutes at maximum speed at 4 °C, and the supernatant was quantified using Bradford assay. Normalized quantities were loaded onto 15% SDS page gels. Anti-HA antibody (clone 3F10, Roche) was

used to assess Reaper levels, and anti-Hsc3 (generated in the lab) or anti-Profilin (Chi1J, Developmental Studies Hybridoma Bank, University of Iowa) were used as loading controls.

Fly strains. Genes were overexpressed in *Drosophila* imaginal discs using the standard Gal4/UAS system.⁴⁰ The Reaper transgenes, as well as the full-length UAS-*dBruce* flies were generated in this study. *uas-Dronc*, *uas-Dark*,²⁶ *uas-dBruce-BIR*,²⁴ *dronc*²⁹ (Xu *et al.*²⁷) and *dBruce*^{E81} and *dBruce*^{E23} (Arama *et al.*²³) were as reported previously. As the molecular nature of the *dBruce*^{E23} allele had not been reported previously, we sequenced this allele and found an 11bp (GCCAAGGACAA) deletion in the coding sequence, generating a frameshift at a.a. 3901, leading to a premature stop codon at a.a. 3906.

Pulldown experiments. 1 ml of induced bacterial extracts of Reaper-CBD (or control plasmids) were bound to 40 μ l of chitin beads (New England Biolabs). Beads were washed three times in binding buffer (PBS1 \times containing 0.1% Igepal CA-630, Sigma, St. Louis, MO, USA). The amount of bound Reaper was estimated by comparing the intensity of Comma assay staining with known amounts of BSA proteins. 10 μ g of each Reaper-CBD proteins was used for binding, and 1 μ g (10%) was saved for input analysis. Bruce^L-GST was purified with glutathione sepharose beads (GE Healthcare), and the fusion protein was eluted using 20 mM glutathione pH8.0 solution. Eluted Bruce^L-GST was quantified as described above, and 1 μ g was used for each incubation. An aliquot of 10% input was saved for input analysis.

10 μ g of Reaper-CBD or control protein bound to chitin beads was incubated with 1 μ g of eluted Bruce^L-GST in a final volume of 300 μ l in binding buffer (PBS1 \times containing 0.1% Igepal CA-630, Sigma) for 3 h at 4 °C. Beads were washed three times in binding buffer. A volume of 30 μ l of 3% SDS was added to the beads, and the supernatant was loaded onto 10% SDS gels. Mouse anti-CBP 1:1000 antibody was used to detect Reaper-CBD fusions (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and affinity-purified rabbit anti-GST antibody (ICL, Newberg, OR, USA) 1:2000 was used to detect Bruce^L-GST.

Conflict of Interest

The authors declare no conflict of interest.

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