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# *Drosophila* Bruce Can Potently Suppress Rpr- and Grim-Dependent but Not Hid-Dependent Cell Death

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## Summary

**Bruce is a large protein (530 kDa) that contains an N-terminal baculovirus IAP repeat (BIR) and a C-terminal ubiquitin conjugation domain (E2) [1, 2]. BRUCE upregulation occurs in some cancers and contributes to the resistance of these cells to DNA-damaging chemotherapeutic drugs [2]. However, it is still unknown whether Bruce inhibits apoptosis directly or instead plays some other more indirect role in mediating chemoresistance, perhaps by promoting drug export, decreasing the efficacy of DNA damage-dependent cell death signaling, or by promoting DNA repair. Here, we demonstrate, using gain-of-function and deletion alleles, that *Drosophila* Bruce (dBruce) can potently inhibit cell death induced by the essential *Drosophila* cell death activators Reaper (Rpr) and Grim but not Head involution defective (Hid). The dBruce BIR domain is not sufficient for this activity, and the E2 domain is likely required. dBruce does not promote Rpr or Grim degradation directly, but its antiapoptotic actions do require that their N termini, required for interaction with DIAP1 BIR2, be intact. dBruce does not block the activity of the apical cell death caspase Dronc or the proapoptotic Bcl-2 family member Debcl/Drob-1/dBorg-1/Dbok. Together, these results argue that dBruce can regulate cell death at a novel point.**

## Results and Discussion

In *Drosophila*, the products of the *reaper* (*rpr*), *head involution defective* (*hid*), and *grim* genes are essential activators of caspase-dependent cell death (reviewed in [3]). We carried out a genetic screen for suppressors of Rpr-, Hid-, and Grim-dependent cell death to identify regulators of their activity. We generated approximately 7000 new insertion lines of the GMREP P element transposon [4]. GMREP contains an engineered eye-specific enhancer sequence (GMR). This sequence is sufficient to drive the expression of linked genes in and posterior to the morphogenetic furrow during eye development. Thus, insertion of GMREP within a region can lead to the eye-specific expression of nearby genes. Each insertion line was crossed to flies that had small eyes due to the eye-specific expression of Rpr (GMR-Rpr flies), Hid (GMR-Hid flies), or Grim (GMR-Grim flies), and the progeny were scored for enhancement or suppression. A number of suppressors were identified (to be described elsewhere). Five lines (GMREP-86A-1–5) mapped to the 86A region (see Figure 1), and each strongly suppressed cell death induced by eye-specific expression of Rpr (Figures 1AII and1AIII) or Grim (Figures 1AIV and 1AV) but not Hid (Figures 1AVI and 1AVII). These lines mapped within a 6-kb interval. We obtained a number of other lines with P-element insertions located in the nearby region. Four of these, EP(3)0359, EP(3)0739, l(3)j8B6, and l(3)06142, mapped within six base pairs of the GMREP-86A-3–5 insertion sites (Figure 1B). None of these, nor a fifth nearby line, l(3)06439, acted as a suppressor of GMR-Rpr-, GMR-Grim-, or GMR-Hid-dependent cell death (data not shown). These results argue that the cell death suppression seen with the GMREP-86A lines was not due to a transposon-induced loss of function, but rather to the GMREP-dependent expression of a nearby gene. All of the GMREP-86A insertions were located 5′ to a gene encoding the *Drosophila* homolog, dBruce, of murine Bruce [1] (also known as Apollon in humans [2]), suggesting this as an obvious candidate. The results of tissue in situ hybridizations with a dBruce probe and immunocytochemistry with a dBruce-specific antibody support this possibility. dBruce transcript and protein were expressed at uniform low levels in wild-type eye discs. However, in the GMREP86A lines, they were expressed at high levels in and posterior to the morphogenetic furrow of the eye disc, which is where the GMR element drives expression [4] (Figure 1C).

To demonstrate that dBruce was responsible for the GMREP-86A-dependent suppression of Rpr- and Grim-dependent cell death, we specifically downregulated levels of the dBruce transcript in the eyes of flies carrying a GMR-Rpr transgene as well as a GMREP-86A element. We focused our analysis on one line, GMREP-86A-1, as all five lines behaved similarly with respect to cell death suppression and dBruce overexpression. We generated flies that carried a dBruce RNA interference (RNAi) construct driven under GMR control (GMR-dBruce-RNAi flies). The eyes of GMR-dBruce-RNAi flies were normal (data not shown). We crossed these animals to flies in which GMR-Rpr-dependent cell death was suppressed by the presence of the GMREP-86A-1 transposon and identified progeny from this cross that carried all three transgenes, GMR-dBruce-RNAi, GMR-Rpr, and GMREP-86A-1. We reasoned that if ectopic expression of dBruce in the eye, driven by the GMREP-86A-1 insertion, was responsible for the suppression of Rpr-dependent cell death, then expression of dBruce-RNAi should downregulate levels of the dBruce sense transcript. This should lead to an attenuation of the GMR-EP-86A-1-dependent suppression of Rpr-dependent cell death, causing a decrease in eye size. Such an attenuation was

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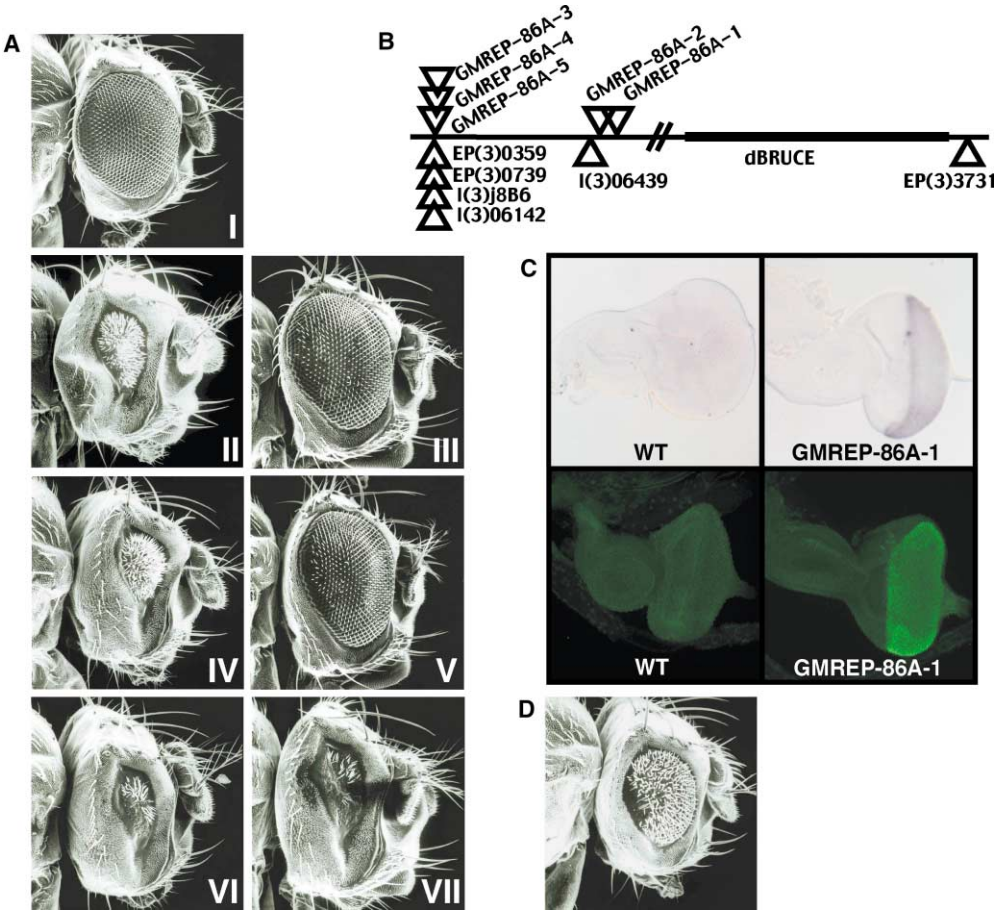


Figure 1. dBruce Expression Suppresses Cell Death Induced by Rpr and Grim but Not Hid

(A) Scanning electron micrographs are shown. The genotypes are as follows: I, wild-type; II, GMR-Rpr/+; III, GMREP-86A-1/GMR-Rpr; IV, GMR-Grim/+; V, GMREP-86A-1/GMR-Grim; VI, GMR-Hid/+; VII, GMREP-86A-1/GMR-Hid. Each of the GMREP-86A insertion lines, which ectopically express dBruce in the eye (Figure 1C), act as strong suppressors of Rpr- and Grim-dependent, but not Hid-dependent, eye cell death. Representative examples are shown for one of these insertions, GMREP-86A-1. Scanning electron microscopy was performed as described in [20].

(B) A diagram of P-element insertions in the 86A region. The P elements shown stacked on top of each other are all within 6 base pairs of each other and are 23 kb upstream of the 5′ end of the dBruce translation start codon. GMREP-86A-1 and -2 and l(3)06439 are within 1 kb of each other and, as a group, are about 18 kb upstream of dBruce. Only the GMREP-86A-1–5 lines suppress GMR-Reaper- and GMR-Grim-induced death. EP(3)3731 is located 1 kb 3′ to the dBruce translation stop codon.

(C) dBruce transcript and protein are ectopically expressed posterior to the morphogenetic furrow in eye discs from all five GMREP-86A lines. In situ hybridizations with a dBruce probe and immunolabeling with a dBruce-specific antibody on eye discs from wild-type larvae and GMREP-86A-1 larvae are shown. In situs were performed as described in [20]. See the Supplementary Material available with this article online for immunolabeling details.

(D) GMREP-86A-1-dependent suppression of GMR-Rpr-induced death (compare with Figure1AIII) is attenuated by coexpression of a dBruce-RNAi construct. A scanning electron micrograph of a fly eye with the genotype GMREP-86A-1, GMR-Rpr/GMR-dBruce-RNAi is shown.

in fact observed (Figure 1D, compare with Figure 1AIII). To rule out the possibility that attenuation of GMREP-86A-1-dependent inhibition of Rpr-dependent cell death by GMR-dBruce-RNAi is simply due to titration of the Glass transcription factor away from the Glass binding sites in the GMREP-86A-1 transposon, we carried out similar crosses with several other GMR-driven transgenes (GMR-dBruce-BIR and GMR-dBruce-UBC). These had no effect on GMREP-86A-1-dependent inhibition of Rpr-dependent cell death (data not shown). These observations, in conjunction with those obtained from studies with dBruce deletion mutants (Figure 2), argue that dBruce can suppress Rpr- and Grim-dependent cell death.

We sequenced cDNAs encompasing the dBruce coding region. This allowed us to assemble an accurate map of the dBruce exon-intron structure, which differs in some respects from that of the BDGP predicted gene (Figure 2A). Overall, dBruce is 30% identical to murine Bruce. However, the dBruce N-terminal BIR domain and the C-terminal E2 domain show much higher degrees of homology, 83% and 86% identity, respectively. *C. elegans* homologs of Bruce were not apparent. We generated mutations in the dBruce gene by carrying out imprecise excision of a P element, EP3731, located 3′ to the dBruce transcript (Figure 2A). We generated two deletions that extended only in one direction, into the 3′ end of the dBruce coding region. E12 deleted a relatively

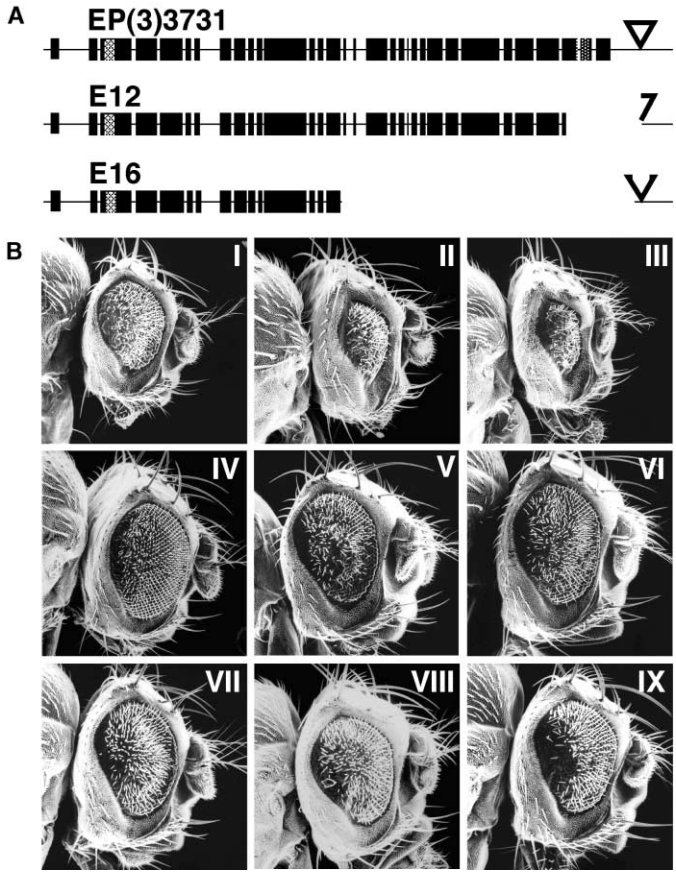


Figure 2. C-Terminal Deletion Mutations of dBruce Enhance Rpr- and Grim-Dependent Cell Death

(A) The genomic structure of the dBruce coding region, and the regions removed in the deletion mutants E12 and E16, are shown. The patterned box in the third exon indicates the location of the BIR; the patterned boxes in the second and third exons from the 3′ end of dBruce indicate the location of the ubiquitin conjugation domain. E12 removes 1.5 kb of dBRUCE genomic DNA, and E16 removes 10 kb. Both deletions remove the ubiquitin-conjugating domain. See the Supplementary Material for details.

(B) dBruce deletion mutants enhance Reaper- and Grim-dependent death. Scanning electron micrographs are shown. The genotypes shown are as follows: I, GMR-RprM/+; II, E12/GMR-RprM; III, E16/GMR-RprM; IV, GMR-GrimM/+; V, E12/GMR-GrimM; VI, E16/GMR-GrimM; VII, GMR-HidM/+; VIII, E12/GMR-HidM; IX, E16/GMR-HidM. GMR-RprM, GMR-GrimM, and GMR-HidM are lines that have slightly larger eyes than the GMR-Rpr, GMR-Grim, and GMR-Hid lines used in Figure 1, and they are used here to score for enhancement.

small region of the C terminus that includes the E2 domain, while E16 deleted approximately the C-terminal half of the dBruce coding region (Figure 2A). Both lines were homozygous viable but male sterile. We cannot exclude the possibility that E12 and E16 represent neomorphic mutations in dBruce. However, we favor the hypothesis that they represent hypomorphs or null mutations, since they had the opposite phenotype of the GMREP-86A dBruce expression lines when in combination with GMR-Rpr, acting as enhancers rather than suppressors of Rpr-dependent cell death in the eye (Figures 2BI–2BIII). E12 and E16 also enhanced GMR-Grim, but this effect was much more modest (Figures 2BIV–2BVI). E12 and E16 had no clear effect on cell death due to expression of Hid (Figures 2BVII–2BIX).

These results argue that endogenous dBruce levels, at least in the eye, are sufficient to act as a brake on Rpr-, and to some extent, Grim-dependent cell death. How does dBruce suppress apoptosis? A number of observations argue that Rpr- and Grim-dependent killing proceeds through distinct mechanisms and/or is regulated differently than that which is due to Hid. These differences are manifest at multiple points. At the level of DIAP1, point mutations of DIAP1 have effects on Rpr- and Grim-dependent cell death that are opposite of those due to Hid [5]. In addition, in a *Drosophila* extract, Hid, but not Rpr and Grim, promotes DIAP1 polyubiquitination [6]. In contrast, in a different set of assays, Rpr and Grim, but not Hid, act as general inhibitors of protein translation [6, 7]. Finally, Rpr and Grim, but not Hid, show strong synergism with the effector caspase DCP-1 in terms of their ability to induce cell death in the eye [8]. Each of these points defines a possible target for dBruce antiapoptotic action.

Because dBruce strongly suppressed cell death induced by Rpr and Grim but not by Hid, one obvious possibility was that dBruce promoted Rpr and Grim ubiquitination and degradation. We tested this hypothesis by generating mutant versions of Grim and Rpr that lacked all lysines, the amino acid to which ubiquitin is added. We introduced these genes into flies under GMR control. GMR-Rpr-lys$^-$ and GMR-Grim-lys$^-$ flies have small eyes, indicating that these mutant proteins are effective cell death inducers (Figure 3). GMREP-86A-1-dependent dBruce expression suppressed this death very effectively, indicating that dBruce cannot be promoting ubiquitin-dependent degradation of Rpr or Grim (Figure 3). Interestingly, however, dBruce expression did not suppress cell death induced by expression of versions of Rpr (GMR-RprC) or Grim (GMR-GrimC) lacking their N termini [9, 10], which are required for their IAP-caspase-disrupting interactions with the DIAP1 BIR2 (reviewed in [11]). This result is important because it argues that dBruce does not act to regulate this relatively uncharacterized death pathway.

The N-terminal dBruce BIR lacks a number of residues thought to be important for binding of Rpr, Hid, and Grim to DIAP1 BIR2 [12]. Thus, it seems unlikely that GMR-driven expression of dBruce inhibits cell death by simply titrating Rpr and Grim away from interactions

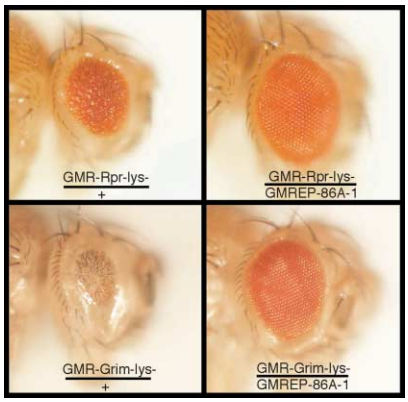


Figure 3. dBRUCE Does Not Suppress Rpr- and Grim-Dependent Cell Death by Promoting Rpr and Grim Ubiquitination

GMREP-86A-1 suppresses death induced by overexpression of versions of Rpr and Grim that lack lysine residues (GMR-Rpr-lys$^-$ and GMR-Grim-lys$^-$) and thus cannot be ubiquitinated.

with DIAP1 BIR2 as a result of similar interactions with the dBruce BIR. Nonetheless, the high degree of conservation between dBruce and mammalian Bruce in the BIR suggests that it is functionally important. To explore this role further, we expressed under GMR control a fragment of dBruce that contained residues 1–531, including the BIR domain (aa 251–321). Flies carrying this construct, GMR-dBruce-BIR flies, had normal appearing eyes, and in crosses to flies expressing GMR-Rpr, -Hid, or -Grim, GMR-dBruce-BIR did not enhance or suppress these eye phenotypes (data not shown). These results do not rule out a role for the dBruce BIR in suppressing Rpr- and Grim-dependent cell death. However, they do suggest that the BIR alone is unlikely to mediate this inhibition.

dBruce overexpression in the eye also did not suppress cell death resulting from GMR-driven expression of the caspase Dronc, which is required for many apoptotic cell deaths in the fly, including those induced by expression of Rpr, Grim, and Hid [13–17] (Figure S1, available with this manuscript online). Dronc most resembles mammalian caspase-9, and its activation is likely to involve interactions with the *Drosophila* Apaf-1 homolog Ark [16, 17]. Thus, this result strongly suggests that dBruce does not block Ark-dependent Dronc activation or Dronc activity. This result is also suggested by the observation that decreasing Ark or Dronc in the eye strongly suppressed Hid-dependent cell death [14–16, 18], which dBruce did not. A similar lack of cell death suppression was seen in the progeny of crosses between GMR-dBruce flies and flies expressing a second long prodomain caspase Strica [19], whose mechanism of activation and normal functions are unknown. Finally, GMREP-86A-1 also failed to suppress the cell death due to GMR-dependent expression of the *Drosophila* proapoptotic Bcl-2 family member known variously as Debcl, Drob-1, dBorg-1, or Dbok (reviewed in [3]) (Figure S1).

## Conclusions

The Bruce gene is found in mammals and flies, but not in the worm *C. elegans*. In humans, it is upregulated in some cell lines derived from gliomas and an ovarian carcinoma, and the results of antisense inhibition of Bruce suggest that it contributes to the resistance of these cells to DNA-damaging chemotherapeutic drugs [2]. Here, we show that the *Drosophila* homolog of Bruce, dBruce, can potently inhibit cell death induced by Rpr and Grim but not Hid. In addition, flies with C-terminal deletions that removed the Bruce ubiquitin conjugation domain, or much larger regions of the coding region, acted as dominant enhancers of Rpr- and Grim-dependent, but not Hid-dependent, cell death. Together, these observations clearly demonstrate that dBruce can function as a cell death suppressor. Our results with the deletion mutants suggest, but do not prove, that dBruce's death-inhibiting activity requires its function as a ubiquitin-conjugating enzyme. Based on the general conservation of cell death regulatory mechanisms, our results, in conjunction with those of Chen et al. [2], argue that mammalian Bruce is likely to facilitate oncogenesis by directly promoting cell survival in the face of specific death signals. One mechanism by which Rpr, Grim, and Hid promote apoptosis is by binding to DIAP1, thereby blocking its ability to inhibit caspase activity (reviewed in [11]). It will be interesting to determine if mammalian Bruce also inhibits cell death induced by the expression of specific IAP binding proteins.

How does dBruce inhibit cell death? It does not promote the ubiquitination and degradation of Rpr and Grim directly. However, we cannot rule out the possibility that it somehow sequesters them from their proapoptotic targets. The fact that it does not inhibit cell death due to Hid or Dronc expression argues that it is unlikely to be acting on core apoptotic regulators such as Ark, Dronc, or DIAP1, which are important for Hid-, Rpr-, and Grim-dependent cell death. An attractive hypothesis is that dBruce, perhaps in conjunction with apoptosis-inhibiting ubiquitin-protein ligases such as DIAP1 or DIAP2, promotes the ubiquitination and degradation of a component specific to Rpr- and Grim-dependent death signaling pathways. What might such a target be? Little is known about how Rpr- and Grim-dependent death signals differ from those due to Hid. However, one possibility is suggested by the recent observation that Rpr and Grim, but not Hid, can inhibit global protein translation [6, 7]. This creates an imbalance between levels of short-lived IAPs and the caspases they inhibit, thereby sensitizing cells to other death signals. Perhaps dBruce targets a protein(s) required for this activity.

Finally, Bruce is a very large protein, and thus its coding region might be expected to be subject to a relatively high frequency of mutation. Truncation of dBruce through the introduction of a stop codon or a frame shift is thus likely to be a relatively common form of Bruce mutation. The results of our deletion analysis show that C-terminal dBruce truncations act to enhance cell death in response to several different signals. Given this, it will be interesting to determine if human Bruce mutations are associated with a predisposition to pathologies that involve an inappropriate increase in cell death.

### Supplementary Material

Supplementary Material including the Experimental Procedures and Figure S1 is available at http://images.cellpress.com/supmat/supmatin.htm.

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**Accession Numbers**

The GenBank accession number for the dBruce exon-intron structure reported in this paper is AF517634.