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Regulation of apoptosis of *rbf* mutant cells during *Drosophila* development

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Abstract

Inactivation of the retinoblastoma gene Rb leads to defects in cell proliferation, differentiation, or apoptosis, depending on specific cell or tissue types. To gain insights into the genes that can modulate the consequences of Rb inactivation, we carried out a genetic screen in *Drosophila* to identify mutations that affected apoptosis induced by inactivation of the *Retinoblastoma-family protein* (*rbf*) and identified a mutation that blocked apoptosis induced by *rbf*. We found this mutation to be a new allele of *head involution defective* (*hid*) and showed that *hid* expression is deregulated in *rbf* mutant cells in larval imaginal discs. We identified an enhancer that regulates *hid* expression in response to developmental cues as well as to radiation and demonstrated that this *hid* enhancer is directly repressed by RBF through an E2F binding site. These observations indicate that apoptosis of *rbf* mutant cells is mediated by an upregulation of *hid*. Finally, we showed that *bantam*, a miRNA that regulates *hid* translation, is expressed in the interommatidial cells in the larval eye discs and modulates the survival of *rbf* mutant cells.

Keywords

Rb family protein Rbf; *hid*; apoptosis; *Drosophila*; eye development; *bantam*

Introduction

The retinoblastoma protein pRb is a prototype tumor suppressor often mutated or inactivated in cancers (reviewed in (Classon and Harlow, 2002; Weinberg, 1995). pRb functions by binding to the E2F family of transcription factors and regulates a variety of normal cellular processes including cell proliferation, differentiation, as well as apoptosis. In mammals, eight E2F, and three DP family members have been identified (reviewed in (DeGregori and Johnson, 2006). These E2F and DP proteins form different complexes with extensive functional overlap within each family; therefore, an analysis of the *in vivo* role of Rb/E2F proteins in mammals is very complicated. In contrast, the Rb/E2F proteins in *Drosophila* are much simpler and yet are highly conserved. There are only one DP (dDP), two dE2F (dE2F1 and dE2F2), and two Rb

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family proteins (RBF and RBF2) in *Drosophila* (Du et al., 1996; Dynlacht et al., 1994; Ohtani and Nevins, 1994; Sawado et al., 1998; Stevaux et al., 2002). The two E2F proteins in Drosophila correspond to the two distinct classes of E2F proteins in mammals: dE2F1 mainly functions as a transcription activator (Du, 2000) similar to the activating E2Fs (E2F1-3), while dE2F2 mainly functions to mediate active repression similar to the repressive E2Fs (E2F4-5) in mammalian systems (Frolov et al., 2001). Similar to mammalian Rb, RBF can bind to both the activating E2F (dE2F1) as well as the repressive E2F (dE2F2), while RBF2 binds specifically to dE2F2, similar to the preferential binding of p107/p130 to the repressive E2F proteins in mammals (Stevaux et al., 2002). Therefore, *Drosophila* provides an attractive model system to study the Rb/E2F proteins.

Although Rb is widely expressed in most cell types, *in vivo* studies of Rb inactivation revealed tissue specific defects in cell proliferation, differentiation, and apoptosis (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). Similarly, RBF is also broadly expressed in all the cells in the *Drosophila* developing eye but inactivation of *rbf* in different cells of the developing eye exhibited distinct effects (Du, 2000; Firth and Baker, 2005). For example, inactivation of *rbf* in the developing eye led to ectopic S phase posterior to the MF but not in the MF (Du, 2000; Firth and Baker, 2005). In contrast, cells near the MF but not in the posterior exhibited significantly increased apoptosis in the absence of RBF (Du, 2000; Moon et al., 2006). Therefore, different cells can respond differently to the inactivation of Rb (RBF) in both flies and in mammalian systems. Understanding the mechanism by which cells respond differently to inactivation of Rb will be critical to our understanding of how Rb functions as a tumor suppressor. In addition, since Rb is functionally inactivated in a majority of human cancers, approaches that specifically increase the apoptosis of Rb mutant cells will have therapeutic potential.

In an effort to identify genes that affected the consequence of Rb inactivation on cell proliferation, differentiation, and apoptosis, we carried out a genetic screen and identified a mutation that blocked apoptosis induced by *rbf* mutation. We found this mutation to be a new allele of *hid*. Additionally, we showed that *hid* expression is increased in *rbf* mutant cells. We identified an enhancer that mediates *hid* expression in response to developmental cues as well as to radiation and showed that this *hid* enhancer is negatively regulated by RBF through an E2F binding site. Finally, we showed that *bantam*, a regulator of *hid* translation, is expressed in the interommatidial cells in the eye discs and modulated the survival of *rbf* mutant cells.

Results

Identification of *w138*, a mutation that blocked apoptosis induced by *rbf* inactivation

Inactivation of *rbf* in the developing eye disc led to ectopic S phase in both the anterior half of the MF and posterior to the MF, and significantly increased apoptosis in and immediately anterior to the MF (Du, 2000;Firth and Baker, 2005;Moon et al., 2006). In the adult compound eye, *rbf* mutant clones occupied slightly smaller areas compared to the control wild-type clones (white ommatidia, Fig. 1A and B) and displayed mild roughness. To identify genes that modulate the effect of *rbf* inactivation, we carried out a genetic screen on the left-arm of the third chromosome (3L) using the *ey*FLP/FRT system (see Materials and Methods). We isolated a mutation, *w138*, which significantly expanded *rbf* mutant clone areas in adult eyes. Double mutant clones of *rbf* and the newly isolated recessive *w138* mutation occupied a large majority of the adult eye tissues (Fig. 1C), whereas the clones of *w138* mutation alone occupied only a little bit more areas than that of the control WT clones (Fig. 1E and B). These observations indicate that *w138* and *rbf* exhibited a synergistic effect on mutant clone size in the adult eyes and suggest that *w138* modulates the effect of *rbf* inactivation.

Since inactivation of *rbf* affects cell proliferation as well as apoptosis, we carried out assays to determine if mutation of *w138* affected cell proliferation or apoptosis induced by *rbf* inactivation. Although BrdU incorporation assay did not reveal striking difference between *rbf* single and *rbf;w138* double mutant clones, apoptosis in the MF observed in *rbf* mutant clones was completely blocked by mutation of *w138*. As shown in Figure 1G, *rbf* mutant clones (marked by the absence of GFP) near the MF exhibited a significant level of apoptosis as shown by increased activated Caspase-3 (Cas3) staining and increased TUNEL positive cells (Fig.1G and data not shown). In addition, the mutant clones near the MF accumulated condensed apoptotic pyknotic nuclei as shown by DAPI staining (white arrows, Fig. 1I). These observations are consistent with the previous reports that inactivation of *rbf* leads to increased apoptosis near the MF (Du, 2000; Moon et al., 2006). In contrast, no increased Cas3 staining, no TUNEL positive cells, and no pyknotic nuclei were observed in *rbf;w138* double mutant clones near the MF (Fig. 1H, J, and data not shown), indicating that mutation of *w138* blocked apoptosis of *rbf* mutant cells near the MF. Previous studies have shown that apoptotic cells in the developing discs induced compensatory proliferation by producing signaling proteins such as Wingless (Wg) (Fan and Bergmann, 2008; Huh et al., 2004; Perez-Garijo et al., 2004; Ryoo et al., 2004). Indeed, ectopic Wg expression was observed in *rbf* mutant cells near the MF and was blocked in *rbf;w138* double mutant clones (data not shown), indicating *w138* is also required for the compensatory proliferation pathway. In conclusion, our data indicate that *w138* mutation blocks apoptosis induced by the inactivation of *rbf*.

w138 is a novel allele of *hid*

To identify the gene that corresponded to the *w138* mutation, we genetically mapped the *w138* mutation. *w138* is a partially lethal mutation, the homozygote survivors exhibited a number of visible phenotypes, including non-transparent wings, degenerated humeral bristles, and additional interommatidial cells in the pupal retina (data not shown). Using the 3L chromosome deficiencies, *w138* was mapped to the chromosomal interval 75C1-2 (between the left breakpoint of *Df(3L)H99* and the right breakpoint of *Df(3L)X25*). This region contains at least 7 annotated genes including the *Drosophila* apoptosis activators *hid* and *grim* according to the FlyBase genome annotation (Grumbling and Strelets, 2006). *hid* and *grim*, as well as *reaper* (*rpr*) encode a family of related pro-apoptotic proteins that inactivate the function of *Drosophila* IAP proteins (Bergmann et al., 2003; Ryoo et al., 2002).

Interestingly, the non-transparent wing, additional interommatidial cells, and degenerated humeral bristles phenotypes of *w138* is similar to those of the viable mutations of the components of the apoptosis pathway, such as mutations of *hid, dronc* and *Dark* (Abbott and Lengyel, 1991; Rodriguez et al., 1999; Xu et al., 2005). Complementation tests showed that the transheterozygotes of *w138/hid*$^{PZ\ 05014}$, *w138/Df(3L)H99, w138/Df(3L)X25*, and *hid*$^{PZ\ 05014}$ */Df(3L)H99* all exhibited reduced viability with survivors that displayed the pupal and adult phenotypes described above. In contrast, the deletion chromosome *Df(3R)X38*, which lacks *rpr* but still retains *hid* and *grim*, complemented *w138* mutation. Taken together, these complementation tests indicate that *w138* is likely an allele of *hid*.

To characterize the *w138* mutation at molecular level, we performed sequencing analysis. A set of DNA fragments corresponding to the *hid* coding regions were amplified by PCR from *w138* adult flies or from transheterozygotes of *w138* and *w300* (*w300* is a different mutant isolated from the same screen). Two different size PCR fragments were obtained from *w138/ w300* genomic DNA while only a single size fragment, corresponding to the smaller PCR fragment was amplified from *w138* homozygote genomic DNA (Fig. 2B). Sequencing analysis of these fragments revealed that the *w138* mutation is associated with a 312bp deletion in the second exon of *hid* (Fig. 2A and C), which results in frame shift and premature stop. Therefore, *w138* is a new allele of *hid*, which we refer to as *hid*^{w138}. *hid*^{w138} can still encode an 176 amino-

acid (a.a.) protein including the RHG domain and two MAPK phosphorylation sites (Ser-121 and Thr-148) (Bergmann et al., 1998;Chai et al., 2003). In contrast the wild-type *hid* encodes a 410a.a. protein with a C-terminus hydrophobic region that required for mitochondria localization which is absent from hid^{w138} (Fig. 2D)

To further compare the effect of hid^{w138} mutation with previously identified *hid* alleles on *rbf*-induced apoptosis, we used a strong loss-of-function allele, $hid^{PZ\,05014}$ (See Materials and Methods). The *rbf;* hid^{PZ} double mutant clones displayed similar phenotypes as that of the *rbf;* hid^{w138} double mutants in adult compound eyes (Fig. 1D and C). Additionally, characterization of the apoptosis phenotype in the third instar eye disc revealed a block of nuclear pyknosis (by DAPI staining) (Fig. 1K) and an inhibition of TUNEL and activation of Caspase 3 in the MF. These phenotypes are identical to those of the *rbf;* hid^{w138} double mutant clones. These observations further support the idea that *w138* is a loss of function allele of *hid*.

Ectopic *hid* expression in *rbf* mutants

The observation that mutation of *hid* blocked *rbf*-induced apoptosis prompted us to examine whether the levels of *hid* mRNA and protein differed in WT and *rbf* mutant eye discs. Using the anti-Hid antibody (Haining et al., 1999), a very low level of Hid protein was detected in WT eye discs (Fig. 3A). Similarly, only a background level of *hid* mRNA was detected in eye discs by *in situ* hybridization or by RT-PCR of total RNA isolated from eye-antennal discs (Fig. 3F and 3L). Coincide with this very low level of *hid* expression, very few apoptotic cells were observed as detected by Acridine Orange (AO) staining (Fig. 3I). In contrast to WT eye discs, transheterozygote of $rbf^{15\Delta/120}$ eye discs exhibited significant increase in apoptosis near the MF as reported earlier (Fig. 3J). The increased apoptosis was correlated with strong punctuated staining of Hid protein in the MF of the $rbf^{15\Delta/120}$ eye discs (Fig. 3B). A high level of Hid protein accumulation was also observed in the *rbf* mutant clones spanning the MF (Fig. 3D, Hid shown in magenta). Interestingly, while blocking apoptosis by using a mutation of *dronc* did not block Hid protein accumulation, Hid staining changed from a punctuate and perinuclear pattern in *rbf* mutant clones to a diffused cytoplasmic pattern in *rbf;dronc* double mutant clones (Fig. 3D, D', E and E'). These observations are consistent with reports that showed Hid protein is localized in the mitochondria (Abdelwahid et al., 2007; Haining et al., 1999) and suggest that the possibility that Dronc is required for the mitochondria localization of Hid protein.

While increased apoptosis and Hid protein were mostly observed near the MF of *rbf* mutant discs, deregulated *hid* mRNA was observed throughout the entire eye disc; although the highest *hid* mRNA level was observed in the MF (Fig. 3G). The observed *hid* mRNA up-regulation in *rbf* mutant eye-antennal eye discs was further confirmed by RT-PCR analysis (Fig. 3L, arrow). In contrast, the level of *rpr* mRNA was not significantly different between WT and *rbf* mutant (data not shown). These observations are consistent with the previous finding that deficiencies that removed *hid* suppressed apoptosis in *rbf* mutant discs while a deficiency that removed both *reaper* and *sickle* did not (Moon et al., 2006). In conclusion, *hid* protein and mRNA is upregulated in *rbf* mutants and is required for apoptosis induced by the inactivation of *rbf*.

An E2F binding site in the *hid* 5' regulatory sequences is occupied by dE2F proteins *in vivo*

To examine the possibility that *hid* is a direct transcriptional target of Rbf/E2F proteins, we analyzed the *hid* non-coding DNA sequence to identify conserved consensus E2F binding sites using the VISTA Browser (http://pipeline.lbl.gov/cgi-bin/gateway2) multiple alignments. The 14 Kb *hid* 5' flanking sequences and 11 Kb intron 1 sequences (Fig. 2A) from *D. melanogaster* and *D. pseudoobscura* were analyzed and two consensus E2F binding sites in

conserved sequence blocks were identified: one at -1415 bp (-5'TTTCGCGC3'-) in the *hid* 5'flanking sequence (*hid* 5'F) and one at +2183 bp (-5'TTTGGCGC3'-) in *hid* intron 1.

To determine if these two consensus E2F binding sites are occupied by dE2F proteins *in vivo*, we used Chromatin Immunoprecipitation (ChIP) assay in eye-antennal disc. As shown in Fig. 4, ChIP with anti dE2F1 antibody led to significant enrichment of the DNA fragment containing the 5' E2F binding site but not the DNA fragment containing the intron 1 E2F binding site (Fig. 4A). These observations indicate that the 5' E2F binding site but not the intron 1 E2F binding site is occupied by dE2F1 *in vivo*. We also carried out ChIP analysis using the anti dE2F2 antibody. In comparison to dE2F1, the ChIP using anti dE2F2 antibody led to modest enrichment of the DNA fragment containing the 5' E2F binding site and not the DNA fragment containing the intron 1 E2F binding site (Fig. 4A). As a positive control for dE2F2 ChIP experiments, we observed the 5' flanking sequence of *Arp53D* (Dimova et al., 2003) was significantly enriched in anti dE2F2 ChIP experiments (Fig. 4A). These data indicate that in eye-antennal disc, the E2F binding site in *hid* 5'F but not the one in intron 1 is occupied by dE2F proteins *in vivo*. It appears that this E2F binding site is bound mostly by dE2F1 and to a lesser degree dE2F2 *in vivo*. This is similar to the observation reported previously in SL2 cells (Moon et al., 2005).

To further demonstrate that dE2F proteins indeed bind specifically to the observed consensus *hid* 5' E2F binding site, we carried out *in vitro* electrophoretic mobility shift assay (EMSA). As shown in Fig. 4B, both dE2F1/dDP and dE2F2/dDP heterodimer bound to the probe containing the WT E2F binding site from the *hid* 5'F (Fig. 4B; lanes 2 and 5) but not to a probe containing the mutated E2F site (Fig. 4B, lanes 3 and 6). These observations demonstrate that the E2F binding site in *hid* 5'F can interact with the two dE2F proteins specifically *in vitro* and is occupied by dE2F proteins *in vivo* in larval eye discs.

An enhancer element that regulates *hid* expression during development and in response to radiation

Since only the E2F binding site in *hid* 5'F is occupied by dE2F proteins *in vivo*, we further characterized the role of this E2F binding site on *hid* transcription. A 2.2Kb fragment containing the *hid* 5' E2F binding site was cloned into the pH-stinger vector (Barolo et al., 2004) to generate WT transgenic lines (Fig. 5A, *hid5'F-WT*) and the GFP reporter expression was examined in transgenic animals during development.

Apoptosis plays little role during larval wing and eye disc development (Milan et al., 1997) and there were very few apoptotic cells in WT eye and wing discs at this stage. Consistent with this, very low levels of GFP reporter expression from the *hid5'F-WT* observed in WT eye-antennal or wing discs (Fig. 5B, C). When these tissues were exposed to exogenous death stimuli such as ionizing radiation, high levels of apoptosis were induced in these discs (Fig. 5H, I) and there was a significantly higher level of GFP reporter expression from *hid5'F-WT* (Fig. 5F and G). Hid induction by ionizing radiation is important for radiation induced apoptosis since apoptosis induced by irradiation in eye and wing discs was significantly blocked in hid^{w138} mutant background (Fig. 5J and K). In addition, although the level of GFP reporter expression in larval stages was quite low, a significantly higher level of the GFP reporter expression from *hid5'F-WT* was observed in pupal stages in tissues with significant amount of developmentally regulated apoptosis such as in ommatidia at the periphery of the eye disc and in interommatidial cells during mid-pupation (Fig. 5R-S'). During retina development, ommatidia at the periphery of the eye undergo apoptosis in response to Wg signaling, which activates the expression of *hid*, *rpr*, and *grim* (Lin et al., 2004). In addition, extra interommatidial cells are removed by apoptosis during pupal stage and this developmental regulated apoptosis is completely dependent on the presence of Hid (Miller and Cagan, 1998; Yu et al., 2002). In conclusion, the 2.2Kb *hid5'F-WT* fragment contains regulatory

elements that control *hid* transcription in response to developmental cues as well as to stress signals such as ionizing radiation.

The E2F binding site in the *hid* 5' regulatory region negatively regulates *hid* expression

To determine if the E2F binding site in *hid* 5'F regulates *hid* transcription, the same base pair substitutions that disrupted the binding of dE2F2 (Fig. 4B) was introduced into the 2.2 Kb *hid5'F-WT* fragment to generate *hid* 5'F with mutated E2F binding sequence (Fig.5A, *hid5'F-E2FMut*). In contrast to *hid5'F-WT*, transgenic flies carrying *hid5'F-E2FMut* displayed significantly increased GFP expression in larval eye and wing discs (Fig. 5D and E). In addition, elevated GFP level was also observed in many other larval cells in *hid5'F-E2FMut* animal, indicating a relative broad requirement of this E2F binding site to repress *hid* expression at this stage (Fig. 5P,Q). To further characterize if RBF repressed the *hid5'F-WT* enhancer activity, we examined the level of GFP reporter expression in *rbf* mutant background. *In situ* hybridization results showed an increased *hid5'F-WT* reporter expression in *rbf* mutant discs, with the highest level observed near the MF (Fig. 5L,M). Similarly, increased β-gal reporter was also observed *rbf* mutant clones (Fig. 5N, β-gal in magenta). These results, in conjunction with the observed effect of the E2F binding site mutation, indicate that the *hid5'F-WT* enhancer is repressed by the RBF/dE2F proteins in the larval stage.

bantam microRNA attenuates *rbf* induced cell death in posterior larval eye disc

Although deregulated hid expression in the absence of *rbf* was most obvious around the MF in the eye disc, a lower level of deregulated *hid* expression was also observed in other parts of the eye disc (Fig. 3G). These observations suggest that additional post-transcriptional mechanisms also contribute to the observed induction of apoptosis near the MF in the absence of *rbf*. In support of this idea, EGFR signaling, which was shown to negatively regulate Hid transcription as well as Hid activity (Bergmann et al., 1998;Kurada and White, 1998), was found to regulate the sensitivity of *rbf* mutant cells to apoptosis (Moon et al., 2006). Since Hid is also regulated at the translational level by *bantam* (Brennecke et al., 2003;Hipfner et al., 2002), we examined the possibility that *bantam* may also regulate the sensitivity of *rbf* mutant cells to apoptosis *in vivo*.

bantam is expressed in a spatio-temporally restricted manner throughout development. We first generated transgenic lines to monitor developmentally regulated *bantam* expression. The important regulatory element for *bantam* was mapped between the restriction enzyme sites BamHI and SpeI (Brennecke et al., 2003) (Fig. 6A). Since all *bantam* enhancer trap lines are clustered within a small region upstream of the *bantam* hairpin sequence (Brennecke et al., 2003; Wilson et al., 2008), we generated *bantam* reporter lines (*ban 5'F-lacZ*) using the BamHI-EcoRV *bantam* 5' flanking fragment and compare the pattern of β-gal expression with that of the *bantam*-sensor. The *bantam*-sensor has been used previously as a reporter for bantam miRNA activity (Brennecke et al., 2003). Cells with low level of *bantam*-sensor would indicate high bantam miRNA activity. Consistent with this, the expression of β-gal reporter from the *ban* 5'F-lacZ transgene was complementary to the previously generated *bantam*-sensor (Brennecke et al., 2003). For example, in posterior eye disc, β-gal from the *ban*5'F-lacZ was highly expressed in interommatidial cells but not in the ommatidia clusters in contrast to the expression of *bantam*-sensor, which is very high in the ommatidia clusters and low in the interommatidial cells (Fig. 6B-B"). The complementary expression pattern between *ban 5'F-lacZ* reporter and *bantam*-sensor also extends to the optic lobes, wing and leg discs (data not shown). *In situ* hybridization of *ban*5'F-lacZ also confirmed the expression of the lacZ reporter in the MF and the posterior interommatidial cells (Fig. 6C). These data indicate that the 2.35Kb *ban 5'F* fragment was sufficient to drive *bantam* expression in the MF and in the interommatidial cells.

To determine whether *bantam* regulates *rbf* dependent cell death in third instar eye disc, we compared the level of apoptosis in *rbf* single and *rbf; ban* double mutant clones in the posterior of larval eye discs. Previous studies showed that although *bantam* is a negative regulator of *hid*, removal of *bantam* alone was insufficient to induce cell death in developing wing disc (Brennecke et al., 2003; Hipfner et al., 2002). Similarly, we did not observe activated Caspase 3 in *bantam* single mutant clones (Fig. 6F-G'). Increased activated Caspase 3 staining in *rbf* single mutant clones was mostly observed in the MF, with very low level in the posterior (Fig. 6H-I"). In contrast, significant increase in the level of activated Caspase 3 staining was observed in *rbf; ban* double mutant clones in posterior (Fig. 6D-E", white arrows). Moreover the fluorescent signal of activated Caspase 3 staining in the MF was also much stronger in *rbf; ban* double mutant clones compare to the *rbf* mutant clones, suggesting that even more cells were under going apoptosis in the MF of *rbf; ban* double mutant clones (Fig. 6D, H). These results indicate that *bantam* antagonizes *rbf*-dependent apoptosis and that the spatial expression of *bantam* provides another level of control for the resistance or sensitivity of *rbf* mutant cells to apoptosis in the developing eye.

Discussion

hid is a key cell death regulator in *Drosophila*, which is upregulated in response to developmental cues as well as to various stress stimuli. In this manuscript, we show that *hid* is also upregulated and responsible for apoptosis in response to *rbf* inactivation. We have identified an enhancer element of *hid, hid5'F*, which regulates *hid* expression in response to developmental cues as well as to ionizing radiation. We demonstrate that RBF directly represses the expression of *hid* through a conserved E2F binding site in this enhancer. Finally we show that removal of *bantam* synergistically increases the apoptosis of *rbf* mutant cells without significantly affecting the apoptosis of WT cells.

Regulators of Hid activity modulates a cell's sensitivity to apoptosis in response to *rbf* inactivation

Although RBF is ubiquitously expressed in developing eye discs, only cells near the MF display significant increased apoptosis in response to *rbf* inactivation, suggesting that RBF plays an important role in preventing apoptosis and that the sensitivity of *rbf* mutant cells to apoptosis is under an additional level of control. We identify *hid* as a key apoptosis target that is repressed by RBF and required for the apoptosis of *rbf* mutant cells. Interestingly, while deregulated *hid* expression can be observed throughout the *rbf* mutant eye discs, the highest *hid* expression was observed near the MF. These results point the contribution of transcriptional mechanisms to the increased sensitivity of MF cells to apoptosis in the absence of *rbf* and further characterization of the *hid 5' F* enhancer will be necessary to elucidate the precise transcriptional mechanisms.

In addition to transcriptional regulation, Hid is also regulated by miRNA *bantam* at translational level and by survival signals such as EGFR signaling at the post-translational level. If *hid* is a key target of *rbf* in apoptosis regulation, it is expected that these additional regulators of Hid will modulate the sensitivity of *rbf* mutant cells to apoptosis. Indeed, removal of *bantam* together with *rbf* induced additional apoptosis in the posterior eye disc cells, indicating that *bantam* played an important role in preventing the apoptosis of *rbf* mutant cells in the posterior eye disc. The observed effect of *bantam* is consistent with its expression pattern, which is in the interommatidial cells in the posterior of the developing eye discs. Interestingly, removal of *bantam* alone was not sufficient to induce apoptosis in eye disc, similar to the observation in wing discs reported previously (Brennecke et al., 2003). These observations are consistent with the very low level of *hid* detected in WT eye or wing discs and suggest that *bantam* is not required during normal larval wing or eye development, but provides another

level of fine tuning in regulating the sensitivity of disc cells to apoptosis in response to stress or developmental signals.

In addition to *bantam*, EGFR signaling, which regulates Hid expression as well as Hid protein activity (Bergmann et al., 1998; Kurada and White, 1998), was shown to be an important survival signal in the posterior eye disc and regulates the apoptosis of *rbf* mutant cells (Baker and Yu, 2001; Moon et al., 2006). EGFR signaling is required for the stepwise recruitment of all the cell types after R8 photoreceptor specification (Freeman, 1996). In contrast to *bantam*, EGFR signaling is required to prevent apoptosis in WT eye discs. Since EGFR activation depends on short range signals from the developing ommatidia clusters, interommatidial cells will likely receive lower level of EGFR signaling and *bantam* expression in these cells may provide an additional level of apoptosis control independent of EGFR signaling. Taken together, we suggest that the activity of Hid, which is regulated by multiple mechanisms at the transcriptional, translational, and post-translational levels, defines a cell's sensitivity to apoptosis upon inactivation of *rbf in vivo*.

Hid, Reaper, and Grim can also induce apoptosis in vertebrate model systems, and their apoptosis activity is subject to regulation by inhibitors of mammalian cell death (Claveria et al., 1998; Evans et al., 1997; Haining et al., 1999; McCarthy and Dixit, 1998). These observations suggest that the proapoptotic function of these genes is conserved. Interestingly, Smac/Diablo, a mammalian functional homolog of Hid, was shown to be a direct target of E2F1 in mammalian cells (Xie et al., 2006) and activation of Ras signaling protected the Rb family null fibroblasts from apoptosis (Young and Longmore, 2004). Therefore, there are striking similarities about the apoptosis regulation of Rb mutant cells between flies and mammalian cells and it will be interesting to determine if the basic principles we learned here in flies can be applied to mammalian cells.

Hid enhancer element

Drosophila contains at least four proapoptotic proteins, Rpr, Hid, Grim and Skl, which are located on the third chromosome 75C region and responsible for most of the apoptosis regulation in flies (Chen et al., 1996; Christich et al., 2002; Grether et al., 1995; Srinivasula et al., 2002; White et al., 1994; White et al., 1996). There are large non-coding sequences between each individual proapoptotic gene, which may provide dynamic regulation of the expression of these genes in response to developmental cues and environmental stresses. The sequences that control *rpr* transcription have been reported previously (Brodsky et al., 2000). A 4 kb genomic fragment containing the p53 responsive element was found to be sufficient to induce *rpr* expression upon ionizing radiation through the direct binding of *Drosophila* p53 homologue (Brodsky et al., 2000). In addition, *rpr* transcription is also regulated by Hox protein Deformed during sculpting of the larval head (Lohmann et al., 2002) and by Ecdysone receptor complex during salivary gland histolysis (Jiang et al., 2000). However, precise transcriptional regulation of other proapoptotic genes, including *hid*, remains to be elucidated.

In this study, we showed that a 2.2 Kb sequence immediately upstream of *hid* 5'UTR was sufficient to drive reporter expression in response to developmental cues and to environmental stress signals. For example, during pupal retina development, excess interommatidial cells are removed by apoptosis. Hid is required for this developmental regulated apoptosis (Yu et al., 2002) and mutation of *hid* led to increased interommatidial cells in the pupal retina. We found that *hid 5'F* enhancer conferred reporter expression specifically in the interommatidial cells of the pupal retinal. Furthermore, while only a background level of *hid* reporter expression was observed in WT larval eye discs, a developmental stage with little apoptosis, *hid* reporter expression was strongly induced in larval eye discs by ionizing radiation, which strongly induces apoptosis in discs that is dependent on the Hid activity. Therefore, the *hid 5'F* enhancer contains regulatory elements that control *hid* expression both in response to developmental

cues as well as to ionizing radiation. Consistent with a previous report that showed Hid is negatively regulated by an E2F site in SL2 cells (Moon et al., 2005), we showed that *hid 5'F* enhancer is repressed by dE2F/RBF complexes through a conserved E2F binding site during development. These observations, however, are inconsistent with the observations that apoptosis observed in *rbf* mutants are suppressed when activation function of dE2F1 was removed (Moon et al., 2006). It is likely that there are additional targets regulated by dE2F1 that are also required for apoptosis in the absence of *rbf*. In addition, it should be pointed out that the *hid 5'F* enhancer is unlikely to contain all the regulatory elements controlling the expression of *hid*. For example, it was shown that an irradiation-responsive enhancer region (IRER) regulates both *hid* and *rpr* expression in response to ionizing radiation during embryogenesis (Zhang et al., 2008). Therefore multiple regulatory elements can control radiation induced *hid* expression possibly during different stages of development.

Mitochondria localization of Hid protein

Hid protein was found to be localized in the mitochondria in apoptotic cells depending on the presence of its C-terminal domain (Haining et al., 1999). Although initial studies suggested that the C-terminal hydrophobic region of Hid was not required for apoptosis induction when high level of Hid was expressed in HeLa cells, a recent study showed that the mitochondria localization played an important role for apoptosis in SL2 cells (Abdelwahid et al., 2007). The observation that hid^{w138}, which lacks the C-terminal hydrophobic region, exhibited similar phenotype as another strong loss of function allele suggested the importance of Hid mitochondria localization *in vivo*. As reported earlier that Hid is localized predominantly in the mitochondria in apoptotic cells (Abdelwahid et al., 2007; Haining et al., 1999), we showed that Hid staining displayed a punctuate and perinuclear pattern in *rbf* mutant clones in near the MF (Fig. 3D, D'). Interestingly, blocking the apoptosis of *rbf* mutant cells by *dronc* mutation led to a lack of mitochondria localization of Hid. As shown in Fig. 3E, E', Hid staining revealed a diffuse and cytoplasmic distribution in *rbf;dronc* double mutant cells. As reported earlier, cotransfection of BclXL blocked Hid mitochondria localization while cotransfection of p35, DIAP1, or XIAP, or treatment with cell-permeant cysteine proteases inhibitor peptide BOC-$_D$-fmk did not (Haining et al., 1999). Since all these treatments inhibited Hid induced apoptosis (Haining et al., 1999), these observations suggest that Dronc is required for the localization of Hid protein to the mitochondria membrane.

Materials and methods

Drosophila genetics

Ethyl methanesulfonate (EMS)-induced *rbf* modifier screening was performed on the left arm of third chromosome (3L) in combination with the *eyFLP/FRT*-mediated mosaic clone technique (Newsome et al., 2000; Xu and Rubin, 1993). 3-day-old *w; p{ry*$^+$*, neoFRT80B}* ry^{506} (BL#1988) males flies were fed overnight with 5mM EMS. These male flies were mated with $rbf^{15a\Delta}$*,w, eyFLP; p{w*$^+$*, rbf-G3} p{w*$^+$*, Ubi-GFP} p{ry*$^+$*, neoFRT80B}* virgin females and cultured at 25°C. The *p{w*$^+$*, rbf-G3}* is a *rbf* genomic rescue transgene integrated in an unknown site on the 3L-chromosome. F_1 male flies that contain larger w^- clones were subsequently crossed with *w; TM3,Ser/TM6b,Tb* balancer virgin females to establish balanced stocks. Balanced males were crossed with $rbf^{15a\Delta}$*,w, eyFLP; p{w*$^+$*, rbf-G3} p{w*$^+$*, Ubi-GFP} p{ry*$^+$*, neoFRT80B}* or *w, eyFLP; p{w*$^+$*, Ubi-GFP} p{ry*$^+$*, neoFRT80B}* virgin females for retest and *rbf* dependence test, respectively. The *w138* (=hid^{w138}) and $dronc^{O1}$ were isolated from this screening. To map the *w138* mutation, following deficiencies were used: 3L chromosome deficiencies from the Bloomington third-chromosome deficiency kit, *Df(3L)H99, Df(3L)XR38, Df(3L)X25*(White et al., 1994) and *Df(3L)W4* (BL#2607). To generate *w; hid* $^{PZ\ 05014}$ *p{ry*$^+$*, neoFRT80B}/TM3 act-GFP, Ser* line, $hid^{PZ\ 05014}$ (W^{05014}-FlyBase, BL#11642) (Grether et al., 1995) was recombined with wild-type chromosome to segregate out unknown lethal

background mutation(s) and subsequently recombined with *w; p{ry*$^{+}$*, neoFRT80B}*. The following fly stocks were used in this study; *rbf*$^{120\text{-}3\text{-}3}$ and *rbf*$^{15a\Delta}$, which was derived from imprecise excision of *rbf*$^{15a\ hop3}$ (Du and Dyson, 1999), *EP(3)3622*, *bantam-sensor* (Brennecke et al., 2003). Fly stocks obtained from the Bloomington Stock Center are shown with Bloomington Stock (BL) number.

Molecular genetics

RT-PCR—Total RNA was prepared from 100 pairs of third instar eye-antenna discs that were dissected in 1xPBS. Transheterozygote of *rbf*$^{120\text{-}3\text{-}3}$*/rbf*$^{15a\Delta}$ female larva were used as *rbf* mutants and *w*^{1118} females as the control. Total RNA was prepared in with TRIZOL Reagent (GIBCO BRL) and RT-PCR (Enhanced Avian RT-PCR kit, SIGMA) reactions were done under the conditions supplied by the manufacturer. Oligo(dT)$_{23}$ reverse primer and *hid* or *GAPDH2* specific primers used for reverse transcription reaction.

Transgenic fly—A 2.2 Kb genomic fragment of *hid* 5'flanking sequence and a 2.35 Kb genomic fragment of *bantam* 5' non-coding DNA sequence were amplified by PCR from *w*^{1118} genomic DNA. Nucleotide substitution of the consensus E2F binding site (from 5'-TTTCGCGC-3' to 5'-TggCtaGC-3') was made as shown previously in (Yamaguchi et al., 1995). Wild-type or mutated *hid 5'F* was subcloned into pH-Stinger or pH-Pelican vector (Barolo et al., 2004). Multiple transgenic lines were established and examined for reporter gene expression.

Immunohistochemistry

The third instar imaginal discs were dissected and fixed as previously described (Tanaka-Matakatsu et al., 2007). Primary antibodies were used at following dilutions: mouse anti-Hid CL1C3, 1:100 (Haining et al., 1999), rabbit anti-Cleaved Caspase-3 (Asp175), 1:500 (Cell Signaling TECHNOLOGY), mouse anti-ß galactosidase JIE7, 1:500 (DSHB) and mouse anti-Wg, 1:10 (DSHB). Dye-conjugated secondary antibodies were from Jackson Immunoresearch, Inc. and were used at 1:500. DNA was counterstained with DAPI 1:1000 (stock 50 ug/ml).

TUNEL analysis was modified for use in eye discs (Lisi et al., 2000) and anti-DIG-Rhodamine used for signal detection. Acridine Orange staining was done by following the Protocol 12.7 in *Drosophila* Protocols (Wolff, 2000). Eye disc *in situ* hybridization was carried out similar to previously described (Du, 2000). All images were taken on Zeiss Axioscop or Zeiss ApoTome with AxioCam CCD cameras. Adult fly eye images were taken using a Leica dissection microscope. Male adult flies were used for data collection.

Electrophoresis mobility shift assay (EMSA)

The sequence of the probes are: Wild-type; 5'-ACTTTGCGCGCGAAAACGCTTGAA-3', E2F mut; 5'-ACTTTGCGCtaGccAACGCTTGAA-3'. Forward and reverse oligonucleotides were annealed, end-labeled, and purified. Binding reactions were as previously described (Tanaka-Matakatsu and Du, 2008).

Chromatin Immunoprecipitation (ChIP)

The third instar larva were dissected, fixed on ice for 15min. Thirty pairs of eye discs were pooled in 250ul of ChIP lysis buffer and were processed essentially as described (Austin et al., 1999). Primer sequences are: *hid 5'F* Forward 5'-ttctctgtcattcccaactttgcgcgcg-3', *hid 5'F* Reverse 5'- cgtaactggcattactttagatccat-3', *hid intron1* Forward 5'-agtaagacattcgactcgagtttggcg-3', *hid intron1* Reverse 5'-gtgctatggctttaagaaagaaacgccg-3', *Arp53D* Forward 5'-gatttttggcctctgtatgtcatggcg-3', *Apr53D* Reverse 5'-

aggagaggttgctgaacgaatcatggtc-3', *rp49* Forward 5'-ccacgaattccca tcacaaacagaagcc-3', *rp49* Reverse 5'-aatatatcgcttggcatcgccatgaacg-3'.

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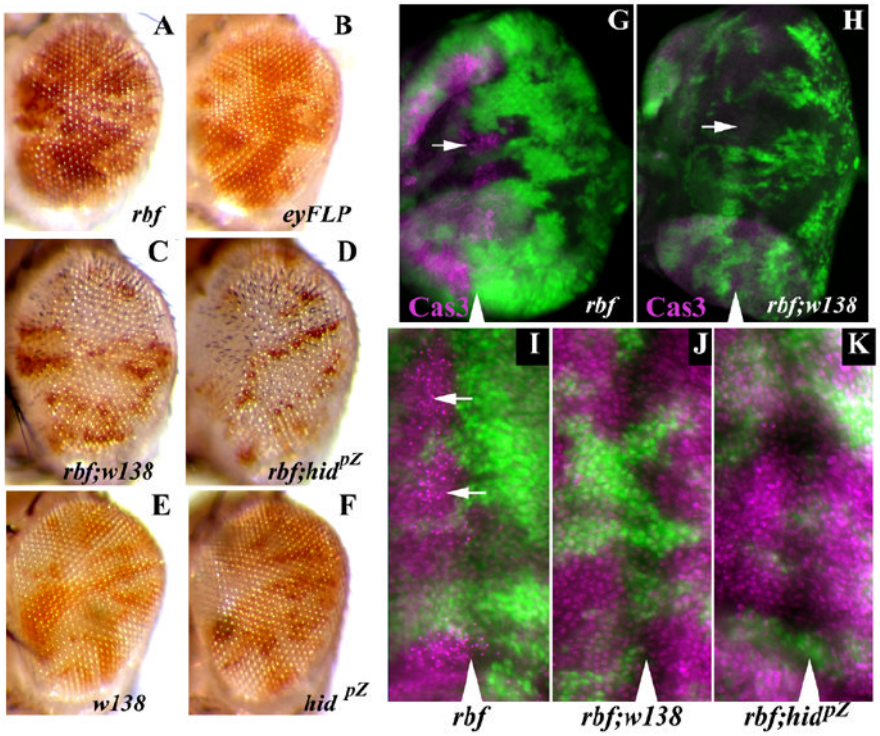

Figure 1. Modification of the *rbf* mutant phenotype by mutations of *w138* and *hid*

A) *rbf* mutant clones (A, white ommatidia) occupied slightly smaller areas in adult eyes as compared to the control wild-type clones (B, white ommatidia). (C and D) Mutation of *w138* or *hid* in conjunction with *rbf* dramatically increased areas occupied by the mutant clones (C, *rbf;w138*, white ommatidia; D, *rbf;hid*, white ommatidia). (E and F) The morphology of *w138*, or *hid* single mutant clones was relatively normal with occasional extra number of interommatidial bristles. Mutant clones were generated using *eyFLP/FRT* method. Anterior of the eye is to the left and dorsal is up. (G-K) Mosaic clone analysis of activated Caspase 3 or pyknotic nuclei in third instar eye disc. Mutant clones were marked by absence of GFP shown in green. (G) *rbf* mutant clones spanning the MF displayed significantly elevated level of anti-activated Caspase 3 staining (Cas3 in magenta, white arrow). (H) *rbf; w138* double mutant clones in the MF lack Cas3 staining. (I-K) High magnification images of DAPI staining to detect nuclear pyknosis in the MF region of the eye discs. (I) *rbf* mutant clones in the MF contained extensive pyknotic nuclei (bright magenta dots, white arrows). (J and K) Nuclear pyknosis was not observed in either *rbf; w138* (J) or *rbf; hid* (K) double mutant clones. In all figures anterior of the eye disc is to the left and dorsal is up. Arrowhead points the MF groove.

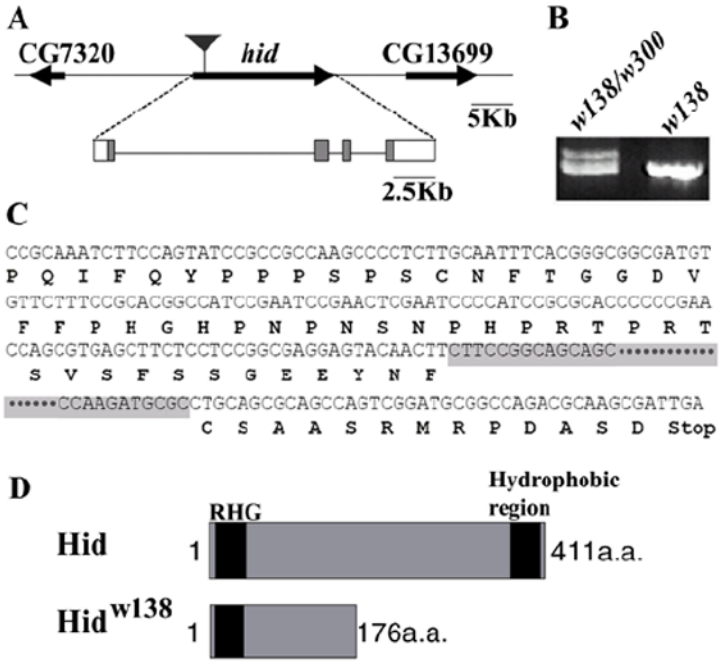

Figure 2. *hid*^{w138} is new allele of *hid*

(A) Schematic representation of the *hid* locus. An approximate 60Kb region surrounding the *hid* locus is shown. A solid black arrow represents the *hid* transcript unit. The black inverted triangle represents the location of the P-element insertion in the *hid*^{PZ} line. The *hid* ORF is shown by shaded boxes and the 5' and 3' UTR are shown by open boxes. (B) PCR product of *hid* exon2-4 from different genomic DNA. Two different size PCR fragments were obtained from *w138/w300* transheterozygotes, a single fragment corresponding to the shorter one was obtained from *w138* homozygote. (C) The sequence of *w138* mutation. Amino acids sequence is shown beneath the nucleotide sequence. Deleted 312bp DNA sequence in the second exon is highlighted in gray. This deletion introduced a frame shift leading to the coding of 13-amino-acids followed by a stop codon. (D) Schematic representation of open reading frames of wild-type Hid and Hid^{w138}.

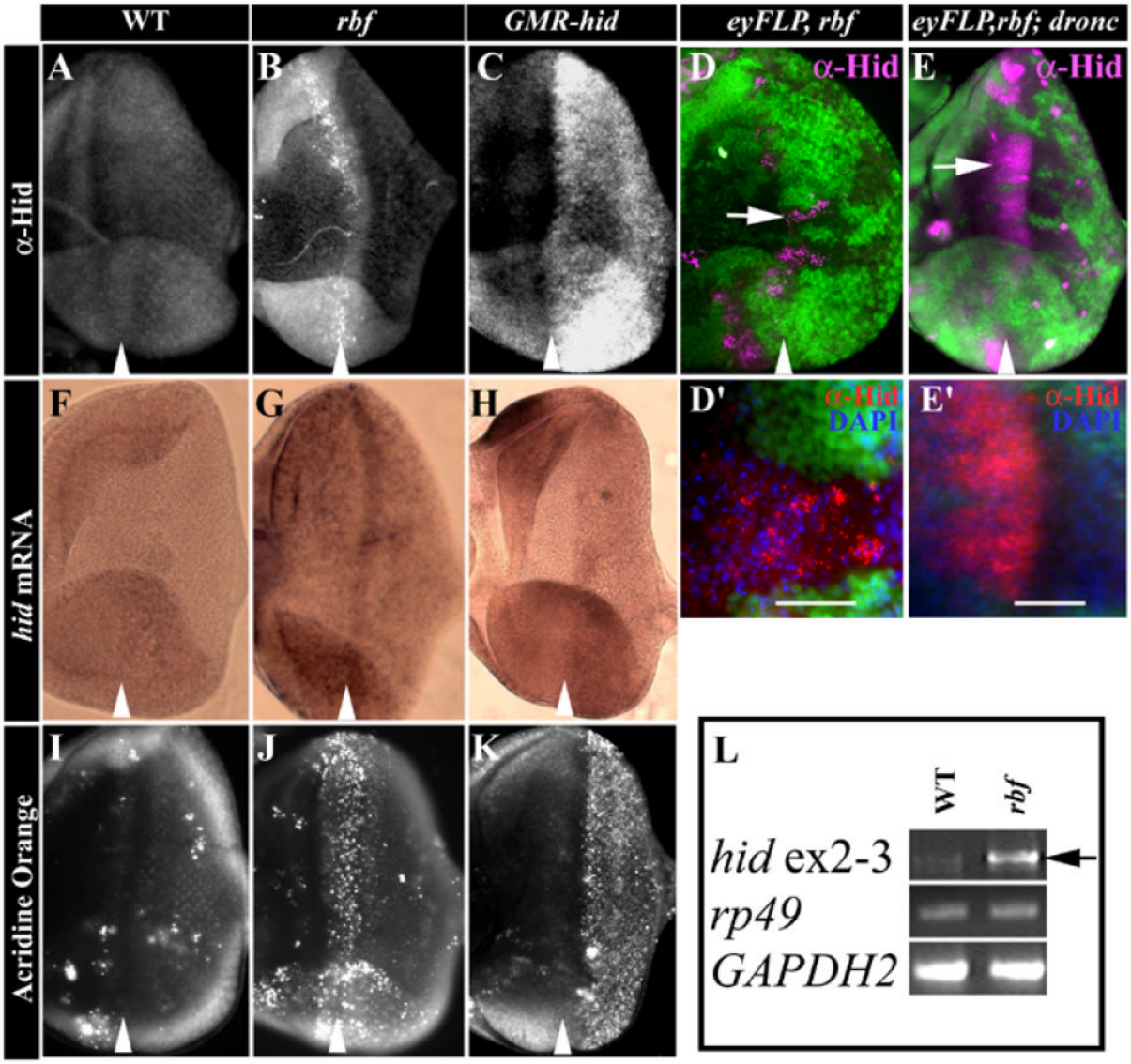

Figure 3. *rbf* mutant discs exhibit increased *hid* expression and elevated level of apoptosis in the MF

(A-E) Immunostaining of anti-Hid (CL1C3) antibody in third instar eye discs. (F-H) *in situ* hybridization to determine *hid* transcripts. (I-K) Acridine Orange staining to detect cell death. (A, F and I) WT eye disc, (B, G and J) $rbf^{15\Delta/120}$ eye discs, and (C, H and K) *GMR-hid* eye discs. While WT eye discs were mostly negative for anti-Hid labeling in eye discs (A), Hid protein was highly accumulated in the MF of *rbf* mutant discs (B), in the mutant clones of *rbf* (D) and *rbf;dronc* (E) that span the MF, and in the in the posterior in *GMR-hid* discs (C). Hid staining was shown in magenta in (D and E). (D' and E') High magnification image that show Hid was localized in punctate pattern and surrounded by pyknotic nuclei in *rbf* clones (D') as compared to a diffused cytoplasmic localization in $rbf;dronc^{O1}$ double mutant clones in (E'). No significant *hid* mRNA is detected in WT eye discs (F) and only scattered AO labeled cells were observed in WT eye disc (I). In contrast, elevated *hid* mRNA was detected throughout the *rbf* mutant eye discs with the highest level observed in the MF (G), which correlated with significantly elevated AO staining in the MF of the *rbf* mutant discs (J). As expected, elevated *hid* expression (H) and AO staining (K) was observed in the posterior of

GMR-hid discs. Arrowhead point the MF groove. In all figures anterior of the eye disc is to the left and dorsal is up. (L) Reverse transcription-PCR analysis demonstrated increased level of *hid* mRNA in *rbf* mutant discs (black arrow). The levels of *ribosomal protein 49* (*rp49*) and *Glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH2*) RNA were used as controls.

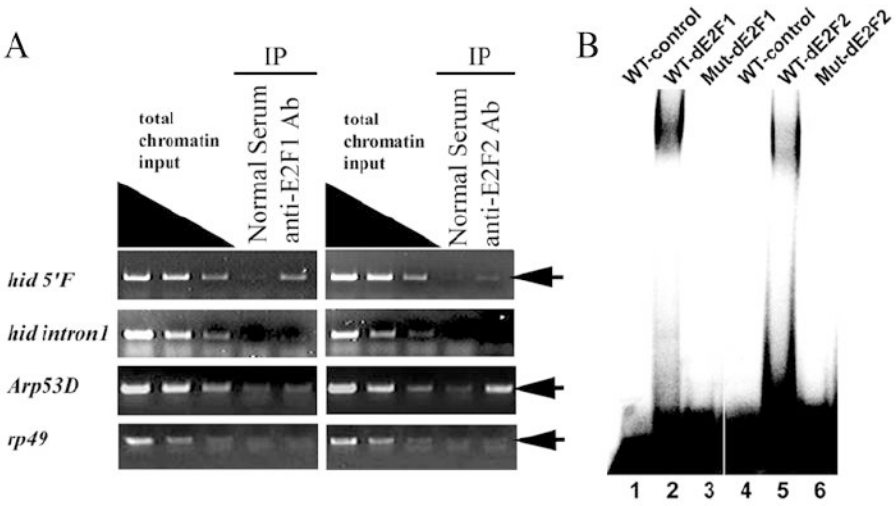

Figure 4. Both dE2F1 and dE2F2 bind to the consensus E2F binding site in the *hid* 5' regulatory region

A) ChIP assay using antibodies against dE2F1 and dE2F2 proteins exhibited different degree of enrichment of the DNA fragment containing the E2F binding site at −1.4Kb in *hid* 5'F (first row, arrow). In contrast, ChIP with either the dE2F1 or the dE2F2 antibody failed to enrich the DNA fragment containing the introns 1 E2F site at +2.1Kb (second row). The E2F binding site in 5' flanking sequence of *Arp53D* was used as a positive control for dE2F2 ChIP assay. Anti-dE2F2 highly enriched target DNA fragment (third row, arrow). *rp49* was used as a negative control. No enrichment of DNA fragment was detected (fourth row, arrow). Nonspecific IgG (Normal Serum) was used as internal negative control. (B) DNA fragments containing either the WT or mutant E2F binding site of the *hid* 5'F locus were used as probes and recombinant dE2F1/dDP (lanes 1-3) or dE2F2/dDP (lanes 4-6) proteins were used in an EMSA assay as indicated. wild-type E2F binding site probe (WT, lanes 1, 2, 4, and 5), E2F binding site mutated probe (Mut, lanes 3 and 6).

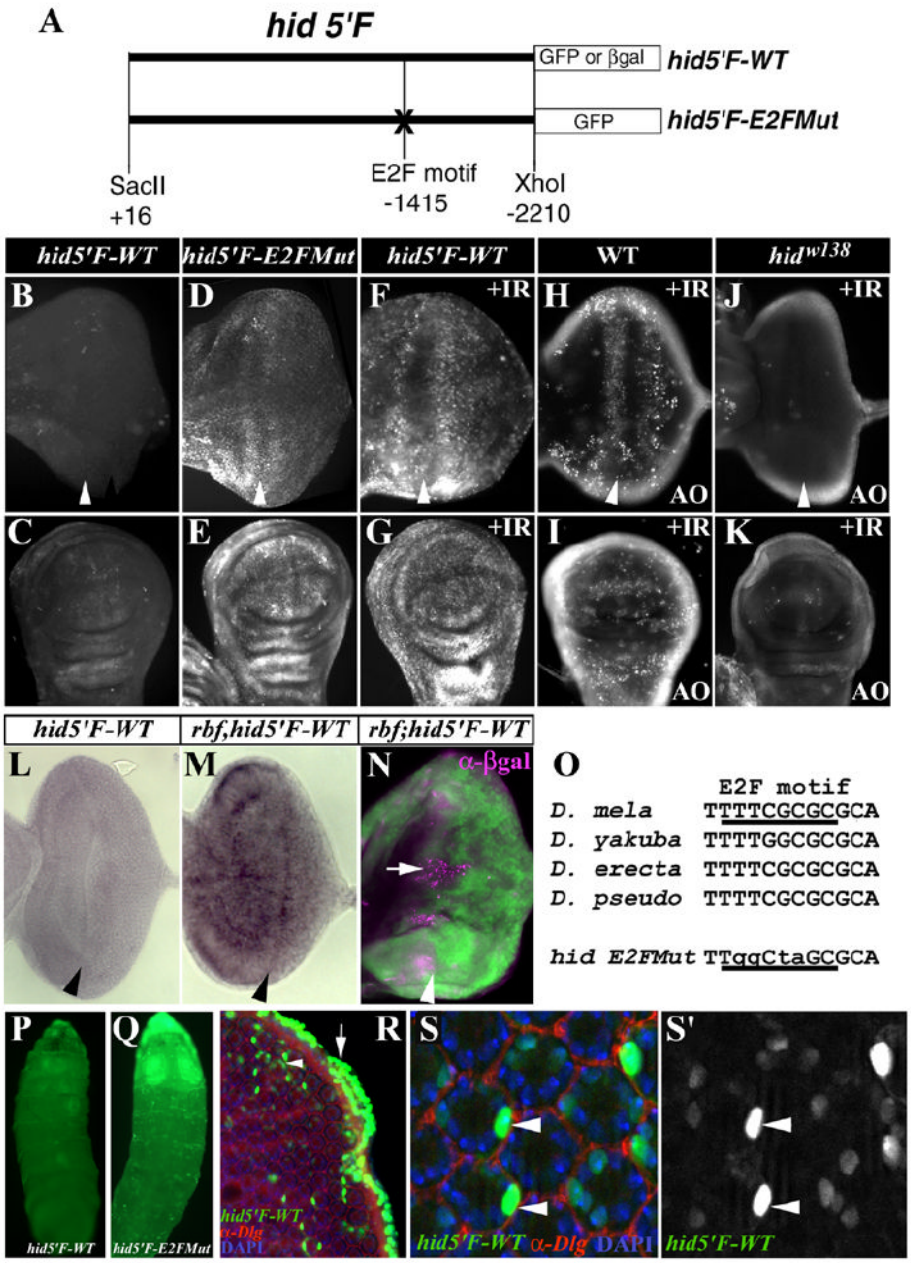

Figure 5. *hid* 5'F drives reporter GFP expression in imaginal discs

(A) Schematic representation of hid enhancer constructs: Black lines indicate non-coding DNA, open boxes indicate reporter GFP and lacZ, "X" represents mutated E2F site. (B and C) Very low level of GFP reporter expression are observed in 3rd instar eye and wing discs from WT constructs (*hid5'F-WT*). (D and E) Mutation of the consensus E2F binding site dramatically upregulated GFP reporter expression in eye and wing discs. (F-K) Eye and wing imaginal discs 4-5 hr after gamma-irradiation (4Gy). (F and G) A high level of GFP reporter expression from *hid5'F-WT* was observed in most cells in the eye disc (F) and wing disc (G). (H and I) Acridine Orange staining of irradiated discs show increased cell death in the eye disc (H) as well as the wing disc (I). (J and K) Irradiation induced cell death in the eye disc (J) and

wing disc (K) was suppressed by removal of *hid*. (L-N) Increased expression of *hid* reporter in *rbf* mutant background. *In situ* hybridizations of GFP reporter expression in WT and $rbf^{15\Delta/120}$ eye discs are shown in (L) and (M), respectively. Increased β-gal reporter level was also observed in *rbf* mutant clones (N, magenta, arrow). The position of the MF groove was indicated by white arrows. Anterior of eye disc is to the left and dorsal is up. Anterior of the wing disc is to the left and ventral is up. (O) Sequence of the conserved *hid 5'* E2F site in *Drosophila* species. E2F binding site is underlined. E2F mutant sequence of mutated E2F binding site is shown at the bottom. Mutated bases are shown in lowercase letters. (P-S') The *hid 5'F* responds various biological cell death stimuli. GFP reporter expression from whole third instar larva from *hid5'F-WT* (P) and *hid5'F-E2FMut* (Q). (R) A 42hr APF pupal retina showed high level of *hid 5'F-WT* GFP reporter expression in ommatidia at the periphery of the eye disc and in some interommatidial cells. (S-S') High magnification image of 42hr APF pupal retina showed upregulation of GPF reporter expression from *hid5'F-WT* enhancer in some interommatidial cells.

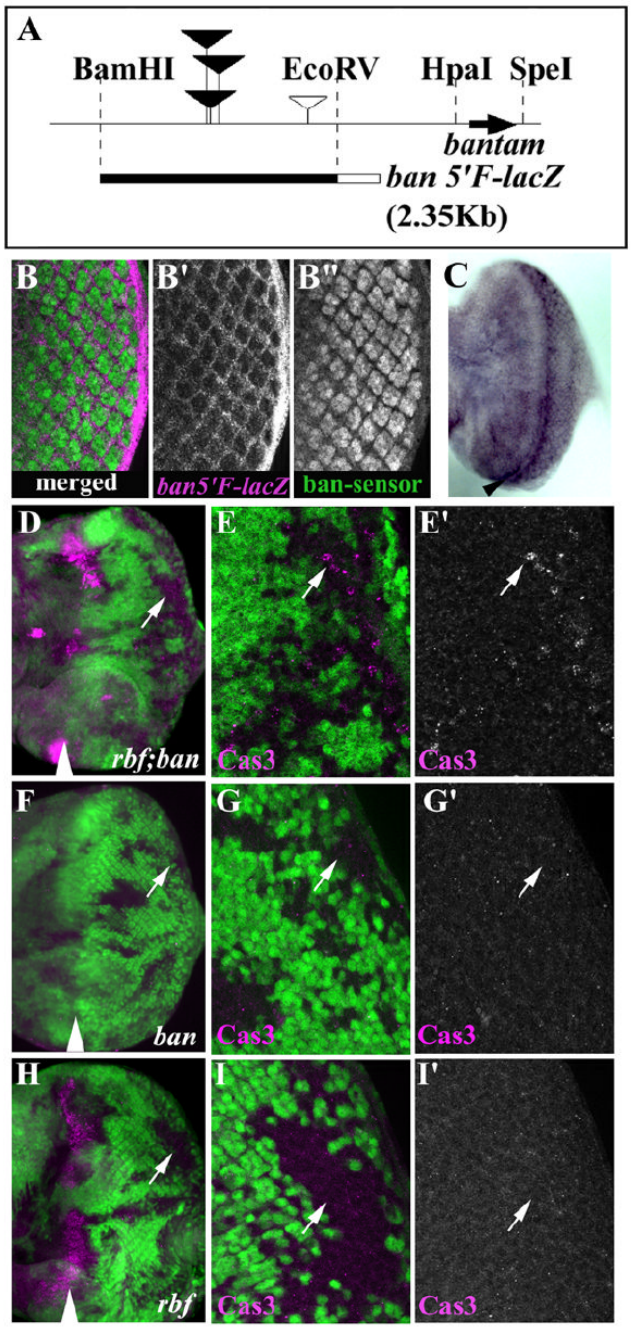

Figure 6. Spatial expression of *bantam* miRNA in developing eye disc and its modulation of *rbf*-induced apoptosis

(A) Schematic representation of *bantam* flanking sequence. *Bantam* hairpin coding region is shown by a black arrow. Filled inverted triangles indicate P-element insertion sites that disrupted *bantam* function. An open inverted triangle indicates P-element insertion site associated non-*bantam* mutation. The 2.35Kb BamHI and EcoRV fragment was used to generate *ban-5'F-lacZ* shown at the bottom (filled black bar). The open box represents the LacZ reporter. β-gal expression from *ban5'F-lacZ* (magenta) and GFP expression from *ban*-sensor-GFP (green) are complementary (B-B"). High bantam expression is seen in interommatidial cells (β-gal, B') while high bantam sensor is seen in photoreceptor cells (GFP,

B”). Merged image is shown in (B). (C) *In situ* hybridization with DIG labeled *lacZ* RNA probe to *ban5’F-lacZ* eye disc. High level of expression in the MF and posterior interommatidial cells were seen. The MF groove indicates black arrowhead. (D-I’) *bantam* miRNA attenuates *rbf* induced cell death. Low (D) and high (E, E’) magnification images of *rbf; ban* double mutant cells showed increased Cas3 staining (magenta) in posterior mutant clones. Low (F) and high magnification (G, G’) images of *bantam* mutant clones showed background level of Cas3 staining. Low (H) and high magnification (I, I’) images of *rbf* mutant clones showed very low Cas3 labeling in the posterior, while clones spanning the MF showed extensive Cas3 labeling.