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ORIGINAL ARTICLE

High levels of dRYBP induce apoptosis in *Drosophila* imaginal cells through the activation of *reaper* and the requirement of *trithorax*, *dredd* and *dFADD*

Inma González[1], Ana Busturia[1]

[1]*Centro de Biología Molecular "Severo Ochoa" CSIC-UAM, c) Nicolás Cabrera 1, Universidad Autónoma de Madrid, Campus de Cantoblanco, 28049 Madrid, Spain*

***Drosophila* RYBP (dRYBP; Ring1 and YY1 Binding Protein) is a Polycomb and trithorax interacting protein, whose homologous RYBP/DEDAF mammalian counterparts exhibit tumor cell-specific killing activity. Here we show that although endogenous dRYBP is not involved in developmental apoptosis, high levels of exogenous dRYBP induce apoptosis in all the imaginal discs of the fly, indicating that dRYBP apoptotic activity is not specific to tumor cells. We also show that dRYBP-induced apoptosis is inhibited by high levels of either p35 or DIAP1 (Drosophila Inhibitor of Apoptosis Protein 1), and requires the function of the pro-apoptotic REAPER, HID and GRIM proteins, the apical caspase DREDD, the adaptor dFADD protein as well as TRITHORAX (TRX), an epigenetic transcriptional regulator. Furthermore, we demonstrate that overexpression of TRX also induces apoptosis in the imaginal discs. Finally, we show that the expression of *reaper-lacZ* is upregulated both upon dRYBP-induced apoptosis and upon TRX-induced apoptosis in imaginal discs and that the *reaper* gene is a direct target of dRYBP in *Drosophila* embryos. Our results indicate that dRYBP triggers in a receptor-mediated apoptotic pathway that also includes TRX-dependent epigenetic regulation of gene expression.**

***Keywords*:** dRYBP, apoptosis, trithorax, Polycomb, *Drosophila*
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Introduction

Apoptosis affects many aspects of development, including shaping of body structures, controlling cell number and eliminating abnormal, misplaced or harmful cells [1, 2]. Moreover, cell killing in response to death signal stimuli is essential to remove cells with neoplastic transformation potential [3, 4]. The pathways controlling apoptosis are highly conserved from flies to humans. The core of the apoptosis cell death machine consists of members of the caspase family of proteases [5] whose activation is regulated either by an extrinsic or by an intrinsic signaling pathway. In *Drosophila*, the intrinsic pathway is stimulated by death-inducing signals that activate the RHG family (reaper, hid, grim, sickle and jafra-2) of pro-apoptotic proteins, leading to the activation of the effector caspases and the induction of apoptosis (reviewed in Domingos *et al*. [6]). Likewise, in mammals the intrinsic pathway is stimulated by intracellular stimuli such as DNA damage and cytotoxic drugs that act inside the cell activating the pro-apoptotic proteins and leading to the activation of caspases [7]. The extrinsic pathway in mammals, also referred to as the receptor-mediated apoptotic pathway, plays an important role in both development and disease [7]. After an apoptotic stimulus, the TNF/TNFR (tumor necrotic factor/tumor necrotic factor receptor) signaling is activated and mediates cell death through both the activation of the JNK pathway and the activation of caspases. Additionally, in mammals, initiation of receptor-mediated apoptosis activates the formation of the death receptor complex, composed of TNFR associated factors (TRAFs), which function with the adaptor protein FADD to recruit and aggregate procaspase-8, thereby activating caspase-8 through

Correspondence: Ana Busturia
Tel: +34-91-196-4689; Fax: +34-91-196-4420
E-mail: abusturia@cbm.uam.es
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mutual cleavage and initiating apoptosis [8]. This pathway, however, is poorly understood in *Drosophila*. The *Drosophila* TNF/TNFR homologs are EIGER/WENGEN [9, 10]. Three *Drosophila* members of the TRAF family have been identified [11, 12]. The *Drosophila* homologs of the DED (Death Domain)-containing protein FADD and caspase-8 are dFADD and DREDD, respectively [13, 14]. However, unlike its mammalian counterpart, the apoptotic effect of EIGER requires neither the activity of the caspase-8 homolog DREDD nor the adaptor dFADD protein, but is completely dependent on the activation of the JNK signaling pathway [10].

The *dRYBP* gene encodes a phylogenetically conserved 150-amino acid protein with a conserved amino terminus, which contains a NZF (Npl4 zinc finger) type zinc-finger [15] (Figure 1L) and a carboxy-terminus with so far no identified functional domains. Recently, a subgroup of NZF domains that includes that of RYBP has been shown to possess ubiquitin binding activity [16]. We have previously shown that loss of *dRYBP* function produces phenotypes that are highly variable in both penetrance and expressivity [17]. Moreover, we showed that dRYBP interacts genetically and molecularly with Polycomb (PcG) and trithorax (trxG) proteins and that its overexpression generates a small wing phenotype [17, 18].

In mammals, high levels of the RYBP protein have been found to have tumor-specific killing activity, a characteristic that could potentially be exploited to develop anti-cancer treatments [19]. Furthermore, the RYBP protein has been found to interact with Apoptin, a protein known to be active in killing tumor cells [20]. As induction of apoptosis is a common mechanism underlying cancer therapy, understanding the mechanisms controlling tumor cell-specific killing may facilitate the development of improved therapies.

We show here that overexpression of dRYBP and overexpression of TRITHORAX, an epigenetic regulator of transcription [21], induce apoptosis in the imaginal discs. Moreover, we show that high levels of dRYBP and high levels of TRITHORAX activate the expression of a *reaper-lacZ* transgene. We also show that the pro-apoptotic gene *reaper* is a direct target of the dRYBP protein and that dRYBP-induced apoptosis requires the function of *reaper* and *trithorax*. These findings reveal the participation of a factor known to be an epigenetic regulator of gene transcription in the control of apoptosis. Furthermore, we show that dRYBP-induced apoptosis is independent of the JNK pathway but requires DREDD and dFADD, two factors previously shown not to be associated with receptor-mediated apoptosis in *Drosophila* [10].

Results

Endogenous dRYBP is not required for developmental apoptosis

Loss of function of the *dRYBP* gene results in phenotypes that are highly variable both in penetrance and in expressivity [17]. In this report, we have investigated whether the inactivation of dRYBP function using $RNAi_{dRYBP}$ transgenic lines and loss of function *dRYBP* mutations have an effect on apoptosis. We first performed caspase-3 [22] and Terminal deoxynucleotidyl transferase-mediated dUTPNick End Labelling (TUNEL) staining in the wing imaginal discs of *sd-GAL4/+*; *UAS-*$RNAi_{dRYBP}$*/UAS-*$RNAi_{dRYBP}$ larvae and the wing imaginal discs of *dRYBP*^{1}*/dRYBP*^{1} larvae and found similar results to those in the wild-type wing imaginal discs. Second, we looked at Acridine Orange staining during embryonic development of the progeny of *dRYBP*^{1}*/CyO-GFP* stock. Compared to wild-type embryos (*Df (1)*^{w67c23}, see Materials and Methods), mutant (non-GFP) embryos displayed no appreciable changes in cell death throughout the central nervous system during nerve cord condensation in stage 16 embryos or in the embryonic head (data not shown). These results suggest that *dRYBP* function is not required for developmental apoptosis.

Exogenous dRYBP expression induces apoptotic cell death

Using the GAL4/UAS system, we observed that overexpression of the dRYBP protein in wing imaginal discs produces small adult wings [17, 18]. We asked whether the reduction of wing size was due to the induction of apoptosis. We have used several GAL4 drivers (*en*-Gal4, *ci*-Gal4, *sd*-Gal4, *nub*-Gal4, *248*-Gal4 and *dll*-Gal4; see Materials and Methods) to induce high levels of dRYBP and found that overexpression of dRYBP induces apoptosis in all imaginal discs as evidenced by the activation of caspase-3 (Figure 1D-1K). We have also investigated whether the murine RYBP/DEDAF protein can induce apoptosis in wing imaginal discs. Similar to the expression of the *Drosophila* protein (Figure 1D), we found that the wing imaginal discs of *sd-Gal4/+; UAS-RYBP murine/+* show induction of apoptosis (not shown). To assess the contribution of apoptosis to the dRYBP overexpression phenotype, we used the baculovirus caspase inhibitor p35 to block apoptosis [23]. Co-expression of p35 and dRYBP in *nub-Gal4/UAS dRYBP; UAS p35/+* represses the dRYBP-induced apoptosis (Figure 1I, 1M and 1N). Moreover, the small-size wing phenotype observed when high levels of dRYBP are expressed in the wing is rescued when p35 is also overexpressed (data not shown). This result indicates that caspases are necessary

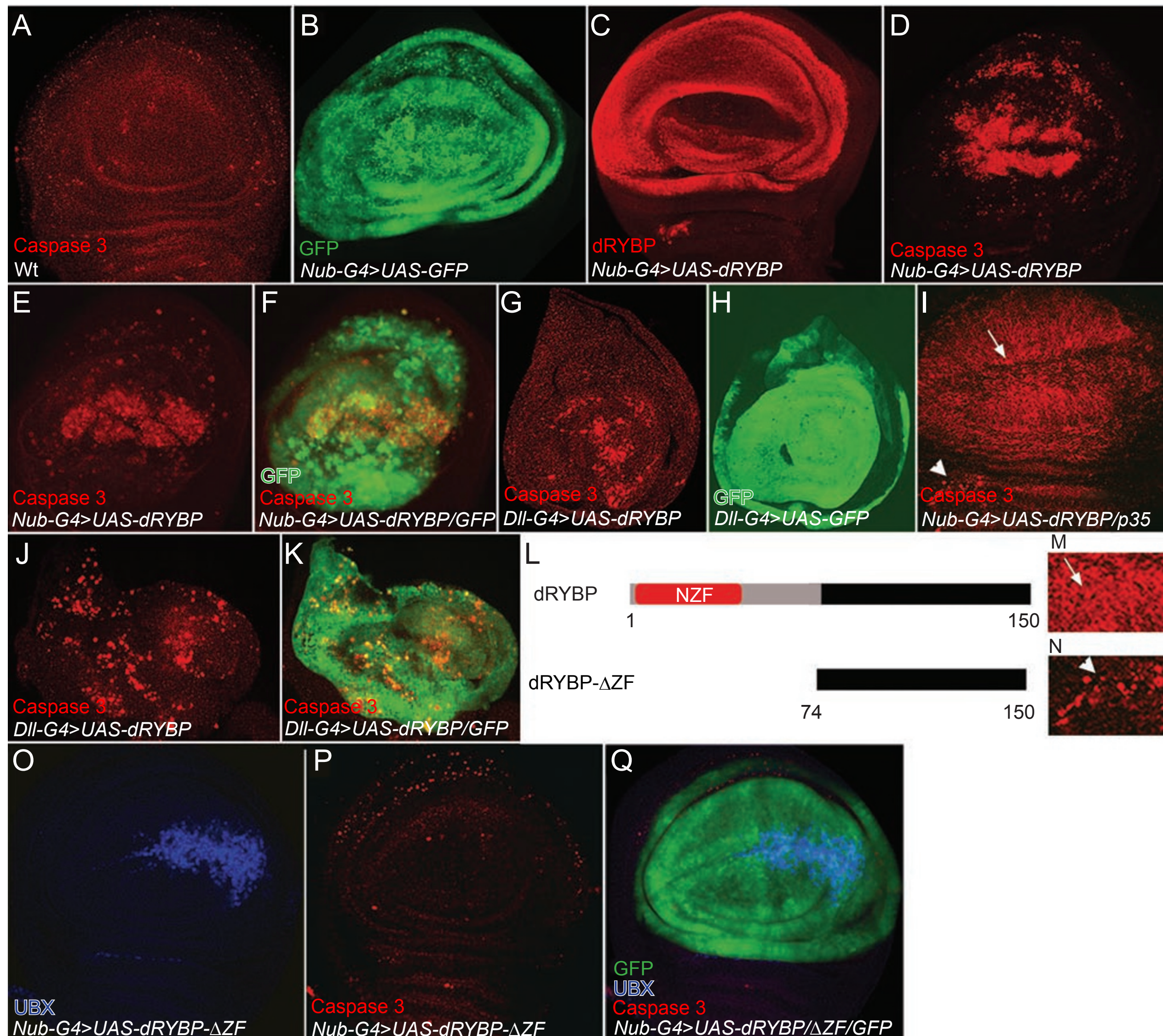

Figure 1 Exogenous dRYBP expression induces apoptotic cell death. **(A)** Activated caspase-3 (C3) staining (red) in a wild-type wing disc. **(B)** GFP expression (green) in a *nub-Gal4, UAS-GFP* wing disc showing the *nub-Gal4* line domain of expression in the wing disc. **(C)** dRYBP expression (red) in a *nub-Gal4, UAS-GFP/ UAS-dRYBP* wing disc. **(D)** C3 staining (red) in a *nub-Gal4, UAS-GFP/UAS-dRYBP* wing disc. **(E)** C3 staining (red) in a *nub-Gal4, UAS-GFP/UAS-dRYBP* haltere disc. **(F)** Merged expression of GFP (green, showing the domain of expression of the *nub-Gal4* line in the haltere disc) and C3 staining (red) in a *nub-Gal4, UAS-GFP/UAS-dRYBP* haltere disc. **(G)** C3 staining (red) in a *dll-Gal4, UAS-GFP/UAS-dRYBP* third leg disc. **(H)** GFP expression (green) in a *dll-Gal4, UAS-GFP/UAS-dRYBP* third leg disc, showing the *dll-Gal4* line domain of expression in the leg disc. **(I)** C3 staining (red) in a *nub-Gal4, UAS-GFP/UAS-dRYBP/+; UAS p35/+* wing disc. This wing disc illustrates the differential staining of the anti-C3 antibody when activated caspases are inhibited by overexpression of *p35* in the *nub-Gal4* domain (arrow, see detail in **M**) and when activated caspases are not inhibited by overexpression of *p35* outside the *nub-Gal4* domain (arrowhead, see detail in **N**). **(J)** C3 staining (red) in *dll-Gal4, UAS-GFP/UAS-dRYBP* antenna disc. **(K)** Merged expression of GFP (green) and C3 staining (red) in *dll-Gal4, UAS-GFP/UAS-dRYBP* antenna disc. **(L)** Scheme of the UAS-dRYBP and UAS-dRYBP-ΔZF constructs. The NZF (indicated in red) is located at the amino-terminus of the protein. **(M)** Detail of **I** – arrow showing staining of C3 in p35-apoptosis inactivated cells. **(N)** Detail of **I** – arrowhead showing staining of C3 in apoptotic cells. **(O)** UBX expression (blue) in *nub-Gal4, UAS-GFP/dRYBP-ΔZF* wing disc. **(P)** C3 staining in a *nub-Gal4, UAS-GFP/dRYBP-ΔZF* wing disc. **(Q)** Merged expression of UBX, C3 and GFP in *nub-Gal4, UAS-GFP/dRYBP-ΔZF*. The de-repression of UBX only takes place in a few cells within the *nub-Gal4* domain.

for dRYBP-induced apoptosis.

We have shown that overexpression of dRYBP in wing discs causes the homeotic Ultrabithorax (UBX) protein [24] to be expressed in a limited number of wing disc cells [17, 18]. This UBX expression is not dependent on the presence of the NZF domain of the dRYBP protein as overexpression of the dRYBP-ΔZF and full-length proteins (Figure 1L) produced the same pattern of UBX expression in wing discs (Figure 1O). Furthermore, in contrast to overexpression of full-length dRYBP, wing discs of *nub-Gal4/UAS-dRYBP-ΔZF* larvae do not show activation of caspase-3 (Figure 1P). As shown in Figure 1, the wing imaginal discs overexpressing the truncated dRYBP-ΔZF protein show expression of the homeotic UBX protein (Figure 1O) but do not show activation of caspase-3 (Figure 1P), indicating that the induction of apoptosis is not a consequence of UBX expression.

These results demonstrate that exogenous expression of either dRYBP or murine RYBP induces apoptosis in the *Drosophila* imaginal disc cells. Moreover, this induction is not a consequence of UBX expression. Finally, this induction of apoptosis does not require the cells to be tumoral as it occurs in normal imaginal disc cells.

dRYBP-induced apoptosis is dependent on both pro-apoptotic and anti-apoptotic factors

To gain insight into the molecular pathways controlling dRYBP-induced apoptosis, we investigated the requirement for pro-apoptotic factors [25] which act by inhibiting the activity of the Drosophila Inhibitor of Apoptosis (DIAP) anti-apoptotic proteins [26]. Three of the pro-apoptotic genes described in *Drosophila* – *reaper*, *hid* and *grim* – are contained within a single genomic deficiency – *Df (3L) H99* ([27]; see Materials and Methods). Imaginal discs from *nub-Gal4/UAS dRYBP*; *Df (3L) H99/+* larvae show a marked reduction in dRYBP-induced apoptosis (Figure 2B) and a partial rescue of the wing size (data not shown). Furthermore, high levels of the DIAP1 protein in *nub-Gal4/UAS dRYBP*; *UAS DIAP1/+* larvae led to a complete inhibition of apoptosis in wing imaginal discs (Figure 2C) and accordingly, rescue of the wing size (data not shown). Together, these results show that dRYBP-induced apoptosis requires the function of the pro-apoptotic Reaper, Hid and Grim proteins, and that this process is inhibited by high levels of DIAP1. This, in turn, suggests that dRYBP-induced apoptosis is dependent on the canonical apoptotic pathways in which these factors function.

The reaper gene promoter is a direct target of the dRYBP protein

To investigate whether dRYBP regulates the expression of apoptotic genes, we studied the effect of dRYBP on the expression of *reaper* by monitoring β-gal expression in *sd-Gal4/+*; *UAS-dRYBP/rpr-lacZ* wing imaginal discs. As shown in Figure 2G-2I, expression of *rpr-lacZ* in the wing discs is activated by dRYBP overexpression, indicating that high levels of dRYBP cause mis-regulation of *reaper* expression.

We next studied whether the *reaper* gene is bound by the dRYBP protein by performing chromatin immunoprecipitation (ChIP) experiments in wild-type *Drosophila* embryos using an antibody against dRYBP. Quantitative PCR was used to identify regions in and around the *reaper* gene enriched in dRYBP ChIP products and that are, therefore, likely to be bound by the dRYBP protein (Materials and Methods). As shown in Figure 2J, the *reaper* gene promoter is enriched in the dRYBP ChIP products compared to the control region.

These results clearly indicate that in wild-type imaginal discs the pro-apoptotic gene *reaper* is a direct target of dRYBP and suggest that its transcription is activated when dRYBP is expressed at high levels.

dRYBP-induced apoptosis is independent of the JNK pathway and is dependent on the DED-domain containing proteins dFADD and DREDD

Activation of the receptor-mediated extrinsic death pathway upon apoptotic stimuli induces cell death through activation of the JNK pathway [9, 10]. In *Drosophila*, activation of the JNK pathway is independent of the DED-domain containing proteins DREDD (caspase-8 in humans) and dFADD (the Fas-associated death-domain containing protein, FADD in humans) [10]. In humans, however, receptor-mediated apoptosis is dependent on FADD and caspase-8 [28]. By monitoring *puckered* expression using *puckered-lacZ* ^{E69} transgenic flies [29], we studied whether dRYBP-induced apoptosis is able to activate the JNK pathway. We found that the β-gal expression pattern is identical in wing imaginal discs from *sd-Gal4/+*; *UAS-dRYBP/+*; *puc-lacZ/+* and from control *puc-lacZ/+* larvae, indicating that exogenous dRYBP does not activate the JNK pathway (not shown).

We next studied the requirement of DREDD and dFADD in dRYBP-induced apoptosis. $dredd^{D44}/dredd^{D44}$; *nub-Gal4/UAS-dRYBP* wing imaginal discs do not show activation of caspase-3 (Figure 2D). Additionally, *nub-Gal4/ UAS-RNAi*$_{FADD}^{M8}$; *UAS-dRYBP/+* wing imaginal discs show no activation of apoptosis (Figure 2E). The overexpression of dRYBP in the absence of either *dredd* or *dFADD* results in flies with wings of a bigger size (data not shown) due to the absence of apoptosis in those mutant conditions. These results indicate that, like receptor-mediated apoptosis in mammals, exogenous dRYBP-

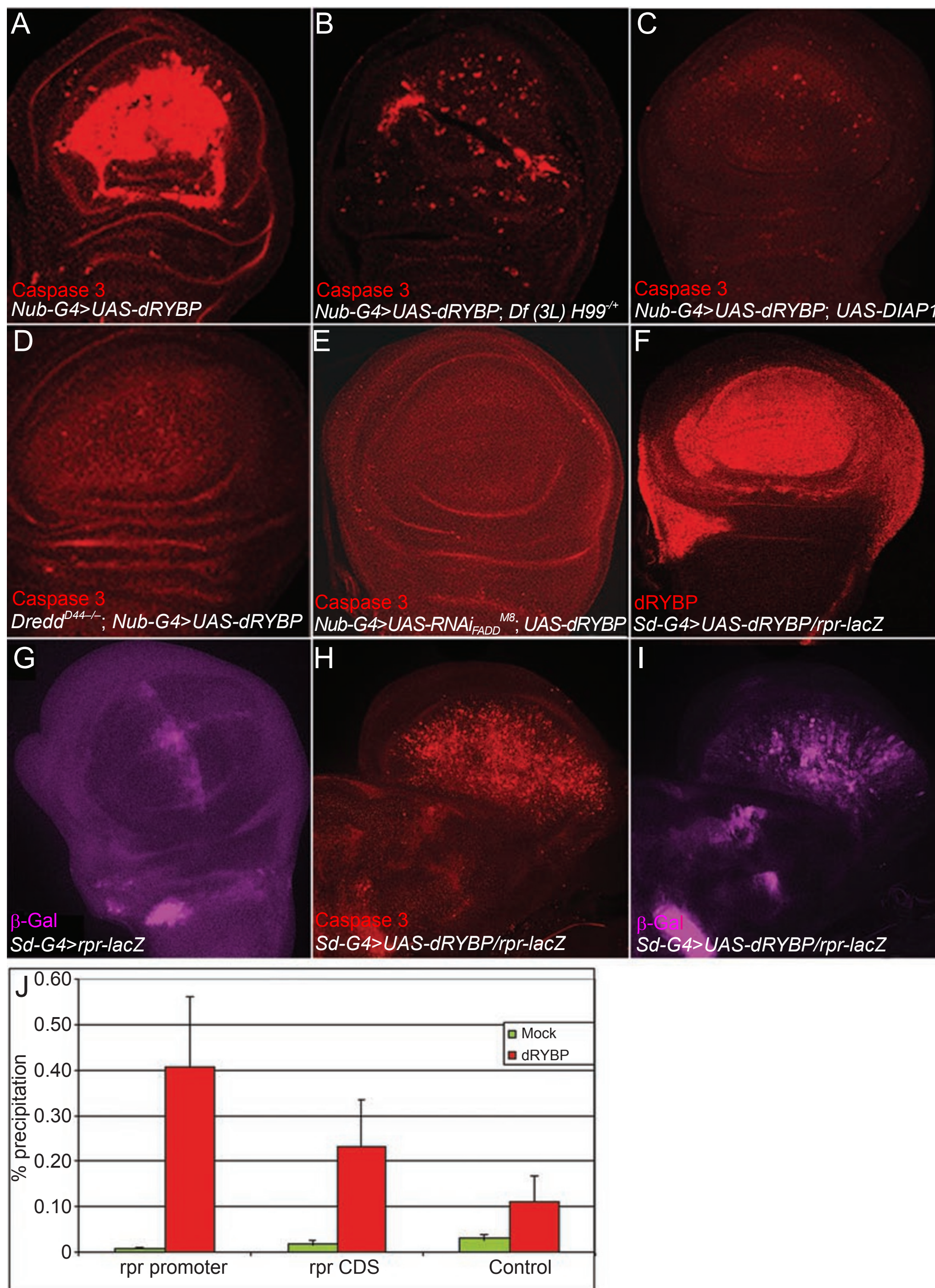

Figure 2 Factors involved in the dRYBP induction of apoptosis. (**A**) C3 staining (red) in *nub-Gal4, UAS-GFP/dRYBP* wing disc. **(B)** C3 staining (red) in *nub-Gal4, UAS-GFP/dRYBP; Df(3L)H99/+* wing disc. Apoptosis is diminished. **(C)** C3 staining (red) in *nub-Gal4, UAS-GFP/dRYBP*; *UAS-DIAP1/+*. Apoptosis is almost absent. **(D)** C3 staining (red) in $dredd^{D44}/dredd^{D44}$; *nub-Gal4*, *UAS-GFP/UAS-dRYBP* wing imaginal disc. Apoptosis is greatly suppressed. **(E)** C3 staining (red) in *nub-Gal4, UAS-GFP/UAS-RNAi*$_{FADD}$*M8*; *UAS-dRYBP/+* wing disc. Apoptosis is nearly completely absent. **(F)** dRYBP expression (red) in a *sd-Gal4/+*; *UAS-dRYBP/rpr-lacZ* wing disc indicating the domain of expression driven by the *sd*-Gal4 line. **(G)** β-Gal expression (purple) of a wing disc *sd-Gal4/+*; *rpr-lacZ* showing the expression of the *reaper-lacZ* construct, which is not affected by the presence of the *sd-Gal4* line. **(H)** C3 staining (red) in *sd-Gal4/+*; *UAS-dRYBP/rpr-lacZ* wing disc. **(I)** β-Gal expression (purple) in a *sd-Gal4/+*; *UAS-dRYBP/rpr-lacZ* wing disc. **(J)** ChIP with dRYBP antibody in 4-12 h embryos. dRYBP antibody (orange bars). Mock (green bars). The immunoprecipitated DNA was analyzed by qPCR using primers for the promoter region of *reaper* (rpr), for coding sequences (CDS) of *reaper* and for *robo 3* gene as a control.

induced apoptosis in fly imaginal discs is dependent on DREDD and dFADD expression.

dRYBP-induced apoptosis is trithorax dependent but Polycomb and Sex Comb extra independent

Several lines of evidence indicate that dRYBP interacts with the Polycomb group (PcG) and trithorax group (trxG) of proteins [17, 18]. We used PcG and trxG mutant backgrounds to investigate whether apoptosis induced by dRYBP is dependent on these proteins. The activation of caspase-3 is clearly diminished in wing imaginal discs of *nub-Gal4/UAS dRYBP*; trx^{E2}/+ larvae (Figure 3B), while there is no change in either *nub-Gal4/UAS dRYBP*; Pc^{3}/+ larvae (Figure 3C) or *nub-Gal4/UAS dRYBP*; Sce^{1}/+ larvae (Figure 3D). These results lead to the conclusion that *trithorax*, but not *Polycomb* or *Sex comb extra*, affects dRYBP-induced apoptosis, although it is possible that lack of only one dose of *Pc* or *Sce* is not sufficient to influence the induction of apoptosis. These results indicate that the trithorax protein functions in dRYBP-induced apoptosis and links the epigenetic regulation mediated by the trxG genes to the processes of programmed cell death.

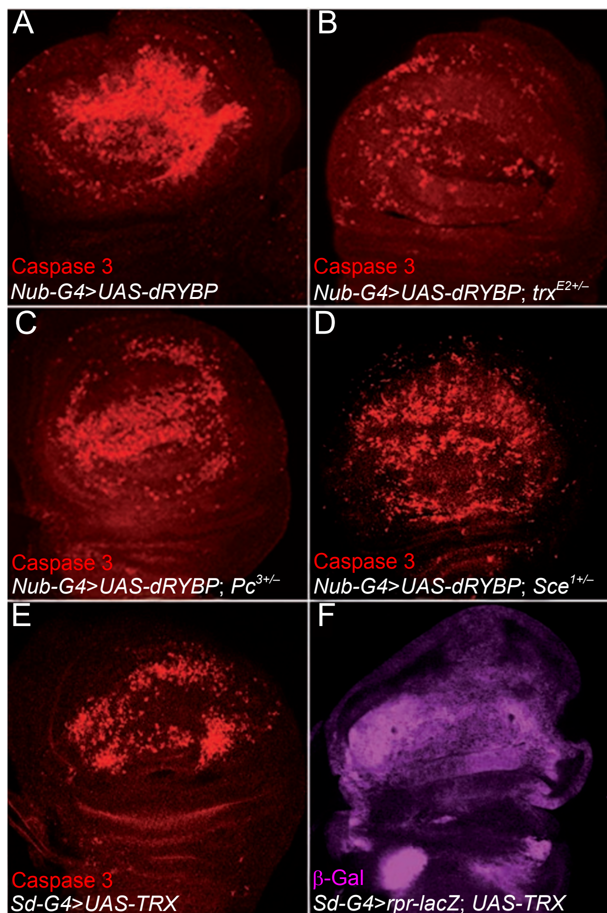

Figure 3 dRYBP-induced apoptosis is *trithorax* dependent and high levels of Trithorax induce apoptosis. **(A)** C3 staining (red) in a *nub-Gal4, UAS-GFP/UAS-dRYBP* wing disc. **(B)** C3 staining (red) in a *nub-Gal4, UAS-GFP/UAS-dRYBP*; trx^{E2}/+ wing disc. Note the decrease, compared with **(A)**, in the number of C3 stained cells. **(C)** C3 staining (red) in a *nub-Gal4, UAS-GFP/UAS-dRYBP*; Pc^{3}/+ wing disc. **(D)** C3 staining (red) in a *nub-Gal4, UAS-GFP/UAS-dRYBP*; Sce^{1}/+ wing disc. **(E)** C3 staining (red) in a *sd-Gal4/+*; *UAS-TRX/+* wing disc. **(F)** β-Gal expression (purple) in a *sd-Gal4/+*; *rpr-lacZ/+*; *UAS-TRX/+* wing disc.

Exogenous TRITHORAX induces apoptosis and activation of reaper

We investigated whether developmental apoptosis is modified in trx^{E2} homozygous mutant embryos and found that activated caspase-3 staining in those embryos is similar to wild-type embryos (data not shown), suggesting that the Trithorax protein is not required in developmental apoptosis. We next investigated whether high levels of POLYCOMB, SEX COMB EXTRA and TRITHORAX are capable of inducing apoptosis. Wing imaginal discs from neither *nub-Gal4/+*; *UAS-SCE/+* nor *nub-Gal4/+*; *UAS-PC/+* larvae showed induction of apoptosis (data not shown). However, a high level of TRITHORAX-induced apoptosis was indicated by the clear activation of caspase-3 in *sd-Gal4/+*; *UAS-TRX /+* imaginal discs (Figure 3E). Moreover, to study whether exogenous TRX is capable of inducing the expression of pro-apoptotic genes, we looked at the β-gal expression in imaginal discs of *sd-Gal4/+*; *rpr-lacZ/+*; *UAS-TRX /+* larvae and found that *rpr-lacZ* expression is activated in the wing imaginal discs (Figure 3F). These results demonstrate the ability of exogenously expressed trithorax protein to both induce apoptosis as well as activate the expression of the pro-apoptotic *reaper* gene.

Discussion

High levels of exogenous dRYBP induce apoptosis

In the present work, we have shown that loss of function of *dRYBP* does not affect the apoptosis associated with development. However, high levels of dRYBP expression induce apoptosis in all the imaginal discs studied (Figure 1). In transfection experiments, murine and human RYBP/DEDAF proteins have been shown to have the ability to specifically kill mammalian tumor cells while leaving normal cells unaffected [19, 20]. We have shown that the induction of apoptosis in *Drosophila* does not require the apoptotic cell to be in the transformed state, indicating that this may be a useful system to study the mechanisms controlling the tumor-specific cell killing.

Factors involved in the induction of dRYBP-mediated apoptosis

We have shown that dRYBP-induced apoptosis depends on the pro-apoptotic *reaper*, *hid* and *grim* genes (Figure 2C) and that it is inhibited by DIAP1 (Figure 2C). How dRYBP-induced cell death is regulated by these factors is not known. However, we have shown that the promoter region of the *reaper* gene is bound by the dRYBP protein (Figure 2J) and that dRYBP overexpression activates the expression of a *reaper-lacZ* construct (Figure 2I). These results suggest that dRYBP regulates the transcription of *reaper* and that dRYBP-induced apoptosis occurs via the canonical cell death pathways.

We have also shown that dRYBP-induced apoptosis is dependent on the DREDD (Figure 2D) and dFADD proteins (Figure 2E). DREDD is the *Drosophila* homolog of the mammalian apical caspase-8 [13], while dFADD is the *Drosophila* homolog of mammalian FADD, an adaptor protein involved in the TNFR signaling complex [14]. Both caspase-8 and FADD are required for the receptor-mediated apoptosis in mammals. However, apoptosis induced by the *Drosophila* TNF homolog EIGER [10] does not require dFADD or DREDD [10]. These results raise the possibility that dRYBP, dFADD and DREDD define a novel *Drosophila* extrinsic apoptotic pathway. Moreover, studying the mechanisms of dRYBP-mediated cell death in *Drosophila* might help to define the cell death functions of dFADD and DREDD as well as to better characterize the TNFR apoptotic pathways.

Finally, our results show that dRYBP-induced apoptosis requires the function of *trithorax* and that TRITHORAX itself is able to induce apoptosis. These findings connect the mechanisms of epigenetic regulation of gene expression with the mechanisms of apoptosis.

Are dRYBP and TRX involved in the epigenetic regulation of stress-induced apoptosis?

Several observations lead us to propose that dRYBP might be involved in apoptosis associated with cellular stress response. First, we have found no evidence indicating dRYBP involvement in developmental apoptosis. Second, loss of function of the *dRYBP* gene results in phenotypes which are highly variable both in penetrance and in expressivity [17]. Third, high levels of RYBP/DEDAF have been found in a number of different cancers, including T-cell and Hodgkin´s lymphomas, pituitary adenomas and oligodendrogliomas [30]. Fourth, it has been shown that the ability of v-Fos transformed cells to invade other tissues is dependent upon repression of RYBP expression. Moreover, re-expression of RYBP in Fos-transformed cells is sufficient to strongly inhibit invasion [31]. Finally, it has been shown in transfection experiments that high levels of RYBP/DEDAF induce tumor cell-specific killing. Therefore, either by induction of apoptosis or by inhibition of invasion, the RYBP protein appears to be able to detect aberrant cell behavior and initiate apoptotic pathways.

We show that high levels of TRX induce apoptosis and, furthermore, that dRYBP-induced apoptosis requires the function of *trithorax*. These results indicate that the TRX protein might be involved in control of apoptosis and suggest that dRYBP and TRITHORAX could function in the regulation of stress-induced apoptosis.

In our model (Figure 4), under normal conditions of development (Figure 4, left panel), the function of the dRYBP protein remains latent and developmental apoptosis takes place through the canonical pathways of apoptosis proceeding through the activation of the effector caspases followed by cell death. Under conditions (Figure 4, right panel) where high levels of dRYBP are induced – and similar to conditions in tumor cells where high levels of RYBP have been found [30] – the *reaper* gene is transcriptionally activated via the dRYBP-dependent recruitment of trxG/PcG complexes. Critical to our model is the establishment of the correct balance between PcG and trxG proteins so that appropriate control of apoptotic gene expression is maintained. This model is consistent with the observed requirement of TRX protein for dRYBP-induced apoptosis (Figure 3B). Although a high level of TRITHORAX can itself activate apoptosis, we propose that the activation of transcription of the apoptotic genes, mediated by the TRX protein, is faster and more accurate when dRYBP is mediating and balancing the recruitment of the PcG and trxG proteins.

We have shown that dRYBP-induced apoptosis requires the function of DREDD and dFADD (Figure 2D and 2E). In mammals the FADD and procaspase-8 proteins are required for the apoptotic response mediated by the TNF/TNFR complex. This is not the case in *Drosophila* as the fly TNF/TNFR homologs EIGER/WENGEN do not require dFADD and DREDD to trigger apoptosis. Moreover, in mammals the RYBP/DEDAF protein mediates the interaction between pro-caspase-8 and FADD to activate caspase-8. This leads us to propose that an additional function of the dRYBP protein is to mediate the interaction between dFADD and DREDD to activate the caspase triggering in the apoptotic pathway. dRYBP and TRITHORAX functions may define a novel apoptotic induction pathway that responds to environmental-stress and mutant-stress signals and that requires dFADD and DREDD.

dRYBP-induced apoptosis does not take place in the absence of pro-apoptotic genes *reaper*, *hind* and *grim* (Figure 2B) or in the absence of DREDD/dFADD (Figure

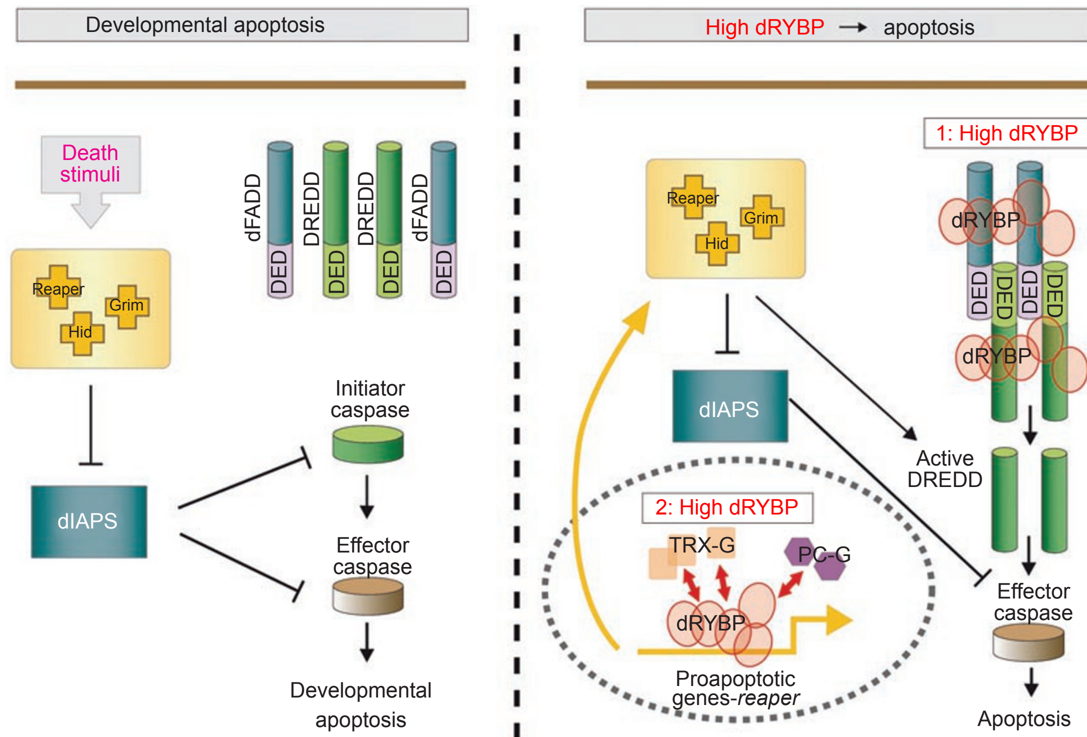

Figure 4 Role of dRYBP and TRX in the epigenetic regulation of apoptosis. Hypothetical model. Developmental apoptosis takes place through the activation, under death stimuli, of the pro-apoptotic genes, which removes the inhibitory action of the DIAP proteins, which in turn allows the initiator and the effector caspases to be activated and induce apoptosis. During developmental apoptosis the function of the dRYBP protein is not required and remains latent. The function of neither dFADD nor the initiator caspase DREDD is required in this process. We hypothesize that the levels of the dRYBP protein are increased, perhaps in response to cellular stress (see text), resulting in the induction of apoptosis. (1) High levels of dRYBP cause the aggregation of the dFADD and the DREDD proteins, thus promoting the activation of DREDD and triggering apoptosis. (2) High levels of dRYBP activate the expression of the pro-apoptotic gene *reaper* through the recruitment of the TRX protein, resulting in the induction of apoptosis.

2E and 2D). It has been previously shown that overexpression of REAPER and overexpression of HID require the presence of *dredd* to induce apoptosis. Thus, DREDD is an effector of REAPER and HID signaling pathways [13]. Moreover, expression of Reaper, Grim and Hid was found to trigger the processing of the DREDD protein precursor [13]. These results support our model and explain why induction of apoptosis by dRYBP is abolished in the absence of either the pro-apoptotic proteins or the adaptor dFADD protein.

How the activation of *reaper* expression and the activation of DREDD processing function in a pathway induced upon dRYBP overexpression is not known. Nor is it known what stress signals would lead to increased levels of dRYBP and thereby promote apoptosis. We have attempted to identify treatments which would result in increased levels of dRYBP expression in the imaginal discs. None of the treatments investigated – X-ray irradiation, overexpression of EIGER [10], overexpression of HEMIPTEROUS [32], overexpression of REAPER [33], overexpression of TRITHORAX or overexpression of p53 [34] – resulted in increased expression of dRYBP (data not shown). The study of factors controlling dRYBP expression might help to clarify the role of the mammalian RYBP gene in cancer development. Furthermore, elucidating the mechanisms of dRYBP-dependent cell killing and the role of epigenetic regulation by TRX might provide a path to the development of new cancer therapies.

Materials and Methods

Drosophila strains and handling

Flies used include stocks containing mutations Pc^3, Sce^1, trx^{E2} and $Df(1)^{w67c23}$ (y^-, w^-), $dredd^{D44}$, *Df(3L)H99* (a deletion that eliminates the pro-apoptotic genes *reaper*, *grim* and *hid*), and they are all described in FlyBase (http://flybase.org). Additionally, the null mutation $dRYBP^1$ [17, 35] was used; the stock $dRYBP^1$/ *CyO-GFP* was used to select homozygous $dRYBP^1$ embryos and larvae.

Transgenic flies containing either *puckered-lacZ* ^{E69} (*puc-lacZ*) [29] or *reaper-4kb-lacZ* (*rpr-lacZ*) [36] were used to detect the expression of *puckered* and *reaper,* respectively. For overexpression experiments, the GAL4/UAS system was used [37] at 25 ºC and 29 ºC with the following lines (described in Calleja *et al*. [38]): *scalloped*-Gal4 (*sd*-Gal4), *nubbin*-Gal4 (*nub*-Gal4), *apterus*-Gal4 (*ap*-Gal4), *distal less*-Gal4 (*dll*-Gal4, MD23 and EM212) and *248*-Gal4. Finally, transgenic lines containing UAS-RNAi$_{dRYBP}$ [18], UAS-RNAi$_{FADD}^{M8}$ [39], UAS-DIAP1 [40], UAS-EIGER [10], UAS-p35 [23], UAS-Dmp53 [34], UAS-TRX (P-EP-TRX, gift of Isabel Guerrero), UAS-SCE [41], UAS-RYBP murine and UAS-PC (both constructed in this work), UAS-dRYBP [18] and UAS-dRYBP-ΔZF [17] were used. For imaginal disc irradiation, eggs were collected during a 24-h laying period, allowed to develop until the formation of second instar larvae and then irradiated. After 24 h, the larvae were collected for dissection and immunostaining. Irradiation was performed using a Philips X-ray instrument at a dose of 1 500R.

Staining procedures

Imaginal discs were dissected in PBS and fixed with 4% paraformaldehyde in PBS for 30 min at room temperature. After blocking in a PBT solution containing 1% bovine serum albumin (BSA) and 0.3% Triton X-100 for 30 min, discs were incubated overnight at 4 °C with the primary antibodies. Following three 10-min washes with PBT (PBS and 0.3% Triton X-100), they were incubated in the dark with the appropriate fluorescently labelled secondary antibody for 1 h at room temperature, washed in PBT and mounted in Vectashield (Vector Laboratories). The primary antibodies used were rabbit anti-dRYBP (1:100) [18], rabbit anti-C3 (1:200) (Cell Signalling Technologies) raised against the cleaved form of human caspase-3, which has been shown to cross-react with cleaved *Drosophila* caspase-3 [22], rabbit anti-β-Gal (1:200) (Promega), mouse anti-β-Gal (1:200) (Promega), mouse anti-UBX [24] and rabbit anti-phospho-Histone H3 (1:100) (Ser10) (Upstate). Images were generated using either a MicroRadiance (Bio-Rad) or an LSM510 META (Zeiss) confocal microscope, and subsequently processed using Adobe Photoshop.

TUNEL staining was performed using imaginal discs dissected, fixed and incubated with the primary and secondary antibodies as described above for immunostaining. After incubation with the secondary antibody, they were incubated at 65 °C with NC-T solution (495 μl of sodium citrate 100 mM, 5 μl of Triton X-100 10%) for 30 min, washed three times for 10 min in PBT, incubated twice with 100 μl of Dilution buffer (Roche) for 5 min and treated with 50 μl of labelling solution (in situ cell death detection TMR Red kit, Roche) for 30 min at 37 °C. Next, 5 μl of terminal transferase (Roche) was added and the reaction was allowed to proceed for 2 h at 37 °C, stopped with PBT, washed three times with PBT and the discs were mounted in Vectashield (Vector Laboratories).

BrdU (bromodeoxyuridine) staining was performed using imaginal discs dissected in cold PBS, incubated in 0.01 mM BrdU for 15 min at 37 °C, washed three times with PBS, fixed for 2 min with Carnoy (3:1-ethanol:acetic acid) and washed four times for 5 min in PBS. Discs were then treated with 2 M HCl for 10 min, washed three times with PBS for 10 min and incubated with the primary antibody at 4 °C (BrdU labelling and detection Kit I-Roche). The anti-BrdU antibody staining was performed according to the protocol of the supplier.

Acridine Orange staining was performed following the procedure of Abrams [42]. Embryos were dechorionated with bleach, rinsed with water and incubated in 500 μl of heptane and 500 μl of 5 mg/ml of Acridine Orange (Sigma). After 5 min of shaking, embryos were removed from the interface and mounted in Halocarbon oil (Merck).

Expression constructs

Procedures for the cloning and generation of transgenic flies containing the p[UAS-dRYBP] and p[UAS-dRYBP-ΔZF] proteins have been described previously [17]. The p[UAS-RYBPmurine] construct was generated by cloning the *Not*I/*Xba*I fragment from the murine RYBP cDNA (accession number AF101779) [43] into the p[UAST] vector [44]. Likewise, p[UAS-PC] was generated by cloning the PC cDNA (NM 079475) *Eco*RI fragment into the p[UAST] vector [44]. Transgenic flies were obtained by standard procedures using *Df (1)* ^{w67c23} flies (y^-, w^-) as hosts.

Chromatin immunoprecipitation

ChIP assays using whole embryos were performed as described previously [45]. Chromatin was immunoprecipitated using a 1:20 dilution of the anti-dRYBP antibody [18]. For quantitative ChIP, following immunoprecipitation and DNA purification, enrichment for specific DNA fragments was analyzed by real-time PCR, using the Roche Light Cycler instrument and accessories as described in Negre *et al*. [45]. Data are expressed as the percentage of input chromatin precipitated for each region examined. The mean values and the standard deviations of two independent ChIP experiments are shown. As a negative control the *robo3* (*roundabout homolog 3*) (http://flybase.org) gene region was included in the RT-PCR experiment. The following primers were used. For reaper CDS: rpr CDS 5′-AGG CGA CTC TGT TGC GGG AG-3′ (loc. 3L: 18391288-18391307; Flybase release 5.10) and rpr CDS 5′-TGC GAT GGC TTG CGA TAT TTG-3′ (loc. 3L: 18391142-18391162; Flybase release 5.10). For reaper promoter: rpr promoter 5′-AAA AAC ACG CTT GGC AAC AG-3′ (loc. 3L: 18391768-18391787; Flybase release 5.10) and rpr promoter 5′-GCT ATT TAT ACC TGG TTC TCT CAC G-3′ (loc. 3L: 18391573-18391597; Flybase release 5.10). For *robo3*: 5′-ATA GCC TCA ACA CTG AGG AAG G-3′ (loc. 2L: 1285910-1285931; Flybase release 5.10) and 3′-GTA GGG TTT GAT TAA CCG GAC C-5′ (loc. 2L: 1286083-1286104; Flybase release 5.10). PCR reactions were performed following Comet *et al*. [46].

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