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Running title : RBF can be pro- or anti-apoptotic

1. Abstract

The retinoblastoma protein, pRb, plays important roles in many processes implicated in cell fate decisions, including cell cycle, differentiation and apoptosis. In cell cycle regulation, pRb interacts principally with the E2F transcription factor family members to inhibit the transcription of many genes controlling cell cycle progression. In this study, we focused on the role of pRb in apoptosis, which is much less clear than its role in cell cycle regulation. Indeed, pRb has been found to be either pro- or anti-apoptotic. To clarify how the proliferative status of the cells impacts the role of pRb in apoptosis, we used *Drosophila* to induce RBF (the pRb fly homologue) expression in different cellular and developmental contexts. We found that RBF expression induces apoptosis in different proliferative tissues in a caspase-dependent manner, whereas this effect was not observed in differentiated post-mitotic cells. Furthermore, RBF-induced apoptosis in proliferating cells was inhibited by co-expression of dE2F1, an antagonistic partner of RBF in cell cycle regulation. These results are in agreement with the view that the apoptotic properties of pRb are tightly linked to, and are probably a consequence of, an effect on cell cycle progression. Moreover, we show for the first time that RBF has a direct anti-apoptotic effect on Dmp53-induced cell death in post-mitotic cells only. Taken together, these data clearly show that RBF can exert a dual role in the control of apoptotic processes, and that its properties depend on the proliferative status of the cells.

2. Key words

Retinoblastoma protein, RBF, *Drosophila*, apoptosis, cell cycle, cell-type specific

3. Introduction

The retinoblastoma protein (pRb) was the first tumor suppressor identified in human cells, and pRB loss of function has been linked to the development of many cancers. pRb is a major element in cell cycle regulation: it exerts its functions principally by interacting with the E2F transcription factor family members, which regulate key cellular events, including DNA synthesis and cell cycle progression (for a review see 1). Binding and subsequent inactivation of E2Fs factors by pRb promotes G1 arrest. Cyclin-dependent kinase (CDK)-mediated phosphorylation of pRb disrupts

pRb/E2Fs interactions, which allows E2Fs activation and subsequent GI/S progression. pRb is conserved between mammals and flies. A *Drosophila* pRb homologue, RBF, has been identified[2] and it has been shown that, like its mammalian counterpart, RBF interacts with *Drosophila* E2F family members and can be regulated during cell cycle progression by CDKs.[2-4]

Although pRb function in cell cycle regulation is well established, its effects on apoptosis remain poorly understood. Indeed, numerous studies using different cellular contexts have led to conflicting results. Knock-out mouse embryos for the *Rb* gene die before day 16 of development, and show ectopic proliferation and extended apoptosis in many tissues.[5, 6] However, it is now clear that most apoptosis observed in $Rb^{-/-}$ embryos is due to abnormal formation of extraembryonic tissues, [7] and is therefore an indirect consequence of *Rb* loss of function. More recent studies in mice have also shown that tissue-specific knock-outs of the *Rb* gene induce ectopic proliferation but do not have any effect on apoptosis in many proliferative tissues (lung, skin, intestine).[8-10] Thus, in proliferating tissues *in vivo*, pRb does not appear to regulate apoptosis. In other cellular contexts, as in cultured mouse embryonic fibroblasts,[11, 12] or in differentiating cells like lens [7] and myoblasts,[13] *Rb* loss of function induces apoptosis cell-autonomously. This observed apoptosis is thought to be an indirect consequence of inappropriate progression of the cell cycle. In the case of differentiating cells, absence of cell cycle arrest impairs proper differentiation and leads to cell death. Like in mammals, *Drosophila RBF* loss of function is lethal during development.[14] *RBF* loss of function clones in proliferative tissues are prone to apoptosis and contain some ectopic S phase cells, both in wing [15, 16] and in eye imaginal discs,[17, 18] the larval tissues giving rise to adult structures. These results support the view that, as in mammals, *Drosophila* RBF is required for cell cycle arrest in G1 phase and that the apoptosis observed could be due to cell cycle deregulation.

In spite of these corroborating results concerning pRb anti-apoptotic properties, numerous studies carried out on human cancer cell lines have raised the possibility that pRb can also be pro-apoptotic in many cellular contexts,[19-24] which is in agreement with its described role as a tumor suppressor. Furthermore, it has been shown recently that pRb can associate together with E2F1 and bind promoters of genes encoding pro-apoptotic factors that are transcriptionally activated in response to genotoxic or oncogenic stress.[25] In *Drosophila*, even if loss of function experiments suggest an anti-

apoptotic role for RBF, the effects of RBF expression on apoptosis has not been studied in detail. Indeed, in many cases, like in some mammalian cells, observed tissue size reduction resulting from RBF expression could also be explained by induction of apoptosis, and not only by cell cycle inhibition as has been the preferred interpretation until now.[4, 26, 27]

The variety of results concerning pRb pro- or anti-apoptotic effects could be a consequence of the complex role of this protein in many cellular events, such as cell cycle, differentiation and apoptosis. Therefore, the cellular response to pRb gain or loss of function could depend to a great extent on the proliferation and differentiation context of the cells. We took advantage of the powerful genetic tools developed in *Drosophila* to better characterize the effects of *Drosophila* RBF on apoptosis *in vivo*, taking into account the proliferation and differentiation status of the cells. Our results provide evidence for a dual role for RBF in apoptotic processes. Indeed, in proliferative cells of wing and eye imaginal discs, RBF induces apoptosis in a caspase-dependent manner, while it does not in differentiated post-mitotic cells. In contrast, we show that in these latter cells, RBF is able to suppress apoptosis induced by Dmp53, the drosophila homolog of p53.

4. Results

4.1 RBF is pro-apoptotic only in proliferative tissues

In order to test the effects of RBF expression in different cellular contexts, we took advantage of an inducible transgenic UAS *Drosophila* strain to precisely choose the expression domain of RBF. To induce *UAS-RBF* expression in a proliferating tissue, we used the *vg-Gal4* driver, which is expressed in wing imaginal discs of third instar larvae, a period of intensive growth. When RBF is expressed in this tissue, resulting adult wings present notches along their margins (Fig 1 E), while control wings have a continuous margin (Fig 1 A). In order to test if RBF expression can also have an effect in another proliferative tissue, we used the *ey-Gal4* driver, which is expressed early in eye imaginal disc development, before the appearance of the morphogenetic furrow and cell cycle arrest. *ey-Gal4/+;UAS-RBF/+* flies present very reduced rough eyes (Fig 1 F) compared to *ey-Gal4/+* control eyes (Fig 1 B), and *ey-Gal4/+;UAS-RBF/+* third instar larvae eye imaginal discs are smaller than control discs (Fig 1 J, M). Thus, RBF expression leads to a loss of tissue in the wing, and to a

reduction in the size of the tissue in both the adult eye and its progenitor, the third instar larval eye disc.

To test if these phenotypes result from RBF-induced apoptosis, we performed TUNEL staining in third instar larval imaginal discs. Few apoptotic cells were detected in *vg-Gal4/+* and *ey-Gal4/+* control discs (Fig 1 I-J). On the contrary, many cells were TUNEL-labeled in *vg-Gal4/+;UAS-RBF/+* wing discs and in *ey-Gal4/+;UAS-RBF/+* eye discs (Fig1 L-M). Similar results were obtained with Acridine Orange staining (data not shown). At the center of the pouch, within the *vg-Gal4* expression domain, few cells were TUNEL-labeled (Fig 1 L, white arrow). This particular zone corresponds to the zone of non-proliferating cells (ZNC), where cells are arrested in G1 and G2 phases of the cell cycle. Furthermore, RBF expression in this zone using the *C96-Gal4* driver did not induce apoptosis (data not shown). These observations suggest that non-cycling cells are more resistant to RBF-induced apoptosis. Thus, RBF expression induces apoptosis, preferentially in proliferating cells.

In addition to apoptosis, the reduction of the size of the tissues could also result from a reduction of cell size. We observed that RBF expression did not reduce cell size neither in wing and eye imaginal discs nor in adult wing (Suppl. Fig. 1). Thus this hypothesis can be excluded. In addition, it has been shown that RBF expression in the wing disc slows cell cycle progression.[28] We cannot exclude that, in proliferative tissues, this cell cycle lengthening could have a cumulative effect with apoptosis on the observed deficit in cells in larval and adult structures.

This observed cell death could seem contradictory with the prevalent view that RBF has anti-apoptotic effects, according to loss of function experiments described elsewhere.[15-18] According to the existing literature, the apoptotic properties of pRb depend on the cellular type, and probably on the cycling and differentiation states of the cells. We thus tested if RBF expression could also induce apoptosis in post-mitotic cells. To do so, we used the pan-neural *elav-Gal4* driver. To specifically observe neurons we focused on the adult eye, an organ principally composed of photoreceptors which are easy to observe. Eyes of adult *elav-Gal4/+;UAS-RBF/+* raised at 25°C have a wild type structure similar to control *elav-Gal4/+* eyes (Fig 1 C, G), and there are 7 photoreceptors visible in each ommatidia (Fig 1 D, H). Since we did not observe any pro-apoptotic effects of RBF in photoreceptors, we verified that the *UAS-RBF* transgene was correctly driven by *elav-Gal4*. Protein detection by western blotting

experiments showed that *elav-Gal4* indeed induces *UAS-RBF* expression under these conditions (Fig 1 O). We increased the breeding temperature of the *elav-Gal/+;UAS-RBF/+* flies to 29°C to enhance the *UAS-RBF* expression rate. Even under such extreme conditions, eye and photoreceptor phenotypes were wild type (data not shown). The *elav-Gal4/+;UAS-RBF/+* eye imaginal discs were also stained by TUNEL and they showed a similar low amount of apoptosis as that in control *elav-Gal4/+* discs (Fig 1 K, N). Thus, RBF expression does not exert any pro-apoptotic activity in these post-mitotic cells, which supports the view that RBF-induced cell death is linked to cell cycle deregulation in proliferative tissues that does not occur in post-mitotic cells.

4.2 Caspase activity, is required for RBF-induced apoptosis

In order to test if apoptosis observed when RBF is expressed in proliferating imaginal disc cells depends on caspase activity, the baculovirus p35 caspase inhibitor protein[29] was induced in wing discs expressing *UAS-RBF* under control of *vg-Gal4.* In the wing, the number of notches present at the margin is correlated with the amount of apoptosis.[30] We classified the wing phenotypes into four categories (wild type, weak, intermediate and strong) according to the number of notches (Fig 2 A, asterisks). We assayed for the strength of the notch phenotype in wings of *vg-Gal4;UAS-RBF* flies in presence or absence of *UAS-p35* (Fig 2). *UAS-p35* expression driven by *vg-Gal4* does not induce any notch phenotype. When p35 was co-expressed together with RBF, distribution of the phenotypes shifted to weaker phenotypes when compared to the expression of RBF alone (Fig 2 B) and the difference is statistically significant (Wilcoxon test, α=7.7E-6, n=493). To ensure that change in phenotype is not due to dilution of Gal4 activity over two UAS , we co-expressed *UAS-RBF* and *UAS-EGFP* transgenes under control of the *vg-Gal4* driver. We did not observe any significant change in phenotype distribution between *vg-Gal4; UAS-RBF* flies and *vg-Gal4; UAS-RBF/UAS-EGFP* flies (data not shown). Thus, these results clearly show that p35 suppresses the notch phenotype induced by RBF in the wing, and therefore that this apoptosis is caspase dependent.

The apical caspase Dronc plays an important role in developmental and stress-induced apoptosis in *Drosophila*,[31] and this caspase is not inhibited by p35.[32] Thus, to test if Dronc is necessary for RBF-induced apoptosis, we used a *dronc* null mutant allele, *dronc*^{I29},[33] to assess the strength of the RBF-

induced notch phenotype in a *dronc* heterozygous mutant context (Fig 2 C). As in the *UAS-p35* expression context, the phenotype induced by RBF in the wings was significantly weaker in a *dronc* mutant context (Wilcoxon test, α=4.7E-07, n=254). Thus, both effector caspases inhibited by p35 and the apical caspase Dronc are required for RBF-induced apoptosis in the wing.

4.3 dE2F1 does not cooperate with RBF to induce apoptosis

The dE2F1 transcription factor is the best characterized target of RBF. When bound to its co-factor dDP, the principal function of dE2F1 is to activate the transcription of major cell-cycle related genes, such as *cyclin E*, and this activity is inhibited by RBF binding.[1] Therefore, RBF and dE2F1 have antagonistic activities in the regulation of the cell cycle. Moreover, dE2F1 overexpression induces apoptosis in *Drosophila* imaginal discs.[34, 35] We thus wondered if dE2F1 cooperates with RBF to induce apoptosis as it has been reported in mammalian cells,[25] or on the contrary, if it counteracts the effects of RBF as it has been observed in cell cycle regulation. To answer this question, we compared the wing phenotypes induced by *UAS-RBF* expression under control of the *vg-Gal4* driver, either in a $dE2F1^{07172}$ heterozygous loss of function context, or together with *UAS-dE2F1,UAS-dDP* (Fig 3). *UAS-dE2F1, UAS-dDP* expression driven by *vg-Gal4* does not induce any notch phenotype. The distribution of the RBF-induced notch phenotypes shifted to stronger phenotypes in a $dE2F1^{07172}$ partial loss of function context (Wilcoxon test, α=4.5E-07 n=462) (Fig 3 A), whereas it shifted to weaker phenotypes in the *dE2F1/dDP* overexpression context (Wilcoxon test, α=0 n=1069) (Fig 3 B). These results therefore show that RBF and dE2F1 have opposite activities and that dE2F1 does not cooperate with RBF to induce cell death in *Drosophila* wing imaginal discs.

4.4 RBF has an anti-apoptotic effect on Dmp53-induced cell death in neurons

As presented above, our results indicate that despite of its pro-apoptotic effects in proliferating cells, RBF alone is not able to induce apoptosis in differentiated cells. We wondered if RBF did not have any influence on apoptotic processes in post-mitotic cells, or if it could enhance apoptosis induced by expression of other pro-apoptotic genes in these cells. We used the *UAS-Dmp53* transgene which is

known to induce apoptosis when driven in the developing eye disc.[36] When we overexpressed *UAS-Dmp53* in neurons in *elav-Gal4/+;UAS-Dmp53/+* flies, we observed apoptosis induction in eye imaginal discs (TUNEL staining, data not shown) and we obtained very strong adult phenotypes: about 85% of the flies died before eclosion and escapers could hardly move and had small altered eyes (Fig 4 B). Strikingly, co-expression of *UAS-RBF* together with *UAS-Dmp53* under control of *elav-Gal4* restored viability significantly (about 43% of the pupae hatched) and reduced the Dmp53-induced eye phenotype (Fig 4 C). As for the *vg-Gal4* driver, expressing *UAS-EGFP* together with *UAS-Dmp53* did not modify *UAS-Dmp53*-induced phenotype using the *elav-Gal4* driver (data not shown) excluding an effect due to potential Gal4 dilution. Therefore, RBF expression has an inhibitory effect on Dmp53-induced apoptotic phenotype in neurons.

Finally, we wondered if RBF could have an anti-apoptotic effect toward Dmp53-induced cell death in proliferating wing disc cells as well. When *UAS-RBF* and *UAS-Dmp53* were co-expressed in the wing disc under control of the *vg-Gal4* driver, apoptosis is enhanced compared to apoptosis induced by Dmp53 alone in *vg-Gal4/UAS-Dmp53* flies (data not shown), indicating an additive effect of RBF on Dmp53-induced apoptosis. Therefore, the observed anti-apoptotic property of RBF is not specific to Dmp53-induced apoptosis, but depends on the cellular context, and most probably on the differentiation status of the cells.

Altogether, our results clearly show that *Drosophila* RBF can be either pro- or anti-apoptotic *in vivo*, and that the balance between these opposite effects strongly depends on the proliferation and differentiation status of the cells.

5. Discussion

In this study, we show that RBF expression can be pro-apoptotic in *Drosophila* in a caspase-dependent manner. The capacity of RBF to be pro-apoptotic depends on the proliferation status of the cells in which it is expressed, as it is observed in proliferating tissues but not in differentiated post-mitotic cells. Non-cell-autonomous mechanisms could explain these results. In this case, RBF-induced effects could be in conflict with proliferating signals sent by surrounding cells. Indeed, in cells of proliferating imaginal discs, RBF expression leads to a lengthening of cell cycle progression,[28]

whereas, at the same time, these cells receive proliferation signals from the surrounding tissue. These conflicting signals could lead to cell death. In addition, it has been shown that cells with a proliferation disadvantage compared to their neighbors can be eliminated from a tissue by a mechanism known as cell competition.[37] It is possible that this process contributes to RBF-induced cell death, even if the effects of RBF expression have never been shown yet to conform to a stringent definition of cell competition. Indeed, according to Moreno,[38] cells carrying a mutation that triggers cell competition are viable when surrounded by cells of the same genotype.

On the other hand, RBF-induced apoptosis could also result from a direct effect of RBF on the cell cycle. In this respect, we have shown that dE2F1/dDP complex has an antagonist effect on RBF-induced cell death. Indeed, our results indicate that dE2F1/dDP overexpression prevents RBF-induced apoptosis, while *dE2F1* loss of function enhances this phenotype. These results corroborate with previous reports concerning dE2F1/dDP and RBF interactions. Indeed, dE2F1/dDP and RBF have opposite effects in cell cycle regulation. Taken together, these data support the view that RBF-induced cell death in proliferating tissues is probably a consequence of cell cycle deregulation. In this case, dE2F1/dDP counters the effects of RBF overexpression on cell cycle control, and therefore decreases subsequent apoptosis. However, a recent study performed in mouse has shown that dE2F and pRb can cooperate to induce the transcription of pro-apoptotic factors,[25] which seems contradictory to our results in *Drosophila*. Nevertheless, this cooperative effect is observed in response to oncogenic and genotoxic stresses, and not when apoptosis is induced by pRb expression. Thus, the experimental settings are very different from those used in our work, and we cannot exclude that such mechanism may exist in *Drosophila* as well. Thus, the RBF partner implicated in induction of apoptosis in our model is probably not dE2F1. It could be that the RBF pro-apoptotic effect depends on interactions of the RBF protein with other partner proteins regulating the cell cycle and/or apoptosis in a more direct way. As we do not observe RBF-induced apoptosis in post-mitotic cells, these partners could be absent or inactive in these cells.

Furthermore, we also show in this study that RBF expression can have anti-apoptotic activity in *Drosophila* neurons when other pro-apoptotic genes are overexpressed. Indeed, when *UAS-Dmp53* is overexpressed in neurons to induce apoptosis, we observed an anti-apoptotic effect of RBF toward

Dmp53-induced cell death. In mammals, pRb can antagonize p53 apoptotic activity in different ways.[39] Mostly, pRb is able to promote p53 degradation by MDM2 and therefore directly suppresses p53-induced apoptosis.[21] However, since no *Drosophila* MDM2 homologue has been discovered yet, this mode of regulation is not likely to be conserved in flies. Furthermore, using the eye-specific *GMR-Gal4* driver, we observed that RBF also counteracted the cell death induced by *UAS-Debcl*, a *Drosophila* pro-apoptotic Bcl-2 family member [40-44] (data not shown). Since there is no data suggesting that Debcl and Dmp53 cooperate to induce apoptosis in *Drosophila*, one could hypothesize that the anti-apoptotic effect of RBF is not specific to Dmp53-induced cell death and occurs downstream in the apoptotic cascade. As is the case in mammals for pRb,[45-47] RBF could act downstream of p53 in regulating the expression of survival factors or anti-apoptotic genes, as well as pro-apoptotic genes. This type of anti-apoptotic regulation by RBF has been described in a very restricted area of the wing imaginal disc. Indeed, at the dorso-ventral boundary of wing imaginal discs, RBF and dE2F1 can cooperate to inhibit the expression of the pro-apoptotic gene *hid* induced by irradiation.[16] It is possible that a similar mechanism of pro-apoptotic gene repression by RBF could occur in neurons and would be enhanced when RBF is overexpressed. Furthermore, dE2F1 expression is maintained in post-mitotic cells of the eye imaginal disc.[48] It would be interesting therefore to test if RBF is also anti-apoptotic in neurons in a dE2F1 loss of function context.

In conclusion, our results show that, like in mammalian cells, Drosophila RBF can have pro- or anti-apoptotic properties depending on the proliferative status of the cells. What are the key elements that determine RBF activity on apoptosis? To answer this question, it will be necessary to identify specific RBF partners in mitotic and post-mitotic cells. This type of information would certainly allow a better understanding of the mechanisms by which RBF controls apoptosis and cellular fate.

6. Materials and methods

6.1 Fly stocks

Flies were raised at 25°C on standard medium. The *dronc^{I29} FRT80/TM3* strain was generated by Xu et al. [33] The *UAS-Dmp53* strain was generated by Ollmann et al.[49] The *UAS-RBF* and *vg-Gal4* strains were generous gifts from J. Silber. The *ey-Gal4/Cyo* strain was generous gift from B. Limbourg-

Bouchon. The *UAS-p35, elav-Gal4, UAS-dE2F1, UAS-dDP/TM6 B* and *dE2F1^{07172}/TM3* strains come from the Bloomington stock center (stock numbers 6298, 458, 4770 and 11717, respectively). The *w^{1118}* strain was used as the reference strain.

6.2 Test of phenotype suppression in the wing

To test caspase implication in RBF-induced apoptosis, the severity of the notch phenotype induced by *UAS-RBF* expression was assayed in a *UAS-p35* co-expression context and in a *dronc^{I29}* heterozygous loss of function context. *UAS-RBF Drosophila* males were crossed with either *vg-Gal4* or *UAS-p35;vg-Gal4/Cyo* females, and *vg-Gal4;UAS-RBF/TM3Sb Drosophila* females were crossed with either *w^{1118}* or *dronc^{I29}/TM3Sb* males. *Concerning* the interaction between dE2F1 and RBF, wing phenotype was assayed in a *UAS-dE2F1, UAS-dDP* co-expression context and in a *dE2F1^{07172}* heterozygous loss of function context. *vg-Gal4;UAS-RBF Drosophila* males were crossed with either *w^{1118}* or *dE2F1^{07172}/TM6B* females, *vg-Gal4 Drosophila* males were crossed with *UAS-RBF* females, and *vg-Gal4;UAS-RBF* males were crossed with *UAS-dE2F1, UAS-dDP/TM6B* females. The progenies of all crosses were classified according to the number of notches on their wings. The data were analyzed by the Wilcoxon test as described previously.[50]

6.3 TUNEL staining of imaginal discs

vg-Gal4, ey-Gal4 and *elav-Gal4* female flies were crossed either with *w$_{1118}$* males for a control or with *UAS-RBF* males. Third instar larvae of the progeny were dissected in PBS pH 7.6, fixed in PBS/formaldehyde 3.7%, washed three times for 20 min in PBT (1XPBS, 0,5% Triton). Discs were then dissected, TUNEL staining was performed following manufacturer's instructions (ApopTag® Red *in situ* apoptose detection kit, Chemicon), then discs were observed with a conventional Leica DMRHC research microscope using the N2.1 filter.

6.4 Western blot analysis

vg-Gal4 and *elav-Gal4* female flies were crossed either with *w$_{1118}$* males for a control or with *UAS-RBF* males. 60 wing and 60 eye imaginal discs of the progeny were dissected in PBS pH 7.6 and

homogenized in 80 µl of NP40 ice cold buffer (50 mM Tris-Cl pH=8, 150mM NaCl, 1% NP40, 1mM DTT, AEBSF^{SC}). Proteins were separated in NuPAGE 4–12 % Bis-Tris polyacrylamide gels according to the manufacturer's instructions (Invitrogen) and transferred onto PVDF membrane (Millipore). Blots were incubated with primary antibodies (rabbit polyclonal anti-RBF,1:500, Custom antibody, Proteogenix and rabbit polyclonal anti-Actin, 1:500, Sigma) overnight at 4°C and were then incubated for 1 h with corresponding peroxidase-conjugated antibody (anti-rabbit immunoglobulin, Biosystem). Immunoreactive bands were detected by Immobilon^{TM} Western Chemiluminescent HRP Substrate (Millipore).

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9. Figures legends

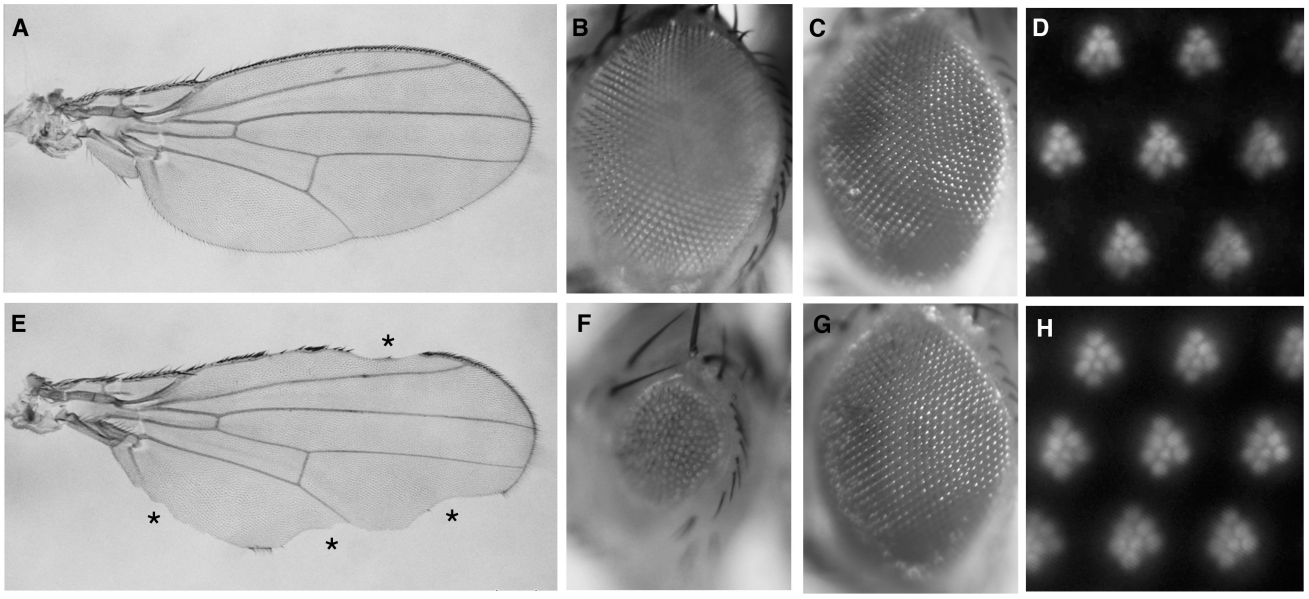
Fig 1: RBF expression induces apoptosis only in proliferating cells and not in post-mitotic cells. RBF expression is driven in proliferating tissues using *vg-Gal4* and *ey-Gal4*, and in post-mitotic cells using *elav-Gal4*. (A-D) Adult structures of control genotypes: *vg-Gal4/+* wing (A), *ey-Gal4/+* eyes (B), *elav-Gal4/+* eyes (C), and *elav-Gal4/+* eye photoreceptors (D). (E-H) Adult phenotypes of RBF expressing flies: *vg-Gal4/+;UAS-RBF/+* wing (E) (asterisks indicate notches in the margin), *ey-Gal4/+;UAS-RBF/+* eyes (F), *elav-Gal4/+;UAS-RBF/+* eyes (G), and *elav-Gal4/+;UAS-RBF/+* photoreceptors (H). (I-N) Visualization of cells that are undergoing apoptosis by TUNEL staining. (I-K). Control imaginal discs: *vg-Gal4/+* wing disc (I), *ey-Gal4/+* (J) and *elav-Gal4/+* (K) eye discs. (L-N) Imaginal discs of RBF expressing larvae: *vg-Gal4/+;UAS-RBF/+* wing disc (L), *ey-Gal4/+;UAS-RBF/+* (M) and *elav-Gal4/+;UAS-RBF/+* (N) eyes discs. White bright patches corresponding to apoptotic cells are principally observed in (L) and (M). (O) Detection of RBF protein in third instar

larvae imaginal discs by western blotting. Endogenous RBF is detected in control wing discs (first lane) and in control eye discs (third lane). *UAS-RBF* overexpression is clearly visible in wing discs under control of *vg-Gal4* (second lane), and in eye discs under control of *elav-Gal4* (fourth lane). Actin is used as a loading control.

Fig 2: RBF-induced apoptosis in the wing is caspase dependent. (A) Phenotypes are grouped into four categories (wild type, weak, intermediate and strong) according to the number of notches observed on the wing margin (asterisks). (B) *UAS-p35* co-expressing flies have phenotypes of weaker strength than those which only express *UAS-RBF* (Wilcoxon test, α=7.7E-06, n=493). (C) $dronc^{I29}$ heterozygous flies expressing *UAS-RBF* have phenotypes of weaker strength than those which express *UAS-RBF* in a wild type background (Wilcoxon test, α=4.7E-07, n=254).

Fig 3: RBF-induced apoptosis in the wing does not require dE2F1. (A) $dE2F1^{07172}$ heterozygous flies have stronger phenotypes than those in flies which only express *UAS-RBF* (Wilcoxon test, α=4.5E-07, n=462). (B) *UAS-dE2F1, UAS-dDP* co-expressing flies have weaker phenotypes than those in flies which only express *UAS-RBF* (Wilcoxon test, α=0, n=1069).

Fig 4: RBF suppresses Dmp53-induced apoptosis in neurons. (A) Wild type *elav-Gal4/+* control eye. (B) *elav-Gal4/+;UAS-Dmp53/+* flies present a reduced eye with altered morphology. (C) Eye size is partially restored in *elav-Gal4/+;UAS-Dmp53/+;UAS-RBF/+* flies.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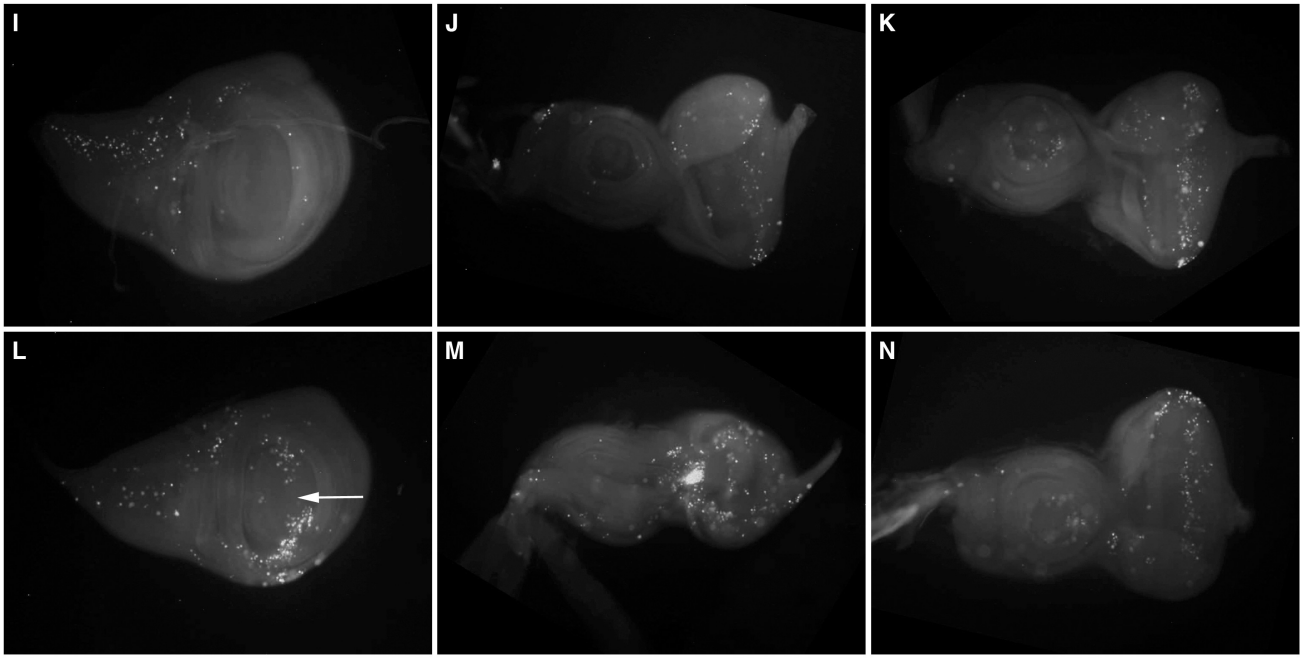
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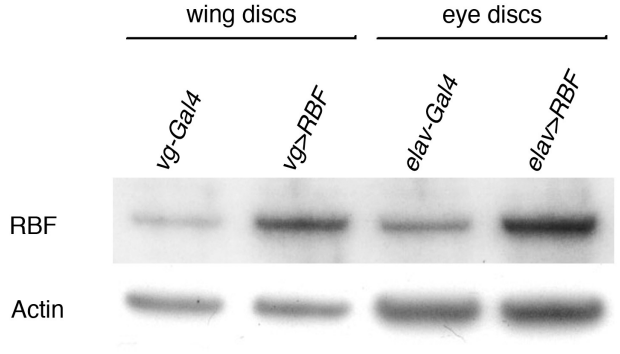
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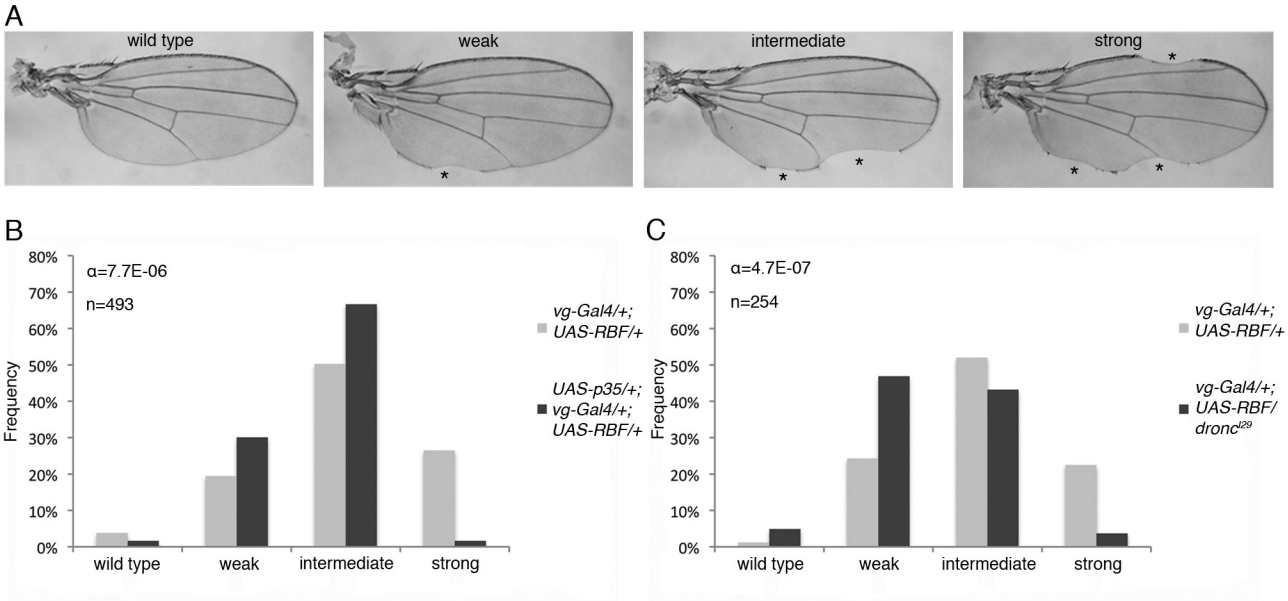
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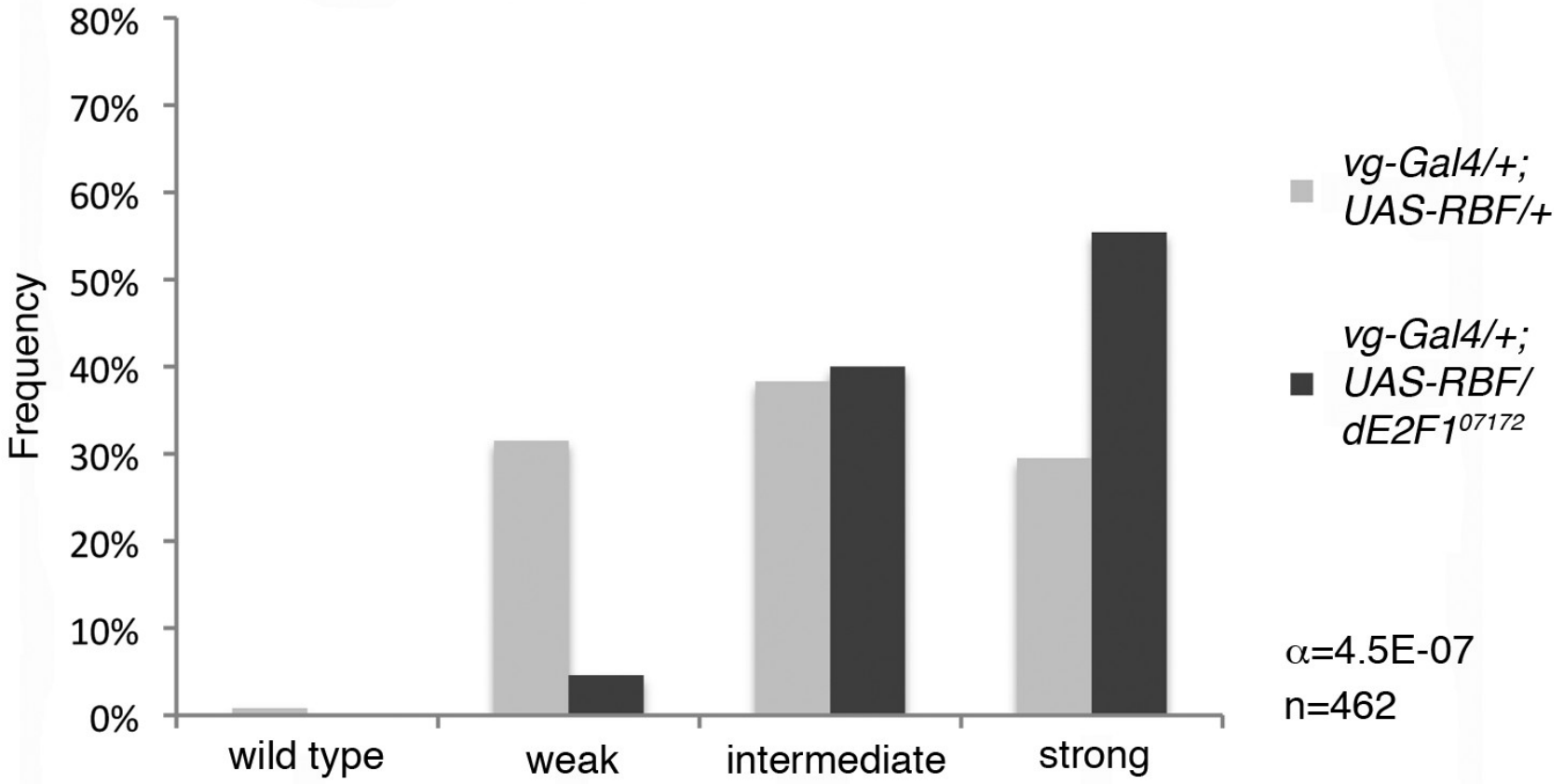
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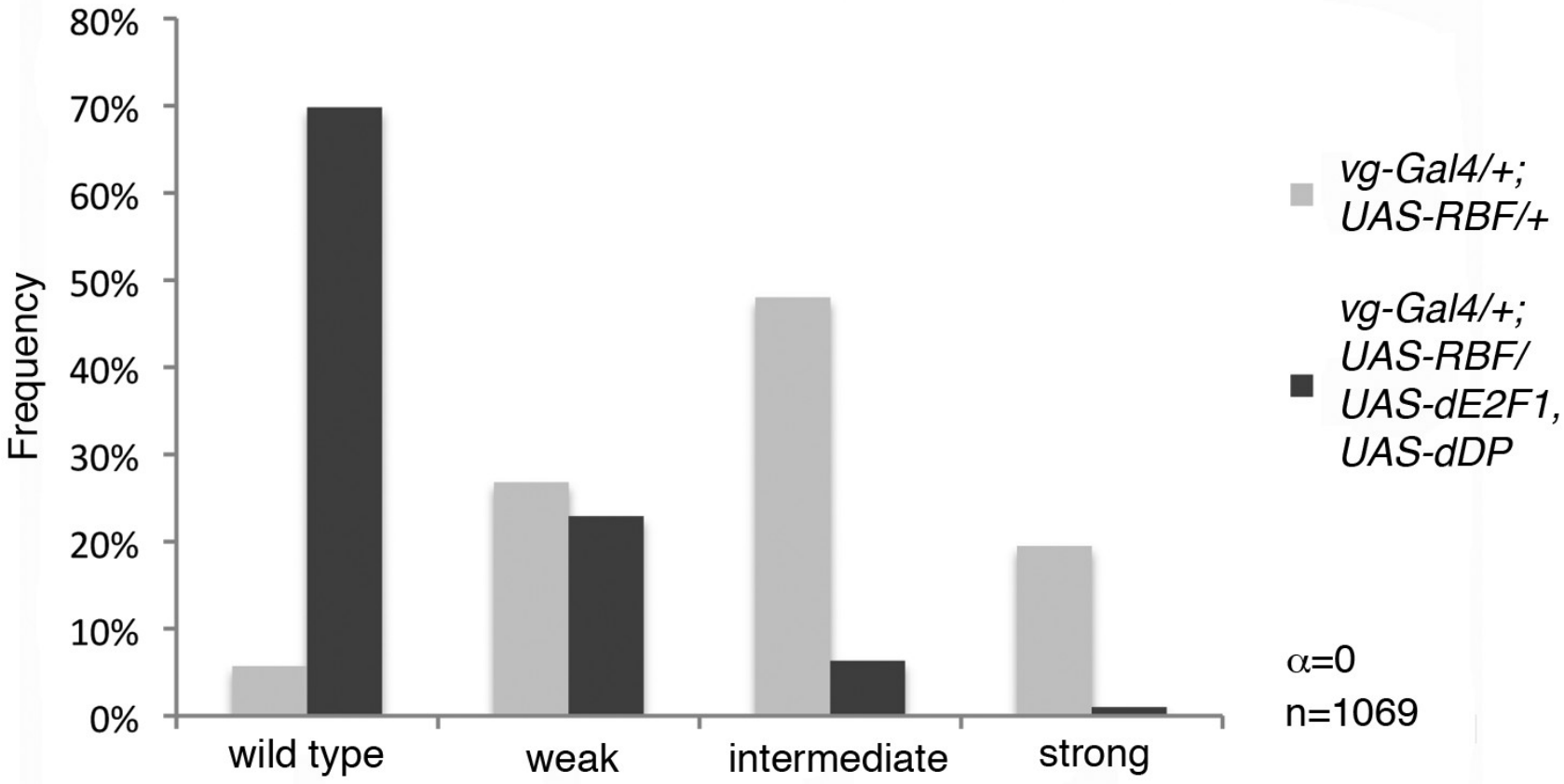
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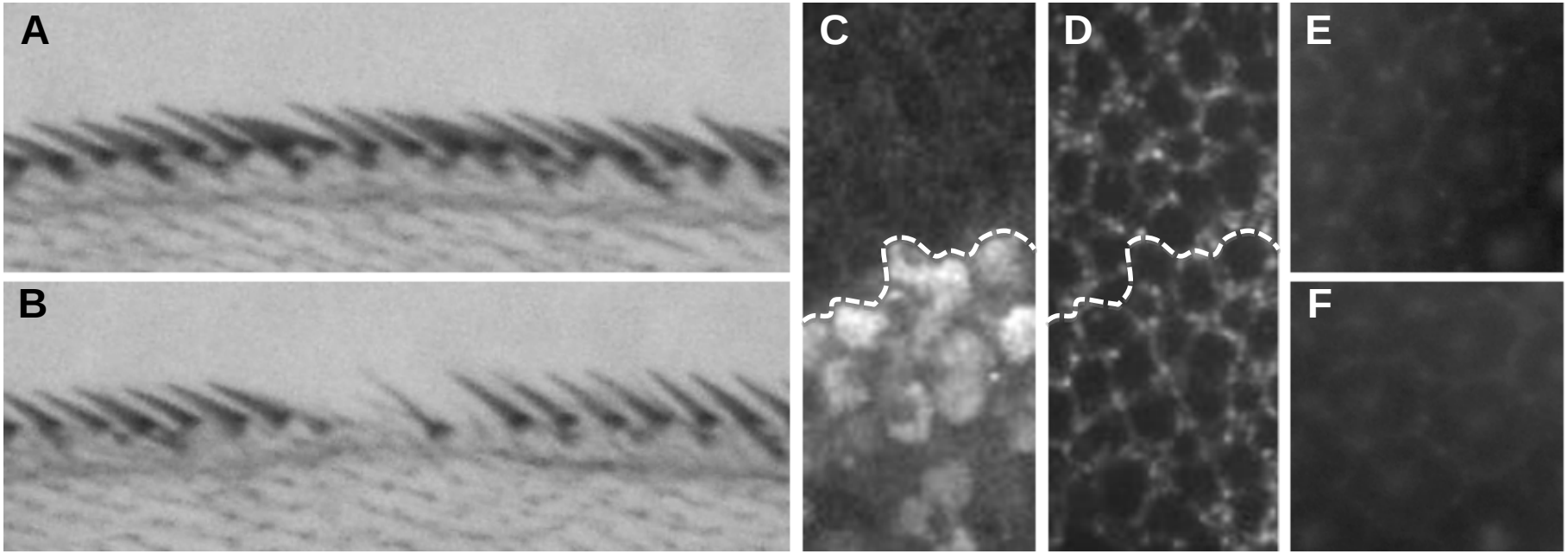

Supplementary Fig 1: RBF expression does not reduce cell size. Anterior wing bristles of *vg-Gal4/+* control flies (A) and *vg-Gal4/+; UAS-RBF/+* flies (B). Wing imaginal disc cells of *en-Gal4/+;UAS-RBF/+* larvae observed by confocal microscopy (C-D). The cells are immuno stained by an anti-RBF antibody (C) or an anti-Armadillo antibody (N2 7A1, DSHB) to visualize cells membrane (D). Images C and D correspond to the same area. The dot line indicates the limit of *RBF* expression domain. Eye imaginal disc cells of *ey-Gal4/+* control larvae or *en-Gal4/+;UAS-RBF/+* larvae, observed by conventional microscopy (E and F respectively). The cells are immuno stained by an anti-Amadillo antibody (D). The size of adult bristles or imaginal disc cells expressing RBF is not reduced compared to control cells.