

Published in final edited form as:

*Dev Biol.* 2011 November 15; 359(2): 190–198. doi:10.1016/j.ydbio.2011.08.018.

## ***RBF* and *Rno* promote photoreceptor differentiation onset through modulating EGFR signaling in the *Drosophila* developing eye**

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### **Abstract**

The retinoblastoma gene *Rb* is the prototype tumor suppressor and is conserved in *Drosophila*. We use the developing fly retina as a model system to investigate the role of *Drosophila* Rb (*rbf*) during differentiation. This report shows that mutation of *rbf* and *rhinoceros* (*rno*), which encodes a PHD domain protein, leads to a synergistic delay in photoreceptor cell differentiation in the developing eye disc. We show that this differentiation delay phenotype is caused by decreased levels of different components of the Epidermal Growth Factor Receptor (EGFR) signaling pathway in the absence of *rbf* and *rno*. We show that *rbf* is required for normal expression of Rhomboid proteins and activation of MAP kinase in the morphogenetic furrow (MF), while *rno* is required for the expression of Pointed (Pnt) and Ebi proteins, which are key factors that mediate EGFR signaling output in the nucleus. Interestingly, while removing the transcription activation function of dE2F1 is sufficient to suppress the synergistic differentiation delay, a mutant form of *de2f1* that disrupts the binding with RBF but retains the transcription activation function does not mimic the effect of *rbf* loss. These observations suggest RBF has additional functions besides dE2F1 binding that regulate EGFR signaling and photoreceptor differentiation.

### **Keywords**

RBF; Rhinoceros; EGFR signaling; differentiation; dE2F1; pointed; Ebi

### **Introduction**

The retinoblastoma gene *Rb* is the prototype tumor suppressor gene that is often mutated or inactivated in cancers (Weinberg, 1995). Investigations into the function of pRb revealed that it plays important roles in diverse biological processes including cell cycle, apoptosis,

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checkpoint control, and differentiation (Du and Pogoriler, 2006; van den Heuvel and Dyson, 2008). pRb exerts these different functions by binding to a large number of interacting proteins. The best studied pRb-interacting proteins are the E2F transcription factors, which are heterodimers composed of a subunit of the E2F family and a subunit of the DP family. In mammals there are three DP and eight E2F family members that can be further divided into activating and repressive subfamilies (DeGregori and Johnson, 2006; Trimarchi and Lees, 2002). Therefore the biology of mammalian Rb/E2F proteins is quite complex.

Although the mechanisms by which pRb regulates cell proliferation and apoptosis are well understood, the role of pRb in cell differentiation is much less well characterized. However this aspect of pRb biology likely also contributes to its tumor suppressor function. The study of pRb's role in differentiation using mouse models is complicated because differentiation defects observed in *Rb*-mutant mice are relatively subtle and are generally associated with deregulation of the cell cycle and/or apoptosis (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). The existence of a large family of E2F proteins in mammalian systems also makes it difficult to examine the contribution of E2Fs to the differentiation function of pRb *in vivo*.

In contrast to the extended family members of the E2F, DP and Rb gene families in mammals, there is only one DP (dDP), two dE2Fs (dE2F1 and dE2F2), and two Rb 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 *Drosophila* E2F proteins behave like the two different classes of mammalian E2Fs: dE2F1 mainly functions as a transcriptional 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). As with pRb, RBF can bind to both the activating dE2F1 as well as the repressive 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 the Rb/E2F pathway is highly conserved but much simpler in *Drosophila*. This feature of the Rb/E2F pathway, in conjunction with the available genetic and developmental tools, makes *Drosophila* an attractive model to study the *in vivo* roles of *Rb*.

The *Drosophila* developing eye has been extensively used as a model system to study cell proliferation and differentiation during development. Photoreceptor differentiation in the *Drosophila* developing eye initiates in the morphogenetic furrow (MF), which moves from the posterior of the eye disc to the anterior during the third larval instar. The first photoreceptor determined is R8 (Wolff and Ready, 1993), which is controlled by expression of the bHLH protein Atonal (Jarman et al., 1994). Following R8 specification, EGFR signaling is required for the stepwise recruitment of additional photoreceptor cells, cone cells, and other accessory cells to form the ommatidia (Freeman, 1996).

The rate-limiting component of EGFR signaling activation is Rhomboid, a seven transmembrane protease expressed in a pattern that prefigures EGFR signaling activation (Wasserman et al., 2000). The EGFR ligand Spitz is activated by direct cleavage within its transmembrane domain by Rhomboid (Urban et al., 2001). Spitz subsequently initiates EGFR signaling to the cytoplasm, leading to activation of MAP kinase. Activated MAP kinase can translocate into the nucleus where it phosphorylates the Yan and Pnt proteins, which are members of the ETS transcription factor family that are critical for mediating EGFR signaling output in the nucleus (Brunner et al., 1994; O'Neill et al., 1994; Rebay and Rubin, 1995). The *pnt* locus encodes two distinct proteins, PntP1 and PntP2, by transcription from alternate promoters. PntP1 is a constitutively active transcriptional activator but its expression is regulated by MAP kinase activity (Gabay et al., 1996; O'Neill et al., 1994). In

contrast, the transcription function of PntP2 is activated by phosphorylation by MAP kinase (Brunner et al., 1994; O'Neill et al., 1994). The transcription activation functions of Pnt proteins are counteracted by the repressor protein Yan, which is in turn inhibited by MAP kinase-mediated phosphorylation (Rebay and Rubin, 1995).

Ttk88 is a transcriptional repressor that blocks neuronal differentiation. Induction of R7 photoreceptor differentiation by EGFR signaling also involves the degradation of Ttk88, which is mediated by PHYL and the ring finger protein SINA (Li et al., 1997; Tang et al., 1997). *ebi*, which encodes a divergent F-box/WD40 protein, was also shown to function in the EGFR signaling pathway (Dong et al., 1999; Tsuda et al., 2002). Ebi contributes to the degradation of Ttk88 by interacting with SINA and PHYL, thereby promoting photoreceptor differentiation (Boulton et al., 2000). In addition Ebi participates in a corepressor complex with SMRTER that can be regulated by EGFR signaling (Tsuda et al., 2006; Tsuda et al., 2002).

In a genetic screen for mutations that modulate the effects of *rbf* inactivation during development (Li et al., 2010; Tanaka-Matakatsu et al., 2009), we identified a mutation in *rno* that induces a synergistic differentiation defect in conjunction with *rbf* loss (Steele et al., 2009). *rno* encodes a nuclear protein with a PHD zinc-finger domain (Voas and Rebay, 2003), a motif commonly found in chromatin-associated proteins (Sanchez and Zhou, 2011). Inactivation of *rbf* and *rno* leads to defective photoreceptor R8 determination, which is due to the synergistic effect of *rbf* and *rno* mutations on the expression of Notch signaling ligand, D1 (Steele et al., 2009).

In addition to the multiple R8 phenotype, mutations in *rbf* and *rno* lead to synergistic photoreceptor differentiation delay after R8 specification. In this study, we show that these effects of *rbf* and *rno* loss are mediated by their regulation of EGFR signaling. Mutation of *rbf* causes a reduced level of Rhomboid and impaired MAP kinase activation in the MF of developing eye discs. On the other hand, mutation of *rno* reduces the expression of Pnt and Ebi proteins, which are required for EGFR signaling output in the nucleus. These results suggest that the observed delay in photoreceptor differentiation results from decreased cytoplasmic and nuclear EGFR signaling caused by mutations of *rbf* and *rno*, respectively. As EGFR signaling induces expression of feedback inhibitors such as Argos and Sprouty (Casci et al., 1999; Golembo et al., 1996), our results also provide new insights into the mechanism by which inactivation of *rno* leads to hyperactivated EGFR signaling (Voas and Rebay, 2003).

## Results and Discussion

### Inactivation of *rbf* and *rno* synergistically delays photoreceptor differentiation

In addition to the multiple-R8 phenotype, *rbf,rno* double mutant clones show a significant delay in photoreceptor differentiation (Steele et al., 2009). To quantitatively compare the extent of this delay in *rbf,rno* double-mutant clones to those of *rbf* or *rno* single-mutant clones, we visualized developing photoreceptors by Elav staining and determined the number of rows photoreceptor differentiation was delayed in mutant clones relative to the neighboring WT tissues. While we found no obvious photoreceptor differentiation delay in *rbf* single mutant clones and only one row in *rno* single mutant clones, we observed around 3 rows of photoreceptor differentiation delay in *rbf,rno* double mutant clones (Fig. 1A–C, H). The observed synergistic differentiation delay is independent of apoptosis since no difference in the level of apoptosis was observed between *rbf* and *rbf,rno* mutant clones located in the MF (Steele et al., 2009). Furthermore, blocking apoptosis by *dronc* mutation does not affect the observed delay in differentiation in *rbf,rno* mutant clones (Fig. 1D,D' H).

Cone cells are recruited after the differentiation of photoreceptors in developing eye imaginal discs, so a delay in photoreceptor differentiation likely leads to delayed cone cell differentiation. To further characterize the synergistic differentiation delay in the *rbf,rno* double mutant clones, we examined the differentiation of cone cells, which can be identified by Cut staining, in *rbf* or *rno* single- as well as *rbf,rno* double-mutant clones. Our previous study in the mid-pupal stage showed cone cells were present in each ommatidia but the number of cone cells/ommatidia is variable in *rbf,rno* double mutant clones (Steele et al., 2009). Interestingly, cone cells were largely missing in *rbf,rno* double mutant clones in larval eye discs (Fig. 1G), suggesting that the differentiation of cone cells is dramatically delayed in the absence of both *rbf* and *rno*. In addition, a significant delay in cone cell differentiation was observed in *rno* single mutant clones and a less pronounced delay was also observed in *rbf* single mutant clones (Fig. 1E–F). To quantify the level of cone cell delay in larval eye discs, we determined ratios of cone cell numbers in mutant clones and compared them to WT tissues of the same area near the mutant clones (Fig. 1I). This analysis indicated that, in addition to the differentiation of photoreceptor cells, cone cell recruitment is synergistically delayed in *rbf,rno* double mutant clones. The dramatically delayed cone cell differentiation likely contributes to the shiny glossy phenotypes that we observed in adult eyes (Steele et al., 2009). As delayed cone cell differentiation is potentially a consequence of delayed photoreceptor differentiation, we focused our effort on characterizing the interactions between *rbf* and *rno* in photoreceptor differentiation.

### ***rbf* is required for *Rhomboid* expression and MAP kinase activation in the morphogenetic furrow**

Photoreceptor differentiation initiates with R8 differentiation followed by recruitment of the remaining photoreceptors. We showed that R8 differentiation is not delayed although multiple R8 cells are often observed in *rbf,rno* mutant clones (Steele et al., 2009). These observations suggest that the observed differentiation delay in *rbf,rno* double-mutant clones is due to defects in photoreceptor cell recruitment after R8 determination.

The EGFR signaling pathway has been shown to be required for the stepwise recruitment of photoreceptor cells and cone cells after R8 determination (Dominguez et al., 1998; Freeman, 1996). Therefore we characterized the effect of *rbf* and *rno* mutations on EGFR signaling by determining the level of activated MAP kinase (dpERK) in single- and double-mutant clones. In wild-type eye imaginal discs, a high level of activated MAPK can be detected in the developing ommatidia clusters within the MF, from which the R8 founder cell will be selected (Gabay et al., 1997; Kumar et al., 1998). This staining pattern requires EGFR activity but is dispensable for the specification of the R8 founder cell (Baonza et al., 2001; Kumar et al., 1998; Yang and Baker, 2001), possibly due to the fact that the activated MAP kinase is predominantly cytoplasmic at this stage and thus unable to regulate nuclear targets (Kumar et al., 2003). After R8 specification, EGFR signaling is required for neuronal differentiation, cell survival, and cell proliferation (Baker and Yu, 2001; Baonza et al., 2001; Yang and Baker, 2001; Yang and Baker, 2003). As shown in Fig. 2, dpERK staining is significantly reduced in *rbf* as well as in *rbf,rno* double-mutant clones. In contrast, the level of dpERK is not significantly altered in *rno* single-mutant clones (Fig. 2A–C). These results show that *rbf* is required for the high level of EGFR signaling in the morphogenetic furrow of the developing retina. A previous study using a viable *rbf* allele did not find a decrease in dpERK levels in the *rbf<sup>d20</sup>* mutants (Moon et al., 2006). It is possible that a low level of RBF is sufficient for the presence of dpERK in *rbf<sup>d20</sup>* mutants.

The rate-limiting components of *Drosophila* EGFR signaling are the Rhomboid family of intramembrane proteases (Lee et al., 2001; Urban et al., 2001). In *Drosophila* developing eye discs, Rhomboid 1 and Roughoid/rhomboid 3 cooperate to activate EGFR signaling (Wasserman et al., 2000). To determine how *rbf* affects EGFR signaling, we analyzed

Rhomboid protein levels using an anti-Rhomboid antibody (Sturtevant et al., 1994). Punctate Rhomboid staining was observed in the eye disc posterior to the MF. The first few rows of Rhomboid staining was significantly reduced in *rbf* as well as in *rbf,rno* mutant clones. In contrast, Rhomboid level was not reduced in *rno* mutant clones (Fig. 2 D–F'). Furthermore, blocking cell death by mutation of *dronc* does not restore the level of Rhomboid levels in *rbf,rno* clones in the MF (Fig. 2G,G'), indicating the reduced level of Rhomboid in *rbf* mutant clones is not due to high level of apoptosis in the MF. Therefore, *rbf* mutation significantly reduced initial Rhomboid expression in the MF of developing eye discs. The reduced level of Rhomboid is consistent with a decrease in dpERK staining observed in the MF of *rbf* mutant clones.

### Rno is required for the expression of the transcription factor *Pointed*

We further characterized the effects of Rno and RBF on the *Pointed* transcription factors, which mediate EGFR signaling output in the nucleus. The *Pointed* locus encodes two different transcripts, *PntP1* and *PntP2*. *PntP1* is normally expressed in developing ommatidia clusters in both the morphogenetic furrow and in the posterior (Brunner et al., 1994; Rawlins et al., 2003). In contrast, *PntP2* is expressed prominently in cells posterior to the morphogenetic furrow but only at low levels in the developing ommatidia clusters located in the morphogenetic furrow (Brunner et al., 1994).

We first determined *PntP1* proteins levels using an anti-*PntP1* antibody (Alvarez et al., 2003). As *rbf* mutant clones show a high level of cell death in the MF (Li et al., 2010; Moon et al., 2006; Tanaka-Matakatsu et al., 2009), where high levels of *PntP1* protein are expressed in the developing eye disc, we crossed the single- or double-mutants into a *dronc*-mutant background to prevent the potential interference of cell death on *PntP1* protein levels. As shown in Fig. 3A, there is a slight reduction of *PntP1* levels in the middle of *rbf* mutant clones but not in cells adjacent to WT cells. As *PntP1* expression is regulated by EGFR signaling (Gabay et al., 1996), the effect of *rbf* mutation on *PntP1* is likely related to decreased EGFR signaling in the MF. Interestingly, a more significant and cell-autonomous reduction of *PntP1* is observed in *rno* as well as in *rbf,rno* mutant clones in the MF (Fig. 3B–C'). These data suggest that Rno is required for the high level of *PntP1* protein that accumulates in the MF.

To further characterize if the effects of *rbf* and *rno* mutations on *PntP1* are due to the regulation of *PntP1* transcription, we determined if *rbf* and *rno* affect the level of a  $\beta$ -gal reporter from a *Pnt-lacZ* enhancer trap line. *Pnt-lacZ* expression appears to reflect a combination of the *PntP1* and *PntP2* expression in the posterior but not the expression of *PntP1* in the MF of the developing retina (Brunner et al., 1994; Frankfort and Mardon, 2004). Significantly decreased  $\beta$ -gal levels from *Pnt-lacZ* enhancer trap were observed in *rno* and *rbf,rno* mutant clones (Fig. 3E–F'). In contrast, no significant change to  $\beta$ -gal levels was observed in *rbf* mutant clones (Fig. 3D–D'). To further examine the effect of *rno* mutation on *PntP1* expression, we used a *Minute* mutation to generate eye discs that contain mostly *rno* mutant tissue and determined the level of *PntP1* expression by *in situ* hybridization. *PntP1* expression in the MF of developing retina is easily detected in WT discs but significantly reduced in eye discs consisting of mostly *rno* mutant clones (Fig. 3G, H). These data provide strong evidence that Rno is required for *Pointed* expression in the developing eye discs.

We have shown that mutation of *rno* leads to decreased *Pnt* protein expression, decreased EGFR signaling output in the nucleus, and a slight delay in photoreceptor differentiation. These results, at first glance, seem to conflict with the reported observation that *rno* mutants exhibit hyperactivated EGFR signaling (Voas and Rebay, 2003). However negative regulators of EGFR signaling, such as *argos* and *sprouty*, are transcriptionally upregulated

by EGFR signaling and form inhibitory feedback loops (Casci et al., 1999; Golembo et al., 1996). Consistent with this, decreased Argos expression is observed in *rno* mutants (Voas and Rebay, 2003). Decreases in the expression of the negative regulators of EGFR signaling will lead to hyperactivated EGFR signaling in the cytoplasm as reported previously (Voas and Rebay, 2003). Therefore mutation of *rno* leads to decreased EGFR signaling output in the nucleus but hyperactivated EGFR signaling in the cytoplasm.

### The differentiation delay phenotypes of *rbf* and *rno* mutants are sensitive to changes in the level of EGFR signaling

The above results suggest that RBF promotes the activation of MAP kinase in developing ommatidia clusters in the MF by regulating the expression of Rhomboid proteins while Rno is required for the expression of EGFR target genes in the nucleus by regulating the expression of Pnt proteins. Inactivation of *rbf* and *rno* leads to synergistically reduced EGFR signaling and delayed photoreceptor differentiation due to both reduced MAP kinase activation and dramatically reduced Pnt protein.

To test this idea, we analyzed the effect of reducing the level of EGFR signaling on the differentiation delay in *rbf,rno* double-mutant clones. While no obvious photoreceptor differentiation delay was observed in *rbf* single-mutant clones, about one row of photoreceptor differentiation delay was observed when the dosage of *pnt-P1* or *pnt-P2* was reduced (Fig. 4A–B', 4I). Similarly, gene dosage reduction of *pnt-P1* or *pnt-P2* also significantly enhanced the delayed photoreceptor differentiation phenotype of *rno* single- and *rbf,rno* double-mutant clones (Fig. 4C–F', 4I). In addition, reducing the gene dosage of *egfr* also significantly enhanced the delay of photoreceptor differentiation in *rno* mutant clones (Fig. 4G, I). Since Rhomboid proteins are the rate-limiting step in EGFR signaling and reduced Rhomboid is observed in *rbf* mutant clones, we determined the effect of Rhomboid expression in *rbf,rno* double-mutant clones. As shown in Fig. 4H, expression of Rhomboid significantly suppressed the photoreceptor differentiation delay phenotypes. The genetic interactions among RBF, Rno, and components of the EGFR pathway support the idea that the synergistic photoreceptor cell differentiation delay in *rbf,rno* double mutant clones is due to a limiting level of EGFR signaling.

### *Ebi* is another target of Rno important for photoreceptor differentiation onset

*Ebi* also functions in the EGFR signaling pathway (Dong et al., 1999; Tsuda et al., 2002). *Ebi* contributes to EGFR signaling-regulated degradation of Ttk88, a repressor of neuronal differentiation (Boulton et al., 2000; Dong et al., 1999) and forms a corepressor complex with SMRTER that can also be regulated by EGFR signaling (Tsuda et al., 2006; Tsuda et al., 2002). In addition, *Ebi* has been shown to enhance the phenotypes of dE2F1 and dDP overexpression (Boulton et al., 2000). Our observation that *rbf* and *rno* mutations affect EGFR signaling prompted us to investigate the possibility that alterations to *Ebi* may also contribute to the synergistic differentiation defects observed in *rbf,rno* mutant clones.

To determine whether Rno and RBF/E2F affect *Ebi* expression in the *Drosophila* developing eye, we generated *rno* mutant clones or dE2F1/dDP flip-out overexpression clones in flies that also carried a myc-*Ebi* genomic construct (Dong et al., 1999) and stained eye imaginal discs with anti-Myc antibodies. As shown in Fig. 5, loss of *rno* significantly blocked expression of *Ebi* protein (Fig. 5A, A'). In contrast, overexpression of dE2F1/DP did not affect *Ebi* levels (Fig. 5B, B'). These observations suggest that Rno, but not RBF/E2F, is important for *Ebi* expression.

To determine if reduced activity of *Ebi* synergizes with *rbf* or *rno* mutation to cause delayed photoreceptor differentiation, we generated *rbf* or *rno* mutant clones in tissues expressing a

dominant-negative form of Ebi to block Ebi function (Dong et al., 1999). As shown in Fig. 5, blocking Ebi causes a significant delay in differentiation onset in *rbf* mutant clones (Fig. 5D, F). In contrast, expression of the dominant negative form of Ebi did not synergize with loss of *rno* in inducing delayed differentiation onset (Fig. 5E–F). This is consistent with the observation that Ebi expression is already lost in *rno* mutant clones, and therefore further expression of the dominant negative form of Ebi has no additional effect.

To further test the contribution of Ebi on the delayed differentiation phenotype of *rbf,rno* double-mutant clones, we determined whether expressing wild type Ebi would be sufficient to suppress the differentiation delay in *rbf,rno* clones. As shown in Fig. 5, expression of Ebi partially suppressed the delayed photoreceptor differentiation phenotype of *rbf,rno* clones (Fig. 5C, F). Therefore, Ebi is a downstream target of Rno that is important for the onset of photoreceptor differentiation in the developing retina.

### Deregulation of dE2F1 is necessary but not sufficient to cause the synergistic differentiation delay observed in *rbf,rno* double mutant clones

dE2F1 is a key target of RBF during *Drosophila* development and *de2f1<sup>i2</sup>*, a mutant allele of *de2f1* that has a premature stop codon that removes the transcription activation domain of dE2F1 (Royzman et al., 1999), can suppress the lethality of *rbf* null mutants (Du, 2000). Therefore we tested whether removing the transcription activation function of dE2F1 using *de2f1<sup>i2</sup>* over a *de2f1* null allele, *de2f1<sup>rm729</sup>* can suppress the observed synergistic differentiation delay of *rbf,rno* double-mutant cells. As shown in Fig. 6, removing the dE2F1 transcription activation function suppressed the differentiation delay of *rbf,rno* mutant clones from around three rows to around one row, which is similar to that of *rno* single mutant clones (Fig. 6A, E). Furthermore suppression of the differentiation delay phenotype is correlated with restored Rhomboid expression in *rbf,rno* double-mutant clones in the MF of the developing retina (Fig. 6C). These observations indicate that deregulated dE2F1 activity is required for the observed synergistic differentiation delay phenotype.

*de2f1<sup>su89</sup>* is an allele of dE2F1 that disrupts the binding between dE2F1 and RBF but retains its transcription activation function (Weng et al., 2003). We used *de2f1<sup>su89</sup>* mutants to determine if *de2f1<sup>su89</sup>*, which cannot be regulated by RBF, can mimic the effect of *rbf* mutation in synergizing with *rno* to induce delayed differentiation. We therefore generated *rno* mutant clones in a *de2f1<sup>su89</sup>* mutant background. As shown in Fig. 6, there is still only one row of delay of Elav staining in *rno* mutant clones in *de2f1<sup>su89</sup>* mutant background, indicating that *de2f1<sup>su89</sup>* does not enhance the photoreceptor differentiation delay phenotype of *rno* mutant clones (Fig. 6B, E). Furthermore, the *de2f1<sup>su89</sup>* mutant also does not affect Rhomboid expression in the MF of the developing eye disc (Fig. 6D). Taken together, our results indicate that while deregulated dE2F1 activity is required for delayed photoreceptor differentiation in *rbf,rno* mutant clones, it is not sufficient to synergize with *rno* mutation to induce the synergistic differentiation delay. These observations suggest that in addition to binding and regulating dE2F1, RBF also targets additional factor(s) that contribute to the regulation of Rhomboid and differentiation onset in conjunction with Rno. It is possible that dE2F2, dCAP-D3, or other RBF-interacting proteins are also involved (Korenjak et al., 2004; Longworth et al., 2008). Further studies will be needed to determine the contributions of these other factors to the differentiation function of RBF.

Block in terminal differentiation is often required for the development of cancer. Our results suggest that although inactivation of *rbf* alone does not show significant differentiation defect, inactivation of *rbf* and *rno* together leads to synergistic differentiation defects, through impacting on the Notch and the EGFR signaling. Since Rb inactivation is common in cancers and since cancer cells generally accumulate a large number of mutations, it is possible that inactivation of Rb in conjunction with a subset of cooperating mutations

contribute to cancer development by blocking differentiation. Such differentiation effects of Rb would define a role in tumorigenesis beyond cell proliferation and apoptosis.

## Materials and Methods

### *Drosophila* stocks

The following fly stocks were used in this study: *rbf<sup>Δ5a</sup>* (Tanaka-Matakatsu et al., 2009), *rno<sup>3</sup>* (Voas and Rebay, 2003), *dE2F1<sup>i2</sup>* (Royzman et al., 1999), *dE2F1<sup>rm729</sup>* (Duronio et al., 1995), *dronc<sup>01</sup>* a CGA to TGA codon mutation that changes Arg195 to stop (Li et al., 2010), *pnt-P1* and *pnt-P2* (O'Neill et al., 1994), *myc-Ebi* and *UAS-Ebi-DN* (Dong et al., 1999), *egf<sup>f2</sup>* (BL-2768), Sca-Gal4 (BL-6479), and *de2f1<sup>su89</sup>* (Weng et al., 2003). Stocks with numbers in parentheses indicated above were obtained from the Bloomington Stock Center.

### *Drosophila* genetics

Flies were cultured at 25°C on standard cornmeal-yeast-agar medium. The genotypes of larvae analyzed in the studies were:

*rbf<sup>Δ5a</sup>*,w, eyFLP (or HsFLP)/Y; RBF-G3, Ubi-GFP, FRT80B/*rno*, FRT80B  
w, eyFLP (or HsFLP)/Y; Ubi-GFP, FRT80B/ *rno*, FRT80B  
*rbf<sup>Δ5a</sup>*,w, eyFLP (or HsFLP)/Y; RBF-G3, Ubi-GFP, FRT80B/FRT80B  
*rbf<sup>Δ15a</sup>*,w, HsFLP/Y; RBF-G3, Ubi-GFP, FRT80B/*rno*,*dronc*,FRT80B  
w, HsFLP/Y; Ubi-GFP, FRT80B/ *rno*, *dronc*,FRT80B  
*rbf<sup>Δ15a</sup>*,w, HsFLP/Y; RBF-G3, Ubi-GFP, FRT80B/*dronc*,FRT80B  
*rbf<sup>Δ15a</sup>*,w, HsFLP/Y; RBF-G3, Ubi-GFP, FRT80B/*rno*, FRT80B, Pnt-LacZ (or *pnt-P1* or *pnt-P2*)  
w, HsFLP/Y; Ubi-GFP, FRT80B/ *rno*, FRT80B, Pnt-LacZ (or *pnt-P1* or *pnt-P2*)  
*rbf<sup>Δ15a</sup>*,w, HsFLP/Y; RBF-G3, Ubi-GFP, FRT80B/FRT80B, Pnt-LacZ (or *pnt-P1* or *pnt-P2*)  
w, eyFLP/Y; Ubi-GFP, M, FRT80B/ *rno*, FRT80B,  
w, eyFLP (or HsFLP)/Y; *egf<sup>f2</sup>*/+; Ubi-GFP, FRT80B/ *rno*, FRT80B  
w, HsFLP/Myc-Ebi; Ubi-GFP, FRT80B/ *rno*, FRT80B  
w, HsFLP/Y; Sca-Gal4/+; Ubi-GFP, FRT80B/ *rno*, UAS-Ebi-DN, FRT80B  
*rbf<sup>Δ5a</sup>*,w, HsFLP /Y; Sca-Gal4/+; RBF-G3, Ubi-GFP, FRT80B/ UAS-Ebi-DN, FRT80B  
*rbf<sup>Δ5a</sup>*,w, HsFLP /Y; Sca-Gal4/UAS-Ebi; RBF-G3,Ubi-GFP, FRT80B/ *rno*, FRT80B  
w, HsFLP/Y; Ubi-GFP, FRT80B,*dE2F1<sup>su89</sup>*/*rno*,FRT80B, *dE2F1<sup>su89</sup>*,  
*rbf<sup>Δ15a</sup>*,w, HsFLP/Y;RBF-G3,Ubi-GFP, FRT80B,*de2f1<sup>729</sup>*/*rno*,FRT80B, *de2f1<sup>i2</sup>*

### Quantification and statistics

To quantify the photoreceptor delay phenotypes, eye discs containing clones of the indicated genotypes were induced via hsFLP and stained with Elav to visualize developing photoreceptor cells. The number of rows of the Elav-positive photoreceptor cells in the center of the mutant clones delayed relative to adjacent WT tissues were determined. A minimum of 10 large mutant clones were counted for each genotype.



## Immunohistochemistry

Unless otherwise indicated, all steps were completed at room temperature. Larval imaginal discs were dissected in 1× PBS, fixed in 4% formaldehyde in 1× PBS for 30 minutes, and incubated in primary antibody diluted in 1× PBS plus 0.3% Triton-X100 (PBSTx) with 10% normal goat serum overnight at 4°C. Primary antibodies used were rat anti-Elav (1:20, DSHB), mouse anti-Cut (1:10, DSHB), dpERK (1:100, cell signaling), Rhomboid (1:500) (Sturtevant et al., 1996), Pointed-P1 (Alvarez et al., 2003). Following incubation with primary antibodies, samples were washed three times (10 minutes each) in PBSTx, and incubated with secondary antibodies from Jackson ImmunoResearch (1:200 to 1:400 dilution). The Cut, Elav, and Rhomboid antibodies were obtained from the Developmental Studies Hybridoma Bank (DSHB), developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242.

## Acknowledgments

We would like to thank Drs. Ilaria Rebay, Jim Skeath, Ethan Bier and Leo Tsuda for generously supplying fly stocks and reagents. We thank the Bloomington *Drosophila* Stock Center, and the Developmental Studies Hybridoma bank at the University of Iowa for providing fly stocks and antibodies. This work was supported by grants from the National Institute of Health GM 074197, CA149275, and AT004418.

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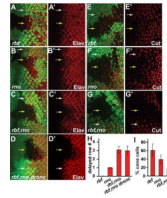
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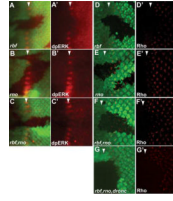
**Highlights**

- > Inactivation of *rbf* and *rno* show synergistic differentiation defect.
- > Inactivation of *rbf* blocks rhomboid expression and MAPK activation in the MF
- > Inactivation of *rno* blocks EGFR signaling in the nucleus by inhibiting Pnt and Ebi
- > deregulated dE2F1 is necessary but not sufficient for the phenotypes of *rbf*



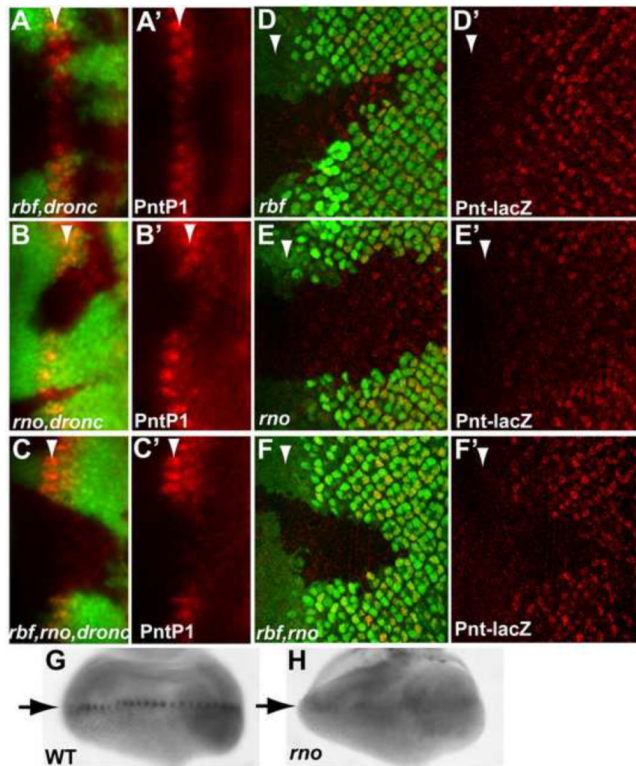
**Figure 1.**

Synergistic differentiation delay in *rbf,rno* double-mutant clones in the developing larval eye discs. (A–D) Images of 3rd instar larval eye discs containing *rbf,rno*, *rbf,rno*, and *rbf,rno,dronc* mutant clones stained for the neuronal marker Elav. White arrows point to the onset of Elav staining in WT tissues while yellow arrows point to the expected location of Elav staining if photoreceptor differentiation were not affected by mutations. (E–G), Images of the 3rd instar larval eye discs containing *rbf*, *rno* single or *rbf,rno* double mutant clones stained for the cone cell marker Cut. White and yellow arrows point to cone cells in the WT or mutant tissues. (H) A diagram showing the average number of rows of photoreceptor differentiation delayed in *rbf*, *rno*, *rbf,rno*, and *rbf,rno,dronc* mutant clones. (I) A diagram showing the percentage of the cone cells in the *rbf*, *rno*, single or *rbf,rno* double mutant clones relative to the cone cells from the same size adjacent WT tissues. For these and all subsequent images of larval eye discs, anterior is to the left, mutant tissues are marked by the absence of GFP, and Elav or Rhomboid staining is shown in Red. Error bars indicate standard deviations.



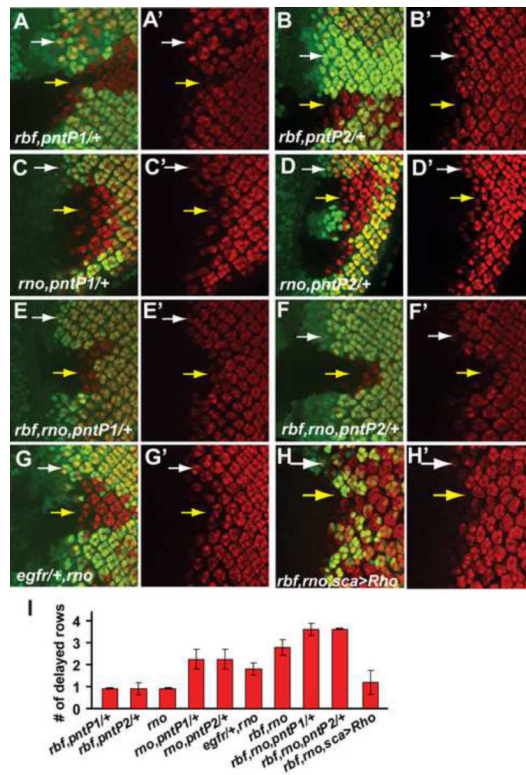
**Figure 2.**

RBF regulates EGFR signaling in the morphogenetic furrow. Eye discs containing *rbf* or *rno* single- and *rbf;rno* double-mutant clones were stained with anti-dpERK antibody (A–C) or anti-Rhomboid antibody (D–G). Clusters of cells with elevated levels of dpERK staining were observed in the morphogenetic furrow in WT or *rno* mutant tissues (A–C). In *rbf* single- or *rbf;rno* double mutant-clones, dpERK staining was significantly reduced (A, A' and C, C'), which is correlated with reduced Rhomboid staining (D, D' and F, F').



**Figure 3.**

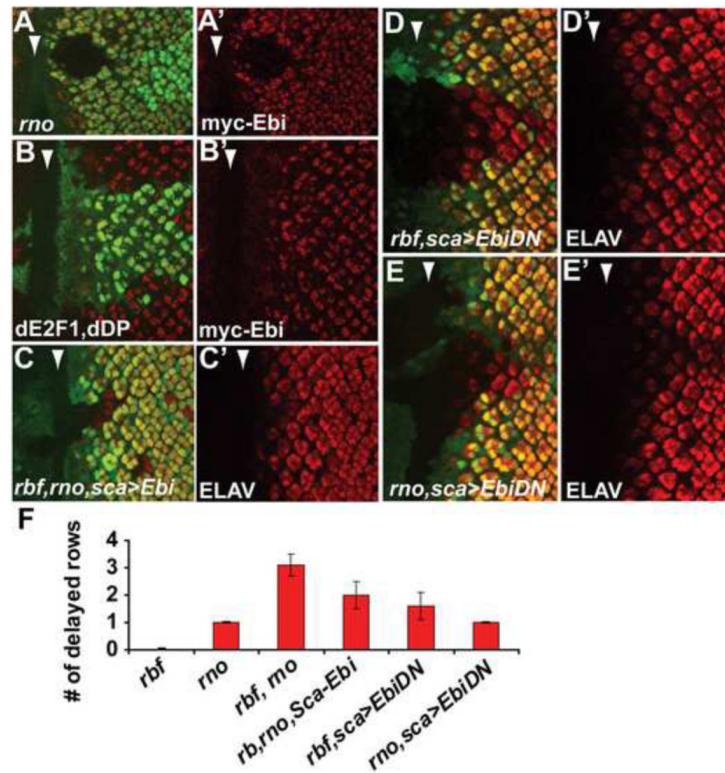
Rno is required for the expression of Pointed. (A–F) Antibody staining to visualize the levels of PntP1 protein (A–C') and Pnt-lacZ reporter expression (D–F') in *rbf, dronc* clones (A, A' and D, D'), *rno, dronc* clones (B, B' and E, E'), and *rbf, rno, dronc* clones (C, C' and F, F'). Mutation of *dronc* was used here to block cell death in the MF induced by *rbf* mutation and does not affect PntP1 levels. (G–H) *in situ* hybridization to detect *Pnt* mRNA in WT (G) or *rno* (H) mutant eye discs. The anterior of these two eye discs are oriented up and arrows point to *PntP1* expression in the MF. *rno* mutant eye discs were generated by inducing *rno* mutant clones with *eyFLP* in a *Minute* background.



**Figure 4.**

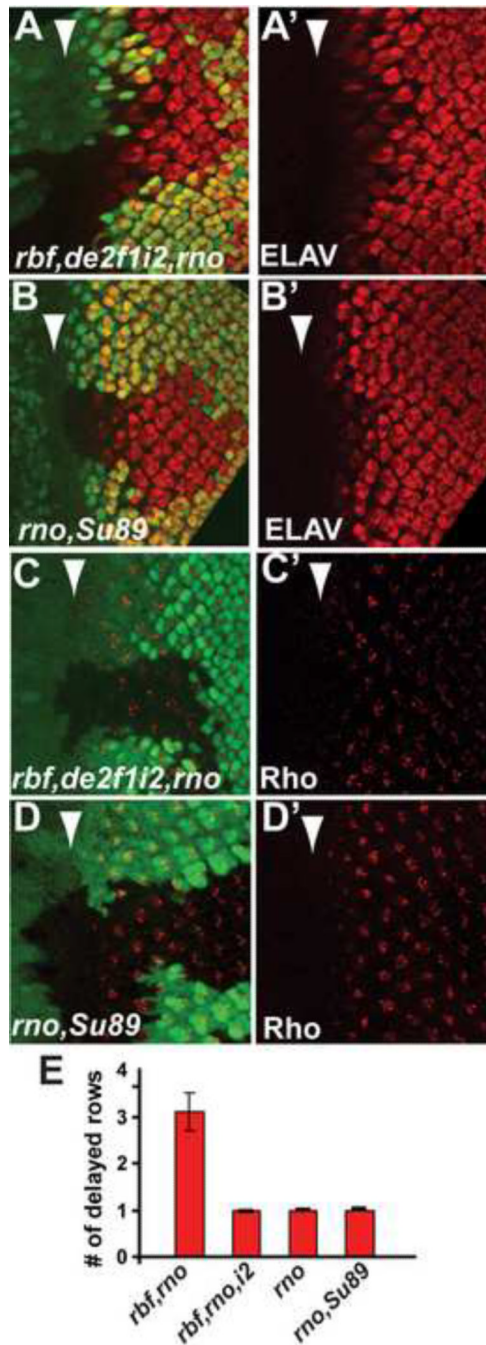
Synergistic differentiation delay phenotype of *rbf,rno* double-mutant clones is sensitive to changes in EGFR pathway signaling. (A–F) Decreasing the gene dosage of *pnt-P1* (A, C, E) or *pnt-P2* (B, D, F) enhances the delay of photoreceptor cell differentiation in *rbf* (A–B) or *rno* (C–D) single-, and *rbf,rno* (E–F) double-mutant clones. (G) Decreasing the gene dosage of *egfr* enhances the differentiation delay of *rno* single-mutant clones. (H) Expression of Rhomboid in *rbf,rno* double-mutant clones significantly decreases the delayed photoreceptor differentiation defect. (I) Diagram showing the number of rows of photoreceptor differentiation delay in eye discs of different genotypes.





**Figure 5.**

Ebi is a target of Rno important for the differentiation delay phenotype of *rbf, rno* double-mutant clones. (A–B) The expression of Ebi from a genomic transgene as detected by anti-Myc antibody in *rno* mutant clones (A) and in *dE2F1/dDP* flip-out overexpression clones (B). (C–E) Effect of expressing WT Ebi (C) or a dominant negative form of Ebi (D and E) on the onset of photoreceptor differentiation in *rbf, rno* (C), *rbf* (D), or *rno* (E) mutant clones. (F) Diagram showing the number of rows of photoreceptor differentiation delay in eye discs of different genotypes, as described above. *dE2F1/dDP* flip-out clones are marked by the presence of GFP.



**Figure 6.**

Deregulated dE2F1 is necessary but not sufficient to synergize with *rno* mutation to both induce the differentiation delay and to affect Rhomboid expression. (A, C) Anti-Elav and anti-Rhomboid staining revealed *de2f1<sup>i2/rm729</sup>* mutation suppresses the photoreceptor differentiation delay (A) and restores Rhomboid expression (C) in the MF in *rbf, rno* clones. (B, D) Anti-Elav and anti-Rhomboid staining reveals that *dE2F1<sup>Su89</sup>* mutation does not enhance the photoreceptor differentiation delay phenotype of *rno* mutant clones (B) or affect Rhomboid expression in the MF. Quantification of the differentiation delay is shown in (E).