A Gradient of Epidermal Growth Factor Receptor Signaling Determines the Sensitivity of *rbf1* Mutant Cells to E2F-Dependent Apoptosis[∇]

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The inactivation of retinoblastoma (Rb) family members sensitizes cells to apoptosis. This cell death affects the development of mutant animals and also provides a critical constraint to the malignant potential of Rb mutant tumor cells. The extent of apoptosis caused by the inactivation of Rb is highly cell type and tissue specific, but the underlying reasons for this variation are poorly understood. Here, we characterize a specific time and place during *Drosophila melanogaster* development where *rbf1* mutant cells are exquisitely sensitive to apoptosis. During the third larval instar, many *rbf1* mutant cells undergo E2F-dependent cell death in the morphogenetic furrow. Surprisingly, this pattern of apoptosis is not caused by inappropriate cell cycle progression but instead involves the action of Argos, a secreted protein that negatively regulates *Drosophila* epidermal growth factor receptor (EGFR [DER]) activity. Apoptosis of *rbf1* mutant cells is suppressed by the activation of *DER*, *ras*, or *raf* or by the inactivation of *argos*, *sprouty*, or *gap1*, and inhibition of DER strongly enhances apoptosis in *rbf1* mutant discs. We show that RBF1 and a DER/ras/raf signaling pathway cooperate in vivo to suppress E2F-dependent apoptosis and that the loss of RBF1 alters a normal program of cell death that is controlled by Argos and DER. These results demonstrate that a gradient of DER/ras/raf signaling that occurs naturally during development provides the contextual signals that determine when and where the inactivation of *rbf1* results in dE2F1-dependent apoptosis.

Retinoblastoma (Rb) family proteins control E2F-dependent transcription and restrict cell proliferation. In the early G₁ phase of the cell cycle, Rb family proteins bind to E2F family members, inhibiting their ability to activate transcription and recruiting repressor complexes to DNA. In late G₁ to S phase, cyclin-dependent kinases (CDK) phosphorylate Rb family proteins, liberating E2F and activating E2F-dependent transcription (reviewed in references 21, 48, and 49). pRB function is compromised in most types of cancer, and the resulting deregulation of E2F is thought to be a critical change that drives the inappropriate cell cycle progression (reviewed in references 57 and 65).

Studies in both mammalian cells and in *Drosophila melanogaster* have identified a single Rb family member (pRB in mammals, RBF1 in flies) that normally binds to activator E2Fs (E2F1 to -3 in mammals, dE2F1 in flies) and directly inhibits E2F-mediated activation of transcription (reviewed in references 3 and 58).

Understanding how cells respond to the inactivation of *Rb* is central to understanding *Rb*'s role as a tumor suppressor (7). Much of our information about the function of *Rb* comes from experiments with tissue culture cells in which pRB is either selectively inactivated or ectopically expressed. It has long been known that cell lines differ greatly in the way that they respond to pRB, but the reasons for these differences are poorly understood. Cell lines carry different sets of mutations, acquired either in vivo or during adaptation to culture condi-

tions, and these may alter the activity of pRB or the cellular response to pRB expression. In addition, differences may exist because the role or importance of *Rb* varies naturally between cell types.

Although pRb is expressed in a broad variety of cell types, the inactivation of *Rb* in vivo gives an assortment of tissue-specific defects (12, 35, 42). These phenotypes include alterations in cell differentiation, cell cycle control, and elevated apoptosis. Because the changes in tissue morphology in *Rb* mutant animals are often quite complex, involving both cell-autonomous and nonautonomous changes (45, 59, 60, 68), explaining why the inactivation of pRB causes these specific phenotypes is often a formidable challenge (reviewed in references 13 and 62).

One of the least-well-understood aspects of in vivo studies of *Rb* function is the fact that the inactivation of *Rb* often sensitizes cells to apoptosis (reviewed in reference 9). *Rb* mutant mice display elevated apoptosis in the central nervous system (CNS), peripheral nervous system, and the eye lens (12, 35, 42, 67). Initially it was proposed that this apoptosis is caused by the deregulation of E2F, particularly E2F1 and E2F3. Overexpression of these E2Fs induces apoptosis, and mutation of *E2f1* or *E2f3* reduces apoptosis in the CNS of *Rb* mutant embryos (39, 63, 76). However, more recent studies have shown that the CNS defects, like many of the developmental phenotypes of *Rb*^{-/-} animals, are not cell autonomous and can be rescued by *Rb* expression in the placenta (68). Apoptosis in the lens of *Rb* mutant animals depends on *E2f1* and, unlike the CNS, appears to reflect a cell-autonomous function of pRB (14, 63, 68).

Since pRB is functionally inactivated in most human cancers, conditions that selectively elevate apoptosis in *Rb*^{-/-} cells could have general therapeutic potential. When, where, and

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how *Rb* mutant cells can be sensitized to apoptosis are important subjects in cancer research (reviewed in references 50 and 56). The ability of p53 mutations to reduce E2F-induced apoptosis is thought to be one of the reasons why lesions in the *Rb* and p53 pathways cooperate in tumorigenesis (32, 67). Recent studies using murine models for retinoblastoma illustrate the cell-type-specific patterns of apoptosis that occur when *Rb* is inactivated in vivo. Several different cell types of the developing retina undergo ectopic division following the mutation of *Rb*; in many cell types, this change is effectively cancelled out by increased apoptosis (11, 46, 52, 74). However, some cell types of the inner nuclear layer, such as horizontal, amacrine, and Müller precursor cells, are intrinsically resistant to cell death following *Rb* inactivation, and it is from these apoptosis-resistant cell types that tumors arise (reviewed in reference 20).

Despite the significance of this topic, virtually nothing is known about the in vivo conditions that determine whether cells undergo apoptosis when *Rb* is mutated or whether they proliferate. What are the intrinsic and extrinsic signals that make *Rb* mutant cells sensitive or resistant to apoptosis, and how can these pathways be manipulated in vivo to induce *Rb* mutant cells to die are questions that remain largely unanswered.

In this study, we have used *Drosophila* eye development as a model system to characterize a developmental context in which *rbf1* mutant cells are transiently hypersensitive to E2F-dependent apoptosis. The results show that cellular sensitivity to E2F-induced apoptosis is independent of cell cycle progression and is generated in vivo by a local, transient, decrease in epidermal growth factor receptor (EGFR) signaling. Hence, *rbf1* mutant cells are not automatically prone to apoptosis, but are sensitized to die by specific signals from the cellular microenvironment. These findings may explain why individual cell types show such different responses to the inactivation of *Rb* in other animal model systems and suggest strategies to target human tumor cells.

MATERIALS AND METHODS

***Drosophila* strains.** The following alleles were used in this study. The *rbf1* mutants *rbf1^{120a}* and *rbf1¹⁴* are described in reference 18. *de2f1* mutants *de2f1^{rm729}*, *de2f1¹²* are described in reference 48. *dronc¹²⁴*, *FRT80B* was a gift from A. Bergmann (70). *sprouty^{Δ5}*, *FRT80B* was a gift from M. A. Krasnow (30). *gap1¹²⁴⁸²*, *FRT80B* was a gift from I. Hariharan. *Argos^{Δ7}*, *FRT80B* was a gift from I. Rebay (64). *UAS-DER^{act}* was provided by N. E. Baker (43). *hs-hid* (29), *hs-reaper* (66), *Df(3l)X14*, *Df(3l)X38*, and *GMR-p35* were gifts from K. White. *UAS-Phl^{80f}*, *UAS-dAkt1* and *UAS-DER^{dn}*; *UAS-DER^{dn}* were obtained from the Bloomington Stock Center.

Clonal analysis. FLP was expressed from the *eyeless* promoter to generate mitotic clones. To examine clones in *rbf1* mutant animals, we used a recombinant X chromosome carrying both the *rbf1^{120a}* mutation and an *eyFLP* transgene. For example, to generate *dronc* mutant clones in *rbf1^{120a}* eye discs, *rbf1^{120a}*, *ey-FLP/* *FM7*, *GFP^{act}*, *GFP^{ubi}*, *FRT80B* virgin females were crossed with *dronc¹²⁴*, *FRT80B* males. Non-*FM7* male progenies at the third instar larval stage were selected and analyzed. A similar approach was used to generate flip-out clones in the background of the *rbf1* mutation using an *hs-FLP* transgene. For example, to generate *DER^{act}*-expressing flip-out clones in *rbf1^{120a}* mutant eye discs, *rbf1^{120a}*, *hsFLP/* *FM7*, *GFP^{act}*, *UAS-DER^{act}* virgin females were crossed with *act5c>CD2>GAL4*, *UAS-GFP* males. Seventy-two hours after heat shock treatment, we collected non-*FM7* male progeny for analysis.

BrdU labeling. For bromodeoxyuridine (BrdU) labeling, eye discs were dissected at third-instar larval stage and incubated in Schneider's medium containing 0.2 mg/ml of BrdU for 1 h at room temperature. Eye discs were fixed overnight with 1.5% formaldehyde and 0.01% Tween 20 in PBS at 4°C. After a washing with PBS, DNA was denatured by treating the eye discs with DNase

(Promega) for 30 min at 37°C. After permeabilization in PBST (0.3% Triton X-100), nuclei with BrdU incorporated were visualized by immunohistochemistry using an anti-BrdU antibody (Becton Dickinson).

Immunohistochemistry and in situ hybridization. The following antibodies were used in this study: anti-C3 (Cell Signaling), anti-ELAV (Developmental Studies Hybridoma Banks [DSHB]), anti-dE2F1 (a gift from Carole Seum), anti-RBF1 (19), anti-cyclin B (DSHB), anti-p-Erk (Sigma and Cell Signaling), anti-Atonal (a gift from Y. N. Jan) (36), anti-Argos (DSHB), and anti-dlg (DSHB). For immunostaining, discs were fixed with 4% formaldehyde for 30 min at 4°C (for p-Erk and Argos, fixation was at room temperature) and washed twice with 0.3% PBST (0.3% Triton X-100 in PBS) for 10 min at room temperature. Discs were incubated with primary antibody in 0.1% PBST (0.1% Triton X-100 in PBS) with 5% normal goat serum at 4°C for 16 h. After being washed five times with 0.1% PBST for 10 min at room temperature, eye discs were incubated with secondary antibody in 0.3% PBST with 5% normal goat serum at 4°C for 16 h. After being washed five times with 0.1% PBST for 10 min at room temperature, eye discs were mounted for confocal microscopic imaging. For in situ hybridization, samples were prepared as previously described (17) and an antidigoxigenin (DIG) antibody conjugated with alkaline phosphatase was used to detect a DIG-labeled antisense RNA probe.

Real-time quantitative PCR. Total RNA was prepared from eye-antenna discs or total larvae using Trizol (Invitrogen) reagent, and reverse transcription-PCR was performed using *Taq* Man reverse transcription reagent (PE Applied Biosystems) according to the manufacturer's specification. Real-time PCR was performed using an ABI Prism 7700 sequence detection system. Relative levels of specific mRNAs were determined using the SYBR Green I detection chemistry system (Applied Biosystems, Foster City, CA). Quantification was performed using the comparative cycle threshold method as described in the manufacturer's procedures manual. *Rp49* was used as normalization control. All primers were designed with Primer Express 1.0 software (Applied Biosystems, Foster City, CA) following the manufacturer's suggested conditions. The following primer pairs were used: RP49-58F (TACAGGCCCAAGATCGTGAAG) and RP49-175R (GACGACTCTGTGTGTCGATACC), Hid-1095F (CATCAGTCAGCA GCGACAGG) and Hid-1196R (ACGAAAACGGTCACAACAGTTG), RNR21180F (CATCTGCCAGATGCTGGTAC) and RNR21282R (GAAGT CCGTAACCCCTTCG) and dCycE-1224F (AGATCTGTGCACCCTGGACG) and dCycE-1324R (AGCGTAAAGCCATCTCCCG).

RESULTS

Drosophila imaginal eye discs provide a powerful system to study the changes that occur as proliferating cells exit the cell cycle and begin to differentiate. In the eye disc, cell cycle exit is coupled to a morphogenetic wave that passes through the disc. This organization allows all of the steps in this process to be visualized, simultaneously, in a single disc.

While characterizing the in vivo functions of RBF1, we examined mutant eye discs in detail. RBF1 protein is expressed ubiquitously in the eye disc. However, discs that are homozygous (or hemizygous) for *rbf1^{120a}*, a hypomorphic allele that expresses a very low level of wild-type RBF1 protein (18), display a distinctive pattern of apoptosis (Fig. 1A). In these discs, a stripe of apoptotic cells occurs within the morphogenetic furrow (MF). This stripe can be visualized using C3, an antibody that recognizes a cleaved form of mammalian caspase 3, or using terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assays (data not shown). To determine whether this stripe reflects a cell-autonomous requirement for RBF1, we examined somatic clones that were homozygous for *rbf1¹⁴*, a null allele of *rbf1* (18). C3-positive cells were detected in *rbf1¹⁴* mutant clones, but not in the wild-type twin spots, in a region between the BrdU-positive cells of the first and second mitotic waves, and were located towards the anterior half of the MF (Fig. 1B). We conclude that *rbf1* is required, cell autonomously, in this region of the eye disc to prevent apoptosis.

FIG. 1. *rbf1* mutant cells are prone to apoptosis at the anterior edge of the MF. (A) Third instar *rbf1*^{120a} eye discs contain a distinctive pattern of apoptosis. Discs were treated with BrdU and immunostained with antibodies to BrdU and C3 to visualize S-phase cells and dying cells, respectively. Note that RBF1 is expressed throughout the disc, but its absence results in a stripe of apoptosis. ELAV stains cells that have initiated neuronal differentiation. (B) The apoptosis triggered by the mutation of *rbf1* is cell autonomous. Mosaic clones of an *rbf1*-null allele, *rbf1*¹⁴, were generated in eye discs using ey-FLP and FRT⁻ *rbf1*¹⁴. *rbf1*¹⁴ mutant clones are marked by the lack of green fluorescent protein (GFP) signal. Apoptosis occurs in both *rbf1*^{120a} discs and *rbf1*¹⁴ mutant clones as *rbf1* mutant cells enter the MF. The MF passes through the discs shown from left to right.

The MF is a physical indentation that sweeps through the eye disc during the third larval instar (reviewed in reference 34). During this process, asynchronously dividing cells are drawn into the MF and become synchronized in the G₁ phase of the cell cycle. Some of these cells are instructed to become photoreceptors and initiate a differentiation program, while others pass synchronously through one more cell cycle, generating a reservoir of uncommitted cells for recruitment into a variety of cell types. The C3 staining pattern in *rbf1*-null clones indicates that RBF1 is needed to prevent cell death at a specific time and place during eye development. Interestingly, RBF1 is primarily

required to prevent apoptosis as cells enter the MF, at a time when cell cycle transitions are precisely controlled and cells are exposed to signals that initiate differentiation. We sought to understand why RBF1 is so important at this particular time and place.

We started by examining the expression pattern of dE2F1. We used the cell cycle marker cyclin B, as a point of reference to mark the position of the 1st and 2nd mitotic waves (Fig. 2A and B). dE2F1 protein is expressed at high levels in a broad swath between the mitotic waves of wild-type and *rbf1*^{120a} eye discs (Fig. 2B) (25). Double staining with antibodies to dE2F1

FIG. 3. Suppression of cell death in *rbf1*^{120a} eye discs does not result in additional S-phase cells. Cell death in the MF of *rbf1*^{120a} eye discs, as assayed by C3 staining or TUNEL (data not shown), was completely inhibited in mutant clones of *dronc*. The distribution of S-phase cells and apoptotic cells in the third instar eye discs was visualized with antibodies to BrdU and C3 as described before; *dronc* mutant clones are marked by the lack of GFP signal. Note the lack of additional S-phase cells in *dronc* mutant clones despite the inhibition of apoptosis. GFP, green fluorescent protein.

and cyclin B confirmed that the levels of dE2F1 are elevated in almost all of the cells between the two mitotic waves. In situ hybridization with an antisense probe against *nmr2*, a known E2F-regulated gene, showed that RBF1 is needed to restrict the activity of E2F1 in this region (Fig. 2C). In wild-type discs, *nmr2* is expressed in a small number of cells and its expression parallels and slightly precedes S-phase entry. In *rbf1*^{120a} eye discs, *nmr2* is abnormally expressed throughout the region of the disc where the level of dE2F1 protein is high. Thus, the normal pattern of *nmr2* expression appears to be determined by the ability of RBF1 to prevent dE2F1 activation and by the release of this inhibitory effect.

Since E2F-dependent transcription is abnormally high in the MF of *rbf1* mutant discs, we asked whether the apoptosis is caused by dE2F1. *de2f1*-null mutants do not reach the third larval instar, and homozygous clones are too small to be informative (8, 54). Therefore, we reduced *de2f1* function using a transheterozygous combination of a null allele, *de2f1*^{rm729}, and

a viable hypomorphic allele, *de2f1*¹², that encodes a truncated dE2F1 protein lacking most of the transactivation domain (53). *de2f1*¹²/*de2f1*^{rm729} transheterozygotes are viable and lack any visible eye defects. Mutation of *de2f1* completely suppressed cell death in *rbf1*^{120a} eye discs (Fig. 2D). Cell death was similarly suppressed in *rbf1*^{120a} discs in *dDP* mutant clones, lacking the heterodimeric partner for dE2F1 (data not shown). We conclude that dE2F1 levels are high in the MF, that dE2F1's transcriptional activity is held in check by RBF1, and that the apoptosis resulting from the mutation of *rbf1* is dependent on dE2F1 and dDP.

In several experimental systems, the inactivation of pRB-related proteins and the activation of E2F-dependent transcription result in unscheduled S phases (reviewed in reference 15). In *rbf1* mutant eye discs, cell death is most apparent at the anterior edge of the MF where cells become synchronized in G₁ (Fig. 1 and 2A). We therefore examined the possibility that the apoptosis seen in *rbf1* mutant eye discs was an indirect

FIG. 2. Mutation of *rbf1* activates dE2F1-dependent transcription, resulting in dE2F1-dependent cell death. Panels A and B show the relative position of apoptotic cells and the distribution of dE2F1 in *rbf1*^{120a} eye discs using the pattern of cyclin B (Cyc B) expression as a reference point. Arrows indicate the position of the MF. The apoptotic cells, detected by anti-C3, are located in the anterior half of the gap between the stripes of cyclin B expression, while dE2F1 expression is elevated in a broad swath that spans the region between the areas of cyclin B expression. (C) In situ hybridization for the E2F target gene, *nmr2*, was used to compare the patterns of dE2F1-dependent transcription in wild-type and *rbf1*^{120a} eye discs. Note that the expression of *nmr2* is elevated and expanded in *rbf1*^{120a} eye discs in a pattern that mirrors the distribution of dE2F1 protein. (D) Apoptosis in *rbf1*^{120a} discs was completely suppressed by mutation of *de2f1*. Eye discs were stained with anti-C3 antibody to visualize the pattern of apoptosis.

FIG. 4. Activation of EGFR suppresses cell death in *rbf1*^{120a} mutant eye discs. (A) In *rbf1*^{120a} eye discs, apoptotic cells (stained with anti-C3) are located adjacent to cells that stain with antibodies to p-Erk. (B) A model showing how fluctuations in EGFR activity might regulate cell death in *rbf1*^{120a} eye discs. The high EGFR activity that is present in differentiating cells and marked by p-Erk may provide a survival signal that protects them from apoptosis. At same time, these cells also express Argos, which inhibits EGFR signaling in the neighboring, uncommitted cells. (C) To test this model, clones of cells that overexpress the activated form of type II EGFR (DER^{act}) were generated in *rbf1*^{120a} eye discs using a heat shock flip-out technique. Seventy-two hours after the heat shock, eye discs were dissected and stained with anti-p-Erk and anti-C3 antibodies. The overexpressing clones are marked by green fluorescent protein (GFP). Strong p-Erk staining in DER^{act}-expressing cells and its neighboring cells was observed in the furrow. Cells that activate p-Erk as a consequence of DER^{act} expression are protected from apoptosis. (D and E) Clones that overexpress activated forms of *Drosophila* Raf, Phl^{gof}, or wild-type AKT1 were generated using a heat shock flip-out technique. Clones were marked by GFP signal. Dying cells in the eye discs were visualized by C3 staining.

consequence of cells failing to arrest and inappropriately entering S phase. The absence of BrdU-positive cells in the MF of *rbf1*^{120a} mutant eye discs might be explained by the fact that apoptosis is a rapid process and that cells inappropriately en-

tering S phase might be quickly eliminated. To test this, we blocked cell death in *rbf1*^{120a} mutant eye discs, which should allow any ectopic S-phase cells to survive and be detected by BrdU staining. *dronc* encodes the *Drosophila* homologue of

FIG. 5. *gap1* or *sprouty* inactivation is sufficient to suppress cell death in *rbf1*^{120a} eye discs. *gap1* or *sprouty* mutant clones were generated in *rbf1*^{120a} eye using ey-FLP/FRT. Eye discs were dissected and stained with anti-C3 (A), anti-p-Erk (B), and anti-Atonal (C) antibodies. Mutant clones are marked by absence of green fluorescent protein (GFP) signals. Note the reduced levels of C3-positive cells in *gap1* or *sprouty* mutant clones and the relatively normal pattern of p-Erk staining in the mutant clones.

FIG. 6. Argos is an important regulator of cell death in *rbf1^{120a}* eye discs. (A) Eye discs from wild-type control third instar larval were dissected and stained with anti-p-Erk and anti-Argos (Aos) antibodies. The p-Erk staining pattern and the boundary of Argos staining follow the MF and moves across the discs shown from left to right. Note that cells on the right of the panel shown will be exposed to Argos before the stripe of p-Erk staining reaches them (B) *argos* mutant clones were generated in *rbf1^{120a}* eye using the ey-FLP/FRT technique. Eye discs were dissected and stained with anti-p-Erk and anti-C3 antibodies. In *argos* mutant clones (marked by the lack of green fluorescent protein [GFP]), C3 staining is greatly reduced. Note that p-Erk staining is increased but has a relatively normal pattern in *argos* mutant clones. (C) The level of Argos expression is unaltered in *rbf1* mutant eye discs. Twenty-five pairs of control and *rbf1^{120a}* eye discs were dissected, and protein levels were compared on Western

mammalian caspase 9 and is required for the activation of several effector caspases (reviewed in reference 51). We found that cell death in *rbf1^{120a}* mutant discs was completely suppressed in *dronc* mutant clones (Fig. 3). However, suppression of apoptosis did not result in the detection of increased numbers of BrdU-positive cells. Hence, the wave of apoptosis that occurs in the *rbf1^{120a}* eye discs is unlikely to be caused by unscheduled or inappropriate S-phase entry.

When comparing the pattern of dE2F1 expression, the distribution of *mnr2* transcripts and the location of dying cells in *rbf1^{120a}* mutant eye discs, we noticed that cell death occurred only at the anterior half of the MF while the levels of dE2F1 protein and its transcriptional target were high throughout the MF (Fig. 1 to 3). This distinction implies that there is a second factor, in addition to the deregulation of dE2F1, which determines the pattern of cell death in *rbf1^{120a}* mutant eye discs. This factor was unlikely to directly inhibit dE2F1 since the transcription of the dE2F1 target genes, *mnr2* and *PCNA*, was up-regulated throughout the region (Fig. 2 and 3) (data not shown). We therefore hypothesized that there is a contextual difference between the anterior and the posterior of the MF that allows cells to be either resistant or sensitive to cell death when RBF1 is lost.

To better understand the context in which apoptosis occurs, we compared the pattern of cell death in *rbf1^{120a}* mutant eye discs with markers of signaling pathways that are important for eye development. In this regard, the distribution of phospho-Erk (p-Erk) was highly informative. p-Erk staining in the MF marks an "intermediate group" (IG) of cells, from which the R8 founder cell will be selected (26, 40). This staining pattern requires DER activity but is dispensable for the specification of the R8 founder cell (5, 72). Previous studies with *der* mutant alleles have shown that DER is required for neuronal differentiation, cell survival, and cell proliferation in the eye disc (4, 5, 71, 72). However, most of the developmental functions ascribed to *der* in the eye disc have been identified in regions posterior to the MF and less is known about the action of DER within or anterior to the MF. When *rbf1^{120a}* eye discs were costained with C3 and p-Erk antibodies, we found that C3-positive cells were immediately adjacent to the IG (Fig. 4 A). Interestingly, although the p-Erk-stained cells fall within the region of the disc that has high levels of dE2F1, these cells do not undergo apoptosis, suggesting that a DER signaling pathway suppresses dE2F1-dependent cell death in *rbf1* mutant discs.

In several different studies, the activation of DER has been shown to induce the expression of Argos, a secreted protein that functions non-cell autonomously in a negative feedback loop to restrict DER signaling during development (28). Argos acts by sequestering Spitz, a DER ligand, preventing it from binding to its receptor (37). Argos controls the amount of

active Spitz and helps to generate a gradient of DER signaling. Mutation of *argos* allows Spitz to activate DER over a greater distance (reviewed in reference 22). Thus, the position of the p-Erk-stained cells suggested a simple model in which activated EGFR/Ras signaling might protect IG cells from dE2F1-induced apoptosis, while the production of Argos might down-regulate EGFR/Ras signaling in cells neighboring the IG, rendering them sensitive to apoptosis (Fig. 4B).

We have tested this model in three ways. First, we asked whether constitutive activation of the EGFR receptor pathway is sufficient to suppress the cell death in *rbf1* mutant cells. To do this, we examined clones of cells that overexpress an activated form of DER (*DER^{act}*) in *rbf1^{120a}* mutant eye discs. As shown in Fig. 4C, DER activation was sufficient to induce ectopic p-Erk-positive cells and suppress cell death. Interestingly, *DER^{act}* expression also induced p-Erk staining in a non-cell-autonomous manner within the MF. None of the cells that showed the activation of this downstream marker, p-Erk, underwent cell death. We also tested downstream signaling molecules of DER, dRaf and dAKT1 (Fig. 4D and E). While expression of activated dRaf, *Ph^{160f}*, suppressed cell death, no decrease was seen when *dAKT1* was tested. Instead, the ectopic expression of *dAKT1* increased the level of C3 staining in *rbf1^{120a}* mutant eye discs.

Hyperactivation of EGFR in eye discs induces ectopic neuronal differentiation (24, 61), and it was possible that the suppression of cell death by *DER^{act}* could have been an indirect consequence of premature differentiation. Because of this, as a second step we asked whether activation of the endogenous EGFR/Ras pathway could suppress apoptosis. We examined mutant clones of *gap1* and *sprouty*, cell-autonomous negative regulators of DER and Ras GTPase, in *rbf1^{120a}* eye discs. Mutation of *gap1* or *sprouty* gave a slight increase in the basal level of p-Erk, but unlike the expression of *DER^{act}*, did not generate patches of ectopic p-Erk-staining cells, indicating that there was no ectopic hyperactivation of DER signaling (Fig. 5B). Furthermore, when we stained eye discs with markers of differentiation processes such as the proneural gene, *Atonal*, or a neuron-specific gene, *Elav*, we saw no marked effect on the expression pattern by *gap1* mutation (Fig. 5C) (data not shown). Nevertheless, the subtle up-regulation of EGFR/Ras signaling that occurs following the mutation of *gap1* or *sprouty* was sufficient to prevent most of the cell death in *rbf1^{120a}* mutant eye discs (Fig. 5A). This result is also in agreement with the idea that cell proliferation, differentiation, and survival in eye discs are controlled by different levels of DER activity (71).

As a third test of the model, we asked whether Argos is important for the induction of apoptosis. Immunostaining experiments confirmed that Argos is expressed in p-Erk-positive IG cells, and the staining pattern extends to cells surrounding

blots. (D) Wild-type and *rbf1^{120a}* eye discs have a similar pattern of Argos expression. (E) Pupal eye discs containing *rbf1¹⁴* mutant clones (marked by the lack of GFP) were dissected, and dying cells were visualized using anti-C3 antibody. Elevated cell death was observed in *rbf1¹⁴* mutant clones at various time points of pupation (illustrated here 24 h after pupal formation). (F) Pupal eye discs at 42 h after pupal formation, which contain *rbf1¹⁴* mutant clones (marked by the lack of GFP), were dissected, and cell boundaries were visualized using anti-disc large antibody (Dlg). The yellow arrows indicate the places where secondary pigment cells or cone cells are missing. (G) Cell death was inhibited by expression of baculoviral protein p35 in pupal eye discs described in panel F, restoring the missing cells to *rbf1¹⁴* mutant clones and revealing the extent of ectopic proliferation.

the IG, including the anterior cells in the region where *rbf1* mutant cells undergo apoptosis (Fig. 6A). When *argos* mutant clones were generated in *rbf1*^{120a} mutant eye discs, the inactivation of *argos* was sufficient to suppress apoptosis in the MF (Fig. 6B). In some clones, a non-cell-autonomous effect of *Argos* could be observed at the clonal boundary, in which the level of cell death in the wild-type cells was lower than normal and some residual apoptotic cells could be seen in the mutant clones. This effect is consistent with the idea that *Argos* can diffuse from the wild-type tissue into the mutant clones, diluting its effect. Control experiments showed that the level and pattern of *Argos* expression are unaltered in *rbf1*^{120a} mutant discs (Fig. 6C and D) and that these mutant discs also have a normal pattern of p-Erk staining (Fig. 7).

During pupariation, a program of cell death occurs in the developing eye that serves to remove surplus cells. *Argos* plays an important role in this process by inhibiting EGFR-mediated survival signals; transgenes that artificially elevate the expression of *Argos* increase cell death, inducing apoptosis in cone cells and in secondary pigment cells that are normally protected at this stage of development (23, 55). Interestingly, in pupal eye discs *rbf1* mutant clones showed an increase in cell death and displayed changes that mimic the effects of elevated *Argos* expression. Increased cell death was already evident in *rbf1* mutant clones by 24 h after pupal formation (Fig. 6E), and at later time points, we observed the specific loss of cone and secondary pigment cells (Fig. 6F). Expression of the baculovirus protein p35, a caspase inhibitor, suppressed these effects and resulted in a surplus of secondary pigment cells, confirming that the loss of cells in *rbf1* mutant clones was indeed due to the excessive cell death (Fig. 6G). Hence, in pupal eye discs, RBF1 is needed to set the normal balance between cell death and survival during a developmentally controlled program of cell death that involves *Argos*-mediated inhibition of EGFR signals.

In summary, these results show that *Argos*, an inhibitor of EGFR/Ras signaling, is required to generate the pattern of apoptosis seen in *rbf1*^{120a} discs and that activation of the EGFR/Ras pathway is sufficient to suppress the dE2F1/dDP-dependent apoptosis that occurs following the mutation of *rbf1*. This indicates that RBF and EGFR/Ras signaling cooperate to suppress E2F-dependent apoptosis during normal development. To test this idea directly, we examined the effect of inhibiting EGFR signaling in an *rbf1* mutant background (Fig. 7). A dominant-negative mutant of DER (*DER*^{dn}) was expressed in the eye disc using the flip-out technique in which Flipase expression was driven by the *eyeless* promoter. In discs with wild-type *rbf1*, *DER*^{dn} expression induced cell death in the first mitotic wave. However, cells between the first and second mitotic waves, in which dE2F1 is inhibited by RBF1 (Fig. 2), did not undergo apoptosis (Fig. 7A). This protection is due to RBF1, since *DER*^{dn} expression in *rbf1*^{120a} mutant eye discs resulted in apoptosis in this region and throughout much of the disc. Discs with mutant *rbf1* and *DER*^{dn} expression were much smaller than wild type, and the level of apoptosis was so high that the region between the first and second mitotic waves, which normally contains high levels of dE2F1, was almost completely eliminated (Fig. 7B). As an alternative way to visualize the disappearance of cells from the MF, the same experiment was performed but discs were stained with antibodies to p-Erk and Atonal. Atonal is normally present in the

MF and is expressed independent of DER activity. As expected, expression of *DER*^{dn} eliminated p-Erk staining of IG cells in both wild-type control and *rbf1* mutant discs. However, cells expressing Atonal were completely eliminated by *DER*^{dn} expression in *rbf1* mutant discs but not in wild-type control discs (Fig. 7C). We conclude that RBF and EGFR cooperate to prevent apoptosis.

How do the functions of RBF1 and EGFR converge? Previous studies showed that dE2F1 can regulate transcription of proapoptotic genes such as *reaper*, *ark*, and *hid* (2, 47, 75). Among these targets, HID-dependent apoptosis stands out because it is known to be suppressed by Ras/Raf/Mapk signaling (reviewed in reference 1). EGFR antagonizes HID function in two ways: by repressing transcription of *hid* and by promoting an inhibitory phosphorylation of HID. This regulation is an unusual property that distinguishes HID from other *Drosophila* inducers of apoptosis such as *Reaper* and *Grim* (6, 41). Since HID is a key regulator of cell death in *Drosophila* and our previous studies have demonstrated that dE2F1 is physically present at the promoter region of *hid* (47), we examined the possibility that RBF1 and EGFR might cooperate to protect cells from apoptosis by regulating HID.

In situ hybridization experiments demonstrate that *hid* expression is elevated in *rbf1*^{120a} eye discs (Fig. 8A). This increase was confirmed by real-time quantitative PCR of *hid* transcripts using mRNA prepared from *rbf1*^{120a} mutant eye discs and *rbf1*^{120a} mutant larvae (Fig. 8B). To test if the increase in *hid* expression was functionally significant, we asked whether reducing the gene dose of *hid* level would alter the level of apoptosis in *rbf1*^{120a} mutant eye discs. A single copy of the *Df(3L)x14* chromosome, which lacks the gene region containing *hid*, was sufficient to suppress most of the ectopic cell death in *rbf1*^{120a} mutant eye discs (Fig. 8C). In contrast, *Df(3L)x38*, which deletes *reaper* and *sickle*, had no effect.

hid transcripts were elevated in *rbf1*^{120a} mutant eye discs in both the MF and in the anterior region of the disc that contains asynchronously dividing cells (Fig. 8A), whereas apoptosis was evident only in the MF. This difference might be due to the presence of other apoptotic RBF1 targets that are specifically deregulated at the MF, or it might be caused by developmental signals that sensitize cells in the MF to HID-induced cell death. To test this, we examined the effects of expressing *hid* using a heat shock (hs)-inducible transgene and asked if ubiquitous expression of *hid* was sufficient to generate a restricted pattern of apoptosis as seen in *rbf1*^{120a} mutant eye discs. When induced at high levels, ectopic *hid* expression was able to induce apoptosis in most cells. However, a low level of *hid* expression from a short pulse of heat shock treatment gave a specific pattern of apoptosis, indicating that some cells are much more sensitive to HID-induced apoptosis than others. Cell death was largely restricted to the MF and was most evident adjacent to the IG (Fig. 8D). In contrast, this portion of the eye disc was relatively insensitive to apoptosis induced from an *hs-reaper* transgene, excluding the possibility that cells in this region are generally more sensitive to apoptosis.

Taken together, these results show that *hid* mRNA is elevated in *rbf1* mutants, that the increased levels of *hid* expression are important for the apoptosis that occurs in the absence of RBF1, and that the region anterior to the IG, where cell death occurs in *rbf1*^{120a} mutant eye discs, is the part of the disc

FIG. 7. RBF1 and EGFR cooperate to protect cells from apoptosis. This figure shows the effects of expressing a dominant-negative mutant of DER (*DER^{dn}*) in wild-type (A) and *rbf1^{120a}* (B) eye discs. Anti-cyclin B (Cyc B) and anti-C3 antibodies were used to visualize the patterns of cell death. The enlarged regions are indicated by the dashed boxes. (C) Eye discs from the indicated genotypes were coimmunostained with anti-Atonal (Ato) and anti-p-Erk antibodies. Expression of *DER^{dn}* inhibits p-Erk staining patterns in both control and *rbf1^{120a}* eye discs. The widespread cell death caused by *DER^{dn}* in *rbf1^{120a}* eye discs causes the almost complete loss of Atonal-expressing cells.

that is most sensitive to *hid*-induced cell death. Taken together with the previously published work (6, 41), these results suggest a simple molecular explanation for the sensitivity of *rbf1* mutant cells to DER/Ras signaling pathways. The absence of RBF1 leads to a slight increase in *hid* transcription. In cells where EGFR signaling is high, these effects can be neutralized by DER/Ras/Raf-induced phosphorylation of HID. However, in cells exposed to Argos, in which DER signaling is low, the elevated level of HID makes *rbf1* mutant cells prone to apoptosis (Fig. 8E).

DISCUSSION

pRB is a key component in most models of cell cycle control, and the pRB pathway is thought to be functionally inactivated in most tumor cells. However, in vivo studies show that mutation of *Rb* has different consequences in different cell types. In many situations, the inactivation of *Rb* renders cells sensitive to apoptosis. This change limits the proliferative capacity of *Rb*^{-/-} cells, and their ability to form tumors. However, the reasons why some *Rb*^{-/-} cell types survive while other cell types die are largely unknown.

Here, we have taken advantage of the observation that the inactivation of *rbf1* in the *Drosophila* eye results in a distinctive pattern of apoptosis that is tightly linked to eye development, and we have used this model system to define a cellular context in which RBF1 is needed to protect cells against dE2F1-dependent cell death. The results show that the cellular response to the inactivation of *rbf1* involves a combination of signals. Deregulated dE2F1 provides one function that is required for apoptosis. However, in most situations, deregulation of the endogenous dE2F1 is not sufficient to induce apoptosis. In addition, a second condition, the down-regulation of an EGFR/Ras/Raf signaling pathway, is also necessary. In the eye imaginal disc, the EGFR/Ras/Raf signaling pathway is down-regulated at the region immediately anterior to the IG. When *rbf1* mutant cells pass through this gradient, they become highly sensitive to dE2F1-dependent apoptosis. Elevation of the level of DER/Ras/Raf signaling by a variety of means suppressed apoptosis in *rbf1* mutant cells. Conversely, expression of a dominant-negative mutant of DER strongly synergized with mutation of *rbf1* to induce apoptosis.

Before starting this work, we considered several different ways in which the inactivation of RBF1 might result in apoptosis. If differentiated/differentiating cells try to reenter the cell cycle following the inactivation of RBF1, then an abnormal or inappropriate S-phase entry might cause apoptosis. Alterna-

tively, one could argue that rapidly proliferating cells contain the highest levels of E2F transcriptional activity, and hence these cells ought to be most sensitive to E2F-induced apoptosis when RBF1 is removed. Although both models were plausible, in fact, neither explanation fits our data. *rbf1* mutant eye discs display little apoptosis in either the population of differentiated cells or in actively cycling cells. Instead, *rbf1* mutant cells are sensitive to apoptosis in the MF, at a time when some cells exit the cell cycle and initiate a differentiation program. This apoptosis was not accompanied by inappropriate cell cycle progression. Indeed, when *rbf1* mutant cells were rescued from apoptosis, they showed no indication of S-phase entry. Hence *rbf1* mutant cells were not dying because they were inappropriately progressing through the cell cycle. Instead, apoptosis was dependent on a specific developmental context. This need for the correct context may be particularly significant when designing cell culture-based screens for treatments that are synthetic lethal with the inactivation of *Rb*.

Several studies have shown that E2F complexes regulate the expression of proapoptotic genes, but why would the effects of losing RBF1 be sensitive to EGFR signaling? While it seems likely that deregulated dE2F1 activates transcription of several proapoptotic targets, our results indicate that an important part of the explanation lies in the regulation of the proapoptotic gene *hid*. *hid* transcripts are up-regulated in *rbf1* mutant eye discs and halving the gene dosage of *hid* dramatically reduced apoptosis. Previous studies have shown that HID-induced apoptosis is highly sensitive to EGFR/Ras/Raf signaling. Signaling through this pathway suppresses transcription of *hid* and is thought to induce an inhibitory phosphorylation on the HID protein. This provides a simple model, in which the loss of RBF1 results in the elevated expression of a proapoptotic protein, which is then held in check by EGFR/Ras/Raf-mediated signaling. Apoptosis would then occur when EGFR signals are reduced. Consistent with this model, we found that the region of the eye disc that is most sensitive to loss of RBF1 is also highly sensitive to low levels of ectopic *hid* expression.

Why does this pattern of apoptosis occur? It is likely that several different factors are needed to establish the gradient of DER activity. Important regulators of DER activity in the eye include Gap1, Sprouty, and Argos. In this particular context, the ability of Argos to diffuse and act at a distance from the p-Erk-positive cells appears to be important. We suggest that the pattern of Argos expression in the developing eye disc generates a zone in which cells that have failed to exit the cell cycle and inappropriately inactivate RBF1 become prone to undergo apoptosis. In essence, this could be viewed as a de-

FIG. 8. *hid* is up regulated in *rbf1* mutant eye discs and is required for apoptosis. (A) In situ hybridization with a riboprobe of *hid* antisense sequences shows that *hid* mRNA is deregulated in *rbf1*^{120a} eye discs. (B) Real-time quantitative PCR was used to measure the change in *hid* mRNA levels in *rbf1*^{120a} eye discs (left panel) or whole larvae (right panel). Two known RBF1/dE2F1 targets, *mnr2* and *cyclin E*, served as positive controls. Expression levels were measured in comparison to the *rp49* housekeeping gene and the changes (fold) in *rbf1*^{120a} samples are shown relative to wild-type controls. Averages were calculated using triplicate samples. (C) Heterozygosity of *hid* suppresses cell death in *rbf1*^{120a} mutant eye discs. Apoptotic cells were visualized with anti-C3 in third instar eye discs of the indicated genotypes. *Df(3L)x14* removes *hid*, while *Df(3L)x38* removes *sickle* and *reaper*. Note the almost complete absence of cell death in *rbf1*^{120a} eye discs heterozygous for *Df(3L)x14*. (D) Cells in the MF are highly sensitive to a low level of *hid* expression. Short heat-shock pulses were used to induce expression from *hs-hid* or *hs-reaper* transgenes. Larvae were placed at 37°C for 10 min and allowed to recover at 25°C for 1 h. The patterns of cell death before and after heat shock were visualized with anti-C3. Note the similar patterns of cell death in *rbf1*^{120a} and *hid*-expressing eye discs. (E) A model illustrating the convergence of RBF1 and DER/Ras on the regulation of HID-induced cell death (see text for details).

developmental failsafe mechanism against inappropriate proliferation. In support of this, we note that E2F1 levels are transiently elevated in G₁ phase cells in the MF, even though E2F regulation is not needed for S phase entry in the second mitotic wave. Consistent with the idea that this region of the disc may be more sensitive to apoptosis, we find that a transient pulse of cyclin E expression, which drives ectopic S phases throughout much of the disc, generates a similar stripe of apoptosis in the MF (data not shown). It is curious that this sensitivity occurs at the time when the role of EGFR is apparently changing from being needed for cell proliferation in the anterior part of the disc to being required for differentiation in the posterior part of the disc (16, 43). It will be interesting to discover whether similarly sensitive regions exist in other discs.

As seen with *rbf1*, the effects of mutating *Rb* in the mouse are most evident at points in development when cells attempt to exit the cell cycle and differentiate. *Rb*-null mouse retinas show increased cell death during the transition from proliferation to differentiation (11, 46). Whether this is due to an analogous interaction between Rb/E2F and EGFR/Ras signaling has not been tested but is an interesting possibility. It is also tempting to speculate that some of the different cellular responses to the inactivation of Rb in the mouse retina may be caused by differences in EGFR/Ras-mediated differentiation signals.

There are several indications that the general phenomenon described here is likely to be conserved in mammalian cells. For example, recent studies have shown that apoptosis in cultured fibroblasts lacking Rb family proteins (TKO) can be suppressed by activation of Ras/Raf (73). Interestingly, a functional homologue of Hid, Smac/Diablo, was recently shown to be a direct target of E2F1 in mammalian cells (69), raising the possibility that mammalian cells may contain a regulatory loop that directly parallels the regulation of Hid. However, a connection between the proapoptotic function of Smac/Diablo and EGFR pathway has yet to be described.

The molecular events underlying the convergence of EGFR signaling and Rb/E2F may be different between flies and humans. We note that Akt activation suppresses E2F1-induced apoptosis in mammalian tissue culture cells (10, 31), while neither the overexpression of dAKT1 nor the mutation of dPTEN was sufficient to prevent cell death in *rbf1* mutant eye discs (Fig. 5) (data not shown). This may reflect a difference between an in vivo analysis and tissue culture conditions, or it may reflect species-specific differences in the regulation of apoptosis. It is known, for example, that caspase activation is regulated differently between species. In vertebrates, cytochrome *c* release from mitochondria is a key step in the promotion of caspase activation, while in *Drosophila*, this step is largely dispensable (reviewed in reference 38). It is possible that EGFR activity converges on E2F-dependent cell death through a previously identified E2F target whose activity is regulated by Raf/Erk- and/or AKT-mediated signals, such as Bim (33, 44). In order to define this circuitry, we first need to identify the appropriate in vivo context in mice or humans in which Rb/E2F and EGFR activity cooperate to regulate cell survival. Once the appropriate context is found, then it may be possible to identify the molecular mechanism linking E2F-dependent cell death to survival signals.

Both EGFR family and Rb pathways are often altered in cancer. Given that developmentally controlled fluctuations in

EGFR signaling have dramatic effects on the sensitivity of *rbf1* mutant cells to apoptosis, we speculate that therapeutic cancer drugs that target EGFR family proteins may induce cell death most efficiently in tumor cells that have the highest levels of E2F1 activity. One of the curious features of human retinoblastoma is that, unlike many other cancers, these tumors rarely contain mutations in p53, suggesting that either these cells do not need to mutate p53 or that they find a more effective way to suppress apoptosis (reviewed in reference 27). Identification of the critical components that protect premalignant *Rb* mutant cells from apoptosis may lead to new ways to target these cells for treatment.

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