The role of RBF in developmentally regulated cell proliferation in the eye disc and in Cyclin D/Cdk4 induced cellular growth

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SUMMARY

During *Drosophila* eye development, cell proliferation is coordinated with differentiation. Immediately posterior to the morphogenetic furrow, cells enter a synchronous round of S phase called second mitotic wave. We have examined the role of RBF, the *Drosophila* RB family homolog, in cell cycle progression in the second mitotic wave. RBF-280, a mutant form of RBF that has four putative cdk phosphorylation sites mutated, can no longer be regulated by Cyclin D or Cyclin E. Expression of RBF-280 in the developing eye revealed that RBF-280 does not inhibit G1/S transition in the second mitotic wave, rather it delays the completion of S phase and leads to abnormal eye development. These observations suggest that RB/E2F control the rate of S-phase progression instead of G1/S

transition in the second mitotic wave. Characterization of the role of RBF in Cyclin D/Cdk4-mediated cellular growth showed that RBF-280 blocks Cyclin D/Cdk4 induced cellular growth in the proliferating wing disc cells but not in the non-dividing eye disc cells. By contrast, RBF-280 does not block activated Ras-induced cellular growth. These results suggest that the ability of Cyclin D/Cdk4 to drive growth in the proliferating wing cells is distinct from that in the none-dividing eye cells or the ability of activated Ras to induce growth, and that RBF may have a role in regulating growth in the proliferating wing discs.

Key words: Cellular growth, Cell proliferation, Cyclin D, Cyclin E, E2F, RB phosphorylation, *Drosophila*

INTRODUCTION

The RB and E2F transcription factors are key regulators of the G1 and S phases of the cell cycle (reviewed by Weinberg, 1995; Dyson, 1998). E2F transcription factors regulate a large set of cell cycle genes, including genes of the DNA replication machinery, as well as regulators of DNA replication such as *Cyclin E*. The prevailing view is that the RB/E2F complex functions primarily to regulate G1/S transition. However, most of these conclusions are based on tissue culture studies. The function of RB in developmentally controlled cell proliferation is still not quite clear.

It is well established that the function of pRB is regulated by phosphorylation. Cyclin D- and Cyclin E-dependent kinases are implicated in the phosphorylation of pRB (Mittnacht, 1998). Recent studies suggest that functional inactivation of pRB requires sequential phosphorylation by both Cyclin D- and Cyclin E-dependent kinases (Lundberg and Weinberg, 1998). Furthermore, the phosphorylation of pRB by different cyclin-dependent kinases inactivates separate functions of pRB (Zarkowska and Mittnacht, 1997; Harbour et al., 1999). However, there are still controversies as to which sites are phosphorylated specifically by Cyclin D, which sites are phosphorylated by Cyclin E and which sites are crucial for the

function of pRB (Kitagawa et al., 1996; Connell-Crowley et al., 1997). Systematic mutagenesis of the phosphorylation sites of pRB showed that multiple phosphorylation sites have a cumulative effect on the function of pRB (Brown et al., 1999). Overexpression of RB that lacks consensus cdk phosphorylation sites can efficiently cause G1 arrest, consistent with a role of the RB/E2F pathway in regulating G1/S transition. Interestingly, the G1 arrest induced by expression of a mutant form of RB lacking all consensus cdk phosphorylation sites can be bypassed by co-expression of Cyclin D- or Cyclin E-dependent kinases (Leng et al., 1997). In addition, these types of studies have also revealed a role for RB in regulating S-phase progression that is independent of G1/S transition (Chew et al., 1998; Knudsen et al., 1998). These studies, while establishing the importance of RB phosphorylation in cell cycle regulation, do not address the consequences of expressing a non-regulated RB on cell growth and proliferation in normal developmental setting in vivo.

The *Drosophila* RB family homolog RBF has been shown to bind to E2F1 and regulate E2F target gene expression both in vivo and in transient transfection assays (Du et al., 1996a; Du and Dyson, 1999). In addition, RBF and E2F1 show an antagonistic relationship in vivo (Du, 2000) similar to the interaction of Rb and E2F1 in mice (Tsai et al., 1998). These

results indicate that the functional relationship between RB and E2F is conserved between *Drosophila* and mammalian systems. In addition, the conserved genes encoding Cyclin D/Cdk4 and Cyclin E/Cdc2c kinases have been identified in *Drosophila*, raising the possibility that regulation of RB by G1 cyclin-dependent kinases is also conserved in *Drosophila*.

The *Drosophila* developing eye provides a nice system with which to study cell proliferation in its normal developmental setting where cell proliferation is coordinated with cell fate determination and pattern formation. In the third instar larval eye disc, photoreceptor cell differentiation initiates within the morphogenetic furrow where cells form preclusters (reviewed by Wolff and Ready, 1993). As these preclusters exit the morphogenetic furrow, five photoreceptor cells (R8, R2, R5, R3, R4) are already determined. Those cells that are not in the preclusters go into a synchronous round of cell proliferation (the second mitotic wave) as they exit the morphogenetic furrow. The second mitotic wave provides the source of cells for the stepwise recruitment of the remaining photoreceptor cells, cone cells, pigment cells and bristle cells into the clusters. Disruption of this pattern of cell proliferation often leads to disruption of normal eye development. For example, ectopic expression of human p21 in the developing eye leads to complete elimination of the second mitotic wave. This results in the development of adult eyes with missing bristles and pigment cells, because there is an insufficient amount of cells available to be recruited into individual ommatidia (de Nooij and Hariharan, 1995).

At present, it is not completely clear what drives cells in the second mitotic wave into S phase. Cells in the second mitotic wave express high levels of E2F target genes, suggesting the possibility that RB and E2F might be responsible for controlling G1/S transition there. However, an equally likely explanation is that E2F-mediated target gene expression in the second mitotic wave is merely a consequence and is not a cause of S-phase entry. As RBF will probably be inactivated by the key S-phase regulator Cyclin E in the second mitotic wave, a form of RBF that can not be regulated by Cyclin E will be needed to determine the role of RBF and E2F in the second mitotic wave cells.

Recent studies have also suggested that Cyclin D/Cdk4 can function to promote growth (Datar et al., 2000; Meyer et al., 2000). Flies that lack Cdk4 are smaller as are cyclin D1 or cdk4 knockout mice (Fantl et al., 1995; Sicinski et al., 1995; Rane et al., 1999; Tsutsui et al., 1999; Meyer et al., 2000). In *Drosophila*, Cyclin D/Cdk4 overexpression in postmitotic cells of the developing eye causes cell enlargement, suggesting that Cyclin D/Cdk4 can promote growth and lead to hypertrophy in non-dividing cells. By contrast, Overexpression of Cyclin D/Cdk4 in the proliferating wing discs leads to increased growth and increased rate of cell proliferation, with normal cell size and cell-cycle phasing (hyperplasia) (Datar et al., 2000). The effect of CyclinD/Cdk4 on cell growth was suggested to be independent of RBF, as wild-type RBF does not block the ability of Cyclin D/Cdk4 to induce growth (Datar et al., 2000). These results, however, are difficult to interpret, as Cyclin D/Cdk4 overexpression will probably inhibit the function of wild-type RBF. A form of RBF that cannot be regulated by Cyclin D is needed to determine the effect of RBF on the ability of Cyclin D to induce growth.

In this report, we show that both *Drosophila* Cyclin D/Cdk4 and Cyclin E/Cdc2c indeed regulate RBF. In addition, we generated a mutant form of RBF, RBF-280, that cannot be regulated by CyclinD/Cdk4 and Cyclin E/Cdc2c. Expression of RBF-280 in the second mitotic wave does not inhibit S phase there. Instead, it delays the completion of S phase and leads to significant defects in *Drosophila* eye development. These results suggest RBF affect primarily S-phase progression instead of G1/S transition in the second mitotic wave. Genetic interactions between RBF-280 and Cyclin D show that Cyclin D cannot suppress the RBF-280-induced phenotypes, and RBF-280 cannot suppress Cyclin D/Cdk4-induced large eye phenotypes, even though RBF-280 blocks ectopic S phase-induced by Cyclin D/Cdk4. These observations are consistent with the idea that in the developing eye Cyclin D/Cdk4 induces cell proliferation through RBF, while Cyclin D/Cdk4 induces cellular growth independently of RBF. In the proliferating wing discs, however, RBF-280 blocks cellular growth induced by Cyclin D/Cdk4 but not by activated Ras, suggesting that activated Ras and Cyclin D/Cdk4 induce growth through distinct mechanisms, and that inactivating RBF is required for Cyclin D/Cdk4 to drive growth in the proliferating wing discs.

MATERIALS AND METHODS

Fly stocks

The following fly strains were used in this study: GMRDap (de Nooij and Hariharan, 1996), GMRCyclin D (Datar et al., 2000), UASCyclin D, UASCdk4 (Meyer et al., 2000) and UAS Ras[V12] (Karim and Rubin, 1998). The GMRRBF[4C]/+ fly used in this paper was generated by hopping the GMRRBF[2]/+ described previously (Du et al., 1996a). The GMRRBF[4C]/+ fly showed similar but weaker phenotypes than the published GMRRBF[4] (=GMRRBF[2]/GMRRBF[2]) phenotypes (Du et al., 1996a). In addition, the GMRRBF[4C]/+ fly showed the same genetic interactions with *de2f1* and *Cyclin E* as the did the GMRRBF[4] fly. As all the P-element insertions in GMRRBF[4C]/+ were on the same chromosome, the GMRRBF[4C]/+ flies were used to test genetic interactions in this study.

Site-directed mutagenesis and generation of transgenic flies

Oligonucleotides with specific mutations were introduced into full-length RBF cDNA by PCR and subcloning. All the mutations were verified by direct sequencing.

To generate transgenic flies, 25 µg of CsCl purified P-element plasmid were mixed with 5 µg of the helper plasmid Δ2-3 in injection buffer (0.1 M sodium phosphate, pH 7.8; 5 mM MgCl₂). These plasmids were injected into 0- to 2-hour-old W1118 embryos to generate transgenic flies. Multiple independent P-element insertions were generated for each mutant form of RBF and the phenotypes shown in this report are not insertion specific.

Transfection, CAT assay and yeast two-hybrid interaction assay

E2F4CAT reporter (2 µg), 4 µg of Copia β-gal, and 1 µg of each of the plasmids dE2F1, dDP, RBF, Cyclin D, Cdk4, Cyclin E and Cdc2c were used as indicated. Total plasmid DNA used in transfection was adjusted to 22 µg with vector plasmids. SL2 cells were plated at 1×10^6 cells/ml 1 day before transfection, and were transfected with the calcium phosphate method (Chen and Okayama, 1988). Transfected cells were harvested 48 hours later, and extracts were made by freezing and thawing three times in 0.25 M Tris buffer (pH 8.0). The β-gal assay was carried out by adding 30 µl of cell extracts, 66 µl of 4 mg/ml ONPG (O-nitrophenyl-β-D-galactopyranoside), 3 µl of

100× Mg buffer (for 1 ml, mix 100 µl 1 M MgCl$_2$, 350 µl β-mercaptoethanol, 550 µl H$_2$O), and 201 µl of 0.1 M sodium phosphate buffer (pH 7). The CAT assay was carried out by mixing 60 µl of extracts with 40 µl of acetyl CoA mix (1.8 µl 5 mM acetyl CoA, 2.0 µl 14C acetyl CoA, 0.46 µl 68 mg/ml chloramphenicol and 35.7 µl H$_2$O).

For the yeast two-hybrid interaction assay, the *Eco*RI fragment of RBF cDNA containing the large pocket domain was fused in frame with the GAL4 DNA-binding domain (in pPC97 vector). dE2F1 cDNA was fused in frame with the GAL4 transactivation domain (in pPC86 vector). These two plasmids were transformed into Mav103 yeast. Transformants were selected on plates lacking Leu and His [sc-L-T]. Interactions were determined by assaying for β-gal activity and the ability to grow on [sc-L-T-H+3AT (10 mM)] plates.

SEM, staining and in situ hybridization

SEM, staining and in situ hybridization were carried out as described previously (Du, 2000). The average number of S-phase cells in the second mitotic wave was determined by counting S phase cells within a 70 µm region along the dorsal/ventral axis in the center of each eye imaginal disc. The sample number is six eye imaginal discs for the wild type and five for GMRRBF-280[4]. The average number of mitosis was determined by counting mitotic cells within a 160 µm region posterior to the morphogenetic furrow of each eye imaginal disc. The sample numbers are ten eye discs each for wild type and GMRRBF-280[4].

Proliferation and growth rate analysis

Overexpression clones were generated as described previously (Datar et al., 2000). Third instar wing discs were dissected out, fixed, mounted and photographed. Clone areas were measured with the histogram function of Adobe Photoshop. The number of GFP positive cells per clone was counted. All data were analyzed with Boxplot. Data points beyond 1.5 IQR (Inter Quartile Range) of the upper or lower quartile were discarded. Two sample T-test were employed to compare the distribution of independent data sets. Cell doubling times were derived using the following formula: (log 2/log n)hour, where n is the average number of cells /clone and hour is the number of hours between the heat shock and disc fixation.

RESULTS

Phosphorylation mutants of RBF

Mammalian RB family proteins are regulated by Cyclin D- and Cyclin E-dependent kinases. Inspection of the *Drosophila* RB family homolog, RBF, revealed several potential consensus cdk phosphorylation sites. To test if these putative cdk phosphorylation sites confer regulation by *Drosophila* Cyclin D- or Cyclin E-dependent kinases, we mutated several of these sites (SP or TP sites) into AP sites (Fig. 1A). A transient transfection assay was used to test if these mutant forms of RBF could still regulate E2F. Transfection of E2F1 and DP strongly activates the expression of a CAT reporter with E2F binding sites; co-transfection of wild-type RBF strongly inhibits such activation (Dynlacht et al., 1994; Ohtani and Nevins, 1994; Du et al., 1996a). RBF-10, -223, -M4 and -280 can inhibit the transactivation by E2F1 (Fig. 1B, lanes 8,11,14,17). By contrast, RBF-30 was defective in the ability to inhibit transactivation by E2F1. E2F1 can increase reporter expression by about twofold in the presence of wild-type RBF (Fig. 1B, lanes 1,5), but can increase reporter expression about 60-fold in the presence of RBF-30. The inability of RBF-30 to inhibit transactivation by E2F1 is due to the inability of

RBF-30 to bind E2F1 (Fig. 1C). We conclude that all the RBF putative phosphorylation site mutants generated, with the exception of RBF-30, retain the ability to regulate dE2F1.

Wild-type RBF is regulated by G1 cyclin-dependent kinases Cyclin D/Cdk4 and Cyclin E/Cdc2c

To test if *Drosophila* Cyclin D- and Cyclin E-dependent kinases regulate RBF, we tested the effect of co-expression of Cyclin D/Cdk4 or Cyclin E/Cdc2c on the ability of RBF to repress transactivation by E2F1. Expression of Cyclin D/Cdk4 or Cyclin E/Cdc2c does not significantly affect transactivation by E2F1/DP in the absence of co-transfected RBF (Fig. 1B, columns 2-4). By contrast, co-expression of Cyclin D/Cdk4 or Cyclin E/Cdc2c together with wild-type RBF significantly impairs the ability of RBF to repress E2F1/DP transactivation, leading to a five- to tenfold increase in E2F reporter activity (Fig. 1B, compare columns 5, 6 and 7). These results suggest that both Cyclin D/Cdk4 and Cyclin E/Cdc2c can regulate RBF. The observed regulation of RBF requires co-expression of Cyclin D and Cdk4, or Cyclin E and Cdc2c. Expression of either cyclin alone or cdk alone does not significantly affect the ability of RBF to repress dE2F1 transactivation (data not shown). In addition, an N-terminal deletion of Cyclin D that lacks the LXCXE motif (Finley et al., 1996; Datar et al., 2000) cannot regulate the function of RBF either (data not shown). In summary, these observations suggest both Cyclin D/Cdk4 and Cyclin E/Cdc2c can regulate the ability of RBF to inhibit E2F1.

Mutating the putative cdk phosphorylation sites in RBF disrupts the regulation by Cyclin D/Cdk4 and Cyclin E/Cdc2c

To test if mutating the potential consensus cdk phosphorylation sites in RBF will disrupt the regulation by D or E type cyclin-dependent kinases, we tested if the putative cdk phosphorylation site mutations described above made RBF resistant to the co-transfected Cyclin D/Cdk4 or Cyclin E/Cdc2c. As shown in Fig. 1B, mutating two putative C-terminal phosphorylation sites into A (760A, 771A) in RBF–223 does not significantly affect its regulation by Cyclin D/Cdk4 or Cyclin E/Cdc2c. By contrast, mutating three putative C-terminal phosphorylation sites (728A, 760A, 771A) in RBF-10 or a single 356A mutation in RBF-M4 significantly affected the regulation of these RBF mutants by Cyclin D/Cdk4 and Cyclin E/Cdc2c (Fig. 1B, columns 11-13 and 17-19). Interestingly, RBF-280, which combines the mutations of RBF-10 and RBF-M4 (356A, 728A, 760A 771A), strongly represses E2F1 transactivation and is completely unresponsive to the co-transfected Cyclin D/Cdk4 or Cyclin E/Cdc2c (Fig. 1B, columns 8-10). We conclude from these results that there are at least two putative phosphorylation sites (T356 and S728) in RBF that contribute to the regulation by Cyclin D/Cdk4 or Cyclin E/Cdc2c. Most importantly, RBF-280, which has these crucial phosphorylation sites mutated, is unresponsive to the regulation by Cyclin D/Cdk4 or Cyclin E/Cdc2c.

Developmental consequences of expressing different mutant forms of RBF

To test the importance of regulation of RBF by Cyclin D and Cyclin E during normal development, we tested the ability of different phosphorylation site mutants of RBF to rescue *rbf*

null lethality as described before (Du, 2000), and we examined the developmental consequences of overexpressing different forms of RBF.

Interestingly, abolishing the regulation of RBF by Cyclin D and Cyclin E does not abolish the ability of RBF to rescue the lethality of *rbf* null mutants. RBF-10, which has three putative phosphorylation sites mutated and retains partial regulation by Cyclin D and Cyclin E (see Fig. 1B), can rescue the lethality of *rbf* null mutants at about 50% of the wild-type RBF level. Furthermore, RBF-280, which cannot be regulated by Cyclin D or Cyclin E, is also able to rescue the lethality of *rbf* null mutants, although the number of *rbf* null flies that are rescued to adults by RBF-280 is significantly fewer, apparently because expression of RBF-280 but not wild-type RBF or other mutant forms of RBF causes significant lethality at pupae stage (only about 30% of the expected number of flies with RBF-280 expression survived to adult stage). The only mutant form of RBF that cannot rescue the lethality of *rbf* null mutants is RBF-30, which is defective in E2F1 binding (Fig. 1C). In addition, RBF-555L and RBF-596W have mutations in the two residues that are conserved between RBF and human RB – the

corresponding mutations in human RB are associated with tumors (Sellers et al., 1998). Both mutations in RBF impair the binding to E2F1 (Fig. 1C) and neither form of RBF can rescue the lethality of *rbf* null mutants. These results suggest that the ability of RBF to bind to E2F1 but not its regulation by Cyclin D and Cyclin E correlates with the ability of RBF to rescue *rbf* null lethality. In addition, regulation of RBF by Cyclin D and Cyclin E appears to be crucial for the expressed RBF to be tolerated during development.

To characterize further the in vivo consequences of expressing different mutant forms of RBF, we expressed these RBF mutant constructs in the developing eye using the eyeless GAL4 driver. Eyeless GAL4 targets expression anterior to the morphogenetic furrow in the third instar eye disc, and in the embryonic central nervous system as well as in the eye-antennal disc primordia (Quiring et al., 1994). Expression of wild-type RBF disrupts normal eye development, resulting in the development of smaller and abnormally shaped eyes (Fig. 2B). Expression of RBF-223, which does not significantly affect regulation by Cyclin D or Cyclin E, induces similar eye phenotypes as does expressing wild-type RBF (Fig. 2C). By
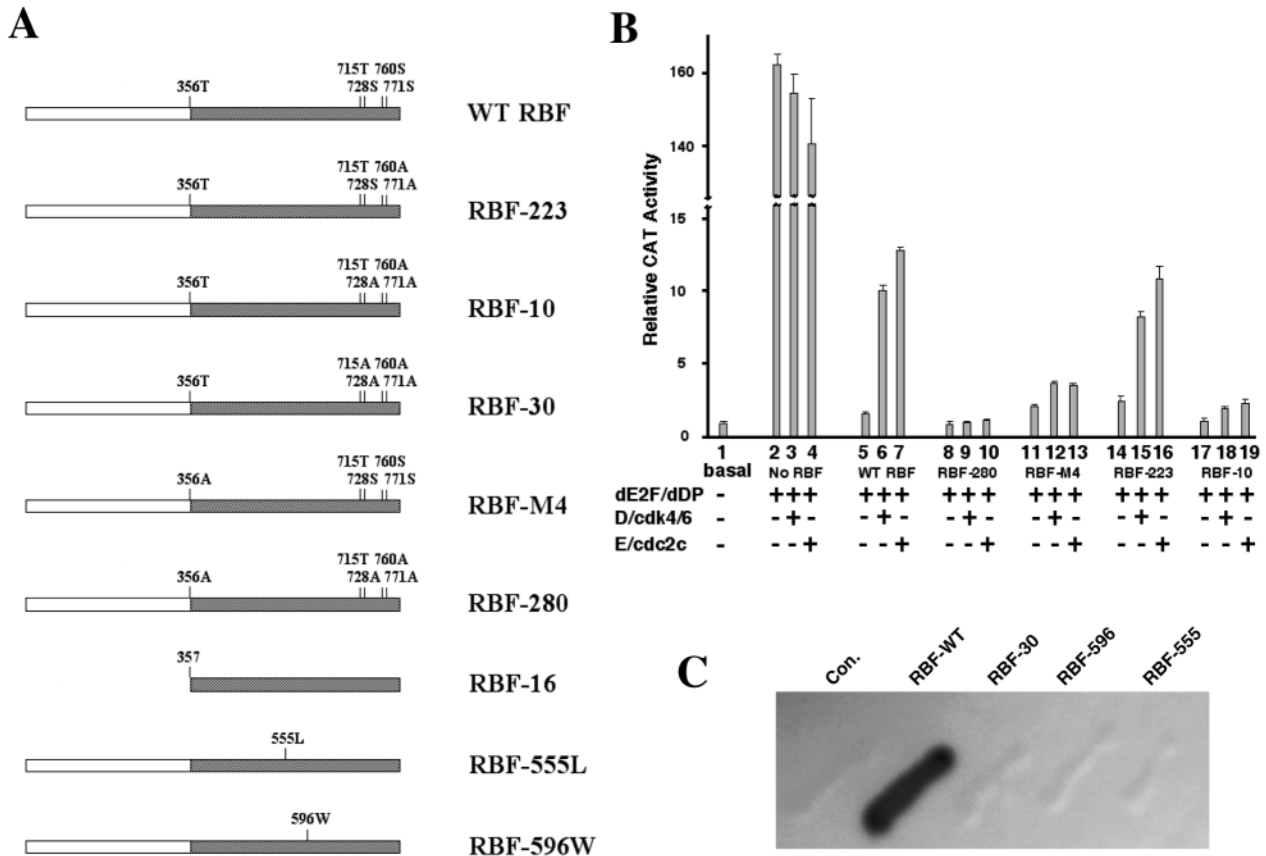

Fig. 1. (A) The putative cdk phosphorylation sites in RBF and the different RBF mutants generated. There are two other potential phosphorylation sites (T83 at the N terminus and T610 in the middle of the pocket domain) in RBF that are not mutated and are not shown. (B) The effects of Cyclin D/Cdk4 and Cyclin E/Cdc2c on wild-type RBF and RBF phosphorylation site mutants. SL2 cells were transfected with the E2F4CAT reporter construct, together with the E2F1/DP, Cyclin D/Cdk4, Cyclin E/Cdc2c, and the different RBF constructs as indicated. Basal indicates cells transfected with E2F4CAT reporter only. D/Cdk4 represents *Drosophila* Cyclin D and Cdk4. E/Cdc2c represents *Drosophila* Cyclin E and Cdc2c. '+' indicates that a given construct was co-transfected; '-' indicates that the construct was not co-transfected. (C) A yeast two-hybrid interaction assay to test the interaction between several RBF mutants and dE2F1. Various patches of yeast contained the pPC86-dE2F1 plasmid and the plasmids as indicated: Control, pPC97; WT RBF, pPC97-RBF(WT); RBF-30, pPC97-RBF-30; RBF-596, pPC97-RBF-596W; RBF-555, pPC97-RBF-555L. Patches of cells growing on plates selective for the presence of both plasmids were tested for the β-galactosidase activity.

contrast, overexpression of RBF-10, which retains only partial regulation by Cyclin D and Cyclin E, results in much smaller eyes and more severe phenotypes (Fig. 2D). Expression of RBF-280, which cannot be regulated by Cyclin D or Cyclin E, causes lethality. Thus, overexpression of RBF in the proliferating region of the developing eye cause significant disruption of normal eye development, and mutations that affect the regulation by Cyclin D and Cyclin E also strongly affect the phenotypes.

We also tested the consequence of expressing several RBF mutants that cannot bind E2F1. As shown in Fig. 2E-G, expression of RBF-30, RBF-555L and RBF-596W in the developing eye failed to cause eye defects. It is worth noting that all three of the putative phosphorylation sites mutated in RBF-10 are also mutated in RBF-30 (Fig. 1A). In addition, the expression levels of RBF-10 and RBF-30 are similar. These observations suggest that the severe eye phenotype induced by RBF-10 overexpression requires E2F1 binding. In addition, expression of RBF-16, which retains E2F1-binding activity, induces similar eye phenotypes as expressing does wild-type RBF (Fig. 2H). Thus, the ability of overexpressed RBF to induce developmental defects requires E2F1 binding, and the severity of the phenotypes is significantly affected by the regulation by Cyclin D and Cyclin E.

Expression of RBF-280 does no inhibit S phase but delays S phase completion in the second mitotic wave

The developing eye was used as a model system to further characterize the function of RB/E2F in development and in developmental regulated cell proliferation. As shown in Fig. 2I, expression of the non-regulated RBF-280 (GMRRBF-280[4]) posterior to the morphogenetic furrow results in the development of a very rough eye. There are a lot of fused ommatidia (Fig. 2J, arrowhead) and almost complete loss of bristles (Fig. 2I,J). In addition, while ommatidia from wild-type eyes have hexagonal shape, the ommatidia from GMRRBF-280[4] flies show variable shapes (Fig. 2J,K, arrow). Similar phenotypes (although less severe) are observed when wild-type RBF (GMRRBF[4]) is overexpressed (Du et al., 1996a). The phenotypes of GMRRBF[4] are shown to be due to missing cone cells, pigment cells and bristles (Du et al., 1996a).

The effect of RBF-280 on E2F target gene expression and cell cycle progression was analyzed. Expression of *PCNA*, an E2F target gene, was analyzed by in situ hybridization in the GMRRBF-280[4] eye discs. As shown in Fig. 3, in wild-type third-instar larval eye discs, *PCNA* is highly expressed in cells anterior to the morphogenetic furrow, as well as in cells corresponding to the second mitotic wave. The high level of *PCNA* expression in the second mitotic wave is repressed in GMRRBF-280[4] eye discs (Fig. 3A-D). We conclude that expression of RBF-280 is

sufficient to block the endogenous E2F target gene expression in the second mitotic wave.

To test if expression of RBF-280 also inhibits S phase in the second mitotic wave, BrdU incorporation assay was carried out. Interestingly, expression of RBF-280 does not block the BrdU incorporation in the second mitotic wave (Fig. 4B,F). However, compared with wild-type eye disc, the second mitotic wave from GMRRBF-280[4] eye disc is broader (compare Fig. 4A,E with Fig. 4B,F) and the BrdU staining is weaker.

As the morphogenetic furrow and the second mitotic wave move from posterior to anterior at a speed of about 1.5 hours/row of ommatidia cluster (Wolff and Ready, 1993), the above observations suggest that expression of RBF-280 may delay S-phase entry in the second mitotic wave or delay the S phase completion. As there is only one round of cell proliferation in the second mitotic wave, one might expect to have more S-phase cells in the second mitotic wave in GMRRBF-280[4] eye discs if S phase entry is not inhibited but S phase completion is delayed. Indeed, counting the number of S-phase cells in the second mitotic wave shows that there is an average of 73±9 S-phase cells in the wild-type eye disc. By contrast, there is an average of 125±13 S-phase cells in the broader second mitotic wave in GMRRBF-280[4] eye discs (80±5 S-phase cells are in the same region as the second
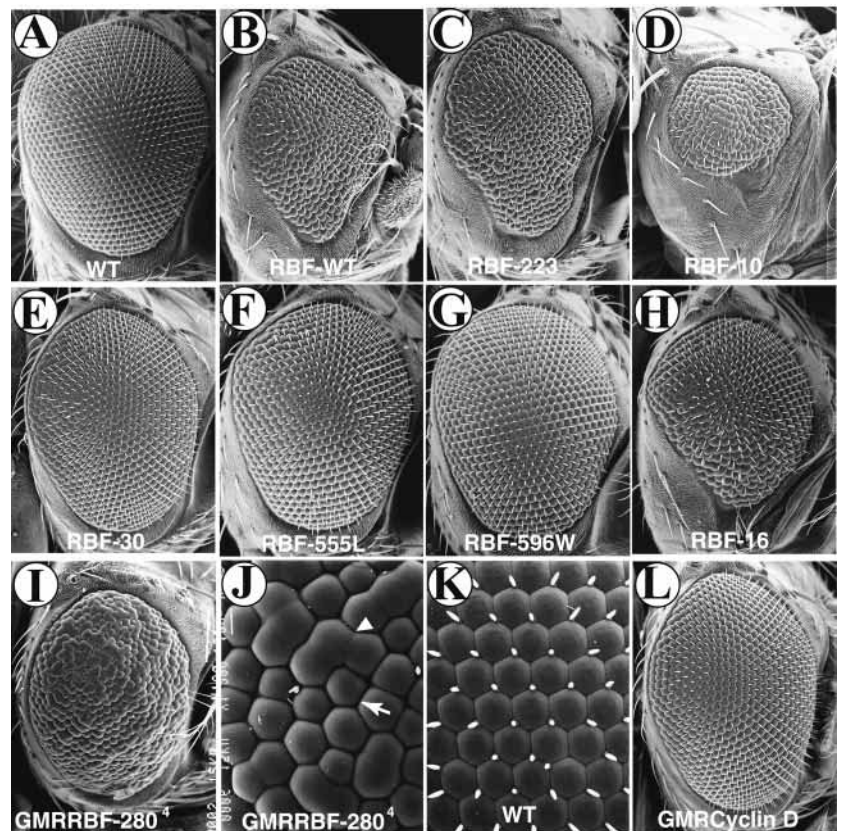

Fig. 2. Scanning electron micrographs of the phenotypes of expressing different mutant forms of RBF. (A) Wild type. (B-H) Different RBF mutant constructs expressed using the eyeless GAL4 driver. (B) Wild type RBF; (C) RBF-223; (D) RBF-10; (E), RBF-30; (F) RBF-555L; (G) RBF-596W; (H) RBF-16. (I,J) GMRRBF-280[4] at low (I) and high (J) magnification. (K) Wild type at high magnification. (L) GMRCyclin D/+. Arrowhead in J shows fused ommatidia; arrow shows an ommatidium with abnormal shape.
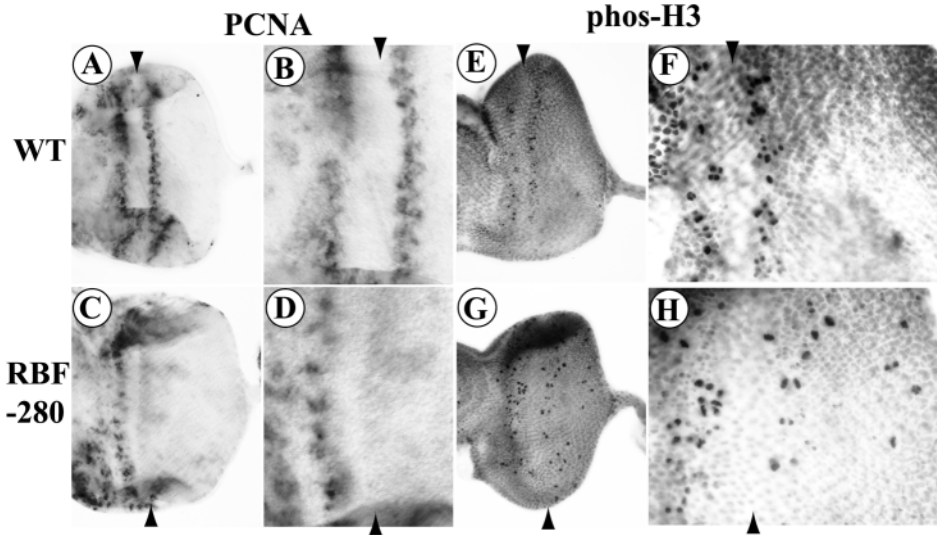
PCNA   **phos-H3**

Fig. 3. The effect of RBF-280 expression on E2F target gene expression and on mitosis. (A-D) In situ hybridization of wild-type and GMRRBF-280[4] eye discs with antisense probes to *PCNA* allows for visualization of E2F target gene expression. (A,C) Low magnification showing whole eye discs. (B,D) High magnification view showing the details of the *PCNA* expression. (E-H) Anti-phospho-Histone H3 antibody staining of eye discs from wild-type and GMRRBF-280[2] eye discs labels cells in mitosis. (E,G) Low magnification showing whole eye discs. (F,H) High magnification view showing the details of mitosis. In all the discs, posterior is oriented towards the right. Arrowheads indicate the morphogenetic furrow.

mitotic wave of wild-type eye discs). These observations are consistent with the idea that entry into S phase in the second mitotic wave is not significantly inhibited, but completion of S phase is delayed in the GMRRBF-280[4] eye disc.

One might predict that the delay in completion of S phase that determines when RBF-280 is expressed will also delay the onset of mitosis in GMRRBF-280 eye discs. To test that prediction directly, an anti-phospho-Histone H3 antibody was used to visualize cells in mitosis. In posterior part of wild-type eye discs, mitotic cells are all very close to the second mitotic wave (Fig. 3E,F), indicating that cells in the second mitotic wave go through S phase and then mitosis synchronously and rapidly in wild-type eye discs. By contrast, mitotic cells in eye discs with RBF-280 expression lose such synchrony. There are mitotic cells scattered throughout the posterior of the eye disc (Fig. 3G,H). Counting the number of M phase cells in the posterior part of eye discs shows that there is an average of 37 ± 3 M-phase cells in the wild-type eye discs, and 32 ± 5 M-phase cells in GMRRBF-280[4] eye discs. We conclude that overexpression of the non-regulated RBF-280 mainly delays the second mitotic wave cells from completing S phase and

entering mitosis. These results are consistent with the idea that the RB and E2F mainly control the rate of S phase progression in this developmental setting.

It has been reported previously that Cyclin E triggers an all-or-nothing transition from G1 to S phase (Duronio et al., 1998). The observation that RBF-280 expression does not inhibit BrdU incorporation in the second mitotic wave suggests that there may be sufficient levels of Cyclin E activity in cells in the second mitotic wave. Such Cyclin E kinase activity could bypass the G1 arrest imposed by the expression of non-regulated RBF-280. If this is the case, one might expect that reducing the activity of Cyclin E should be sufficient to block cells from entering S phase. As predicted, expression of Dap, an inhibitor of Cyclin E kinase activity, together with RBF-280, completely inhibited BrdU incorporation in the second mitotic wave (Fig. 4C,G). By contrast, expression of Dap at this level alone does not significantly affect the BrdU incorporation (Fig. 4D,H).

Genetic interactions between RBF and Cyclin E

RBF-280, which is no longer regulated by Cyclin D or Cyclin

WT   **RBF-280**   **RBF-280+Dap**   **Dap**
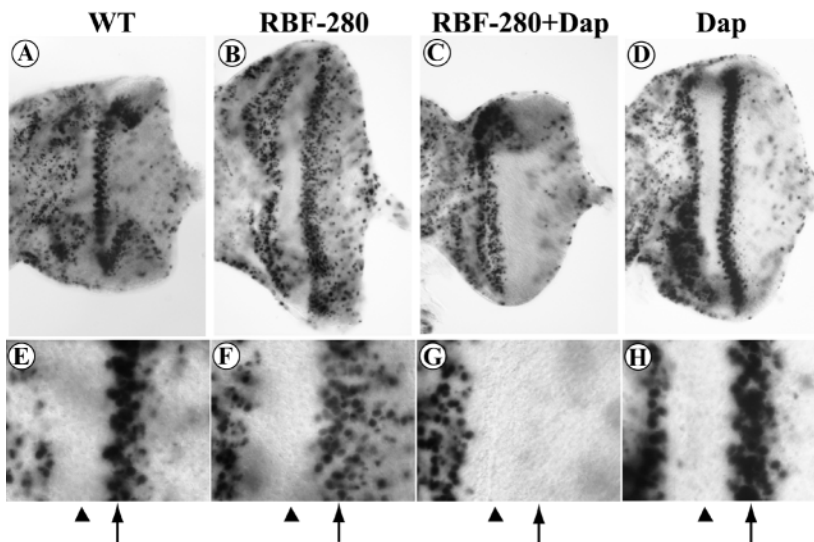

Fig. 4. The effect of RBF-280 expression on S phase in the second mitotic wave. (A-D) Low-magnification view; (E-H) High-magnification view. (A,E) A wild-type eye disc; (B,F) A GMRRBF-280/GMRRBF-280 eye disc; (C,G) A GMRRBF-280/GMRDap eye disc; (D,H) A GMRDap/+ eye disc. The BrdU-positive cells in the second mitotic wave in wild-type eye discs are clustered together, indicating that cells finish S phase rapidly and synchronously. In GMRRBF-280[4] eye discs, the second mitotic wave is broader and the BrdU staining is weaker in the second mitotic wave, indicating a prolongation of S phase and a loss of synchrony in the completion of S phase. (A,B,E,F) 1 hour of BrdU incorporation was used instead of 30 minutes to visualize the weaker staining in the second mitotic wave of GMRRBF-280[4]. In all the discs, posterior is oriented towards the right. Arrows indicate the second mitotic wave and arrowheads indicate the morphogenetic furrow.

E, provides the ideal tool with which to test the genetic interaction between RBF and Cyclin D and Cyclin E. The phenotypes induced by RBF-280 expression are dose sensitive, two copies of GMRRBF-280 (GMRRBF-280^2) lead to much weaker phenotypes compared with four copies of GMRRBF-280 (GMRRBF-280^4; Fig. 2I,J and Fig. 5F). As Cyclin E activity has an all-or-none effect on S-phase entry (Duronio et al., 1998), the observation that GMRRBF-280 delays S phase completion rather than inhibits S phase entry suggest that the phenotypes associated with GMRRBF-280 are not due to an insufficient amount of Cyclin E activity. Consistent with such an idea, an increase the level of cyclin E, caused by expressing Cyclin E together with GMRRBF-280^2, does not suppress the phenotypes of GMRRBF-280^2; the resulting eye shows phenotypes of both Cyclin E and RBF-280 overexpression (Fig. 5E,J), which is consistent with the observations that RBF-280 can block S-phase progression, even in the presence of ectopic Cyclin E expression, and Cyclin E can induce ectopic S phase entry and cell death in the presence of RBF-280 (data not shown). Similarly, increasing the Cyclin E activity by reducing one copy of *dap* gene dose does not suppress the GMRRBF-280^2 phenotypes, although it does partially suppress the GMRRBF^{4C} phenotypes (Fig. 5C,H). These observations are also consistent with the idea that Cyclin E can regulate wild-type RBF but not RBF-280 (Fig. 1B). However, inhibiting the Cyclin E kinase activity by co-expression of Dap

strongly enhances the phenotypes of both GMRRBF^{4C} and GMRRBF-280^2 (Fig. 5D,I). Expression of Dap by itself has no phenotypes (Fig. 5M). The observed enhancement is consistent with the finding that expression of Dap with either wild-type RBF or RBF-280 results in the complete elimination of the second mitotic wave (Fig. 4C,G) (de Nooij and Hariharan, 1996). Similarly, reducing the gene dose of *Cyclin E* by 50% also strongly enhances the phenotypes of both GMRRBF-280^2 and GMRRBF^{4C} (Fig. 5N,O). These observations are consistent with the established role of Cyclin E in promoting S-phase entry in addition to regulating RBF.

Genetic interactions between RBF and Cyclin D

While overexpression of Cyclin D alone in the developing eye (GMRCyclin D^2) does not disrupt normal eye development (Fig. 2L), GMRCyclin D^2 completely suppresses phenotypes that result from overexpression of wild-type RBF (GMRRBF^{4C}; Fig. 5A,B,K,L). However expression of Cyclin D does not affect the GMRRBF-280^2 phenotypes (Fig. 5F,G). These observations are consistent with the finding that Cyclin D/Cdk4 can regulate wild-type RBF but not RBF-280 (Fig. 1B).

In contrast to expressing Cyclin D alone, overexpression of Cyclin D together with Cdk4 leads to significantly larger and slightly rough eyes (Fig. 6G,I,J). These phenotypes are primarily due to the ability of Cyclin D/Cdk4 to induce cellular
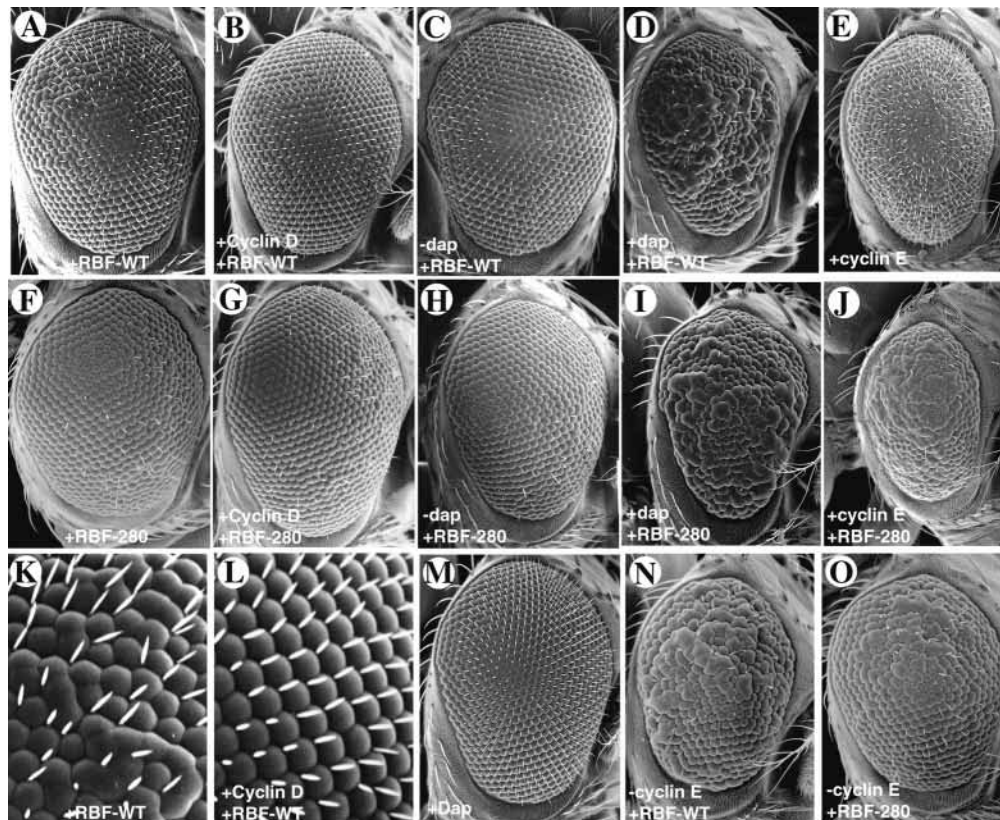

Fig. 5. Scanning electron micrographs (SEM) showing the adult eye phenotypes of GMRRBF-280^2 and its interactions with Cyclin D and Cyclin E. (A) GMRRBF^{4C}; (B) GMRRBF^{4C}/GMRCyclin D; (C) *dap*/+;GMRRBF^{4C}; (D) GMRRBF^{4C},GMRDap/+; (E) GMRGAL4/+;UASCyclin E/+; (F) GMRRBF-280^2/+; (G) GMRRBF-280^2/GMRCyclin D; (H) *dap*/+;GMRRBF-280^2; (I) GMRRBF-280^2,GMRDap; (J) GMRGAL4/+;UASCyclin E/GMRRBF-280^2; (K,L) high magnification view of GMRRBF^{4C} (K) and GMRRBF^{4C}/GMRCyclin D (L); (M) GMRDap/+; (N) *cyclin E*/+; GMRRBF^{4C}/+; (O) *cyclin E*/+; GMRRBF-280^2/+.

growth (leading to larger cells) in the non-dividing cells in the developing eye (Datar et al., 2000). We found that in addition to promoting cellular growth, overexpression of Cyclin D and Cdk4 can also induce ectopic S phase in the posterior of the developing eye but not in the morphogenetic furrow (Fig. 6A-C). Expression of RBF-280 blocks ectopic S phase induced by Cyclin D/Cdk4 overexpression in the posterior part of the developing eye (Fig. 6D-F), these observations indicate that Cyclin D/Cdk4 induces ectopic S phase through inactivating RBF. Interestingly, although RBF-280 blocks ectopic S-phase induced by Cyclin D/Cdk4 overexpression, eyes expressing RBF-280 together with CyclinD/Cdk4 show phenotypes of both RBF-280 overexpression (missing bristles and fused ommatidia) and Cyclin D/Cdk4 overexpression (large and bulge eyes) (Fig. 6G-K). Thus, while Cyclin D/Cdk4 cannot inhibit the phenotypes induced by RBF-280, RBF-280 cannot inhibit the large eye phenotypes induced by Cyclin D/Cdk4 either. These results are consistent with the idea that Cyclin D/Cdk4 induces cellular growth in the non-dividing eye cells through targets that are independent of RBF (Datar et al., 2000).

RBF-280 blocks Cyclin D/Cdk4 but not activated Ras induced cellular growth in the developing wing

Cellular growth induced by Cyclin D/Cdk4 in the proliferating wing discs is distinct from growth induced in the eye. In the proliferating wing discs, Cyclin D/Cdk4 increases cellular growth and cell division rate proportionally, so that cell size or cell cycle profile is not affected (Datar et al., 2000). To test if RBF mediates the ability of Cyclin D/Cdk4 to induce growth, RBF-280, which cannot be regulated by Cyclin D/Cdk4, was expressed together with Cyclin D/Cdk4 in clones in the developing wing discs (Fig. 7C). As shown in Fig. 7A, overexpression of RBF-280 together with Cyclin D/Cdk4 strongly inhibits the ability of Cyclin D/Cdk4 to induce cellular growth. In fact, the average area (Ave=806) occupied by the RBF-280+Cyclin D/Cdk4 cell clones is not significantly different from the area (Ave=767) occupied by the RBF-280 cell clones (P=0.68). In addition, RBF-280 also blocks the Cyclin D/Cdk4 induced cell proliferation. As shown in Fig. 7A, RBF-280+Cyclin D/Cdk4 clones have similar number of cells as the RBF-280 clones (P=0.32), indicating a similar cell doubling rate. Thus RBF-280 inhibits both cell growth and proliferation induced by Cyclin D/Cdk4 in the proliferating wing discs.

Expression of activated Ras leads to increased cell size and growth rate (Prober and Edgar, 2000). To test if RBF-280 also inhibits the cellular growth induced by activated Ras in the proliferating wing discs, we overexpressed RBF-280 together with

Ras^{V12}. As show in Fig. 7B, while the sizes of Ras^{V12}+RBF-280 cell clones are significantly smaller than the Ras^{V12} cell clones ($P<0.0001$), Ras^{V12}+RBF-280 cell clones are much larger than RBF-280 cell clones ($P<0.0001$). These results indicate strongly that Ras^{V12} can still induce growth in the presence of RBF-280 in the developing wing discs. The Ras^{V12}+RBF-280 cells are noticeably larger than the RBF-280 cells (Fig. 7C). In situ measurement of cell size shows that the ratio of average cell sizes between RBF-280+Ras^{V12} cells and RBF-280 cells is 2.55 (Fig. 7C). By contrast, RBF-280+Cyclin D/Cdk4 cells are not significantly different in size from RBF-280 cells; the ratio of the average cell size between RBF-280+Cyclin D/Cdk4 and RBF-280 is 0.93 (Fig. 7C). These results are consistent with the observations that the Cyclin D/Cdk4+RBF-WT cells are similar in size to the RBF-WT cells (Datar et al., 2000), while the Ras^{V12}+RBF-WT cells are much larger than the RBF-WT cells (Prober and Edgar, 2000). In addition, Ras^{V12}+RBF-280 clones also show a few more cells than do RBF-280 clones ($P=0.0002$), indicating a faster cell doubling rate (Fig. 7B). Taken together, these results indicate that Ras^{V12} can stimulate cellular growth in the presence of RBF-280 while Cyclin D/Cdk4 cannot. It remains possible, however, that as the growth stimulatory effect of Ras^{V12} is significantly stronger than Cyclin D/Cdk4, the level of RBF-280 expression might not be sufficient to repress all the Ras^{V12} effect.
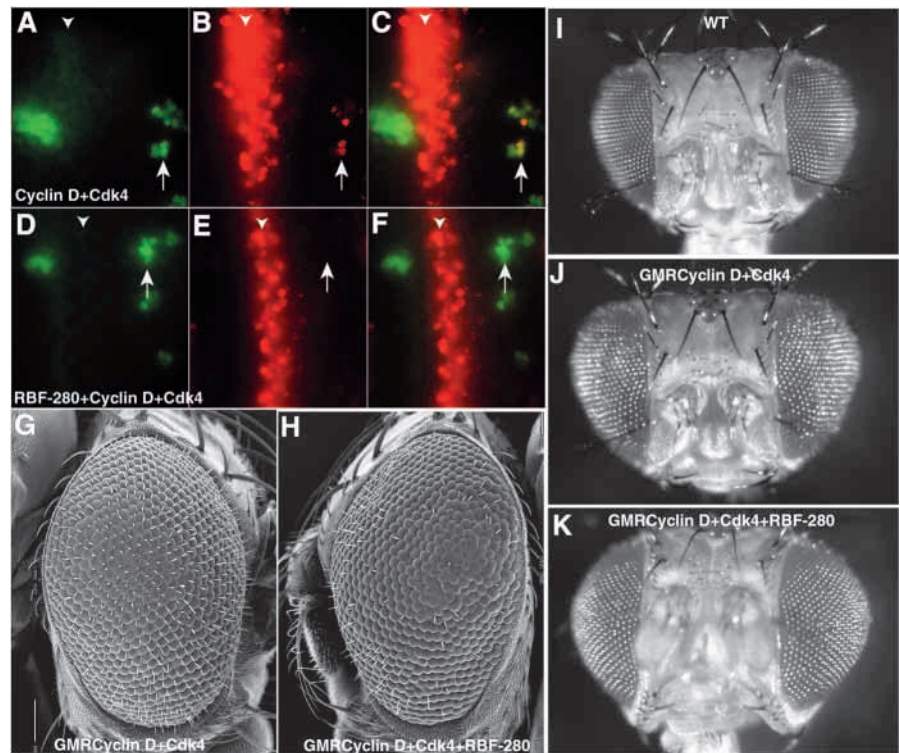

Fig. 6. RBF-280 inhibits CyclinD/Cdk4 induced ectopic S phase but not the large eye phenotypes in the *Drosophila* eye. BrdU staining (red) of eye discs with Cyclin D/Cdk4 overexpression (A-C), or RBF-280+Cyclin D/Cdk4 overexpression (D-F) showing RBF-280 blocks CyclinD/Cdk4 induced ectopic S phase. Clones of cells with CyclinD/Cdk4 or RBF-280+CyclinD/Cdk4 overexpression are marked by GFP (green). Arrowheads point to the second mitotic wave and arrows point to GFP marked clones. (G-K) Phenotypes of fly eyes with CyclinD/Cdk4 or RBF-280+CyclinD/Cdk4 overexpression as indicated.
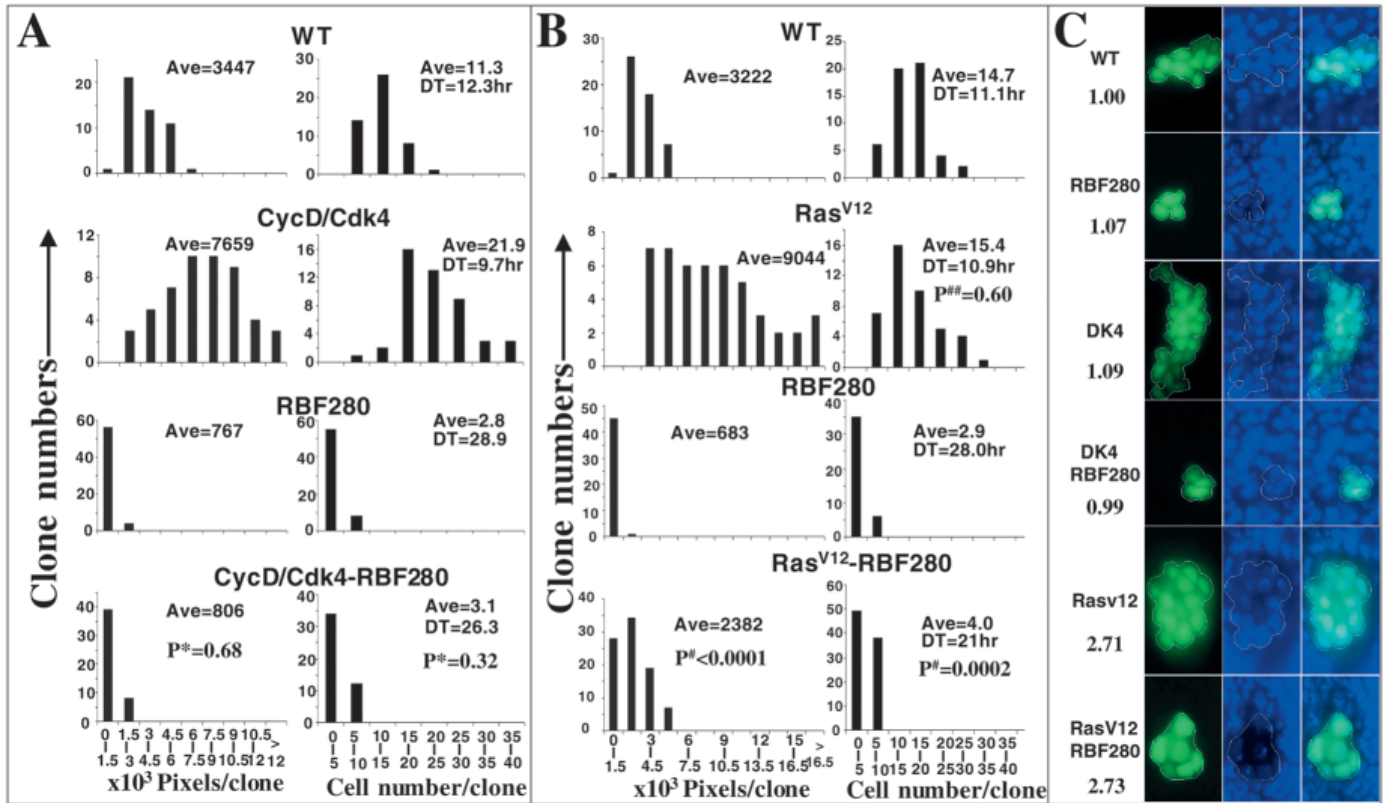
Fig. 7. RBF-280 inhibits CyclinD/Cdk4 induced growth and proliferation but not activated Ras-induced cellular growth in the proliferating wing disc cells. (A) Distribution of clone size and cell number/clone for the genotype given is shown. Cell doubling times (DT) are shown. Clones were analyzed 43 hours post-induction. Total number of clones analyzed (*n*): control, 48; Cyclin D/Cdk4, 51; RBF-280, 60; RBF-280+Cyclin D/Cdk4, 47. P*, comparison of clone area or cell number/clone between RBF-280+Cyclin D/Cdk4 and RBF-280. Clones with RBF-280+Cyclin D/Cdk4 expression occupy similar areas (P*=0.68) and have similar number of cells (P*=0.32) as the RBF-280 clones. Comparison of the clone area or cell number/clone between other genotypes, *P*<0.0001. (B) RBF-280 does not block Ras^{V12}-induced cellular growth. Clones were analyzed 43 hours post induction. Total number of clones analyzed (*n*): control, 52; Ras^{V12}, 47; RBF-280, 46; RBF-280+Ras^{V12}, 88. P#, comparison of clone area or cell number/clone between RBF-280+Ras^{V12} and RBF-280; P##, comparison of cell number/clone between Ras^{V12} and wild type. Comparison of the clone area or cell number/clone between other genotypes, *P*≤0.0001. (C) Examples of cell clones of each genotype are shown as indicated. Clones are marked by GFP (green). DAPI staining that labels the nuclei is shown in blue. Cell sizes were estimated by the average of clone area/cell number per clone for all genotypes. The ratio between cell sizes of indicated genotype over wild-type cell size is shown.

DISCUSSION

We have generated a form of RBF, RBF-280, that cannot be regulated by Cyclin D- and Cyclin E-dependent kinases, and have tested the role of RBF in developmentally regulated cell proliferation and in Cyclin D/Cdk4-induced cellular growth. We show that inhibiting the E2F target gene expression in the second mitotic wave of the developing eye mainly delays S-phase completion instead of inhibiting S phase entry (Figs 3,4). These results suggest that cells in the second mitotic wave are driven into S phase through an RB/E2F-independent mechanism. In addition, we find that while RBF-280 completely inhibits cellular growth induced by Cyclin D/Cdk4 in the proliferating wing discs, RBF-280 cannot block cellular growth induced by activated Ras in the wing disc cells or Cyclin D/Cdk4 in the non-dividing eye cells. These observations indicate that the ability of Cyclin D/Cdk4 to induce growth in the proliferating wing discs is distinct from the ability of activated Ras to induce growth or the ability of Cyclin D/Cdk4 to induce growth in the non-dividing eye cells.

In addition, these results suggest that RBF may have a role in inhibiting growth.

We have shown previously that *e2f1* null mutant eye discs have second mitotic wave when the level of RBF is reduced (Du, 2000), indicating that transcription activation by E2F1 is not required for S-phase entry in the second mitotic wave. There are two possible explanations for this observation: one possibility is that derepressed basal E2F target gene expression in the second mitotic wave is sufficient to drive S-phase entry. Alternatively, it is possible that cells in the second mitotic wave are driven into S phase through an E2F-independent mechanism. The results presented in this report suggest that S-phase entry in the second mitotic wave is probably driven by an E2F-independent mechanism, as overexpression of a non-regulated RBF inhibits E2F target gene expression but does not inhibit S phase there. These results are consistent with our recent observation that Hh signaling is required for S phase entry in the second mitotic wave through direct induction of Cyclin E (M. Duman-Scheel, L. Weng, S. Xin and W. Du, unpublished). As Hh signal is known for its role in neuronal

differentiation and in pattern formation of the developing eye (Heberlein et al., 1993), it appears that developmental regulated G1/S transition in the second mitotic wave is controlled by the same signal that also controls differentiation and pattern formation to coordinate the cell proliferation with differentiation. Although RB/E2F does not control the S-phase entry in this case, RB and E2F appear to be important for the rapid progression through S phase in the second mitotic wave. The observation that RBF-280 delays S-phase completion in the second mitotic wave and severely disrupts normal eye development indicates the importance of coordinating the rate of cell proliferation and differentiation in the developing eye.

Besides our current observation that RB and E2F play important roles regulating S-phase progression in the developing eye, RB/E2F has also been shown to affect S-phase progression in the developing embryos (Royzman et al., 1997; Duronio et al., 1998) and wing discs (Neufeld et al., 1998). Thus, RB/E2F appear to regulate S-phase progression in multiple developmental settings. Similarly, RB/E2F have also been shown to regulate G1/S transition in a number of other developmental settings (Asano et al., 1996; Du et al., 1996b; Tsai et al., 1998; Du and Dyson, 1999). The question is when do RB/E2F regulate G1/S transition and when do they regulate S phase progression? It appears that RB/E2F often play important roles in the G1 arrested cells (such as the G1 arrested cells in the embryos and in the eye discs) to prevent ectopic S phase entry. In these cases, Rb and E2F probably function through inhibiting the expression of Cyclin E. By contrast, developmentally regulated cell proliferation (G1/S transition) appears to be tightly linked to the developmentally regulated transcription of cyclin E, which is controlled by a large *cis*-regulatory region containing tissue- and stage-specific components (Jones et al., 2000). Temporal and tissue specific Cyclin E expression will drive cells into S phase and lead to the inactivation of RB and the coordinated E2F target gene expression, which might be required for the timely progression through S phase. Our observation that RBF-280 expression inhibits E2F target gene expression and delays the completion of S phase supports a role for RB/E2F in S phase progression in the second mitotic wave.

There are at least two possible mechanisms that may contribute to the function of RBF in regulating S-phase progression. One mechanism is through the inhibition of E2F target gene expression besides cyclin E. Because several E2F target genes such as *PCNA*, *RNR2*, *Orc1* and *DNA pol α* are components of the DNA replication machinery, inhibition of E2F target gene expression may result in an insufficient amount of DNA replication machinery, which may delay the completion of S phase. A second possibility is that RBF may regulate DNA replication directly. Recently, it has been shown that the E2F1/RBF complex is localized to the DNA replication origin, and interacts with ORC proteins directly (Bosco et al., 2001). In addition, mammalian RB can interact with MCM7 and regulate DNA replication directly (Sterner et al., 1998). Thus, it is possible that RBF can regulate S-phase progression directly by controlling firing at replication origins.

It is interesting to note that delaying the completion of S phase leads to the development of adult eyes with missing bristles and fused ommatidia, similar to the adult eyes developed when the second mitotic wave is inhibited (de Nooij

and Hariharan, 1995). These observations suggest that not only cell proliferation, but also the rate of cell proliferation, need to be tightly coordinated with differentiation in certain developmental settings. How might delaying the completion of S phase lead to phenotypes similar to inhibition of S phase? It is possible that there is only a very short time window that a specific cell type can be recruited into the ommatidia clusters from the surrounding cells. Delaying the completion of S phase may lead to a lack of cells that can be recruited locally, resulting in the phenotype of missing cone cells, pigment cells and bristles. In addition, it is possible that specific phases of the cell cycle (such as S phase and M phase) are incompatible with the ommatidia recruitment process. Thus, those ommatidia clusters that are surrounded by cells that are still in S phase will not be able to recruit additional cells into the ommatidia. There are some reports that support this idea. For example, Tribbles is required to prevent premature mitosis by inducing specific degradation of String and Twine during *Drosophila* embryogenesis. Failure to prevent the premature mitosis leads to defects in gastrulation (Grosshans and Wieschaus, 2000; Mata et al., 2000; Seher and Leptin, 2000). Similarly, failure to have G1 arrest in *roughex* mutant eye discs results in defects in cell fate determination, as well as abnormalities in the adult eye (Thomas et al., 1994; Thomas et al., 1997). Further studies will be needed to directly test the relationship between cell differentiation and cell cycle phasing.

Cyclin D/Cdk4 was shown recently to be able to drive cellular growth in addition to induce cell proliferation. Interestingly, the consequence of Cyclin D/Cdk4 expression is different in different cell types: Cyclin D/Cdk4 primarily induces growth and lead to larger cells in the non-dividing differentiated eye cells (hypertrophy); Cyclin D/Cdk4 induces increased DNA endoreplication and increased cell size (hypertrophy) in the salivary gland cells; Cyclin D/Cdk4 induces growth and division coordinately without affecting cell size in the proliferating wing discs, leading to more rapid cell cycle and more cells (hyperplasia) (Datar et al., 2000). To test if these biological effects are mediated by RBF, we tested the effect of RBF-280, a form of RBF that cannot be regulated by Cyclin D, on cellular growth and proliferation induced by Cyclin D and Cdk4.

We show that RBF is an important target of Cyclin D/Cdk4 in G1/S regulation. RBF-280 blocks the ability of Cyclin D to induce S phase in G1 arrest eye disc cells (Fig. 6A-F) and the excessive DNA endoreplication in the salivary gland cells (data not shown). In addition, RBF-280 also blocks the ability of Cyclin D/Cdk4 to increase the rate of cell proliferation in the proliferating wing discs (Fig. 7A). These results demonstrate that the ability of Cyclin D/Cdk4 to induce cell proliferation (G1/S transition) is mediated through inactivation of RBF. By contrast, the ability of RBF-280 to block Cyclin D/Cdk4 induced growth varies in different cell types. In the proliferating wing disc cells, Cyclin D/Cdk4 expression leads to more rapid cell cycle and more cells (hyperplasia). RBF-280 completely blocks the effect of Cyclin D/Cdk4. The average size of RBF-280 clones is not significantly different from the average size of Cyclin D+Cdk4+RBF-280 clones (*P*=0.68). In addition, the number of cells in the clone is also not significantly different (*P*=0.32). These observations indicate that RBF-280 blocks both cell growth and proliferation induced by Cyclin D/Cdk4 in the

proliferating wing discs. The effect of RBF-280 on Cyclin D/Cdk4-induced growth is distinct from the effect of RBF-280 on activated Ras induced growth. Activated Ras induces cellular growth and leads to larger cells without affecting the rate of cell doubling in the wing discs (hypertrophy). We found that the average area of Ras^{V12}+RBF-280 clones is significantly larger than the area of RBF-280 clones ($P<0.0001$). The observed increase in clone size is mainly due to increased cell size. Although we are unable to get enough cells for Facs analysis to determine the cell size directly, because clones with RBF-280 expression are extremely small, cell size estimation using the average cell sizes derived from clone area/cell number showed that RBF-280+Ras^{V12} cells is noticeably larger than RBF-280 cells, while the size of RBF-280+Cyclin D+Cdk4 cells is similar to the size of RBF-280 cells (Fig. 7C). These results are consistent with the reported cell size effect of Cyclin D/Cdk4 and activated Ras on RBF-WT. It was shown that the Cyclin D/Cdk4+RBF-WT cells are similar in size as the RBF-WT cells (Datar et al., 2000), while the Ras^{V12}+RBF-WT cells are much larger than the RBF-WT cells (Prober and Edgar, 2000). Taken together, this evidence supports the notion that Ras^{V12} can stimulate cellular growth in the presence of functional RBF, while Cyclin D/Cdk4 induces growth at least in part through inactivation of RBF in the developing wing discs.

Similar to activated Ras, Cyclin D/Cdk4 also induces growth and leads to large eyes as a result of increased cell size in the non-dividing eye cells (hypertrophy). Consistent with the idea that the large eye phenotypes are the consequence of cellular growth induced by Cyclin D/Cdk4, which is mediated through targets distinct from RBF (Datar et al., 2000), RBF-280 blocks Cyclin D/Cdk4-induced ectopic S phase in the eye discs (Fig. 6A,B) but not Cyclin D/Cdk4 induced large eye phenotype (Fig. 6I-K). It is likely that Cyclin D/Cdk4 drives growth through distinct targets in the non-proliferating eye cells and in the proliferating wing disc cells. The target in the non-proliferating eye cells can drive growth in the presence of RBF-280, while the target in the proliferating wing disc cells are either RBF itself or a target that can drive growth only when RBF is inactivated. Further studies will be needed to identify the targets that mediate the ability of Cyclin D/Cdk4 to induce growth.

In addition to increase cell sizes, the Ras^{V12}+RBF-280 clones also have more cells (faster cell doubling time) than the RBF-280 clones ($P=0.0002$). These observations suggest that activated Ras can increase proliferation in addition to growth in the presence of RBF-280. This seems to be contradictory to the observations that Ras^{V12} expression alone does not appear to affect the rate of cell doubling significantly (Fig.7B) (Prober and Edgar, 2000). Interestingly, although no statistical difference is observed in cell doubling rate between Ras^{V12} and wild-type control ($P=0.60$), Ras^{V12} overexpression clones do have a few more cells (Fig. 7B) than do the wild-type control clones. A plausible explanation is that the observed increase in cell number by Ras^{V12} is RBF independent, and the contribution of this difference in cell number may become statistically significant when the total number of cells in each clone is reduced in the presence of RBF-280. It is possible that this observed small increase in cell doubling might be the consequence of increased growth by activated Ras.

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