The EMBO Journal Vol. 21 No. 18 pp. 4927–4937, 2002

Distinct mechanisms of E2F regulation by *Drosophila* RBF1 and RBF2

Olivier Stevaux, Dessislava Dimova, Maxim V.Frolov, Barbie Taylor-Harding, Erick Morris and Nicholas Dyson[1]

Massachusetts General Hospital Cancer Center, Laboratory of Molecular Oncology, Charlestown, MA 02129, USA

[1]Corresponding author
e-mail: dyson@helix.mgh.harvard.edu

RBF1, a *Drosophila* pRB family homolog, is required for cell cycle arrest and the regulation of E2F-dependent transcription. Here, we describe the properties of RBF2, a second family member. RBF2 represses E2F transcription and is present at E2F-regulated promoters. Analysis of *in vivo* protein complexes reveals that RBF1 and RBF2 interact with different subsets of E2F proteins. dE2F1, a potent transcriptional activator, is regulated specifically by RBF1. In contrast, RBF2 binds exclusively to dE2F2, a form of E2F that functions as a transcriptional repressor. We find that RBF2-mediated repression requires dE2F2. Moreover, RBF2 and dE2F2 act synergistically to antagonize dE2F1-mediated activation, and they co-operate to block S phase progression in transgenic animals. The network of interactions between RBF1 or RBF2 and dE2F1 or dE2F2 reveals how the activities of these proteins are integrated. These results suggest that there is a remarkable degree of symmetry in the arrangement of E2F and RB family members in mammalian cells and in *Drosophila*.

Keywords: cell cycle/*Drosophila*/E2F/RBF/Retinoblastoma

Introduction

In mammalian cells, arrest in the G_1 phase of the cell cycle requires the family of pocket proteins that includes pRB, the Retinoblastoma protein, and two close homologs, p107 and p130 (Dyson, 1998). When overexpressed in a subset of cancer cell lines, pRB, p107 and p130 arrest cell cycle progression, causing an accumulation of cells in the G_1 phase of the cell cycle (Goodrich *et al*., 1991; Hinds *et al*., 1992). Conversely, cells lacking pRB family members have a short G_1 phase and are largely unresponsive to cell cycle withdrawal signals (Dannenberg *et al*., 2000; Sage *et al*., 2000). In mouse knockout experiments, heterozygous $RB^{+/-}$ mice are predisposed to pituitary tumors and homozygous $RB^{-/-}$ embryos display a broad array of phenotypes, including elevated apoptosis and failure to arrest cellular divisions in the nervous system (Jacks *et al*., 1992; Lee *et al*., 1992).

Pocket proteins are thought to govern the G_1 to S phase transition mainly through their interaction with E2F transcription factors (Nevins, 2001). E2F regulates the transcription of a large set of genes involved in all aspects of S phase initiation and progression, such as the *mcm* genes, *cyclin E*, *pcna*, and *DNA Pol*α (Ishida *et al*., 2001; Ren *et al*., 2002). Pocket proteins bind to E2F and are proposed to recruit histone deacetylases (Brehm *et al*., 1998; Magnaghi *et al*., 1998) or other chromatin-remodeling factors to repress E2F target genes expression (Harbour and Dean, 2000). Pocket proteins are inactivated by phosphorylation in late G_1 by cyclin/CDK complexes (Dyson, 1998), allowing a synchronized expression of this large set of E2F-regulated genes.

The mammalian E2 factor is a heterodimer composed of one E2F and one DP subunit. Six E2F and two DP genes have been characterized in mammalian cells, and distinct E2F/pocket protein complexes exist *in vivo* (Trimarchi and Lees, 2002). pRB interacts with E2F1, E2F2, E2F3 and E2F4, whereas p107 is only found in complexes with E2F4, and p130 with E2F4 and E2F5. Synchronized cell experiments suggest that E2F proteins can be divided into two sub-families based on their presence at E2F-regulated promoters at different phases of the cell cycle (Takahashi *et al*., 2000; Wells *et al*., 2000). Activator E2Fs (E2F1, E2F2 and E2F3), on the one hand, are induced in late G_1 and in S phase, and are detected on E2F-regulated promoters at times when E2F target genes are actively transcribed. Knockout cells deleted for E2F1, E2F2, and E2F3 are unable to enter S phase and fail to express many E2F-regulated genes (Wu *et al*., 2001). On the other hand, the repressor E2Fs (E2F4 and E2F5) represent most of the E2F-binding activity during G_0 and most of G_1 at times when E2F target genes are not induced. These repressor E2Fs are required for several types of pocket protein-mediated cell cycle arrest in G_1 (Gaubatz *et al*., 2000). The G_1 to S phase transition coincides with a shift in the occupancy of E2F promoters from repressor to activator E2F complexes (Takahashi *et al*., 2000; Wells *et al*., 2000).

A similar mechanism of S phase entry regulation exists in *Drosophila*, where a pRB-related protein, RBF, associates with a DP homolog, dDP, and with two fly E2F proteins, dE2F1 and dE2F2. In many ways, dE2F1 appears to be a homolog of the mammalian activator E2Fs. In tissue culture experiments, dE2F1 is a potent transcriptional activator (Frolov *et al*., 2001). Tissues of transgenic animals expressing high levels of dE2F1 and dDP show extensive upregulation of E2F targets, increased BrdU incorporation, and elevated apoptosis (Asano *et al*., 1996). Mutant *de2f1* animals cease to grow after completion of embryogenesis and die as small larvae unable to sustain normal cell proliferation (Duronio *et al*., 1995). In contrast, dE2F2 appears to function primarily as a repressor E2F. It represses E2F-dependent transcription when expressed in cultured cells, and E2F target genes are upregulated in *de2f2* mutant animals (Cayirlioglu *et al*.,

© European Molecular Biology Organization

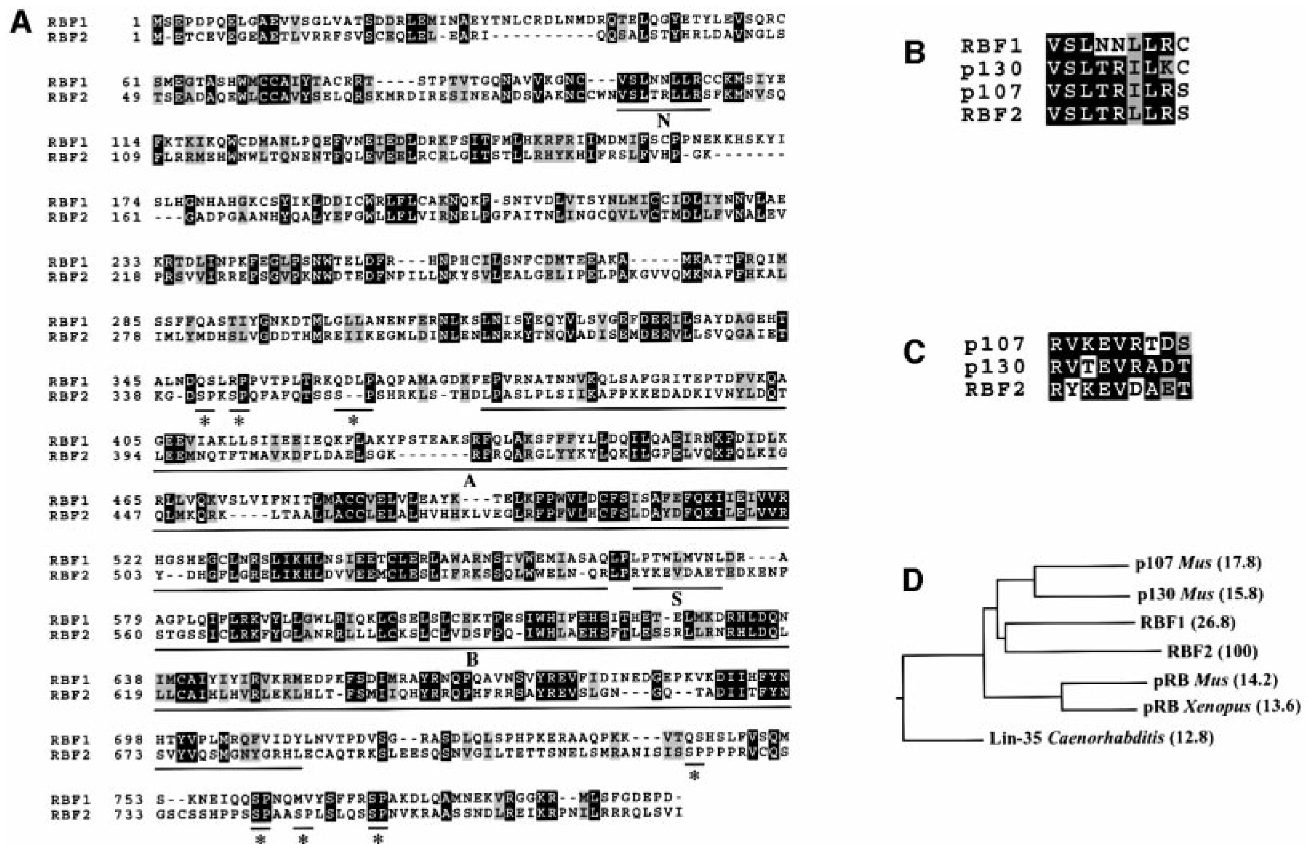

Fig. 1. RBF2 is an RBF1 homolog more closely related to p107 than to pRB. (**A**) Alignment of RBF1 and RBF2. Conserved domains between RBF2 and other pocket proteins are underlined: the A and B halves of the pocket, multiple cyclin/CDK phosphorylation sites (*), and the N-terminal (N) and spacer (S) domains that are conserved in p107 and p130, but not in pRB. (**B**) Alignment between the N-terminal domain of RBF2 and conserved regions of p107 and p130. (**C**) Alignment between the spacer sequence of RBF2 and portions of the p107 and p130 spacer sequences. (**D**) Phylogenetic tree of RB-related proteins. The percentages of identity between each pocket protein and RBF2 are shown in parenthesis.

2001; Frolov *et al.*, 2001). In double *de2f1;de2f2* mutant animals, the loss of *de2f2* rescues the growth defect of the *de2f1* mutant larvae (Frolov *et al.*, 2001) and restores the level, but not the normal pattern, of expression of many E2F-regulated genes. These results suggest that E2F regulation in *Drosophila* is a balance between dE2F1 activation and dE2F2 repression. When dE2F1 is removed, the balance shifts towards E2F repression and cell cycle arrest, and when dE2F2 is ablated, the balance shifts towards E2F activation (Frolov *et al.*, 2001).

RBF interacts directly with the transcriptional activation domain of dE2F1. Consistent with this, RBF blocks cell proliferation and suppresses phenotypes induced by dE2F1 when overexpressed in transgenic animals (Du *et al.*, 1996a). Mutant *rbf* animals display phenotypes that are highly reminiscent of those reported for $RB^{-/-}$ mouse embryos: elevated apoptosis, ectopic entry into S phase, and deregulated E2F transcription (Du and Dyson, 1999). The observation that the phenotypes of *rbf* mutant animals are almost completely suppressed by a truncated allele of *de2f1* that can no longer activate transcription or induce apoptosis supports the claim that the principal function of RBF is to restrain dE2F1 activity (Du, 2000). This interaction is similar to the genetic interactions between *RB* and *E2f* alleles observed in mice where the neuronal defects in $RB^{-/-}$ embryos are strongly reduced in an $E2f1^{-/-}$ mutant background (Tsai *et al.*, 1998).

In this study, we describe RBF2, the second pocket protein of *Drosophila melanogaster*. To understand the role of this protein, we have compared the biochemical and functional characteristics of RBF1 and RBF2. We find that RBF1 and RBF2 interact with different subsets of E2F proteins and that this distinction enables the RBF proteins to regulate E2F-dependent transcription in distinct ways.

Results

RBF2, a second RB family member in Drosophila

While searching the *Drosophila* EST database, we noted two entries (LD02737 and LD15806) that presented similarities to the N-terminal portions of RBF and human p107. Sequencing of these cDNAs and the corresponding genomic region confirmed the presence of an RBF-related gene (DDBJ/EMBL/GenBank accession No. AF197059 and AF195899). For simplicity, we will refer to the original retinoblastoma family member as *rbf1*, and to this later isolate as *rbf2*.

rbf2 is an intronless gene coding for a 782 amino acid protein with extensive homology to RBF1 and mammalian pocket proteins (Figure 1). RBF2 contains sequences corresponding to the A and the B halves of the pocket-domain, and has potential CDK phosphorylation sites on both sides of the pocket region (Figure 1A). RBF2 also contains an N-terminal sequence (the N-box in Figure 1A

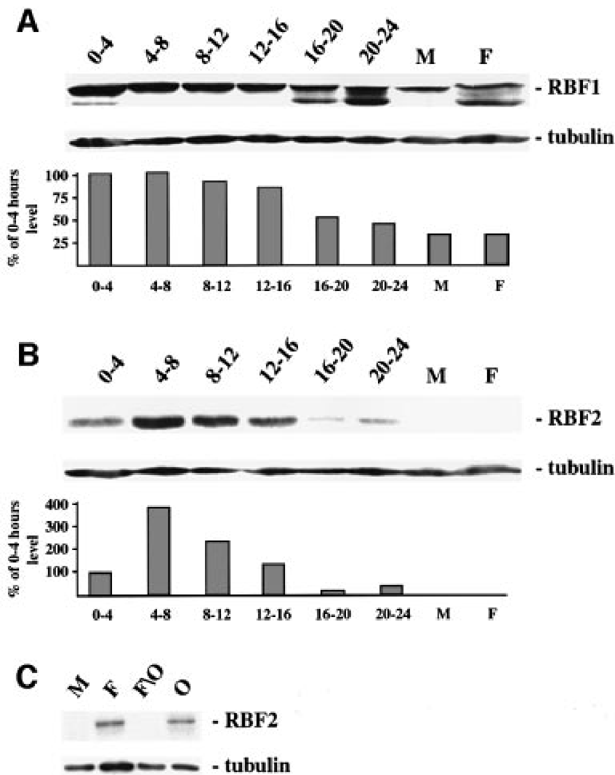

Fig. 2. RBF1 and RBF2 are differentially expressed throughout development. *W1118* embryos were collected at intervals of 4 h. Six collections covered the 24 h of embryogenesis at 25°C. Protein extracts from the staged embryos and from male or female adults were analyzed by western blotting with monoclonal antibodies specific for (**A**) RBF1 or (**B**) RBF2 and with a tubulin antibody for loading control. The charts show the levels of the RBF1 and RBF2 as a percentage of the level in the earliest extract, normalized for tubulin levels. (**C**) Analysis of protein extracts, corresponding to one adult male (M), one adult female (F), one adult female after resection of ovaries (F\O), and dissected ovaries from one female (O), by western blotting with an RBF2 or a tubulin antibody.

and B) that is highly conserved in human p107, human p130 and both *Drosophila* pocket proteins, but is absent in pRB. In addition, and unlike RBF1, RBF2 contains a short spacer sequence between the two halves of the pocket domain that is homologous to sequences in the p107 and p130 spacer regions (Figure 1C). However, RBF2 lacks sequences homologous to the high affinity cdk-binding sites found in the N-terminal and the spacer regions of p107 and p130. Thus, the RBF2 protein shares more sequence identity with RBF1 than with any other pocket protein, and like RBF1, appears more closely related to p107 and p130 than to pRB (Figure 1D).

An N-terminal portion of RBF2 was expressed in bacteria and used to generate monoclonal antibodies that specifically recognize RBF2 and do not cross-react with RBF1. On SDS–PAGE, RBF2 migrates slightly faster than RBF1 and separates as a single band of ~85 kDa. The expression of RBF1 and RBF2 was examined by western blot analysis using extracts prepared at various stages of *Drosophila* development. As shown in Figure 2A, the levels of RBF1 are relatively constant; the highest level of RBF1 is seen in 0- to 4-h-old embryos, and this drops slightly during the later stages of embryogenesis. In contrast, RBF2 levels vary considerably during development. RBF2 levels increase 4-fold in the first 8 h of development, and drop by one to two orders of magnitude at later stages of embryogenesis. Comparatively low levels of RBF2 were detected in whole larval extracts, and on long exposures of the western blots, in adult females, but not in males (Figure 2C; data not shown). Higher levels of RBF2 were found in dissected larval imaginal discs (see Figure 9H, lane 1) and in tissue culture cells. These patterns suggest that RBF2 may be most highly expressed in rapidly cycling cells, a fact that has previously been noted for p107 (Hurford *et al*., 1997). Analysis of extracts prepared from dissected ovaries, and the adults from which ovaries had been resected, shows that the RBF2 present in female extracts was provided exclusively by the ovary (Figure 2C), raising the possibility that RBF2 may function during oogenesis.

RBF2 can repress E2F-dependent transcription and is found at E2F-regulated promoters

Repression of E2F transcription is a hallmark of RB family proteins. To determine whether RBF2 regulates E2F-dependent transcription, we assayed its ability to repress E2F-regulated reporter constructs in SL2 cells. RBF1 and RBF2 expression constructs were generated and transfected into SL2 cells together with a *PCNA* reporter that had been previously used to measure the activity of dE2F1 and dE2F2 (Frolov *et al*., 2001). In order to monitor the expression levels from both constructs, RBF1 and RBF2 were HA-tagged on their N-terminal ends. Titration experiments showed that RBF2 repressed transcription from the wild-type *PCNA* promoter but had no effect on the mutant *PCNA* reporter construct lacking E2F-binding sites (Figure 3A). We then compared the repression properties of RBF1 and RBF2. Western blot analysis was performed on the transfected cells to ensure that the activities of RBF1 and RBF2 were compared at similar levels of protein expression. As shown in Figure 3B, equivalent levels of HA-RBF1 and HA-RBF2 were detected following transfection of 4 μg of PIE4-*HARBF2* and 0.05 μg of PIE4-*HARBF1*. Under these conditions, RBF2 repressed transcription from the *PCNA* promoter (Figure 3B), as well as the *MCM3* and *DNA Pol*α promoters, two other E2F-regulated genes (Figure 3C). In these reporter assays, RBF1 proved a more effective repressor than RBF2, when expressed at the same level. The reason why RBF1 and RBF2 expression plasmids give such different levels of protein expression is not known. This difference may reflect a property of the endogenous proteins, since quantitative blots show that SL2 cells contain ~30 times more RBF1 than RBF2 (data not shown). We conclude that RBF2 can repress E2F-dependent transcription, but in a less efficient manner than RBF1.

To determine whether RBF1 and RBF2 are present at these promoters *in vivo* under physiological conditions, a chromatin immunoprecipitation (ChIP) assay was used with specific RBF1 or RBF2 antibodies. As shown in Figure 3D and E, DNA sequences from the *PCNA* and the *DNA Pol*α promoters were selectively enriched in the RBF1 and RBF2 immunoprecipitations. No enrichment was observed with primers to the *RP49* control

promoter that lacks E2F-binding sites, or with non-specific control antibodies.

We conclude that RBF1 and RBF2 are able to repress transcription from E2F-regulated promoters, and that the endogenous RBF1 and RBF2 proteins are normally found at these promoters *in vivo*. The presence of both *Drosophila* pocket proteins at E2F promoters suggests that RBF1 and RBF2 may have overlapping functions in the regulation of E2F targets genes.

RBF2 fails to block dE2F1-mediated activation

Since the overexpression of RBF2 was able to repress E2F-dependent transcription, it seemed likely that RBF2 would repress dE2F1-mediated activation in a manner similar to that previously shown for RBF1 (Du *et al.*, 1996a). To test this possibility, we co-transfected RBF2 and dE2F1 expression constructs together with a *PCNA* reporter plasmid in SL2 cells. To ensure that dE2F1 was not saturating, we used small quantities of the dE2F1 expression plasmid that resulted in a 4.5-fold activation of the *PCNA* reporter (Figure 4). Co-transfection of HA-RBF1 completely blocked this dE2F1-induced transcriptional response, and a significant degree of repression was observed when a low amount of HA-RBF1 was transfected.

Surprisingly, however, HA-RBF2 had no effect on dE2F1-activated transcription (Figure 4). RBF2 also failed to repress the dE2F1 activation of the *DNA Polα* reporter (data not shown). In keeping with these transfection results, we noted that in transgenic animals, contrary to RBF1, RBF2 overexpression failed to suppress phenotypes caused by elevated levels of dE2F1 in various tissues (data not shown). Since overexpression of RBF2 does not inhibit dE2F1-driven transcription and does not suppress dE2F1-induced phenotypes *in vivo*, it appears that RBF1 and RBF2 regulate E2F-dependent transcription in a distinct manner.

Endogenous dE2F–RBF protein complexes

To understand the relationship between RBF1, RBF2, and the E2F proteins, we examined the pattern of dE2F–RBF protein interactions that exist in *Drosophila* SL2 cells. Specific antibodies for dDP, dE2F2, dE2F1, and non-specific control antibodies were used to immunoprecipitate protein complexes from SL2 extracts. These immune complexes were analyzed by western blotting with monoclonal antibodies specific for RBF1 or RBF2. A single 85 kDa band was detected by an anti-RBF2 monoclonal antibody in dDP and dE2F2 immunoprecipitates, but not in the dE2F1 or the control immunoprecipitates (Figure 5A, RBF2 line). The blot was stripped and re-probed with an anti-RBF1 monoclonal antibody and, as expected, RBF1 was detected in each of the test lanes and in none of the control lanes (Figure 5A, RBF1

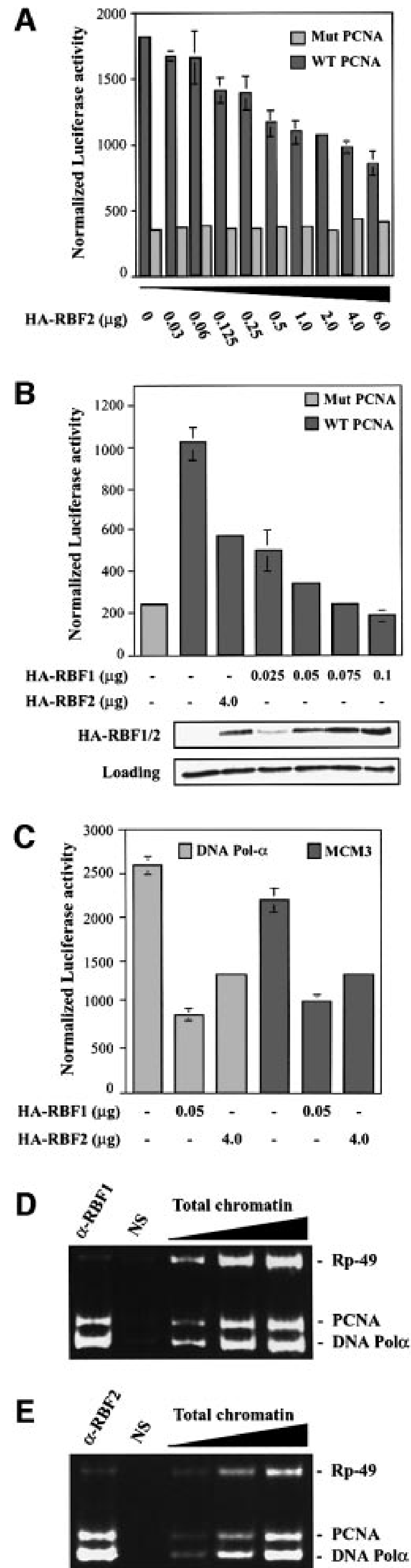

Fig. 3. RBF1 and RBF2 both repress E2F transcription. (**A**) SL2 cells were transfected with wild-type or mutant *PCNA* reporters and with various amounts of PIE4-*HARBF2*. (**B**) SL2 cells were transfected with the *PCNA* reporter and with 4 μg of PIE4-*HARBF2* and various amounts of PIE4-*HARBF1*. An HA antibody was used to monitor the expression of HA-RBF1 and HA-RBF2 in transfected cells. (**C**) A similar experiment was conducted using the *DNA Polα* (light bars) and the *MCM3* (dark bars) reporter constructs. (**D** and **E**) RBF1 and RBF2 occupy E2F-regulated promoters *in vivo*. Chromatin samples precipitated with RBF1, RBF2 or non-specific (NS) antibodies were used as templates for the amplification by PCR of the *PCNA*, the *DNA Polα* and the control *RP49* promoter regions with specific primers. Increasing amounts of total chromatin were used to ensure the linearity of the PCR amplification.

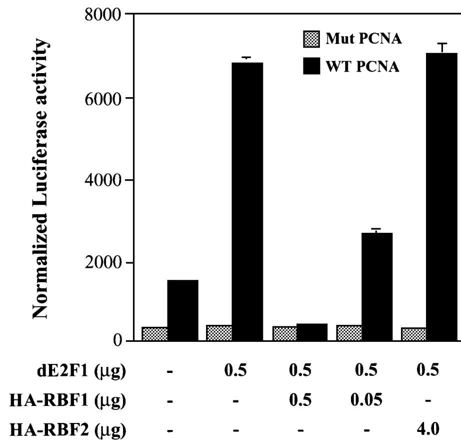

Fig. 4. RBF2 does not inactivate dE2F1-induced transcription. PIE4-*mycdE2F1* (0.5 μg) was transfected with the *PCNA* reporter and various amounts of PIE4-*HARBF1* or PIE4-*HARBF2*. Clear bars are transfected with the mutant *PCNA* reporter, dark bars are transfected with the wild-type construct. In the absence of PIE4-*mycdE2F1*, the activation of the wild-type *PCNA* promoter is due to the endogenous E2F activity in SL2 cells.

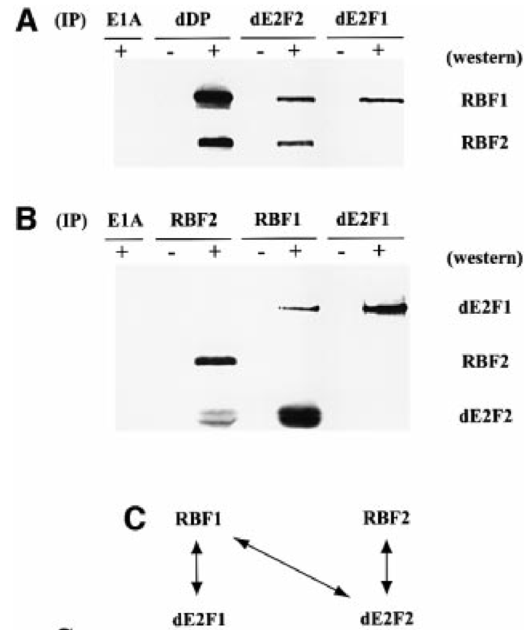

Fig. 5. Protein interactions between RBF and dE2F proteins. (**A**) RBF2 co-immunoprecipitates with dE2F2 but not with dE2F1. α-dDP, α-dE2F1, α-dE2F2 or control (α-E1A) antibodies were used to prepare immune complexes from SL2 cell extracts (+). Western blot analysis was performed using an α-RBF2-specific monoclonal antibody. After stripping, the membrane was re-probed with an α-RBF1-specific monoclonal antibody. (–) represents control immunoprecipitations using lysis buffer. (**B**) dE2F1 co-immunoprecipitates with RBF1 but not with RBF2. Immune complexes were prepared from SL2 cell extracts (+) using antibodies specific for RBF1, RBF2, dE2F1 or a control (E1A). The western blot was probed with a guinea pig α-dE2F1 antibody, then stripped and re-probed with an α-RBF2 antibody. This immunoprecipitation was subsequently repeated and the western blot was probed with an α-dE2F2 rabbit polyclonal antibody. (**C**) Summary of the interaction pattern between dE2Fs and RBFs.

line). In the reciprocal experiment, dE2F1 was detected in RBF1 immune complexes, but not in RBF2 immune complexes, whereas dE2F2 was found in both RBF1 and RBF2 immune complexes (Figure 5B). These results indicate that, under physiological conditions, RBF1 forms complexes with dE2F1 or dE2F2. RBF2, however, does not bind dE2F1, the activator *Drosophila* E2F, but associates exclusively with the repressor *Drosophila* E2F, dE2F2 (Figure 5C).

RBF2-mediated repression requires dE2F2

This pattern of interactions could explain, in a very simple way, why RBF2 is unable to block dE2F1-mediated activation. This arrangement also predicts that the effects of RBF2 on E2F-regulated transcription are likely to be mediated via dE2F2. To test this hypothesis, we assessed the ability of RBF2 to repress transcription in cells depleted for dE2F2 by RNA-mediated interference (RNAi). Cells were treated with control or dE2F2-specific double-stranded (ds) RNA and subsequently transfected with an E2F reporter construct and RBF1 or RBF2 expression plasmids. Western blots of the dsRNA-treated cells showed that the level of dE2F2 was substantially reduced by the RNAi treatment after 4 days (Figure 6A), and confirmed that equivalent levels of HA-RBF1 and HA-RBF2 were expressed. As shown in Figure 6A, the ability of RBF2 to repress transcription from the *MCM3* promoter was completely inhibited in cells treated with dE2F2 dsRNA, but was unaffected in cells treated with a control RNA targeting the *white* gene. In contrast with RBF2, the ability of RBF1 to repress transcription was unaffected by the depletion of dE2F2, presumably because of the presence of dE2F1, the other E2F partner of RBF1. This experiment indicates that dE2F2 is necessary for the proper repression of E2F target genes by RBF2. To test whether dE2F2 is required for the detection of RBF2 at E2F-regulated promoters, we performed ChIP assays on cells treated with control or dE2F2 dsRNA. As shown in Figure 6B, the depletion of dE2F2 from SL2 cells eliminated the binding of dE2F2 and RBF2 to the *DNA Polα* promoter, while leaving dE2F1 binding unaffected.

These experiments show that RBF2 can no longer be localized to E2F-regulated promoters or repress transcription in the absence of the DNA-binding activity provided by dE2F2. While the levels of HA-RBF2 expressed in transient transefction assays were unaffected by depletion of dE2F2 (Figure 6A), we noticed that levels of endogenous RBF2 protein were slightly reduced when dE2F2 was depleted by RNAi (Figure 6C). Interestingly, western blots of larval extracts prepared from wild-type and *de2f2* mutant larvae show that a long-term consequence of removing dE2F2 is that RBF2 becomes barely detectable (Figure 6D). The reduction of RBF2 protein is due to post-transcriptional effects, since *rbf2* transcripts are readily detectable in total RNA preparations from *de2f2* mutant larvae and, indeed, are present at elevated levels in the *de2f2* mutants (Figure 6E). The most likely explanation is that RBF2 becomes less stable in the absence of its binding partner. Such a link between stability and functional cooperativity has been previously noted in other pairs of interacting proteins such as TSC1 and TSC2 (Benvenuto *et al*., 2000). At present, we cannot exclude the formal possibility that dE2F2 influences the synthesis of RBF2, and further experiments are needed to determine precisely how the synthesis and turnover of RBF2 change in the absence of dE2F2. Nevertheless, these

observations all support the notion that the function of RBF2 depends on the presence of dE2F2.

RBF2 and dE2F2 cooperate to antagonize dE2F1-mediated activation and block S phase entry

Previous studies have revealed that dE2F1 and dE2F2 have distinct biochemical and functional properties (Frolov *et al.*, 2001). Both proteins can be found at endogenous E2F-regulated promoters, but dE2F1 functions primarily as an activator of transcription, whereas dE2F2 is a transcriptional repressor. Studies of *de2f1*, *de2f2* and *de2f2;de2f1* double mutants demonstrate that the normal expression patterns of E2F-target genes depends on the integrated activities of both dE2F1 and dE2F2. Thus far, we have observed that (i) RBF2 interacts specifically with dE2F2, (ii) dE2F2 is able to antagonize dE2F1 in a manner dependent upon its interaction with RBF proteins (Cayirlioglu *et al.*, 2001), and (iii) RBF2-mediated repression requires dE2F2. These results suggest that transcriptional repression by RBF2 is not mediated via dE2F1. Rather, RBF2 appears to form an RBF2–dE2F2 complex that antagonizes dE2F1 indirectly, by altering the balance between the transcriptional activities of dE2F1 and dE2F2.

We tested this idea directly in SL2 cells, where we challenged constant levels of transfected dE2F1 with increasing amounts of transfected dE2F2. We compared the overall level of transcription generated by both dE2Fs at the *PCNA* promoter with and without co-transfected RBF2. As shown in Figure 7, the combined expression of dE2F2 and RBF2 was able to reduce dE2F1-mediated activation of the *PCNA* reporter far more effectively than dE2F2 alone. Thus, in SL2 cells, RBF2 and dE2F2 cooperate to antagonize the transcriptional activity of dE2F1.

Given that RBF2 and dE2F2 together can antagonize dE2F1-driven transcription and that dE2F1 activity is needed for normal cell cycle progression, we tested whether the co-expression of dE2F2 and RBF2 might block cell cycle progression *in vivo*. Indeed, we were able to recapitulate the synergy between RBF2 and dE2F2 in transgenic flies. For these experiments, UAS-*rbf2* transgenes were generated and crossed to an extensive collection of tissue- or pattern-specific Gal4 drivers. No visible phenotypes were observed when RBF2 was expressed alone. Previous studies had also found that the over-expression of dE2F2 *in vivo* had minimal effects (Cayirlioglu *et al.*, 2001). However, when UAS-*rbf2* and UAS-*de2f2* transgenes were combined, we found that the co-expression of RBF2 and dE2F2 gave clearly visible phenotypes in both the wing and the eye (Figure 8). Two lines containing both a UAS-*de2f2* and a UAS-*rbf2* transgene were used to exclude non-specific effects (lines a and b). Western blot analysis using extracts from dissected wing discs of third instar larvae showed that RBF2 and dE2F2 were overexpressed 3.5- and 11-fold,

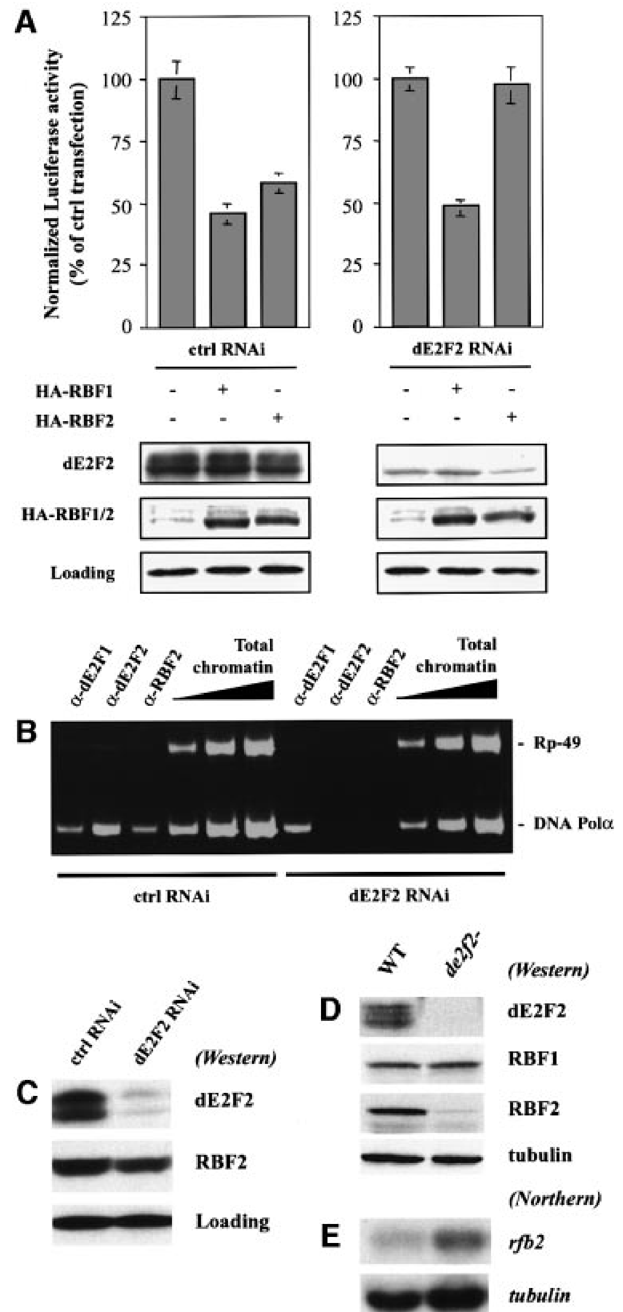

Fig. 6. dE2F2 is required for RBF2 repression, localization, and expression. (**A**) SL2 cells were treated with *white* (ctrl) or *de2f2* dsRNA and transfected with the *MCM3* reporter together with either 0.05 μg of PIE4-*HARBF1* or 4 μg of PIE4-*HARBF2*. Depletion of dE2F2 increases transcription from the PCNA reporter by 35%. To compare the ability of RBF1 and RBF2 to repress in these different conditions, the levels of luciferase activity are expressed as a percentage of the luciferase activity observed in the absence of any RBF expression plasmid. Western blot analysis with an α-dE2F2 or an α-HA antibody confirms the specific depletion of dE2F2, and the expression of equivalent levels of HARBF1 and HARBF2. (**B**) RBF2 occupancy of the *DNA Polα* promoter requires dE2F2 in *vivo*. Chromatin samples precipitated with dE2F1, dE2F2, or RBF2 antibodies were used as templates for the amplification by PCR of the *DNA Polα*, and the control *RP49* promoter regions with specific primers. (**C**) RBF2 levels are only slightly reduced in cells treated with dE2F2 RNAi. (**D**) dE2F2 is required for the maintenance of RBF2 protein levels *in vivo*. Western blotting analysis of protein extracts from wild-type or *de2f2* mutants larvae using α-dE2F2, α-RBF1, α-RBF2 or α-tubulin antibodies. (**E**) The loss of the RBF2 protein in *de2f2* mutants is not due to transcriptional changes. Northern blot analysis of total RNA from wild-type or *de2f2* mutant larvae using an *rbf2* or a *tubulin* radiolabeled antisense riboprobe.

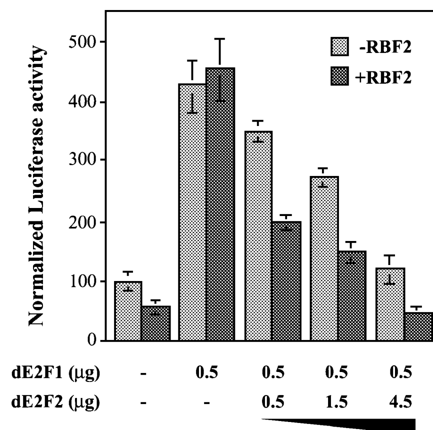

Fig. 7. RBF2 and dE2F2 cooperate to antagonize dE2F1-induced transcription. SL2 cells were transfected with the *PCNA* reporter, 0.5 μg of PIE4-*mycdE2F1*, and increasing amounts of PIE4-*mycdE2F2*. E2F-dependent transcription was detected in either the presence (4 μg of PIE4-*HARBF2*; dark gray bars) or absence (4 μg of empty PIE4; light gray bars) of exogenous RBF2.

respectively, in line a, and 5.5 fold and 18-fold in line b (Figure 9H). Consistent with this, the strongest phenotypes were seen using line b.

In Figure 8, results are shown using two distinct Gal4 drivers: *apterous*-Gal4 (*ap*-Gal4), in which Gal4 is expressed in wing discs, and *eyeless*-Gal4 (*ey*-Gal4), a transgene that drives Gal4 expression in eye discs. The UAS-*lacZ* control, UAS-*de2f2* or UAS-*rbf2* transgenes (Figure 8A–C) gave no visible phenotype when combined with *ap*-Gal4. However, visible phenotypes were observed when both UAS-*de2f2* and UAS-*rbf2* were present. These effects varied in severity, from a small, upward-curved wing (line a) to a severe atrophy of the wing (line b; Figure 8D and E). We were unable to compare the effects of RBF2/dE2F2 with RBF1 using this driver because the *ap*-Gal4/UAS-*rbf1* transgene combination is lethal.

In a similar way, no visible phenotype was observed when *ey*-Gal4 flies were crossed to UAS-*lacZ*, UAS-*de2f2* or UAS-*rbf2* alone (Figure 8F–H). However, the combined expression of dE2F2 and RBF2 generated a small rough eye phenotype (Figure 8I). This small eye phenotype was similar to, but weaker than, what we observed with the *ey*-Gal4/UAS-*rbf1* transgenic line (Figure 8J).

Wing discs overexpressing RBF2 and dE2F2 were examined to determine the physiological basis of the observed phenotype. As shown in Figure 9E and F, GFP detection revealed that the *ap*-Gal4 transgene drives Gal4 expression in the lower part of the wing disc. BrdU incorporation assays indicated that the co-expression of RBF2 and dE2F2 causes a significant decrease in DNA synthesis (Figure 9C and D) compared with the *ap*-Gal4/UAS-*lacZ* control (Figure 9A and B). No change in BrdU incorporation is seen at the edges of the disc where the *ap*-Gal4 transgene is not expressed (arrow in Figure 9C–E). Overexpression of RBF2 and dE2F2 gave a phenotypic range that varied from discs that were shrunken and abnormal to those discs that have relatively normal morphology but displayed reduced BrdU incorporation, as shown in Figure 9. *Ap*-Gal4 and *ey*-Gal4 were expressed early in development, yet the effects of RBF2–E2F2 expression were often not evident until the late larval stages. We interpret this, and the lack of phenotypes from other Gal4 drivers, to mean that the defects do not appear until sufficient amounts of dE2F2 and RBF2 have accumulated in sensitive tissues.

To further assess the effects of RBF2–E2F2 expression on the cell cycle, clones of dE2F2–RBF2-overexpressing cells were generated in the wing discs and marked with GFP. The cell cycle profile of these cells was determined by FACS analysis and compared with wild-type cells from the same discs (Neufeld *et al.*, 1998). The overexpression of dE2F2 or RBF2 alone had no effect on cell cycle distribution (data not shown). However, the co-expression of dE2F2 and RBF2 caused a significant increase in the population of cells with a G_1 DNA content, and a decrease of S phase and G_2 cells (Figure 9G). Taken together, these experiments demonstrate that, when overexpressed, dE2F2 and RBF2 act synergistically to antagonize dE2F1-mediated transcriptional activation and to block S phase entry *in vivo*.

Discussion

In order to understand how E2F regulation provides cell cycle control, it is necessary to know the properties of each E2F component, and to discover how these components work together. This is a daunting task using mammalian cells, given the complexity of mammalian E2Fs. *Drosophila melanogaster* appears to encode just two RB family members (*rbf1* and *rbf2*), one DP gene (*dDP*), and two E2F family members (*de2f1* and *de2f2*), and provides a simpler system to examine the mechanisms underlying E2F regulation.

In this study, we describe the properties of *rbf2*, the second Retinoblastoma family gene to be found in *Drosophila*. Chromatin immunoprecipitation experiments reveal that the endogenous RBF1 and RBF2 proteins are both normally present at known E2F-regulated promoters. In previous studies, we have found that dE2F1, dE2F2 and dDP are also detected at these same promoters (Frolov *et al.*, 2001). These biochemical data are consistent with genetic studies showing that E2F-regulated promoters are deregulated in the absence of dE2F1, dE2F2, dDP or RBF1 (Duronio and O'Farrell, 1995; Duronio *et al.*, 1995; Royzman *et al.*, 1997; Du and Dyson, 1999; Cayirlioglu *et al.*, 2001; Frolov *et al.*, 2001). Taken together, these results demonstrate that the normal pattern of gene expression from known E2F-regulated promoters depends on the integrated activity of multiple proteins, rather than any one E2F or RBF component alone.

Although RBF1 and RBF2 are both able to repress E2F-dependent transcription, they appear to act in markedly different ways. RBF1 was originally identified by virtue of its ability to physically interact with the transcriptional activation domain of dE2F1 (Du *et al.*, 1996a). RBF1 is a potent inhibitor of dE2F1-mediated activation and it readily suppresses dE2F1-induced phenotypes *in vivo*. Unlike RBF1, RBF2 does not associate with dE2F1 *in vivo*, and it is unable to suppress the effects of overexpressed dE2F1. *In vivo*, RBF2 associates specifically with dE2F2. The recruitment of RBF2 to E2F-regulated promoters, and its ability to repress transcription, requires dE2F2. In support of the idea that RBF2 acts in a stable complex with

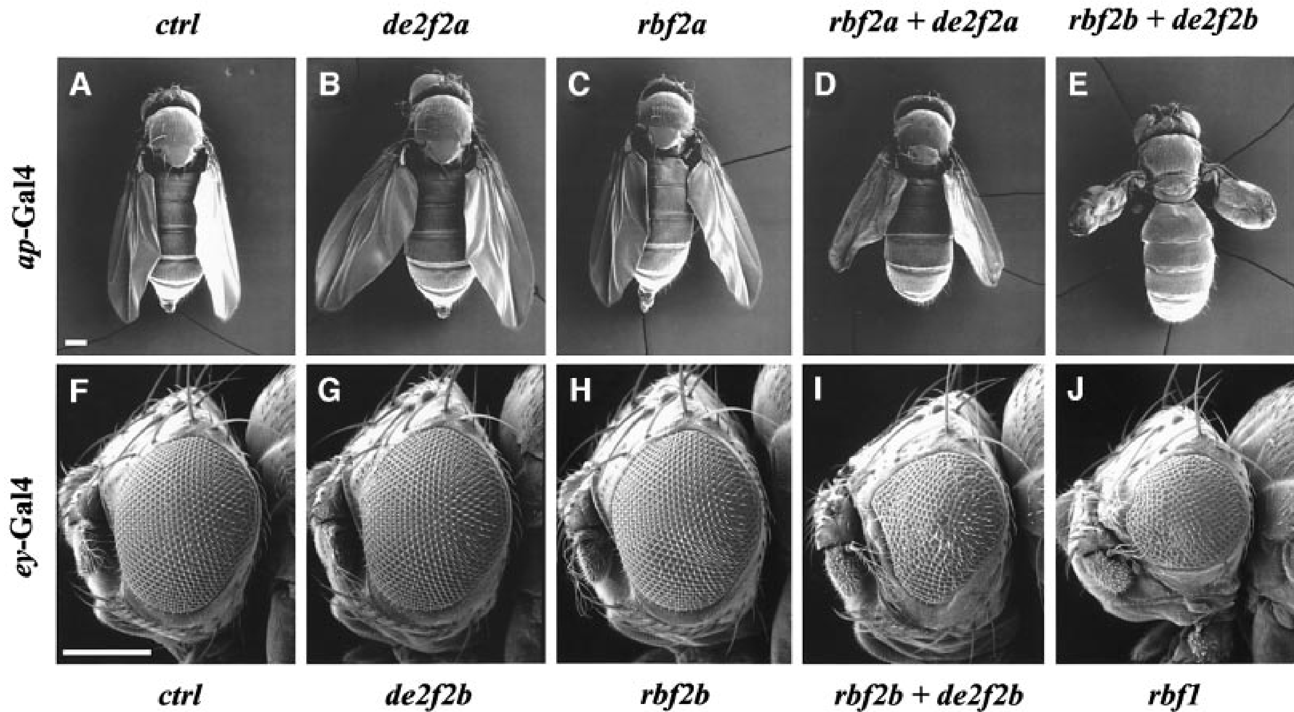

Fig. 8. RBF2 and dE2F2 transgenes have a synergistic effect *in vivo*. Scan electron microscopy (SEM) of transgenic flies of the following genotypes: (**A**) *ap-Gal4/+;*UAS-*lacZ/+*; (**B**) *ap-Gal4/+;*UAS-*de2f2a/+*; (**C**) *ap-Gal4/*UAS-*rbf2a*; (**D**) *ap-Gal4/*UAS-*rbf2a;*UAS-*de2f2a/+*; (**E**) *ap-Gal4/+;*UAS-*de2f2b*-UAS-*rbf2b/+*; (**F**) *ey-Gal4/+;*UAS-*lacZ/+*; (**G**) *ey-Gal4/+;*UAS-*de2f2b/+*; (**H**) *ey-Gal4/+;*UAS-*rbf2b/+*; (**I**) *ey-Gal4/+;*UAS-*de2f2b*-UAS-*rbf2b/+*; (**J**) *ey-Gal4/*UAS-*rbf1*. SEM (A–E), 5 kV, 32×; SEM (F–J), 10 kV, 200× (Scale bars = 1 mm).

dE2F2, we find that these proteins act synergistically when overexpressed in SL2 cells or in transgenic animals, and that RBF2 levels are strongly reduced in *de2f2* mutant larvae.

The pattern of interactions between *Drosophila* pocket proteins and E2F proteins is illustrated in Figure 10. Of the four proteins shown, only dE2F1 activates transcription (Figure 10A). We suggest that RBF proteins modulate dE2F1-mediated activation in two distinct ways. First, dE2F1 can be directly regulated through a physical interaction with RBF1 (Figure 10B). Secondly, dE2F1-mediated activation can be antagonized by the presence of RBF2–dE2F2 and RBF1–dE2F2 repressor complexes at the promoter of E2F-regulated genes (Figure 10C). Because E2F-regulated promoters often contain multiple E2F-binding sites, it is unclear whether these complexes compete for the same binding element or whether they act antagonistically through adjacent sites. The results described here and in Frolov *et al*. (2001) suggest that there is a hierarchy of effects, with RBF1 being a stronger antagonist of dE2F1 than either of the dE2F2-containing complexes. Nevertheless, the level of dE2F1-dependent transcription is influenced by dE2F2 and RBF2, and changing the levels of RBF2 alters the balance between dE2F1-mediated activation and dE2F2-mediated repression. One of the implications of this arrangement is that a pocket protein does not need to bind directly to an E2F subunit in order to influence its activity (Figure 10C).

Strikingly, a similar arrangement of E2F/pocket proteins exists in mammalian cells (Figure 10D). Although mammalian cells contain multiple E2F and pRB family members, recent studies have suggested that the different forms of E2F can be subdivided into two groups depending on whether they appear to be primarily involved in activation or repression. Intriguingly, pRB, like RBF1, interacts with both sets of E2Fs, whereas p107 and p130, like RBF2, interact specifically with the co-repressor E2Fs. Consistent with this, the loss of RBF1 function induces phenotypes that are remarkably similar to the effects of mutating pRB (deregulation of E2F, ectopic S phases, increased apoptosis), and the mutation of dE2F1 gives phenotypes that are very similar to those recently described for the combined mutation of the murine E2F1, E2F2 and E2F3 genes (G_1 arrest and loss of E2F-dependent transcription; Wu *et al*., 2001). Furthermore, the genetic interactions observed between *RB* and *E2f1* or *RB* and *E2f3* alleles in mice are reminiscent of the genetic interactions between *rbf* and *de2f1* in flies. The logical extension of this homology is that the roles of RBF2–E2F2 in *Drosophila* may be similar to the repressor complexes formed by p107/p130 and E2F4/E2F5 in mammalian cells. Despite the attractions of this analogy, we note that the phylogenetic tree of Retinoblastoma-related proteins shows that RBF1 and RBF2 are more closely related to one another than they are to any mammalian protein. This suggests that RBF1 and RBF2 were generated by a gene duplication event, most likely from an ancestral protein that resembled p107 or p130. Consequently, similarities between the arrangement of *Drosophila* and mammalian RB–E2F complexes are more likely to result from a convergent evolutionary process, rather than the conservation of functional differences between pocket proteins.

What is the advantage of such an arrangement of complexes, and why might it be selected? The distinction

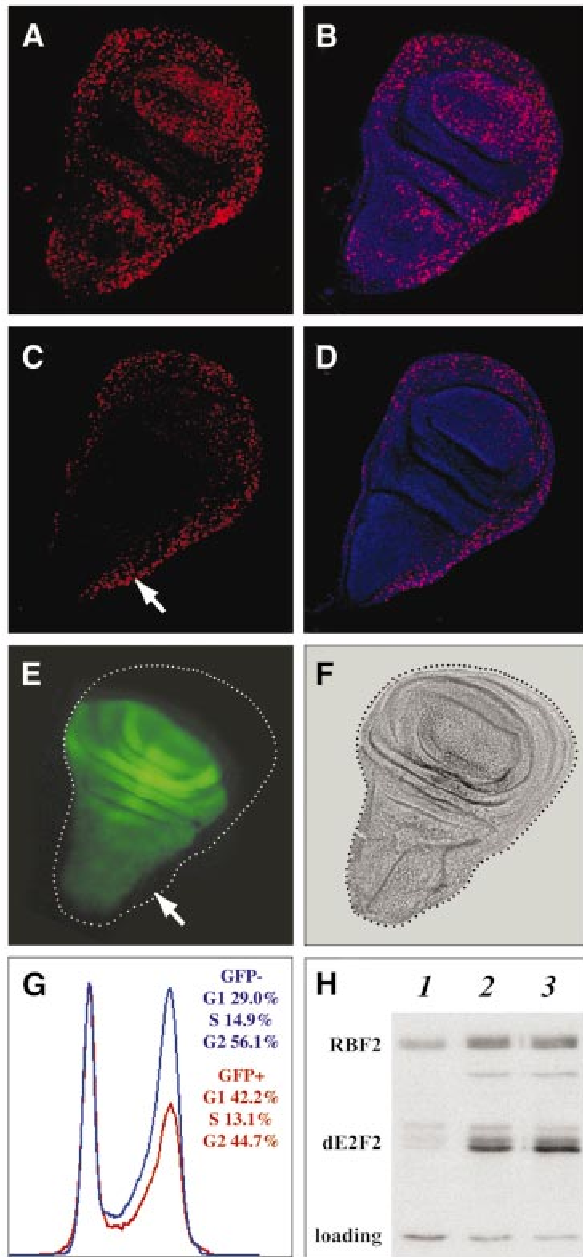

Fig. 9. Overexpression of RBF2 and dE2F2 blocks S phase entry and causes cells to accumulate in the G_1 phase of the cell cycle. BrdU incorporation in third instar larval wing discs of the following genotype: (**A** and **B**) *ap*-Gal4/+;UAS-*lacZ*/+; (**C** and **D**) *ap*-Gal4/UAS-*rbf2a;*UAS-*de2f2a*/+. Red, anti-BrdU staining; blue, yo-yo DNA staining. (**E**) EGFP pattern of expression in an *ap*-Gal4/+;UAS-*EGFP*/+ third instar larval wing disc. (**F**) Nomarski imaging of the same disc as in (E). (**G**) FACS profile of the GFP-negative wild-type cells (blue graph) as compared with GFP-positive cells overexpressing dE2F2 and RBF2 (red graph). (**H**) Western blot analysis with RBF2 or dE2F2 antibodies of protein extracts from ten third instar larval wing disc of the following genotypes: *ap*-Gal4/+;UAS-*lacZ*/+ (lane 1); *ap*-Gal4/UAS-*rbf2a;*UAS-*de2f2a*/+ (lane 2); *ap*-Gal4/+;UAS-*de2f2b-UAS-rbf2b*/+ (lane 3).

between activator and repressor E2Fs is most important if one considers the effects when the complexes are disrupted. Since dE2F2 appears to be unable to activate transcription, the release of RBF1 or RBF2 from a dE2F2 complex is predicted to de-repress E2F target genes. In contrast, the release of RBF1 from a dE2F1-containing

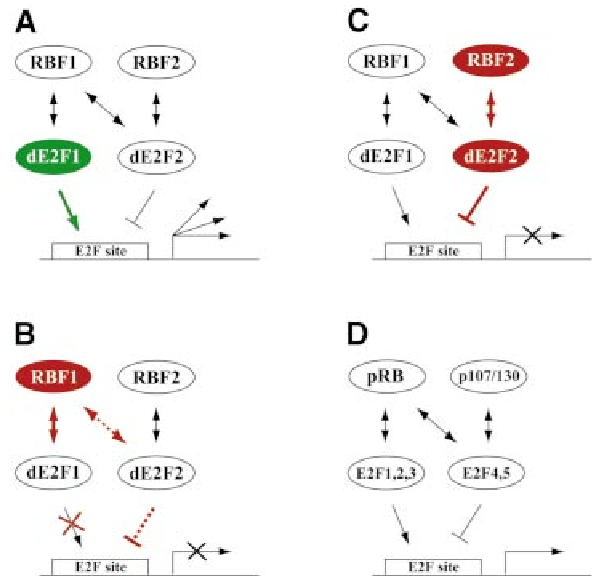

Fig. 10. Model of E2F transcriptional regulation by pocket proteins in *Drosophila*. The transcriptional output from known E2F-regulated promoters depends on the relative activity of activator and repressor complexes. Activation is depicted in green, repression in red. (**A**) The expression of dE2F1, or its release from RBF1, strongly induces transcription. (**B**) RBF1 binds to the activation domain of dE2F1 and inactivates dE2F1-induced transcription. RBF1 can also interact with dE2F2, but this interaction is not required for RBF1 to repress E2F-dependent promoters. (**C**) RBF2, however, does not associate with dE2F1. RBF2 forms complexes with dE2F2 that antagonize dE2F1-induced transcription indirectly and causes a shift towards repression. (**D**) The pattern of dE2F–RBF interactions in *Drosophila* parallels the arrangement of the mammalian E2F/pocket protein pathway.

complex would liberate a strong activator of transcription. These types of E2F complexes therefore allow three different types of E2F regulatory transitions: (i) from repression to de-repression (in which RBF2–dE2F2 or RBF1–dE2F2 complexes are disrupted and removed from the promoters); (ii) from repression to activation (in which RBF1–dE2F1 complexes are disrupted by phosphorylation liberating dE2F1, a strong activator of transcription); and (iii) from repression to de-repression to activation (in which RBF2–dE2F2 or RBF1–dE2F2 repressors are disrupted and replaced by dE2F1).

The evolution of multiple E2F–RB complexes may offer several additional advantages. Individual complexes may preferentially regulate different subsets of targets. This specificity might be achieved by different DNA-binding subunits (e.g. E2F1–RBF1 versus E2F2–RBF1) or by different protein–protein interactions with adjacent factors at the promoter. Indeed, several studies have suggested that mammalian E2F/pocket proteins may target specific promoters (Hurford *et al*., 1997; Takahashi *et al*., 2000; Wells *et al*., 2000). A second possibility is suggested by the fact that many chromatin-remodeling activities have been linked to pocket proteins, potentially allowing a wide variety of activities to be recruited to E2F-regulated promoters (Brehm and Kouzarides, 1999; Harbour and Dean, 2000). Perhaps RBF1 and RBF2 provide a bridge to different types of complexes. A third possibility is that the pocket protein–E2F complexes may be differentially regulated. pRB appears to be uniquely

required for DNA damage-induced cell cycle arrest (Harrington *et al*., 1998), and Zhang *et al*. (1999) have shown that pRB is specifically required in TGFβ and p16-induced cell cycle arrest. In a similar way, RBF2 may be required for cell cycle arrest at a specific stage of development or in particular tissues. The high levels of RBF2 protein in early embryos and in dissected ovaries may reflect specific roles in embryonic cell cycle and oogenesis. Alternatively, RBF1- and RBF2-containing complexes might occupy E2F sites during different phases of the cell cycle. E2F has recently been shown to control the expression of genes encoding mitotic functions whose transcription is induced later than the G_1 to S transition (Ishida *et al*., 2001; Ren *et al*., 2002). RBF2, like p107, is expressed at higher levels in actively cycling cells and is a likely E2F target gene. Potentially, this newly synthesized pocket protein may provide a repressor activity during S phase on mitosis-specific promoters or during G_2 at S phase-specific promoters.

The answer to many of these questions will stem from a careful comparison between *rbf1* mutants, *rbf2* mutants and *rbf1;rbf2* double mutants. This analysis will be needed to separate the specific functions of RBF1 and RBF2 *in vivo* and to uncover the functions that are redundant between the two *Drosophila* pocket proteins.

Materials and methods

Characterization of the rbf2 cDNA and genomic region

The two ESTs with similarities to p107, LD02737 and LD15806 were analyzed with the matchbox software at www.fundp.ac.be/sciences/biologie/bms/matchbox_submit.html. The two ESTs were full-length clones with identical sequences (DDBJ/EMBL/GenBank accession No. AF197059). The genomic sequence (DDBJ/EMBL/GenBank accession No. AF195899) revealed that *rbf2* is intronless. To obtain the 5′ and 3′ boundaries of the *rbf2* cDNA, a RACE protocol was performed using the Marathon cDNA amplification kit (Frolov *et al*., 2001). Multiple alignments were performed with the Clustal method, PAM250 matrix, and the alignment was processed at www.ch.embnet.org/software/BOX_form.html with boxshade.

Preparation of antibodies, western blot analysis and immunoprecipitation assays

Mice were immunized with the His-tagged N-terminal half of the RBF2 protein expressed in *Escherichia coli* (amino acids 1–324). The RBF2 monoclonal antibodies DR-3 (used for RBF2 IP) and DR-6 (used for RBF2 western blots) were prepared as described previously (Harlow and Lane, 1999). A polyclonal antiserum to RBF2 was raised by injecting mice with a mix of the recombinant GST-tagged N- and C-terminal halves of RBF2. Protein work was performed as described previously (Harlow and Lane, 1999). Anti-dDP (Yun-3 and 6), anti-dE2F1 (Hao-4 and guinea pig polyclonal), anti-dE2F2 (mei-3 and -8, rabbit polyclonal) and anti-RBF1 (DX-3) antibodies have been described previously (Brook *et al*., 1996; Du *et al*., 1996a,b; Bosco *et al*., 2001; Frolov *et al*., 2001). Our tubulin antibody is the E7 monoclonal (Developmental Studies Hybridoma Bank). ChIP assays were performed as described previously (Frolov *et al*., 2001).

Plasmid construction

All standard DNA manipulations were performed as described previously (Sambrook *et al*., 1989). The RBF1 and RBF2 open reading frames were HA tagged on their 5′-end and cloned into the pIE4 expression vector (Novagen). The *PCNA*–luciferase reporter was kindly provided (Yamaguchi *et al*., 1995). The *MCM3* –515 to +138 and the *DNA Polα* –368 to +22 promoter regions containing E2F consensus sites were cloned in the pGL2 luciferase vector (Promega). The full-length *rbf2* cDNA was cloned in the pUASt vector (Brand and Perrimon, 1993).

Transient transfections

Drosophila SL2 cells were transfected with CellFectin (Invitrogen) with 10 μg of plasmid DNA. All reporter assays were performed in triplicate. In some graphs, data points are shown without error bars. This is because the variation on these points was very small and did not reach the threshold (2% of the highest value on the graph) needed to be displayed by the graphing program (Cricket Graph III). In reporter assays, 2 μg of reporter plasmid were used along with 2 μg of normalizing pIE4-lacZ plasmid and various amounts of dE2F/RBF pIE4 plasmids, except for Figure 7, where 0.5 μg of reporter and 0.5 μg of *lacZ* plasmids were used. Empty pIE4 plasmid was used as filler to reach 10 μg. β-galactosidase and luciferase assays were performed as described previously (Frolov *et al*., 2001).

RNA interference

RNA interference assays were performed as described previously (Worby *et al*., 2001). In Figure 6, cells were transfected 4 days after standard RNAi treatment.

Fly stocks

UAS-*rbf2* transgenic animals were obtained as described previously (Spradling and Rubin, 1982). Other stocks have been described previously (Neufeld *et al*., 1998; Frolov *et al*., 2001). The following stocks were used in this study: UAS-*de2f2*a = UAS-*de2f2* 14.9 (third chromosome); UAS-*de2f2*b = UAS-*de2f2* 18.11/*TM6B, Hu Tb*; UAS-*rbf2*a = UAS-*rbf2*.5 (second chromosome); UAS-*rbf2*b = UAS-*rbf2*.4/*TM6B, Hu Tb*; *line a* = UAS-*rbf2*a; UAS-*de2f2*a/*T* (*2; 3*) *CyO TM6B, Tb Cy*; *line b* = UAS-*de2f2*b – UAS-*rbf2*b/*TM6B, Hu Tb*; *ap*-Gal4 = *ap*-Gal4/*T* (*2; 3*) *CyO TM6B, Hu Tb* (modified B-3041); UAS-*lacZ* (third chromosome) = B-1777; *ey*-Gal4/*CyO* = B-5535; UAS-*rbf1* = UAS-*rbf1*.4/*CyO*; UAS-*EGFP*(5a.2) = B-5431.

For generating clones overexpressing *GFP*, *de2f2* and *rbf2*: HS-FLP/Y; UAS-*de2f2*b – UAS-*rbf2*b/*TM6B*, *Hu Tb* males were crossed with Act5c>CD2>*Gal4;* UAS-*GFP* females, and after heat shock, wing discs from non-*tubby* third-instar larvae were dissected, dissociated and analyzed by FACS as described previously (Neufeld *et al*., 1998).

Scanning electron microscopy

Samples were prepared as described previously (Kimmel *et al*., 1990).

BrdU labeling

Wing discs from third instar larvae were labeled in Schneider media with 0.2 mg/ml BrdU (Sigma) at room temperature for 30 min. Wing discs were fixed and BrdU was detected with a mouse α-BrdU antibody (Becton Dickinson). Cy3 donkey α-mouse (Jackson Immunolaboratories) was used as a secondary antibody. DNA staining was obtained with the yo-yo dye (Molecular Probes). Images were collected on a Zeiss LSM510 confocal microscope.

Acknowledgements

We thank Bill Fowle and Joanne Yetz-Aldape for their expert assistance with SEM and FACS, respectively, the Bloomington *Drosophila* Center for fly strains, Giovanni Bosco and Terry Orr-Weaver for providing the dE2F1 antiserum, the Hariharan and Harlow laboratories for help and fruitful discussions, and Katie Nolan and Fred Dick for the critical reading of this manuscript. The support of Professor Vandenhaute at the Unité de Recherche en Biologie Moléculaire in Namur, Belgium, is gratefully acknowledged by O.S., who was supported in part by the Rose and Jean Hoguet Foundation for Cancer Research and the Belgian American Educational Foundation. This work was supported in part by NIH post-doctoral fellowship F32 CA93045 (to D.D.) and F32 CA88474 (to E.M.), by NIH grant GM53203 (to N.D.) and by a Tosteson fellowship from the MBRC (to M.V.F.).

References

Asano,M., Nevins,J.R. and Wharton,R.P.E. (1996) Ectopic E2F expression induces S phase and apoptosis in *Drosophila* imaginal discs. *Genes Dev*., **10**, 1422–1432.

Benvenuto,G. *et al*. (2000) The tuberous sclerosis (TSC1) gene product hamartin suppresses cell growth and augments the expression of the TSC2 product tuberin by inhibiting its ubiquitination. *Oncogene*, **19**, 6306–6316.

Bosco,G., Du,W. and Orr-Weaver,T. (2001) DNA replication control

through interaction of E2F-RB and the origin recognition complex. *Nat. Cell Biol.*, **3**, 289–295.

Brand,A.H. and Perrimon,N. (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development*, **118**, 401–415.

Brehm,A. and Kouzarides,T. (1999) Retinoblastoma protein meets chromatin. *Trends Biochem. Sci.*, **24**, 142–145.

Brehm,A., Miska,E.A., McCance,D.J., Reid,J.L., Bannister,A.J. and Kouzarides,T.E. (1998) Retinoblastoma protein recruits histone deacetylase to repress transcription. *Nature*, **391**, 597–601.

Brook,A., Xie,J.E., Du,W. and Dyson,N. (1996) Requirements for dE2F function in proliferating cells and in post-mitotic differentiating cells. *EMBO J.*, **15**, 3676–3683.

Cayirlioglu,P., Bonnette,P.C., Dickson,M.R. and Duronio,R.J. (2001) *Drosophila* E2F2 promotes the conversion from genomic DNA replication to gene amplification in ovarian follicle cells. *Development*, **128**, 5085–5098.

Dannenberg,J.-H., van Rossum,A., Schuijff,L. and te Riele,H. (2000) Ablation of the Retinoblastoma gene family deregulates G_1 control causing immortalization and increased cell turnover under growth-restricting conditions. *Genes Dev.*, **14**, 3051–3064.

Du,W. (2000) Suppression of the *rbf* null mutants by a *de2f1* allele that lacks a transactivation domain. *Development*, **127**, 367–379.

Du,W. and Dyson,N. (1999) The role of RBF in the introduction of G_1 regulation during *Drosophila* embryogenesis. *EMBO J.*, **18**, 916–925.

Du,W., Vidal,M., Xie,J.-E. and Dyson,N. (1996a) *RBF*, a novel RB-related gene that regulates E2F activity and interacts with cyclin E in *Drosophila*. *Genes Dev.*, **10**, 1206–1218.

Du,W., Xie,J.-E. and Dyson,N. (1996b) Ectopic expression of dE2F and dDP induces cell proliferation and death in the *Drosophila* eye. *EMBO J.*, **15**, 3684–3692.

Duronio,R.J. and O'Farrell,P.H. (1995) Developmental control of the G_1 to S transition in *Drosophila*: cyclin E is a limiting downstream target of E2F. *Genes Dev.*, **9**, 1456–1468.

Duronio,R.J., O'Farrell,P.H., Xie,J.-E., Brook,A. and Dyson,N. (1995) The transcription factor E2F is required for S phase during *Drosophila* embryogenesis. *Genes Dev.*, **9**, 1445–1455.

Dyson,N. (1998) The regulation of E2F by pRB-family proteins. *Genes Dev.*, **12**, 2245–2262.

Frolov,M.V., Huen,D.S., Stevaux,O., Dimova,D., Balczarek-Strang,K., Elsdon,M. and Dyson,N.J. (2001) Functional antagonism between E2F family members. *Genes Dev.*, **15**, 2146–2160.

Gaubatz,S., Lindeman,G.J., Jakoi,L., Nevins,J.R., Livingston,D.M. and Rempel,R.E. (2000) E2F4 and E2F5 play an essential role in pocket protein-mediated G_1 control. *Mol. Cell*, **6**, 729–735.

Goodrich,D.W., Wang,N.P., Qian,Y.W., Lee,E.Y. and Lee,W.H. (1991) The retinoblastoma gene product regulates progression through the G_1 phase of the cell cycle. *Cell*, **67**, 293–302.

Harbour,J.W. and Dean,D.C. (2000) The Rb/E2F pathway: expanding roles and emerging paradigms. *Genes Dev.*, **14**, 2393–2409.

Harlow,E. and Lane,D. (1999) *Using Antibodies: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Harrington,L.J., Bruce,J., Harlow,E. and Dyson,N. (1998) pRB plays an essential role in cell cycle arrest induced by DNA damage. *Proc. Natl Acad. Sci. USA*, **95**, 11945–11950.

Hinds,P.W., Mittnacht,S., Dulic,V., Arnold,A., Reed,S.I. and Weinberg, R.A. (1992) Regulation of retinoblastoma protein functions by ectopic expression of human cyclins. *Cell*, **70**, 993–1006.

Hurford,R., Cobrinik,D., Lee,M.-H. and Dyson,N. (1997) pRB and p107/p130 are required for the regulated expression of different sets of E2F responsive genes. *Genes Dev.*, **11**, 1447–1463.

Ishida,S., Huang,E., Zuzan,H., Spang,R., Leone,G., West,M. and Nevins,J.R. (2001) Role for E2F in control of both DNA replication and mitotic functions as revealed from DNA microarray analysis. *Mol. Cell. Biol.*, **21**, 4684–4699.

Jacks,T., Fazeli,A., Schmitt,E., Bronson,R., Goodell,M. and Weinberg,R. (1992) Effects of an Rb mutation in the mouse. *Nature*, **359**, 295–300.

Kimmel,B.E., Heberlein,U. and Rubin,G.M. (1990) The homeodomain protein rough is expressed in a subset of cells in the developing *Drosophila* eye where it can specify photoreceptor cell subtype. *Genes Dev.*, **4**, 712–729.

Lee,E.Y., Chang,C.Y., Hu,N., Wang,Y.C., Lai,C.C., Herrup,K., Lee,W.H. and Bradley,A. (1992) Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis. *Nature*, **359**, 288–294.

Magnaghi,L., Groisman,R., Naguibneva,I., Robin,P., Lorain,S., Le Villain,J.P., Troalen,F., Trouche,D. and Harel-Bellan,A. (1998) Retinoblastoma protein represses transcription by recruiting a histone deacetylase. *Nature*, **391**, 601–604.

Neufeld,T.P, de la Cruz,A.F., Johnston,L.A. and Edgar,B.A. (1998) Coordination of growth and cell division in the *Drosophila* wing. *Cell*, **93**, 1183–1193.

Nevins,J.R. (2001) The Rb/E2F pathway and cancer. *Hum. Mol. Genet.*, **10**, 699–703.

Ren,B., Cam,H., Takahashi,Y., Volkert,T., Terragni,J., Young,R.A. and Dynlacht,B.D. (2002) E2F integrates cell cycle progression with DNA repair, replication and G_2/M checkpoints. *Genes Dev.*, **16**, 245–256.

Royzman,I., Whittaker,A.J. and Orr-Weaver,T.L. (1997) Mutations in *Drosophila* DP and E2F distinguish G_1–S progression from an associated transcriptional program. *Genes Dev.*, **11**, 1999–2011.

Sage,J., Mulligan,G., Attardi,L., Miller,A., Chen,S., Williams,B., Theodorou,E. and Jacks,T. (2000) Targeted disruption of the three Rb-related genes leads to loss of G_1 control and immortalization. *Genes Dev.*, **14**, 3037–3050.

Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Spradling,A.C. and Rubin,G.M. (1982) Transposition of cloned P elements into *Drosophila* germ line chromosomes. *Science*, **218**, 341–347.

Takahashi,Y., Rayman,J. and Dynlacht,B. (2000) Analysis of promoter binding by the E2F and pRB families *in vivo*: distinct E2F proteins mediate activation and repression. *Genes Dev.*, **14**, 804–816.

Trimarchi,J.M. and Lees,J.A. (2002) Sibling rivalry in the E2F family. *Nat. Rev. Mol. Cell. Biol.*, **3**, 11–20.

Tsai,K.Y., Hy,Y., Macleod,K.F., Crowley,D., Yamasaki,L. and Jacks,T. (1998) Mutation of E2F1 suppresses apoptosis and inappropriate S-phase entry and extends survival of Rb-deficient mouse embryos. *Mol. Cell*, **2**, 293–304.

Wells,J., Boyd,K., Fry,C., Bartley,S. and Farnham,P. (2000) Target gene specificity of E2F and pocket protein family members in living cells. *Mol. Cell. Biol.*, **20**, 5797–5807.

Worby,C.A., Simonson-Leff,N. and Dixon,J.E. (2001) RNA interference of gene expression (RNAi) in cultured *Drosophila* cells. *Sci. STKE*, **2001**, PL1.

Wu,L. *et al*. (2001) The E2F1–3 transcription factors are essential for cellular proliferation. *Nature*, **414**, 457–462.

Yamaguchi,M., Hayashi,Y. and Matsukage,A. (1995) Essential role of E2F recognition sites in regulation of the proliferating cell nuclear antigen gene promoter during *Drosophila* development. *J. Biol. Chem.*, **270**, 25159–25165.

Zhang,H.S., Postigo,A.A. and Dean,D.C. (1999) Active transcriptional repression by the Rb–E2F complex mediates G_1 arrest triggered by p16, TGFβ and contact inhibition. *Cell*, **97**, 53–61.

Received March 11, 2002; revised July 19, 2002; accepted July 31, 2002