The EMBO Journal Vol.18 No.4 pp.916–925, 1999

The role of RBF in the introduction of G_1 regulation during *Drosophila* embryogenesis

Wei Du[1,2,3] and Nicholas Dyson[1]

[1]MGH Cancer Center, Building 149, 13th Street, Charlestown, MA 02129 and [2]Ben May Institute for Cancer Research and Center for Molecular Oncology, University of Chicago, JFK R314, 924 E. 57th Street, Chicago, IL 60637, USA

[3]Corresponding author
e-mail: wdu@ben-may.bsd.uchicago.edu

The first appearance of G_1 during *Drosophila* embryogenesis, at cell cycle 17, is accompanied by the down-regulation of E2F-dependent transcription. Mutant alleles of *rbf* were generated and analyzed to determine the role of RBF in this process. Embryos lacking both maternal and zygotic RBF products show constitutive expression of *PCNA* and *RNR2*, two E2F-regulated genes, indicating that RBF is required for their transcriptional repression. Despite the ubiquitous expression of E2F target genes, most epidermal cells enter G_1 normally. Rather than pausing in G_1 until the appropriate time for cell cycle progression, many of these cells enter an ectopic S-phase. These results indicate that the repression of E2F target genes by RBF is necessary for the maintenance but not the initiation of a G_1 phase. The phenotype of RBF-deficient embryos suggests that *rbf* has a function that is complementary to the roles of *dacapo* and *fizzy-related* in the introduction of G_1 during *Drosophila* embryogenesis.

Keywords: cell cycle/embryogenesis/*Drosophila*/RBF/transcription factor E2F

Introduction

In mice and humans, most somatic cells progress through a cell cycle in which DNA synthesis (S-phase) and mitosis (M-phase) are separated by two gap phases (G_1 and G_2). However, cycles that differ from the G_1/S/G_2/M cycle also occur in many species, including mammals. Polyploid cells, for example, have been observed in a wide variety of plants, animals and ciliates (for examples, see Nagl, 1978; Brodsky and Uryvaeva, 1984; MacAuley *et al*., 1998). In *Drosophila*, non-G_1/S/G_2/M cell cycles are widespread throughout development. The first 13 cell cycles, that occur synchronously and rapidly in the embryo, consist only of alternating S-phases and M-phases without any significant gap phases. G_2 phase first appears in cell cycle 14, but G_1 regulation is not apparent until the completion of mitosis of cell cycle 16 (Foe and Alberts, 1983; Foe, 1989; Edgar and O'Farrell, 1990; Smith and Orr-Weaver, 1991). Endoreduplication cycles, consisting of alternating gap and S-phases, have been observed in many, if not most, larval and adult tissues (Spradling and Orr-Weaver, 1987). During *Drosophila* embryogenesis, progression through the cell cycle and changes in cell cycle composition occur in precise temporal and spatial patterns that have been described in great detail (reviewed in Foe *et al*., 1993). The synchrony and reproducibility of this process has facilitated investigations into the regulatory mechanisms responsible for these transitions.

How are different types of cell cycle regulation imposed? The best understood transition is the introduction of G_2 regulation. The appearance of G_2 in cell cycle 14 results from the degradation of maternally supplied string, and a subsequent requirement for *de novo* string synthesis (Edgar and O'Farrell, 1989, 1990; Edgar and Datar, 1996). The *string*-encoded phosphatase promotes M-phase entry by activating Cdc2-containing kinases. String synthesis is regulated developmentally in a complex pattern, and cycles 14, 15 and 16 vary considerably in length between cell types.

The mechanisms responsible for the imposition of G_1 regulation are less well understood, and it is unclear how many factors are required for this process. Two different mutants have been described in which cells fail to arrest in G_1 at the appropriate time: *dacapo* (*dap*) (de Nooij *et al*., 1996; Lane *et al*., 1996) and *fizzy-related* (*fzr*) (Sigrist and Lehner, 1997). In wild-type embryos, cells of the epidermis leave mitosis of cell cycle 16 relatively synchronously and enter a sustained period of quiescence, the G_1 phase of cell cycle 17 [G_1(17)]. These cells do not normally enter S-phase until the embryo has hatched and the larva has begun to feed. In *dap* mutant embryos, epidermal cells do not arrest following mitosis of cell cycle 16 but continue through an additional cycle (cell cycle 17) before arresting in G_1 of cell cycle 18 (de Nooij *et al*., 1996; Lane *et al*., 1996). *dap* encodes a cyclin-dependent kinase (cdk) inhibitor with homology to human p21 and p27 cdk inhibitors. It has been proposed that the additional cycle results from a failure to inactivate the cyclin E–Cdc2c kinase. Several lines of evidence support the idea that inactivation of cyclin E is important for G_1 to be established. Cyclin E is broadly expressed in the early embryo but is down-regulated as cells reach G_1 (Richardson *et al*., 1993; Knoblich *et al*., 1994). Moreover, the ectopic expression of cyclin E drives cells from G_1(17) into S-phase (Knoblich *et al*., 1994; Richardson *et al*., 1995).

In *fzr* mutant embryos, like *dap* mutant embryos, epidermal cells progress through an additional cell cycle following mitosis of cell cycle 16 (Sigrist and Lehner, 1997). *Fizzy* (*fzy*) and *fzr* promote the destruction of A- and B-type cyclins (Dawson *et al*., 1995; Sigrist *et al*., 1995). Studies of mutant embryos lacking Fzy and/or Fzr indicate that Fzr is required specifically for the down-regulation of cyclins A, B and B3 in G_1 when epidermal cells cease to proliferate, or in G_2

preceding salivary gland endoreduplication (Sigrist and Lehner, 1997).

In both *dap* and *fzr* mutant embryos, epidermal cells complete only one additional cycle before entering G_1, suggesting that other regulatory mechanisms can override the proliferative stimulus to these cells. The identity of the other regulators is unclear. One potential target of this regulation is the E2F transcription factor. dE2F and dDP, two components of E2F, are broadly expressed in *Drosophila* embryos (Duronio *et al*., 1995; Hao *et al*., 1995). However, the expression of *RNR2* and *PCNA*, two genes whose transcription requires dE2F and dDP, is down-regulated in wild-type embryos as cells enter G_1 (Duronio and O'Farrell, 1994; Duronio *et al*., 1995). It is unclear whether this decline in E2F activity is important in establishing G_1 control, or simply a consequence. Although ectopic expression of dE2F and dDP can drive cells from G_1(17) into S-phase (Duronio and O'Farrell, 1995; Duronio *et al*., 1996), the analysis of *dDP* and *dE2F* mutant embryos shows that regulated S-phase entry can occur in the absence of measurable expression of E2F target genes (Royzman *et al*., 1997).

The abundance and activity of E2F complexes are subject to multiple levels of control. Studies of E2F in mammalian cells have illustrated how E2F activity is altered by changes in E2F gene expression, subcellular location, phosphorylation, ubiquitination and by protein association (reviewed in Dyson, 1998). In particular, pRB family proteins act to repress E2F-dependent transcription and are thought to provide an important level of regulation. Mammalian cells contain at least three pRB family members, and the analysis of cells lacking these proteins has been complicated by evidence that there is extensive functional overlap and/or functional compensation between family members in knockout cells (Mulligan and Jacks, 1998).

RBF, a *Drosophila* protein with homology to the pRB family of proteins, has a sequence and structural organization that is intermediate between that of human pRB, p107 and p130, raising the possibility that RBF might represent the archetypal family member (Du *et al*., 1996a). RBF associates with dE2F/dDP and inhibits the effects of dE2F/dDP overexpression (Du *et al*., 1996b). Here, we have generated mutant alleles of *rbf* and used these to investigate the role of RBF in the embryonic cell cycles. The phenotype of mutant embryos lacking both maternal and zygotic RBF products reveals that RBF is dispensable for the early cell cycles but plays an essential role in the introduction of G_1 control during development. The cell cycle defects observed in RBF-deficient embryos are strikingly different from those described previously in *dap* and *fzr* mutant embryos, and suggest that Dacapo, Fizzy-related and RBF provide distinct functions that are required for the timely cessation of cell cycle progression.

Results

Chromosomal deletions in the 1B–2A region modify eye phenotypes that result from altered cell proliferation

The *rbf* gene was mapped by *in situ* polytene hybridization to the cytological region 1CD. Stocks carrying deletions of the 1B–2A region were analyzed and two deficiencies were identified that delete *rbf* [*Df(1)AD11* and *Df(1)su(s)83*]. Consistent with the mapping data, deficiencies that extend from 1E to 2B [*Df(1)A94* and *Df(1)S39*] leave *rbf* intact. Previously, we have shown that the overexpression of dE2F and dDP in the developing eye generates a rough eye phenotype that is suppressed by the co-expression of RBF (Du *et al*., 1996a). We found that the two deficiencies that delete *rbf* caused a moderate enhancement of the *GMRdE2FdDP* eye phenotype (Figure 1). While individual ommatidia are relatively normal in the eyes of *GMRdE2FdDP* flies (Figure 1C and D), the introduction of either *Df(1)AD11* or *Df(1)su(s)83*, that remove one copy of *rbf*, resulted in abnormal, variably shaped ommatidia and, in places, additional bristles (Figure 1E and F, and data not shown). Expression of human p21 in the eye (*GMRp21*) blocks the second mitotic wave during eye development, resulting in abnormal eyes that are characterized by missing cone cells, pigment cells and bristles (de Nooij and Hariharan, 1995). Interestingly, these deficiencies that delete the *rbf* gene strongly suppressed the eye phenotypes caused by the expression of human p21 (Figure 1K and L). These genetic interactions suggest that this region contains an important negative regulator of eye cell proliferation. Although both *Df(1)AD11* and *Df(1)su(s)83* are large deletions and are likely to remove many genes, *rbf* represented the most likely candidate for the critical gene. We sought mutants within this region that specifically affect *rbf* using these observations.

Generation of mutant alleles of RBF

Pre-existing mutants that had been mapped to the 1B–1DE interval were obtained, but none enhanced the *GMRdE2FdDP* phenotype. Overlapping cosmids of the cytological region 1B–1DE were obtained from the European Genome Mapping project and used to characterize the *rbf* genomic locus further. Colony hybridization identified two overlapping cosmids (158H9 and 26B3) that map to cytological region 1C and contain *rbf* sequences (data not shown; see Figure 6 for a diagram). Lines carrying P-elements inserted in the 1C region were obtained from the Bloomington Stock Center and the Berkeley *Drosophila* Genome Project. To identify P-elements inserted in the vicinity of the *rbf* gene, probes corresponding to the genomic sequences flanking the P-element insertion sites were generated by an inverse PCR approach (Dalby *et al*., 1995) and screened for hybridization to the 158H9 and 26B3 cosmids. By this method, one P-element line, $P[w+]cx31A.2^{wd1}$ (see Materials and methods), generated probes that hybridized with the cosmid 26B3 but not with 158H9 (data not shown; see Figure 6 for a diagram of the *rbf* genomic locus). This P-element insertion is not lethal and fails to enhance the *GMRdE2FdDP* phenotype. Western blot analysis showed that the P-element does not alter the level of RBF protein (data not shown), and Southern blot analysis indicated that the P-element lies at least 20 kb 3′ to the RBF coding sequences (data not shown).

A local hopping strategy was used to generate P-element insertions in the *rbf* locus. The P-element in $P[w+]cx31A.2^{wd1}$ was mobilized by the introduction of

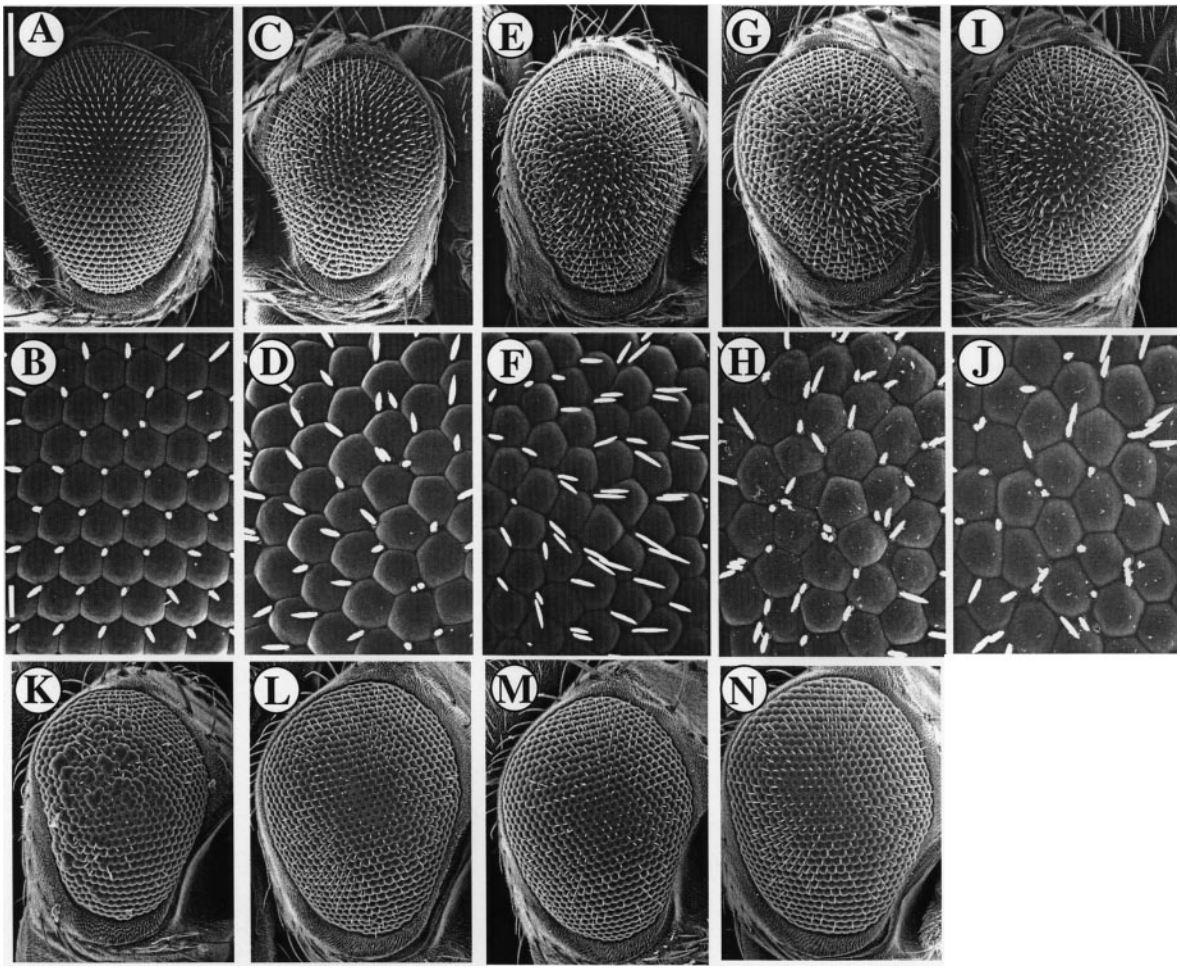

Fig. 1. *rbf* mutants enhance the phenotypes of *GMRdE2FdDP* and suppress the phenotypes of *GMRp21*. Scanning electron micrographs of adult eyes. (A), (C), (E), (G), (I) and (K–N) were at the same magnification, the white bar in (A) corresponding to 100 μm. (B), (D), (F), (H) and (J) were at the same magnification, the white bar in (B) corresponding to 10 μm. Genotypes: (**A** and **B**) wild-type; (**C** and **D**) *GMRdE2FdDP/+*; (**E** and **F**) *Df(1)su(s)83/+;GMRdE2FdDP/+*; (**G** and **H**) *P[w+]wd* ^{120a}*/Y; GMRdE2FdDP/+*; (**I** and **J**) rbf^{14}*/+;GMRdE2FdDP/+*; (**K**) *GMRp21/+*; (**L**) *Df(1)su(s)83/+;GMRp21/+*; (**M**) *P[w+]wd* ^{120a}*/+;GMRp21/+*; and (**N**) rbf^{14}*/+;GMRp21/+*. Note that the *GMRdE2FdDP* phenotype (in C and D) is enhanced in (E), (G) and (I), and in (F), (H) and (J), whereas the *GMRp21* phenotype (in K) is suppressed in (L), (M) and (N).

a Δ2-3 transposase, and two complementary screening strategies were used to identify insertions in the vicinity of *rbf* as described in Materials and methods. A total of three new P-element lines ($P[w+]wd^{15a}$, $P[w+]wd^{38a}$, and $P[w+]wd^{120a}$) were obtained.

Analysis of $P[w+]wd^{15a}$, $P[w+]wd^{38a}$ and $P[w+]wd^{120a}$ revealed that in each case the P-element was inserted 5′ to the *rbf* open reading frame. This is illustrated by the PCR analysis shown in Figure 2. PCR reactions using a primer from the P-element and a primer from the *rbf* 5′-untranslated region (Figure 2A) specifically amplified DNA fragments from DNA prepared from each of these lines (Figure 2B). The size of these fragments indicates that the P-elements lie ~700 bp from the translation start codon. Flies homozygous for any one of these new insertions were viable, and Western blot analysis showed that RBF levels varied between 50 and 100% of wild-type (data not shown), indicating that $P[w+]wd^{15a}$, $P[w+]wd^{38a}$ and $P[w+]wd^{120a}$ are not null alleles of *rbf*.

To generate null alleles of *rbf*, these new P-element lines were used to carry out imprecise excision. After crossing with flies carrying the transposase Δ2-3, new lines were established and *rbf* was analyzed by Southern blot analysis (see Figure 2C and D for an example). rbf^{11}, rbf^{14} and rbf^{16} are three alleles obtained by this method that contain complete deletions of the RBF coding sequence.

RBF mutations enhance a phenotype caused by the overexpression of dE2F/dDP and suppress a phenotype caused by the overexpression of p21

Mutant alleles of *rbf* were assessed for interactions with the *GMRdE2FdDP* and *GMRp21* phenotypes as predicted from the interactions observed using the large deficiencies. As shown in Figure 1, reducing the gene dosage of *rbf* by either the null allele RBF^{14} or the viable weak allele $P[w+]wd^{120a}$ both strongly suppressed the *GMRp21* phenotype (Figure 1K–N). In addition, reducing the gene dosage of *rbf* also enhanced the *GMRdE2FdDP* phenotype (Figure 1C–J). These interactions indicate that $P[w+]wd^{120a}$ indeed behaves like a viable weak allele of RBF, and showed that the activity of ectopic dE2F/dDP is limited by endogenous RBF protein and that the p21-mediated cell cycle arrest depends on RBF. These results strongly suggest that RBF

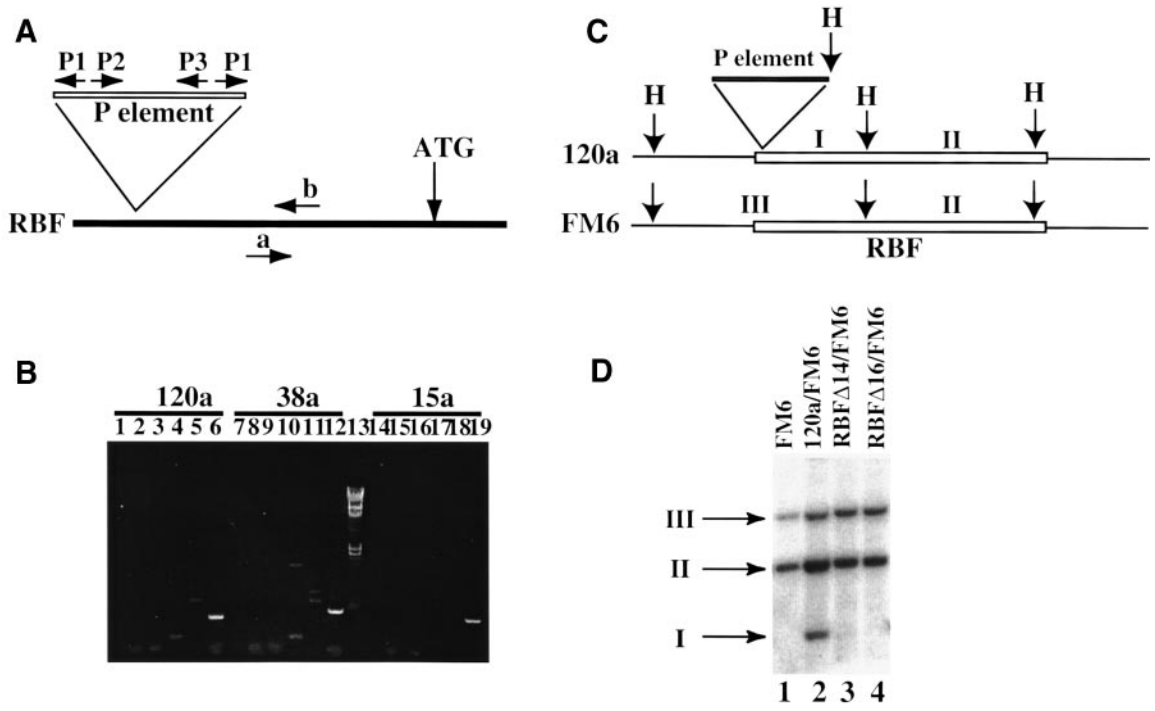

Fig. 2. PCR and Southern blotting analysis illustrating P-element insertion in the *rbf* locus and its subsequent excision to generate mutant alleles of *rbf*. (**A**) Diagram showing the relative position of the P-element insertion in *P[w+]wd*^{120a} and the position of primers used for PCR. P1, P2 and P3 are primers from the ends of the P-element; a and b indicate the primers from the RBF 5′-untranslated region. Arrows indicate the direction of the primers from 5′ to 3′. ATG indicates the start of translation. (**B**) PCR products from the P-element insertion lines. DNA isolated from the P-element lines *P[w+]wd*^{120a}, *P[w+]wd*^{38a} and *P[w+]wd*^{15a} are in lanes 1–6, 7–12 and 14–19, respectively. Lane 13 is a molecular weight marker. Primer pairs: (P3 and a) are used in lanes 1, 7 and 14; (P2 and a) in lanes 2, 8 and 15; (P1 and a) in lanes 3, 9 and 16; (P3 and b) in lanes 4, 10 and 17; (P2 and b) in lanes 5, 11 and 18; and (P1 and b) in lanes 6, 12 and 19. Note that PCR products were generated from each of the lines by the combination of primers P1 and b. (**C**) Diagram showing the position of *Hin*dIII sites at the *rbf* locus in *P[w+]wd*^{120a} and *FM6*. In *FM6* (as in wild-type), the RBF cDNA hybridizes to two *Hin*dIII fragments (fragment II and III). The P-element insertion in *P[w+]wd*^{120a} (labeled 120a) introduces a new *Hin*dIII site, resulting in the appearance of an additional short fragment I. Thus the two fragments that hybridize to RBF in *P[w+]wd*^{120a} are fragments I and II, and the two fragments that hybridize to RBF in *FM6* are fragments II and III. (**D**) Southern blot analysis illustrating a P-element insertion in the RBF locus and its subsequent excision in *RBF*^{14} and *RBF*^{16}. The genotypes of the DNA samples: lane 1, *FM6*; lane 2, *P[w+]wd*^{120a}*/FM6*; lane 3, *RBF*^{14}*/FM6*; and lane 4, *RBF*^{16}*/FM6* as indicated. The full-length cDNA RBF was used as a probe. Note that the additional *Hin*dIII fragment (fragment I) introduced in *P[w+]wd*^{120a} is deleted in *RBF*^{14} and *RBF*^{16}. In addition, the intensity of fragment II is also reduced by 50% in lanes 3 and 4 compared with lane 2, whereas the fragment III that is derived solely from the FM6 chromosome is unchanged (compare lanes 2, 3 and 4). Lane 1 contains less DNA than lanes 2–4.

normally acts as a negative regulator of cell proliferation during eye development.

Expression of RBF cDNA rescues the lethality of RBF mutants

Mutations resulting in the complete deletion of *rbf* were lethal as homozygotes, hemizygotes or trans-heterozygotes. To demonstrate that the lethality of these *rbf* alleles is due to the lack of RBF activity, rescue experiments were carried out by expressing the RBF cDNA under the control of a heat shock promoter. The lethality of a trans-heterozygous combination of *rbf*^{11} and *rbf*^{14}, two null alleles in which the RBF coding sequence is completely deleted, was rescued efficiently by expression of the RBF cDNA from the heat shock-regulated transgene *hsRBF* (see Materials and methods). Similarly, the *hsRBF* transgene also allowed rescue of viable males carrying *rbf*^{14} or *rbf*^{11}. For these experiments, vials were incubated at 37°C for 1 h/day throughout development. No viable *rbf*^{11}/*rbf*^{14} females, or *rbf*^{11} or *rbf*^{14} male adult flies were observed without heat shock treatment or by heat shock in the absence of the *hsRBF* transgene. Thus, *rbf* is the only essential function missing in *rbf*^{14} and *rbf*^{11}.

RBF is an essential regulator of E2F-dependent transcription in the embryo

Previous work has demonstrated that the transcription of *PCNA* and *RNR2*, two genes that are coordinately expressed as cells enter S-phase, requires both *dE2F* and *dDP* and is induced by ectopic expression of dE2F/dDP (Duronio and O'Farrell, 1994, 1995; Duronio *et al*., 1995, 1996, 1998; Royzman *et al*., 1997). In wild-type embryos, *RNR2* is expressed uniformly during early stages but is down-regulated following cell cycle 16 in cells entering G_1, the first time that G_1 regulation is apparent (Duronio and O'Farrell, 1994). Periodic expression of *RNR2* is seen in the gut and peripheral nervous system (PNS) as cells enter S-phase. *RNR2* expression persists in the CNS that contains actively dividing cells, but *RNR2* is not re-expressed in epidermis of the embryo following mitosis of cell cycle 16 as these cells remain quiescent until the first larval instar.

While no *rbf*^{11}/*rbf*^{14} trans-heterozygous adult flies were found in the absence of the *hsRBF* transgene, egg counts showed that most *rbf*^{11}/*rbf*^{14} trans-heterozygous embryos hatched (data not shown). Embryos homozygous for mutant alleles for *rbf* were assayed by *in situ* hybridization

using a probe for *RNR2* expression, but no abnormalities were apparent. In particular, *RNR2* expression was repressed after mitosis 16 in *rbf* mutant embryos in a manner that appeared identical to the wild-type embryos (data not shown). These observations might suggest that RBF is neither required for the repression of *RNR2* expression nor has an essential function during embryogenesis; alternatively, RBF may have essential functions that can be performed by maternally supplied products. To test this, we generated *rbf* mutant embryos that were derived from germline clones and lacked both maternal and zygotic RBF. As described below, such RBF-deficient embryos displayed a variety of phenotypes that reveal essential roles for RBF in E2F regulation and cell cycle control during embryogenesis.

In situ hybridization experiments show that the expression of *RNR2* is strongly deregulated in the RBF-deficient embryos. In these embryos, *RNR2* was expressed ubiquitously and uniformly in both early stages of embryogenesis and also in later stage embryos following germband retraction (Figure 3E–H, and data not shown). The expression of *PCNA* was altered similarly. In RBF-deficient embryos, the level of *PCNA* expression in the epidermis is equivalent to that seen in the PNS, where PCNA staining is detected in wild-type embryos at this stage (Figure 3A–D). Using *RNR2* and *PCNA* expression as a measure of E2F activity, these results suggest that E2F-dependent transcription is constitutively elevated in the absence of RBF. Thus, RBF is required for the developmental down-regulation of E2F that occurs when G_1 regulation is introduced during *Drosophila* embryogenesis.

Defective G_1 regulation in the absence of RBF

Crosses between females bearing *rbf* germline clones and wild-type males gave viable females, indicating that expression of the wild-type *rbf* gene from the paternal X chromosome was sufficient for viability. As the first 13 cell cycles are driven exclusively by maternally encoded products, the paternal rescue indicates that RBF has no essential functions during these early stages.

Bromodeoxyuridine (BrdU) incorporation was used to monitor DNA synthesis in the RBF-deficient embryos. Aberrant cell cycle control was observed in cells of the midgut in stage 12–14 embryos. In wild-type embryos, pulses of BrdU incorporation are observed in the midgut due to differential timing of G_1(17) in subsets of cells. In RBF-deficient embryos, the pulses of BrdU incorporation that normally distinguish the central and anterior midgut were weaker, and BrdU incorporation occasionally was seen throughout the midgut region (data not shown). However, the most dramatic changes in DNA synthesis were seen in the epidermis. In wild-type embryos, cells of the epidermis complete cell cycle 16 in late stage 11; these cells enter G_1 of cell cycle 17 and no longer incorporate BrdU (Figure 4A and C). DNA synthesis is evident in mid-stage 12 in the PNS cells that lie just below the epidermis (Figure 4A). No defect was observed in the pattern of S-phases of RBF-deficient embryos until mid-stage 12 when the germband was partially retracted. In these embryos, ectopic S-phase cells were first observed in the dorsal epidermis. In stage 13 and stage 14 embryos, ectopic S-phases had become more abundant and extended to the ventral epidermis (Figure 4B and D). In all RBF-deficient embryos examined, BrdU incorporation was observed in only a subset of the epidermal cells. These observations suggest that epidermal cells initially enter G_1 following mitosis 16 but, in the absence of RBF, a significant proportion of the cells were unable to remain in G_1 and entered S-phase. *In situ* hybridization with a probe for cyclin E showed that the level of cyclin E mRNA is significantly elevated in the epidermis of RBF-deficient embryos (Figure 4G and H). In these embryos, cyclin E expression is only partially deregulated, consistent with previous evidence that dE2F and dDP provide only one of several activities that regulate cyclin E expression (Duronio and O'Farrell, 1995; Royzman *et al*., 1997; Duronio *et al*., 1998). Interestingly, cyclin E expression was slightly higher in epidermal cells near the segment boundaries, in a pattern that resembled the pattern of BrdU incorporation. It is possible that RBF is more important for cyclin E regulation in these cells. Alternatively, these cells may be more sensitive to deregulation of E2F, and the elevated level of cyclin E expression may be caused by S-phase entry.

To determine whether ectopic S-phases resulted in cell proliferation, RBF-deficient embryos were stained with an antibody to phosphorylated histone H3 that detects mitotic chromosomes (de Nooij *et al*., 1996). No extra mitotic cells were found in the epidermis in RBF-deficient embryos (data not shown), indicating that the epidermal cells that entered S-phase did not complete a mitotic cell cycle. The disparity between the S-phase and M-phase markers prompted us to assess the level of apoptosis in these embryos. Wild-type embryos show a low but significant level of apoptosis in the epidermis that can be detected by the TUNEL assay. In contrast, extensive apoptosis was found in the epidermis of stage 13 RBF-deficient embryos (Figure 4E and F). Although the majority of cells incorporating BrdU were located near the segment boundaries, TUNEL-positive cells were distributed throughout the segment. This difference suggests that, in many cells, induction of apoptosis is unlinked to S-phase entry. Taken together, these observations indicate that epidermal cells initially stop in G_1 following mitosis of cell cycle 16, but that these cells are unable to maintain this arrest effectively. With time, an increasing proportion of cells enter S-phase. In addition, many cells are eliminated by apoptosis.

Discussion

RBF-deficient embryos provide a third example, in addition to *dap* and *fzr* mutants (de Nooij *et al*., 1996; Lane *et al*., 1996; Sigrist and Lehner, 1997), where epidermal cells are unable to stop cell cycle progression following mitosis of cell cycle 16. Although the same cells enter an ectopic S-phase in each case, the phenotypes of these embryos are quite different. First, in *dap* and *fzr* mutants, epidermal cells entering an ectopic S-phase complete a mitotic cell cycle; in RBF mutants, no additional mitotic cells were detected. A second distinction lies in the persistence of ectopic S-phases. In *dap* and *fzr* mutants, ectopic S-phases were seen for a short time window; once epidermal cells complete an additional cycle, they remain arrested in G_1. In RBF-deficient embryos, ectopic S-phases persisted in the epidermis and even increased as the

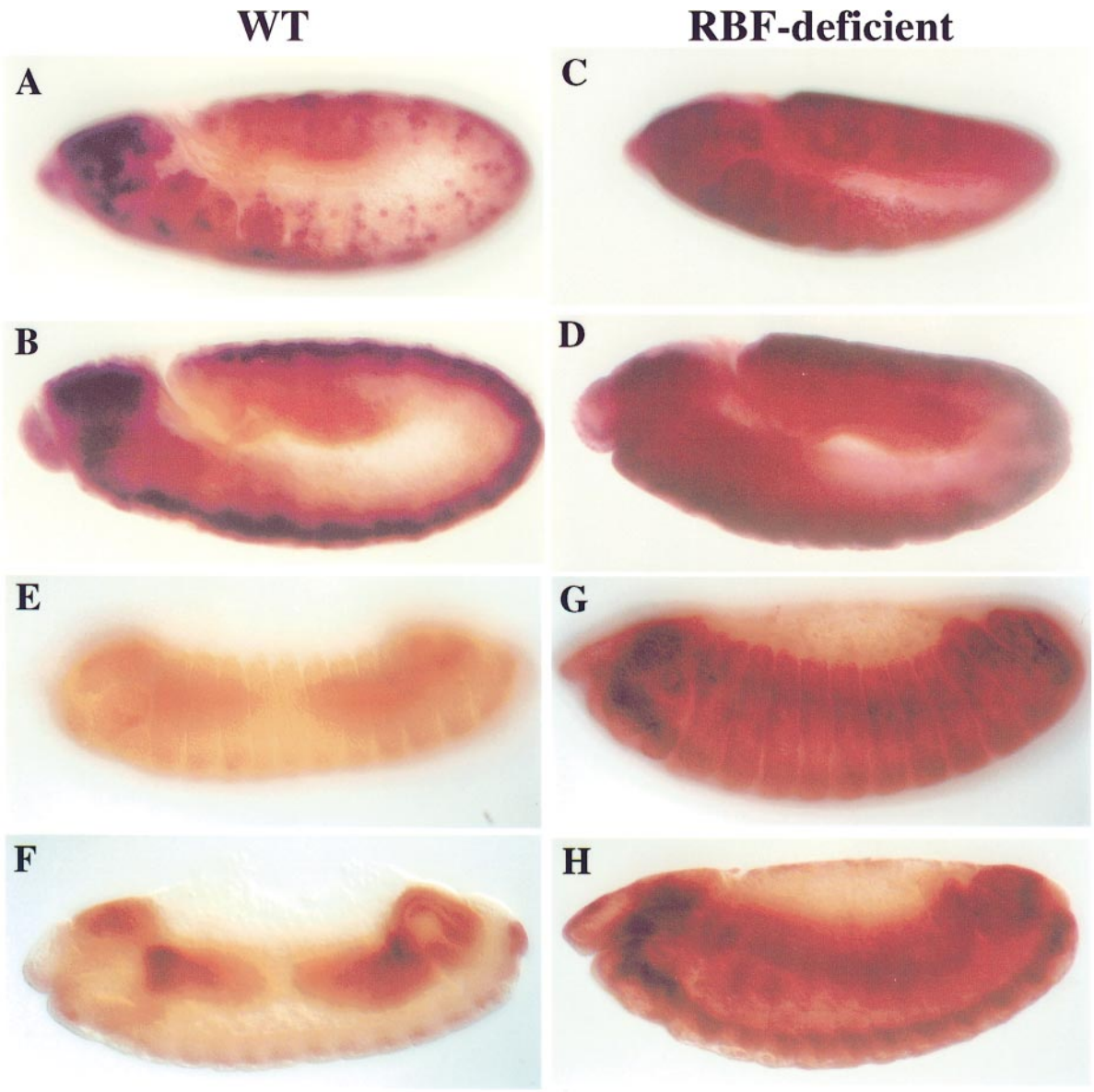

Fig. 3. Deregulation of E2F activity in embryos lacking RBF. Endogenous E2F activities were detected by *in situ* hybridization with antisense probes to *PCNA* (A–D) or *RNR2* (E–H); the same patterns of expression were detected for these two genes. Two different stage of embryos are shown. (A–D) Embryos at the beginning of germband retraction; (E–H) embryos with a completely retracted germband. *RNR2* and *PCNA* are expressed ubiquitously at high levels at each of the stages shown in RBF-deficient embryos. (**A** and **B**) A wild-type embryo at the beginning of germband retraction; the image was focused on the epidermis in (A) and on the midline in (B). (**C** and **D**) An RBF maternal and zygotic null embryo; the image was focused on the epidermis in (C) and on the midline in (D). (**E** and **F**) A germband-retracted wild-type embryo; the image was focused on the epidermis in (E) and on the midline in (F). Note that the anterior and posterior midgut staining in (F) is out of focus in (E). (**G** and **H**) A germband-retracted RBF maternal and zygotic null embryo; the image was focused on the epidermis in (G) and on the midline in (H).

embryos aged. Third, in RBF-deficient embryos, most epidermal cells initially enter G_1(17). Because epidermal cells of *dap* mutant embryos enter S-phase synchronously and rapidly following mitosis of cell cycle 16, it has been unclear whether these cells enter G_1(17) or whether they progress directly from mitosis to S-phase (de Nooij *et al*., 1996; Lane *et al*., 1996). In mid-stage 12 RBF-deficient embryos, only a subset of cells incorporate BrdU, indicating that the majority of cells initially remained in G_1(17). Cells of the dorsal epidermis were already post-mitotic by this stage in control embryos.

We infer from these observations that RBF plays an important role in the imposition of G_1 regulation during *Drosophila* development. It is well established that the appearance of G_1(17) correlates with the down-regulation of E2F-dependent transcription (Duronio and O'Farrell, 1994). As the overexpression of dE2F and dDP is able to drive cells from G_1 into S-phase, it appeared likely that this inhibition of E2F activity would be essential for the appearance of G_1. The analysis of RBF-deficient embryos argues against this conclusion. *RNR2* and *PCNA*, the two genes that have been used most widely in previous studies to provide a measure of endogenous E2F activity, are constitutively expressed in RBF-deficient embryos. Nevertheless, the majority of epidermal cells are able to enter G_1, and it is only as the embryo ages that large numbers of these cells enter ectopic S-phases. Thus, most epidermal cells require neither RBF nor the repression of E2F-dependent transcription to enter G_1. Instead RBF's role appears to lie in the maintenance of the G_1 phase.

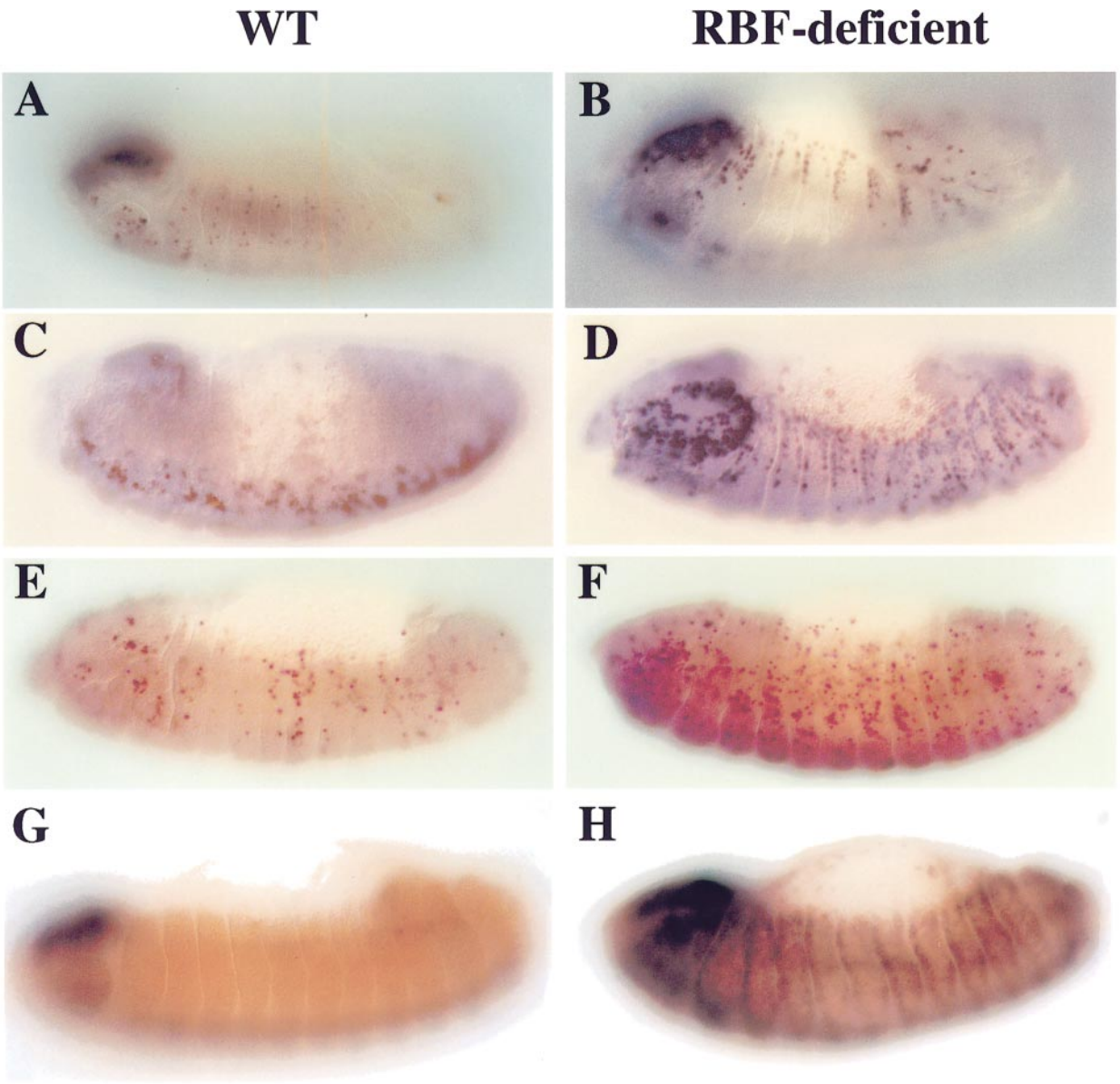

Fig. 4. Ectopic S-phases and increased apoptosis in RBF-deficient embryos. BrdU incorporation of wild-type embryos (A and C) or RBF maternal and zygotic null embryos (B and D). (**A** and **B**) Germband partially retracted stage 12 embryos. Cells in the epidermis were not labeled with BrdU in wild-type embryos (A); the cells that are labeled with BrdU in the middle of each segment are PNS cells just below the epidermis. In contrast, cells in the epidermis in RBF-deficient embryos were labeled with BrdU (B); note that the cells that are labeled are on the dorsal epidermis and are adjacent to the segment boundary. (**C** and **D**) Germband completely retracted embryos. Cells in the epidermis were not labeled with BrdU in wild-type embryos (C); the dark shadow in the middle of the embryo is due to BrdU staining of midgut and hindgut. CNS cells at the ventral side of the embryo are labeled strongly; these cells are still proliferating at this stage. Cells in the epidermis in RBF-deficient embryos were labeled with BrdU (D); note that cells in the ventral epidermis also incorporate BrdU. CNS cells are below this focal plane. (**E** and **F**) TUNEL staining of a wild-type embryo shown in (E) and an RBF-deficient embryos shown in (F). Note that the number of TUNEL-stained cells is significantly increased; however, these cells do not appear to have the same pattern of BrdU-labeled cells (compare B, D and F). (**G** and **H**) Cyclin E expression was detected by whole-mount *in situ* hybridization. A germband-retracted wild-type embryo is shown in (G); cyclin E transcripts were not detected in the epidermis. A similar staged RBF-deficient embryo is shown in (H); a significant level of cyclin E RNA was detected in the epidermis. Note that cyclin E expression is elevated in cells adjacent to the segment boundary, resembling the pattern of BrdU incorporation in these embryos.

The properties of *dap* mutant embryos and RBF-deficient embryos are highly consistent with a model in which the complementary roles of RBF and Dacapo in G_1 control are due to their complementary roles in the regulation of cyclin E activity (Figure 5). In this model, G_1 is triggered by the inhibition of the cyclin E–Cdc2c kinase. Dacapo is required for this process (de Nooij *et al*., 1996; Lane *et al*., 1996), although it is not certain that it is the only mechanism involved. Previous studies have shown that Dacapo is only transiently expressed as cells exit the cell cycle (de Nooij *et al*., 1996; Lane *et al*., 1996). Our results suggest that the repression of cyclin E and other E2F-regulated genes by RBF is not important at this stage, but becomes essential later, as the expression of Dacapo declines. In RBF-deficient embryos, the elevated expression of cyclin E in cells that lack Dacapo will lead to the accumulation of an active kinase, and eventually to S-phase entry. In *dap* mutants, however, RBF repression of cyclin E expression would limit its ability to drive epidermal cells through multiple cycles.

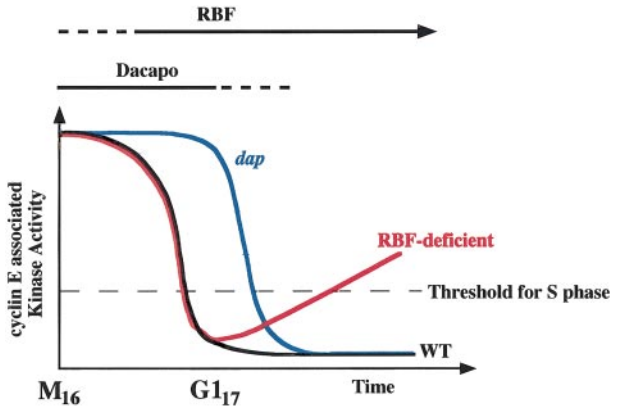

Fig. 5. RBF and Dacapo cooperate to regulate cyclin E kinase activity and to establish G_1. The cyclin E-associated kinase activity after mitosis 16 in wild-type, *dap* or RBF-deficient embryos are drawn in black, blue and red, respectively. Lines on top show the time at which RBF and Dacapo are important. In this model, we suggest that the functions of *rbf* and *dap* converge on the regulation of cyclin E, Dacapo acting to inhibit the residual cyclin E kinase activity following mitosis 16, and RBF acting to repress cyclin E expression. In *dap* mutants, residual cyclin E-associated kinase activity is sufficient to drive cells into S-phase following mitosis 16. Only one additional cycle occurs because cyclin E-associated kinase activity drops below the threshold due to the repression of cyclin E expression by RBF. In RBF-deficient embryos, the expression of Dacapo inhibits the cyclin E-associated kinase, initially causing a G_1 arrest. However, in the absence of RBF, cyclin E is mis-expressed and cells accumulate cyclin E kinase activity as Dacapo levels decline and the cyclin E level increases. Once the cyclin E kinase activity passes the threshold for S phase, these cells will initiate DNA replication.

Why do the ectopic S-phase cells in RBF-deficient mutants fail to progress to mitosis? We suggest that this may occur for several reasons. The levels of cyclins A and B drop rapidly once cells enter G_1 (Lehner and O'Farrell, 1989, 1990). In *dap* mutant embryos, where cells enter S-phase rapidly after mitosis of cell cycle 16, there may still be sufficient A- and B-type cyclins to allow these cells to progress to mitosis 17. Given the delay before ectopic S-phase cells appear in RBF-deficient embryos, the levels of mitotic cyclins may be insufficient for cell cycle progression. In wild-type animals, epidermal cells will leave $G_1(17)$ to enter an endoreduplication S-phase. By the time that ectopic S-phase cells appear in RBF-deficient embryos, these cells may already be committed to an endoreduplication cycle. In addition, there is evidence that the down-regulation of E2F activity is important for cells to exit S-phase (Krek *et al*., 1995). Potentially, the failure to down-regulate E2F or, alternatively cyclin E expression (Follet *et al*., 1998; Weiss *et al*., 1998), may arrest cells either in S-phase or post-S-phase, in RBF-deficient embryos. Finally, the overexpression of E2F genes induces apoptosis in a variety of experimental systems (Qin *et al*., 1994; Shan and Lee, 1994; Wu and Levine, 1994; Kowalik *et al*., 1995; Asano *et al*., 1996; Du *et al*., 1996b). Although our results suggest that S-phase entry is not a prerequisite for apoptosis in the RBF-deficient embryos, cells that enter S-phase inappropriately may be especially sensitive to the effects of elevated E2F activity and may not survive to complete the cell cycle.

The changes in expression of E2F-regulated genes caused by the absence of RBF provide new insights into the regulation of E2F activity in the *Drosophila* embryo. Previous studies have shown that *PCNA* and *RNR2* are not expressed in *dE2F* or *dDP* mutant embryos (Duronio *et al*., 1995, 1998; Royzman *et al*., 1997). These results suggested that the induction of gene expression that occurs in wild-type embryos as cells enter S-phase was due primarily to transcriptional activation by a dE2F–dDP complex. However, the finding that these genes are constitutively expressed in RBF-deficient embryos adds a level of complexity. This result indicates that RBF actively represses the expression of *PCNA* and *RNR2* in G_1 phase cells and suggests that the pulses of gene expression seen as cells enter S-phase could be due largely to the release of repression. If RBF, dE2F and dDP are common components of a repressor complex, one wonders why *PCNA* and *RNR2* are not constitutively expressed in *dE2F* and *dDP* mutant embryos? One possible explanation is that dE2F and dDP are not only co-repressors with RBF but are also required to activate the *RNR2* and *PCNA* promoters. An alternative possibility is that RBF repression of *PCNA* and *RNR2* expression is not mediated by dE2F/dDP, but by a different RBF-binding protein. The recent discovery of dE2F2, that associates with dDP and RBF in *Drosophila* embryos (D.Huen, W.Du, Y.Chen, and N.Dyson, unpublished observations), opens up a variety of potential explanations. It is also evident that *RNR2*/*PCNA* and cyclin E represent two different types of E2F target genes. Although expression of all three genes can be induced by ectopic expression of dE2F and dDP, *RNR2* and *PCNA* were completely derepressed by the absence of RBF, whereas cyclin E was deregulated in a more subtle manner. The level of cyclin E expression observed in the epidermis of RBF-deficient embryos remains considerably lower than the level of cyclin E expressed in the central nervous system and brain. In some cell types, expression of cyclin E is independent of both *dE2F* and *dDP* (Duronio and O'Farrell, 1995; Royzman *et al*., 1997; Duronio *et al*., 1998), and it seems likely that the expression of cyclin E, like that of string and other critical cell cycle regulators, is under the control of multiple enhancer elements.

The phenotype of RBF-deficient embryos has several features in common with the phenotype of $Rb^{-/-}$ mice (Clarke *et al*., 1992; Jacks *et al*., 1992; Lee *et al*., 1992, 1994). Both mutants are characterized by ectopic S-phases, the expression of E2F-regulated genes and high levels of apoptosis. Such similarities emphasize that these homologous proteins serve analogous functions. Interpretation of the phenotypes of pRB, p107 and p130 knockout mice is complicated by the fact that these proteins share overlapping functions. Even in studies of double knockout mice, these animals progress through many stages of mouse development before cell cycle defects become apparent (Cobrinik *et al*., 1996; Lee *et al*., 1996; Mulligan and Jacks, 1998). It has been unclear whether pRB family proteins are unimportant for many cell cycles or whether they have a redundant but critical function. The phenotype of RBF-deficient embryos reveals that RBF is important at the very first cell cycle in *Drosophila* development where G_1 regulation is introduced. Interestingly, RBF is required for the maintenance of G_1, but not for its initial appearance. By analogy, one of the initial roles of mammalian pRB family members may be to allow rapidly proliferating cells to pause in G_1.

Materials and methods

Fly stocks

Deficiency lines *Df(1)AD11/FM7* and *Df(1)su(s)83, y^1 cho^1 ras^1 v^1/ Dp(1;Y)y^2 sc/C(1)DX, y^1 f^1*, the P-element stock P1318 (*P[w+] cx31A.2*) and the FLP, FRT lines for germline clonal analysis were obtained from the Bloomington Stock Center. *P[w+; hsp70-RBF]* was generated by subcloning of the RBF full-length cDNA (Du *et al*., 1996a) into pCaSpeR-hs vector. A *P[w+; hsp-RBF]* on the X chromosome was used for the rescue experiments.

Characterization of the RBF chromosomal region

Cosmid contigs between the 1B and 1DE polytene region were obtained from the European Genome Mapping Project, and were hybridized using the RBF cDNA as a probe. Two overlapping cosmids, 158H9 and 26B3, contain RBF sequences and were used to identify P-elements in the vicinity of *rbf*. P-element lines were obtained from the Bloomington Stock Center and the Berkeley *Drosophila* Genome Project. An inverse PCR method was used to make probes from the genomic sequences adjacent to the P-element insertions. These probes were hybridized to the two cosmids that contained RBF sequences. Stock number P1318 (*P[w+]cx31A.2*) gave probes that hybridize to the cosmid 26B3 but not to 158H9. Since this stock contains two P-element insertions on the X chromosome, this stock was crossed with w^{1118}, and the resulting two different classes of lighter eye color flies were established. One such line, *P[w+]cx31A.2^{wd1}*, was shown to retain the P-element in the vicinity of *rbf* and was used in subsequent experiments.

Embryo analysis

In situ hybridization. Digoxigenin-labeled antisense RNA probes were prepared by *in vitro* transcription reaction. Properly aged embryos were collected and fixed. Hybridization was carried out at 70°C as described (Duronio and O'Farrell, 1994).

BrdU staining. Properly aged embryos were collected, dechorionated in 50% Chlorox, permeabilized in octane, and incubated in 1 mg/ml BrdU in Schneider's medium for 20 min. Embryos were fixed in an equal volume of 4% formaldehyde and heptane. BrdU was detected using a mouse anti-BrdU antibody (Becton Dickenson, 1:100).

TUNEL assay. Collected and fixed embryos were treated with proteinase K (10 μg/ml) for 5 min, washed twice with PBT and post-fixed with 4% formaldehyde for 20 min. These embryos were processed further using the Apotag kit from ONCOR (Gaithersberg, MD) according to the instructions provided.

Generation of RBF mutants

The scheme used for hopping is shown in Figure 6. Females from 20 different lines (one female each line) were pooled for inverse PCR to generate probes. These probes were hybridized to the 26B3 and 158H9 cosmid DNA digested with *Not*I. A total of 1600 lines were established and tested using cross A (Figure 6); one line was found to have a new P-element insertion in cosmid 158H9. Four hundred lines were established using cross B (Figure 6) and two lines were found to have new P-element insertions in cosmid 158H9. Southern and PCR analysis showed that all three P-elements were inserted in the 5′-untranslated region of the RBF cDNA, ~700 bp from the start codon ATG. To generate null alleles of RBF, these three P-element lines isolated (*P[w+]wd^{15a}*, *P[w+]wd^{38a}* and *P[w+]wd^{120a}*) were crossed to the transposase Δ2-3 and new lines were established. The deletions of the *rbf* coding sequences were determined by Southern analysis. All lines with deletions of the *rbf* coding sequences were found to be lethal.

Generation of germline clones

rbf mutants were recombined onto an X chromosome carrying an FRT site inserted at 14AB. Germline clone females were generated as described (Soto *et al*., 1995). Virgin females with *rbf* germline clones were collected and were crossed with males carrying *Eve-lacZ* on the X chromosome. Embryos with the wild-type *rbf* gene were identified by anti-β-gal staining.

Rescue of homozygous RBF mutants

For the rescue experiments, *rbf^{14},P[w+; hsp70-RBF]/DP(1:Y)* males were crossed to *rbf^{11}/FM7* females. Once eggs were laid, the vials were heat-shocked for 1 h at 37°C per day. *rbf^{11}/rbf^{14},P[w+; hsp70-RBF]* females represented ~20% of the adults. Theoretically, 25% of the progeny from this cross are expected to be the mutant class; however, the FM7 male is usually under-represented and, taking this into account, complete rescue of the mutant class could provide up to 30% of the adults.

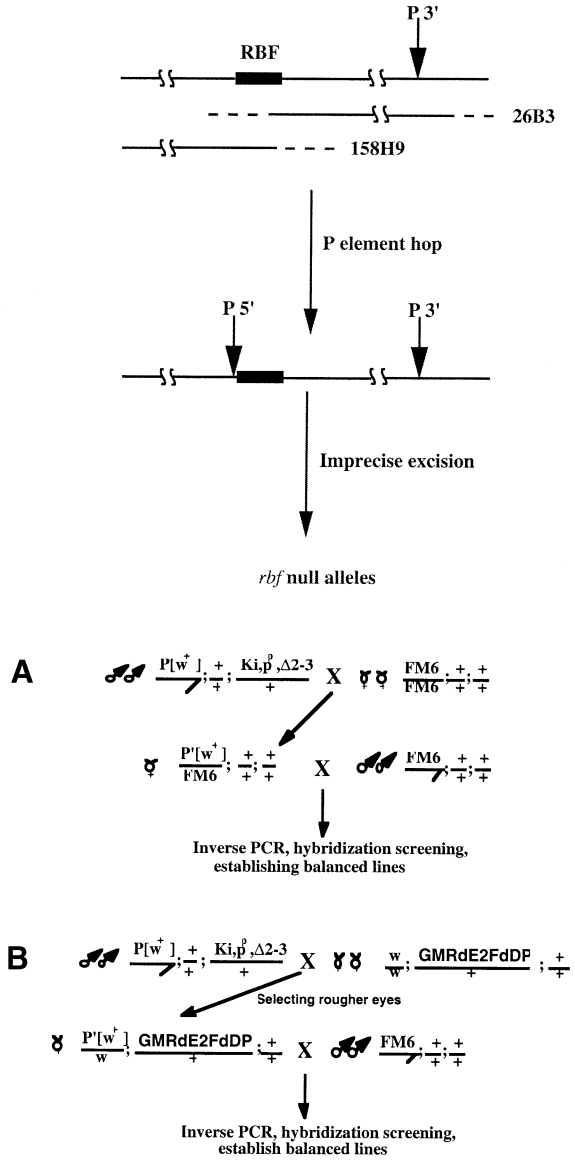

Fig. 6. The scheme for hopping. Two different crosses were used to generate *rbf* mutants. A transposase Δ2-3 was crossed to the *P[w+]cx31A.2^{wd1}* line. Resulting males were either crossed to a first chromosome balancer *FM6* to establish lines in (**A**) or crossed to *GMRdE2FdDP* to identified flies with slightly enhanced eye phenotypes in (**B**).

Acknowledgements

We thank the Bloomington Stock Center and the Berkeley *Drosophila* Genome Project for the fly stocks used in this study, and the European Genome Mapping Project for the cosmid contigs. We are grateful to Iswar Hariharan for stimulating discussions and for the GMRp21 stocks. We thank Kristin White for advice on TUNEL staining, Ed Seling for help with the SEM, and Simon Boulton and Iswar Hariharan for comments on the manuscript. W.D. was a recipient of a Leukemia Society Fellowship, and currently is a scholar of the Kimmel Foundation for Cancer Research. This work was supported by NIH grant R01GM53203.

References

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

Brodsky,V.Y. and Uryvaeva,I.V. (1984) *Genome Multiplication in Growth and Development: Biology of Polyploid and Polytene Cells*. Cambridge University Press, New York, NY.

Clarke,A., Maandag,E., van Roon,M., van der Lugt,N., van der Valk,M., Hooper,M., Berns,A. and te Riele,H. (1992) Requirement for a functional *Rb-1* gene in murine development. *Nature*, **359**, 328–330.

Cobrinik,D. *et al*. (1996) Shared role of the pRB-related p130 and p107 proteins in limb development. *Genes Dev.*, **10**, 1633–1644.

Dalby,B., Pereira,A.J. and Goldstein,L.S. (1995) An inverse PCR screen for the detection of P element insertions in cloned genomic intervals in *Drosophila melanogaster*. *Genetics*, **139**, 757–766.

Dawson,I.A., Roth,S. and Artavanis-Tsakonas,S. (1995) The *Drosophila* cell cycle gene *fizzy* is required for normal degradation of cyclins A and B during mitosis and has homology to the *CDC20* gene of *Saccharomyces cerevisiae*. *J. Cell Biol.*, **129**, 725–737.

de Nooij,J.C. and Hariharan,I.K. (1995) Uncoupling cell fate determination from patterned cell division in the *Drosophila* eye. *Science*, **270**, 983–985.

de Nooij,J.C., Letendre,M.A. and Hariharan,I.K. (1996) A cyclin-dependent kinase inhibitor, dacapo, is necessary for timely exit from the cell cycle during *Drosophila* embryogenesis. *Cell*, **87**, 1237–1247.

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. (1994) Developmental control of a G_1–S transcriptional program in *Drosophila*. *Development*, **120**, 1503–1515.

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.

Duronio,R.J., Brook,A., Dyson,N. and O'Farrell,P.H. (1996) E2F-induced S phase requires cyclin E. *Genes Dev.*, **10**, 2505–2513.

Duronio,R.J., Bonnette,P.C. and O'Farrell,P.H. (1998) Mutations of the *Drosophila* dDP, dE2F, and cyclin E genes reveal distinct roles for the E2F-DP transcription factor and cyclin E during the S-phase transition. *Mol. Cell. Biol.*, **18**, 141–151.

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

Edgar,B.A. and O'Farrell,P.H. (1989) Genetic control of cell division patterns in the *Drosophila* embryo. *Cell*, **57**, 177–187.

Edgar,B.A. and O'Farrell,P.H. (1990) The three post-blastoderm cell cycles of *Drosophila* embrogenesis are regulated in G_2 by *string*. *Cell*, **62**, 469–480.

Edgar,B.A. and Datar,S.A. (1996) Zygotic degradation of two maternal Cdc25 mRNAs terminates *Drosophila*'s early cell cycle program. *Genes Dev.*, **10**, 1966–1977.

Foe,V.E. (1989) Mitotic domains reveal early commitment of cells in *Drosophila* embryos. *Development*, **107**, 1–22.

Foe,V.A. and Alberts,B.M. (1983) Studies of nuclear and cytoplasmic behavior during the five mitotic cycles that precede gastrulation in *Drosophila* embryogenesis. *J. Cell Sci.*, **61**, 31–70.

Foe,V.E., Odell,G.M. and Edgar,B.A. (1993) Mitosis and morphogenesis in the *Drosophila* embryo: point and counterpoint. In Bate,M. and Martinez-Arias,A. (eds), *The Development of Drosophila melanogaster*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 149–300.

Follet,P.J., Duronio,R.J. and O'Farrell,P.H. (1998) Fluctuations in cyclin E levels are required for multiple rounds of endocycle S-phase in *Drosophila*. *Curr. Biol.*, **8**, 235–238.

Hao,X.F., Alphey,L., Bandara,L.R., Lam,E.W.-F., Glover,D. and La Thangue,N.B. (1995) Functional conservation of the cell cycle-regulating transcription factor DRTF1/E2F and its pathway of control in *Drosophila melanogaster*. *J. Cell Sci.*, **108**, 2945–2954.

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.

Knoblich,J.A., Sauer,K., Jones,L., Richardson,H., Saint,R. and Lehner,C.F. (1994) Cyclin E controls S-phase progression and its down-regulation during *Drosophila* embryogenesis is required for the arrest of cell proliferation. *Cell*, **77**, 107–120.

Kowalik,T.F., DeGregori,J., Schwarz,J.K. and Nevins,J.R. (1995) E2F1 overexpression in quiescent fibroblasts leads to induction of cellular DNA synthesis and apoptosis. *J. Virol.*, **69**, 2491–2500.

Krek,W., Xu,G. and Livingston,D.M. (1995) Cyclin A-kinase regulation of E2F-1 DNA binding function underlies suppression of an S phase checkpoint. *Cell*, **83**, 1149–1158.

Lane,M.E., Sauer,K., Wallace,K., Jan,Y.N., Lehner,C.F. and Vaessin,H. (1996) Dacapo, a cyclin-dependent kinase inhibitor, stops cell proliferation during *Drosophila* development. *Cell*, **87**, 1225–1235.

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.

Lee,E.Y.-H.P., Hu,N., Yuan,S.-S.F., Cox,L.A., Bradley,A., Lee,W.-H. and Herrup,K. (1994) Dual roles of the retinoblastoma protein in cell cycle regulation and neuron differentiation. *Genes Dev.*, **8**, 2008–2021.

Lee,M.-H., Williams,B.O., Mulligan,G., Mukai,S., Bronson,R.T., Dyson,N., Harlow,E. and Jacks,T. (1996) Targeted disruption of p107: functional overlap between p107 and Rb. *Genes Dev.*, **10**, 1621–1632.

Lehner,C.F. and O'Farrell,P.H. (1989) Expression and function of *Drosophila* cyclin A during embryonic cell cycle progression. *Cell*, **56**, 957–968.

Lehner,C.F. and O'Farrell,P.H. (1990) The roles of *Drosophila* cyclins A and B in mitotic control. *Cell*, **61**, 535–547.

MacAuley,A., Cross,J.C. and Werb,Z. (1998) Reprogramming the cell cycle for endoreduplication in rodent trophoblast cells. *Mol. Biol. Cell*, **9**, 795–807.

Mulligan,G. and Jacks,J. (1998) The retinoblastoma gene family: cousins with overlapping interests. *Trends Genet.*, **14**, 223–229.

Nagl,W. (1978) *Endopolyploidy and Polyteny in Differentiation and Evolution: Towards an Understanding of Quantitative and Qualitative Variation of Nuclear DNA in Ontogeny and Phylogeny*. North-Holland Publishing Co., New York, NY.

Qin,X.-Q., Livingston,D.M., Kaelin,W.G.J. and Adams,P. (1994) Deregulated transcription factor E2F-1 expression leads to S-phase entry and p53-mediated apoptosis. *Proc. Natl Acad. Sci. USA*, **91**, 10918–10922.

Richardson,H.E., O'Keefe,L.V., Reed,S.I. and Saint,R. (1993) A *Drosophila* G_1-specific cyclin E homolog exhibits different modes of expression during embryogenesis. *Development*, **119**, 673–690.

Richardson,H., O'Keefe,L.V., Marty,T. and Saint,R. (1995) Ectopic cyclin E expression induces premature entry into S-phase and disrupts pattern formation in the *Drosophila* eye imaginal disc. *Development*, **121**, 3371–3379.

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.

Shan,B. and Lee,W.-H. (1994) Deregulated expression of E2F-1 induces S-phase entry and leads to apoptosis. *Mol. Cell. Biol.*, **14**, 8166–8173.

Sigrist,S.J. and Lehner,C.F. (1997) *Drosophila fizzy-related* down regulates cyclins and is required for cell proliferation arrest and entry into endocycles. *Cell*, **90**, 671–681.

Sigrist,S.J., Jacobs,H., Stratmann,R. and Lehner,C.F. (1995) Exit from mitosis is regulated by *Drosophila* fizzy and the sequential destruction of cyclins A, B and B3. *EMBO J.*, **14**, 4827–4838.

Smith,A.V. and Orr-Weaver,T.L. (1991) The regulation of the cell cycle during *Drosophila* embryogenesis: the transition to polyteny. *Development*, **112**, 997–1008.

Soto,M.C., Chou,T. and Bender,W. (1995) Comparison of germline mosaics of genes in the polycomb group of *Drosophila melanogaster*. *Genetics*, **140**, 231–243.

Spradling,A. and Orr-Weaver,T.L. (1987) Regulation of DNA replication during *Drosophila* development. *Annu. Rev. Genet.*, **21**, 373–403.

Weiss,A., Herzig,A., Jacobs,H. and Lehner,C.F. (1998) Continuous cyclin E expression inhibits progression through endoreduplication cycles in *Drosophila*. *Curr. Biol.*, **8**, 239–242.

Wu,X. and Levine,A.J. (1994) p53 and E2F-1 cooperate to mediate apoptosis. *Proc. Natl Acad. Sci. USA*, **91**, 3602–3606.

Received December 2, 1998; accepted December 18, 1998