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Requirements for dE2F function in proliferating cells and in post-mitotic differentiating cells

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The transcription factor E2F is a target of the retinoblastoma tumor suppressor protein (pRB) and may mediate pRB regulation of S phase entry in mammalian cells. The recent identification of mutant alleles of the *Drosophila E2F* gene (*dE2F*) has shown that dE2F is required for embryogenesis. *dE2F*-mutant embryos lack a co-ordinated program of gene expression which accompanies S phase entry and DNA synthesis declines to levels that are barely detectable. We have investigated the role of the *dE2F* gene at later stages of development. dE2F is expressed in several larval tissues and is required for cell proliferation in the eye imaginal disc. Surprisingly, dE2F expression persists in post-mitotic cells of the eye disc of third-instar larvae. The loss of dE2F function in these cells causes a novel phenotype, characterized by loss of photoreceptors and abnormal rhabdomere cell morphology. These results show that dE2F is required at multiple stages of development and suggest that E2F may have an important function in post-mitotic cells in addition to its role during cell proliferation.

Keywords: differentiation/*Drosophila*/E2F/pRB/proliferation

Introduction

Studies in mammalian cells have suggested that the retinoblastoma tumor suppressor protein is a pivotal regulator of the G_1–S transition. In several different experimental systems the overexpression of pRB in tissue culture cells that lack pRB is sufficient to arrest these cells in late G_1 (Goodrich *et al.*, 1991; Hinds *et al.*, 1992). Investigations into the mechanisms by which pRB arrests the cell cycle have focused on the association between pRB and the transcription factor E2F. The retinoblastoma tumor suppressor protein is a repressor of E2F-dependent transcription (Dalton, 1992; Hamel *et al.*, 1992a; Hiebert *et al.*, 1992; Zamanian and La Thangue, 1992; Flemington *et al.*, 1993; Helin and Harlow, 1993), and the release of E2F from pRB and pRB-related proteins is thought to be a critical event in promoting G_1–S progression. In normal cells the release of E2F appears to be determined, at least in part, by the phosphorylation state of pRB (Chellappan *et al.*, 1991; Helin *et al.*, 1992; Kaelin *et al.*, 1992; Shan *et al.*, 1992). pRB phosphorylation by cyclin-dependent kinases leads to the release of E2F and the activation of E2F-dependent transcription. E2F-binding sites are found in several genes that are expressed during the transition from G_1 into S phase and encode proteins required for cell proliferation (reviewed in Nevins, 1992; Helin and Harlow, 1993; La Thangue, 1994; Farnham, 1995). Moreover, in a number of experimental systems, events that artificially elevate the level of E2F activity promote entry into S phase (Johnson *et al.*, 1993; Zhu *et al.*, 1993; Qin *et al.*, 1994, 1995; Shan and Lee, 1994; Wu and Levine, 1994; Kowalik *et al.*, 1995).

Experiments investigating the consequence of loss of E2F function have provided further insights into the normal role of E2F. The analysis of *Drosophila* embryos that are homozygous for mutant alleles in the *dE2F* gene revealed that dE2F is required for the co-ordinate expression of DNA polymerase α, ribonucleotide reductase subunit 2, and PCNA during G_1–S progression and for the initiation of DNA synthesis (Duronio *et al.*, 1995). All mutant *dE2F* alleles described to date are embryonic-lethal and fail to initiate DNA synthesis in the 17th embryonic cell cycle. The loss of *dE2F* function leads to an almost complete cessation of DNA synthesis by stage 13 of embryogenesis indicating that, at least at this stage of development, dE2F is essential for cell proliferation (Duronio *et al.*, 1995).

Mammalian cells contain a family of *E2F* genes that share a number of common properties. To date, five *E2F* genes have been identified (Helin *et al.*, 1992; Kaelin *et al.*, 1992; Shan *et al.*, 1992; Ivey-Hoyle *et al.*, 1993; Lees *et al.*, 1993; Beijersbergen *et al.*, 1994; Sardet *et al.*, 1995). These encode proteins with extensive sequence similarity that heterodimerize with DP subunits and activate transcription from reporter constructs containing consensus E2F DNA-binding sites. While these genes are expected to play specific roles, the functional differences between forms of E2F are not yet clear. *dE2F* contains only limited sequence homology with mammalian *E2F* genes and is not markedly closer in sequence to any individual mammalian E2F (Dynlacht *et al.*, 1994; Ohtani and Nevins, 1994). Thus, the early lethal phenotype of *dE2F*-mutant embryos might suggest that *Drosophila* contains a single *E2F* gene. Alternatively *Drosophila*, like mammalian cells, may contain additional *E2F* genes that are required at later stages of development or in specific cell types.

Mutational analysis of human promoters have shown that E2F-binding sites can confer both activation and repression of transcription, depending on cell cycle position (Dalton, 1992; Lam and Watson, 1993; Hsiao *et al.*, 1994; Johnson *et al.*, 1994; Neuman *et al.*, 1994). Transcriptional repression is thought to be due to the recruitment of pRB or a pRB-related protein to the promoter. The overexpression of pRB represses promoters containing E2F-binding sites (Hamel *et al.*, 1992b; Weintraub *et al.*,

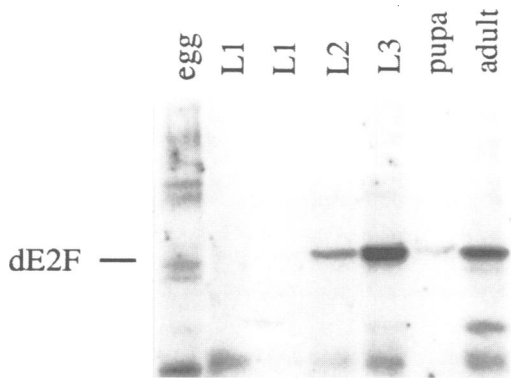

Fig. 1. dE2F expression is high in third-instar larvae. Western blot of *Drosophila* extracts (2 μg total protein/lane) probed with antiserum to dE2F.

1992). Furthermore, fusion of E2F-1 sequences to the Gal4 DNA-binding domain enabled pRB to repress the activity of heterologous promoters that contained Gal4-binding sites (Adnane *et al.*, 1995; Weintraub *et al.*, 1995). Since E2F sites confer a potent transcriptional repression in G_1 phase cells, E2F may be necessary for functions that are carried out in G_1. However, this notion has been difficult to investigate due to lack of an experimental system.

To investigate the function(s) of E2F we have used somatic mosaic analysis to examine the role of dE2F during *Drosophila* eye development. Cells of the eye imaginal disc divide asynchronously during the larval stages. During the third-larval instar a morphogenetic wave traverses the eye disc and is visible as a furrow in the disc. In the furrow, cells are synchronously arrested in G_1, and a subset of these becomes committed to a cell fate, forms preclusters and expresses differentiation markers (reviewed in Wolff and Ready, 1993). As cells leave the furrow, uncommitted cells are stimulated to undergo a final cell division. This final division occurs relatively synchronously and generates a pool of post-mitotic cells that serves as a reservoir for subsequent recruitment and differentiation events. Thus the analysis of *dE2F*-mutant clones in the eye disc allows an investigation of E2F function in proliferating cells and also in post-mitotic cells.

Results

E2F is expressed in proliferating cells and in post-mitotic cells

We have examined the expression pattern of dE2F. Although dE2F is required for embryogenesis, and embryos might be expected to contain the highest proportion of rapidly dividing cells, the level of dE2F protein seen in Western blots of *Drosophila* embryo extracts was low and barely detectable. In contrast, considerably higher levels of dE2F were found at later stages of development (Figure 1). To study the later expression of dE2F in more detail we used two alleles of *dE2F*, $dE2F^{729}$ and $dE2F^{7172}$, that were generated by P element insertion and which act as enhancer traps (Duronio *et al.*, 1995). In both alleles the P element is inserted near to the translation start site. In $dE2F^{729}$ the *lacZ* gene of the P element is in the same orientation as the *dE2F* gene and gives a strong pattern of β-galactosidase (β-gal) staining. In $dE2F^{7172}$, the *dE2F* and *lacZ* genes are in opposite orientations and the pattern of *lacZ* expression, while similar to that of $dE2F^{729}$, is considerably weaker in intensity.

Using $dE2F^{729}$ and $dE2F^{7172}$ alleles, patterned expression of *lacZ* was observed in several third-larval instar tissues. High levels of expression were apparent in the central nervous system, in salivary glands and in eye, leg and wing imaginal discs (Figure 2A and B and data not shown). Because the development of the eye imaginal disc has been studied extensively we have focused on the pattern in this region. Double staining was performed to compare the presumptive *dE2F* expression pattern with DNA synthesis (Figure 2C). As determined from the distribution of β-gal relative to the morphogenetic furrow, no *dE2F* transcription was found in the anterior portion of the disc that contains asynchronously dividing cells. A pulse of *dE2F* transcription was observed anterior to the furrow, and a strong pulse of *dE2F* transcription was observed posterior to the furrow that corresponded to the second waves of S phases. β-gal staining persisted in post-mitotic cells posterior to the second wave of S phases.

E2F has been considered primarily as a factor that promotes cell proliferation. The suggestion that high levels of dE2F might persist in post-mitotic cells was surprising. To compare these patterns with the distribution of the endogenous protein, eye discs were stained with an anti-dE2F monoclonal antibody (Figure 3). The expression of dE2F protein closely resembled the enhancer trap staining pattern. A stripe of strong nuclear staining was seen anterior to the furrow and posterior to the furrow in the region corresponding to the second wave of S phases. In addition, an elevated level of dE2F was present in all the post-mitotic cells found posterior to the furrow. Taken together the enhancer trap and antibody staining patterns suggest that cells proliferating in the anterior of the disc contain dE2F, but that the levels increase anterior to the furrow as a result of new gene transcription. Intriguingly, elevated levels of dE2F are maintained in cells that have exited the cell cycle and are undergoing differentiation.

E2F is required for cell proliferation in the larval eye disc

To investigate the function of dE2F during eye development we used the FLP recombination system to generate *dE2F*-mutant clones in flies heterozygous for *dE2F* (+/$dE2F^{729}$ or +/$dE2F^{7172}$) (Golic and Lindquist, 1989; Golic, 1991; Xu and Rubin, 1993; Xu and Harrison, 1994). Following induction of FLP recombinase in the first-larval instar, adult eyes showed the presence of large twin spots (+/+ clones marked with two copies of the *mini-white* pigment), but no mutant clones ($dE2F^{729}/dE2F^{729}$ or $dE2F^{7172}/dE2F^{7172}$, lacking the *mini-white* gene) were readily apparent (Figure 4A). Similar results were seen following induction of recombinase in the second-larval instar (Figure 4B). These results showed that the *dE2F*-mutant cells were at a significant proliferative disadvantage compared with the wild-type twin-spot cells.

To determine if *dE2F*-mutant clones were present in third-instar larvae, we examined imaginal discs for microscopic clones. Somatic recombination was induced following heat-shock treatment of first- and second-instar larvae, and eye discs dissected from third-instar larvae were

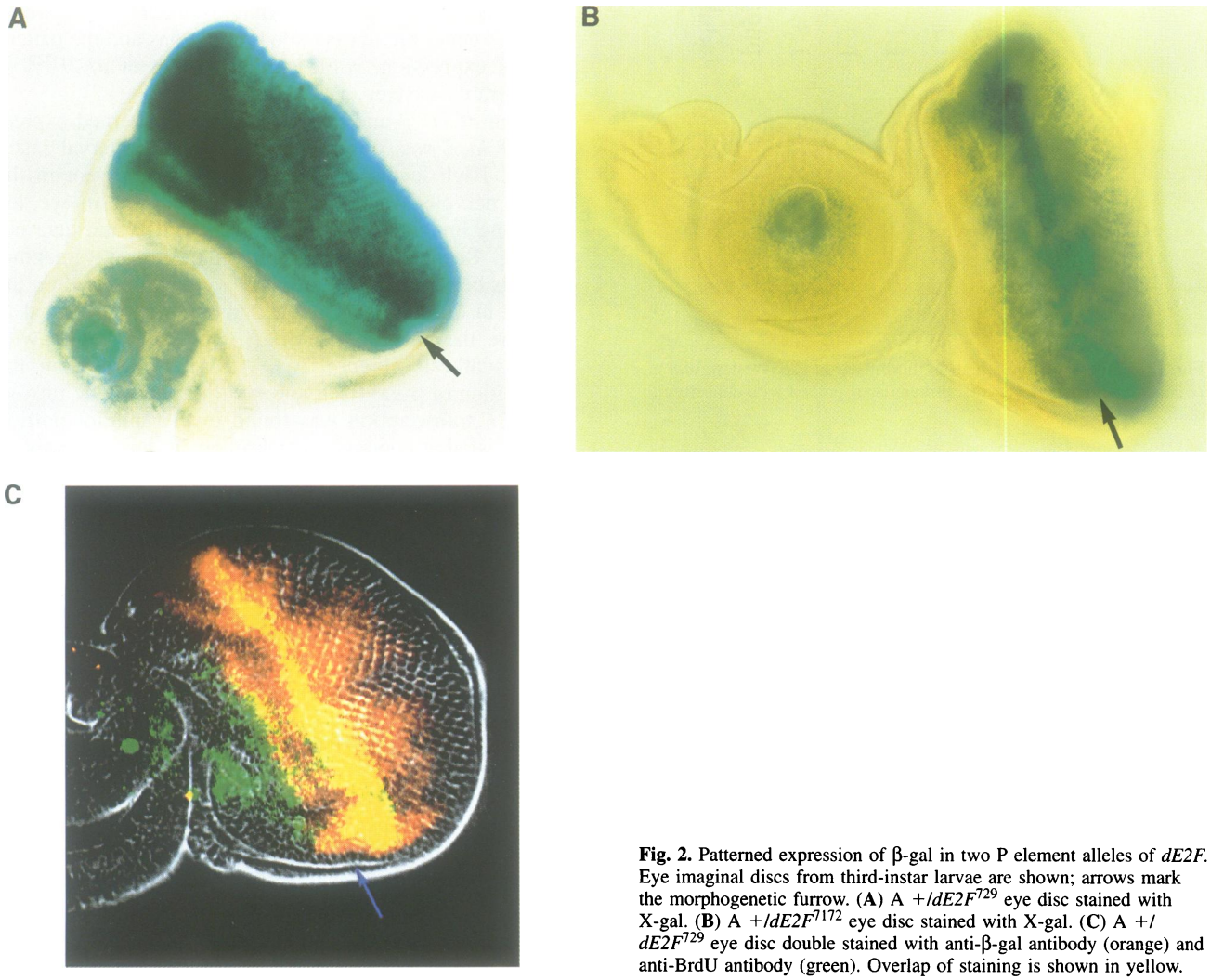

Fig. 2. Patterned expression of β-gal in two P element alleles of *dE2F*. Eye imaginal discs from third-instar larvae are shown; arrows mark the morphogenetic furrow. (**A**) A $+/dE2F^{729}$ eye disc stained with X-gal. (**B**) A $+/dE2F^{7172}$ eye disc stained with X-gal. (**C**) A $+/dE2F^{729}$ eye disc double stained with anti-β-gal antibody (orange) and anti-BrdU antibody (green). Overlap of staining is shown in yellow.

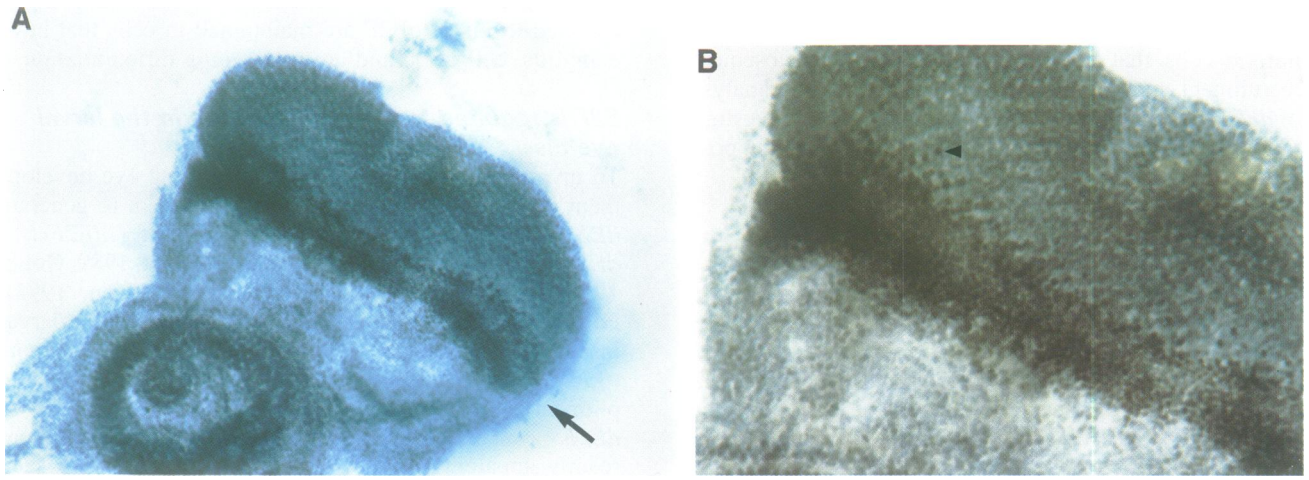

Fig. 3. A wild-type eye disc stained with an anti-dE2F monoclonal antibody. The morphogenetic furrow is marked by the black arrow. (**A**) dE2F is detected throughout the eye disc. A low level of dE2F is seen in the anterior portion of the disc. dE2F staining increases anterior to the furrow and this elevated level of dE2F is maintained to the posterior of the disc. (**B**) Strongest dE2F staining is seen in two stripes on opposite sides of the furrow. Individual nuclei that stained strongly posterior to the furrow (arrowhead) may represent S phase cells.

stained for β-gal expression from the $dE2F^{729}$ allele. One hundred and sixty-four twin spots were examined in detail; in 76 cases no adjacent clone of mutant cells could be found; in 88 cases $dE2F^{729}$ clones were found, but these clones were much smaller than the adjacent twin spots (Figure 4C and D). These $dE2F^{729}$ clones contained between one and 14 cells, with a mean of 4.3 cells per clone. The disparity in the sizes of wild-type and mutant

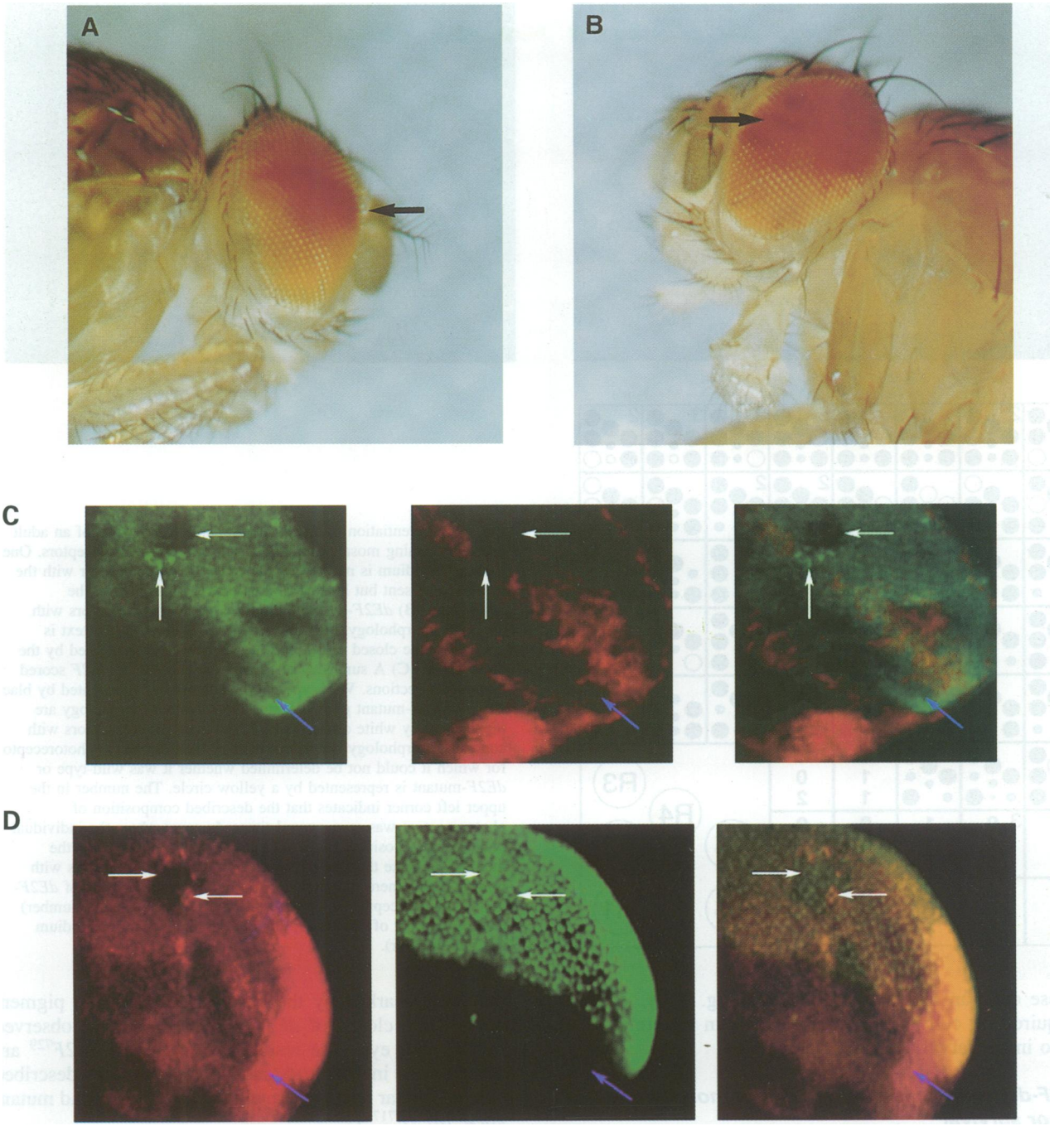

Fig. 4. *dE2F* is required for cell proliferation in the larva. FLP stocks carrying $dE2F^{729}$ or $dE2F^{7172}$ were generated following the scheme of Xu and Harrison (1994). Somatic recombination was induced by heat-shocking for 1 h at 37°C during the first-larval instar (**A**), or the second-larval instar (**B**). Twin spots appearing as dark red patches (arrowed) against the orange heterozygous background were evident in the adult eyes but no adjacent clones containing *dE2F*-mutant cells were visible. (**C**) Cyclin B expression was not detected in E2F-deficient clones. Somatic recombination was induced during the first-larval instar and discs taken from third-instar larvae were stained with anti-β-gal antibody (green) to visualize the clone and anti-cyclin B antibodies (red). Cells in the heterozygous background have one copy of the *lacZ* gene, cells that are $dE2F^{729}/dE2F^{729}$ have two copies of the *lacZ* gene and increased β-gal staining, and +/+ cells in the twin spot lack the *lacZ* gene and have no β-gal staining. The clone of $dE2F^{729}/dE2F^{729}$ cells is marked by a vertical white arrow, the twin spot by a horizontal white arrow and the furrow by a blue arrow. (**D**) elav is expressed normally in E2F-deficient clones. Following somatic recombination eye discs carrying *dE2F*-mutant clones were double stained with anti-β-gal antibody (red) to visualize the clone and with a rat anti-elav antibody (green). A clone of $dE2F^{729}/dE2F^{729}$ cells is marked by the leftward pointing white arrow, the twin spot (+/+ cells) by the rightward pointing white arrow and the furrow by the blue arrow.

clones was consistent with failure of the *dE2F*-mutant cells to proliferate because of cell cycle arrest. In support of this interpretation, no cyclin B expression was detected in $dE2F^{729}/dE2F^{729}$ cells in the third-instar discs (Figure 4C), regardless of the position of the cells relative to the morphogenetic furrow. Cyclin B has been used as a marker of G_2/M phase cells in the eye (Whitfield *et al.*, 1990; Knoblich and Lehner, 1993). From these observations we infer that *dE2F*-mutant cells are capable of a limited number of cell divisions but by the third-larval instar

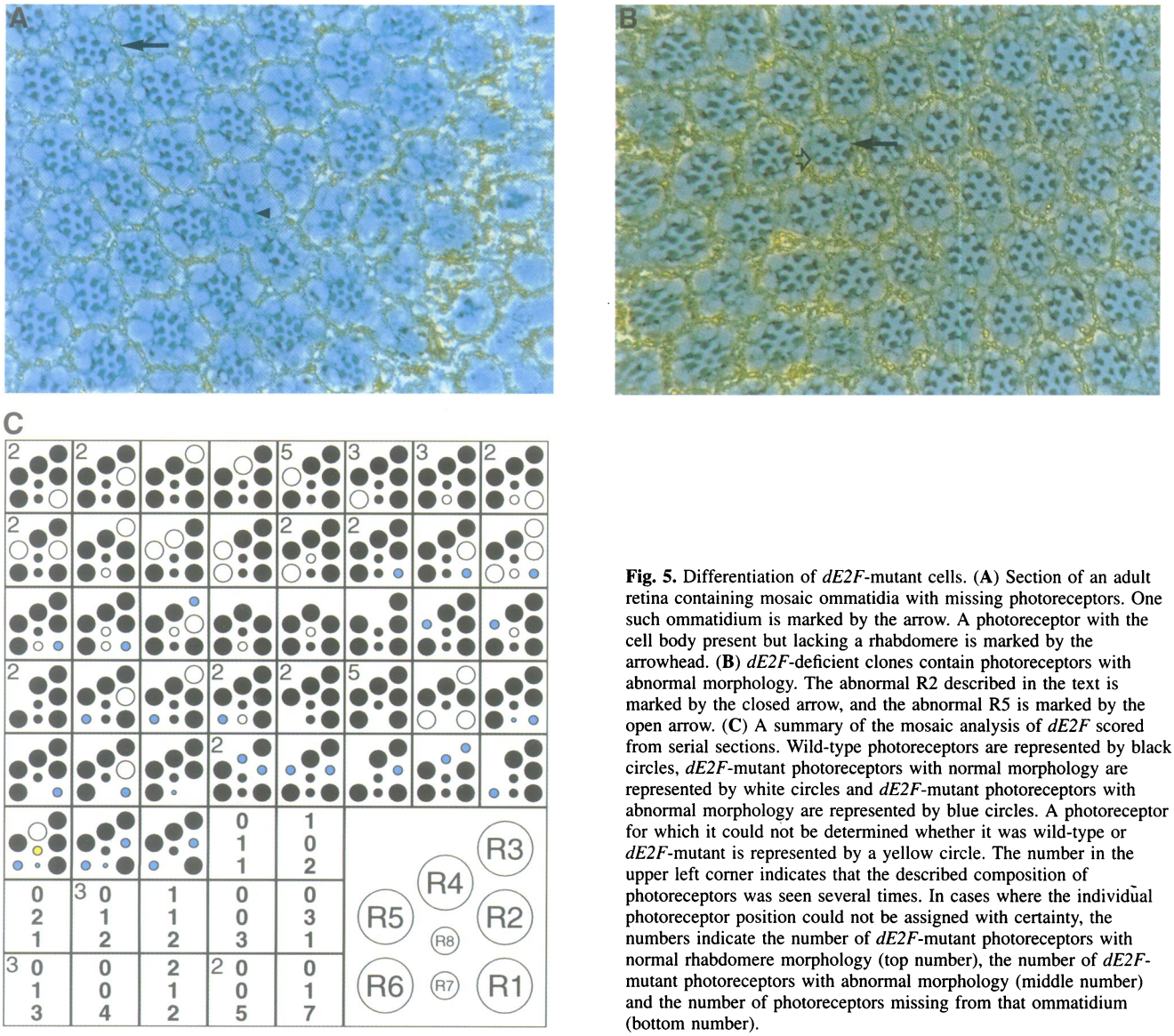

Fig. 5. Differentiation of *dE2F*-mutant cells. (**A**) Section of an adult retina containing mosaic ommatidia with missing photoreceptors. One such ommatidium is marked by the arrow. A photoreceptor with the cell body present but lacking a rhabdomere is marked by the arrowhead. (**B**) *dE2F*-deficient clones contain photoreceptors with abnormal morphology. The abnormal R2 described in the text is marked by the closed arrow, and the abnormal R5 is marked by the open arrow. (**C**) A summary of the mosaic analysis of *dE2F* scored from serial sections. Wild-type photoreceptors are represented by black circles, *dE2F*-mutant photoreceptors with normal morphology are represented by white circles and *dE2F*-mutant photoreceptors with abnormal morphology are represented by blue circles. A photoreceptor for which it could not be determined whether it was wild-type or *dE2F*-mutant is represented by a yellow circle. The number in the upper left corner indicates that the described composition of photoreceptors was seen several times. In cases where the individual photoreceptor position could not be assigned with certainty, the numbers indicate the number of *dE2F*-mutant photoreceptors with normal rhabdomere morphology (top number), the number of *dE2F*-mutant photoreceptors with abnormal morphology (middle number) and the number of photoreceptors missing from that ommatidium (bottom number).

these cells are not actively proliferating. Thus, *dE2F* is required for cell proliferation not only in the embryo but also in the larval eye disc.

E2F-deficient cells have abnormal morphology and poor survival

The failure of homozygous *dE2F*-mutant cells to proliferate in the eye disc provided strong evidence that these cells lacked functional dE2F. We then asked whether such E2F-deficient cells could differentiate normally. FLP-recombinase was induced in second-instar larvae, and third-larval instar eye discs containing clones of *dE2F*-mutant cells were stained with an antibody to elav (Robinow and White, 1988), an early marker of neuronal differentiation. As shown in Figure 4D, *dE2F* clones express elav normally, indicating that dE2F was not required for cells to be recruited to a cell fate nor for the initiation of neuronal differentiation.

To determine the fate of E2F-deficient cells, we sectioned adult eyes following somatic recombination in the second- and early third-larval instars and examined serial sections for evidence of microscopic *dE2F*-mutant clones that were marked by the absence of the white pigment allele. Rare clones of *dE2F*-mutant cells were observed in the adult eye. The results obtained with $dE2F^{729}$ are summarized in Figure 5 and Table I and are described below. Similar results were obtained with a second mutant allele ($dE2F^{7172}$).

From an analysis of serial sections, 69 ommatidia were identified that contained between one and five $dE2F^{729}$/$dE2F^{729}$ or $dE2F^{7172}$/$dE2F^{7172}$ cells. Of these mosaic ommatidia, 22 had missing photoreceptors (an example is shown in Figure 5A). The number and position of missing photoreceptors were variable. In addition, a further 13 ommatidia were observed adjacent to twin spots that had missing photoreceptors but lacked *dE2F*-mutant cells. This frequency of missing photoreceptors is unusual and was not seen in parts of the eye that were distant from the twin spots nor in non-heat-shocked eyes. These observations indicate that the absence of dE2F leads to poor cell survival.

Approximately 45% of the *dE2F*-mutant photoreceptors identified had abnormal cell morphology. Three unusual features were found. First, rhabdomeres were seen in the position of outer photoreceptors that were unusually small

Table I. The composition of mosaic ommatidia with no more than one missing photoreceptor

Photoreceptor	No. of times missing	No. of *dE2F*-mutant cells with abnormal morphology	No. of *dE2F*-mutant cells with normal morphology
R1	1	9	5
R2	0	6	9
R3	2	2	5
R4	1	3	3
R5	4	3	9
R6	2	8	8
R7	9	4	12
R8	0	0	6

in diameter; in some clones such outer photoreceptors appeared smaller than normal inner photoreceptors. Second, outer photoreceptors were found with rhabdomeres that projected centrally, a feature which is normally only seen in inner photoreceptors. Third, unlike normal outer photoreceptors, the abnormal photoreceptors did not extend the full length of the retina. A mosaic ommatidium containing abnormal photoreceptors is shown in Figure 5B. In the clone indicated, the rhabdomeres of the photoreceptors at the R2 and the R5 positions, are unusually small. The R2 rhabdomere appears to project centrally, and the R5 rhabdomere also partially projects. These rhabdomeres extend basally past the level of the R7–R8 boundary but do not span the retina. Morphological changes of *dE2F*-mutant cells were not restricted to individual photoreceptor types; in other mosaic ommatidia R2 and R5 were normal, but other photoreceptors had these unusual morphological characteristics (Figure 5C and Table I). In each case, the photoreceptors with abnormal rhabdomeres lacked pigment, indicating that this phenotype is restricted to *dE2F*-mutant cells and is caused by the absence of dE2F.

Discussion

Much of the information about the function of E2F is derived from overexpression experiments showing that increased levels of E2F drive cell proliferation. The identification of mutant *dE2F* alleles allowed the effects of loss of E2F activity to be examined and revealed that dE2F is necessary for DNA synthesis during embryogenesis. dE2F appears to be relatively unstable in embryos suggesting that dE2F turnover may be an important element of E2F regulation; dE2F protein expressed in embryos from a heat-shock inducible transgene has a half life of ~40 min (data not shown). Analysis of dE2F expression patterns shows that the level of dE2F is relatively low in embryo extracts but increases dramatically during the larval stages. Enhancer trap lines and immunostaining show that dE2F is expressed in many larval tissues, including the eye imaginal disc of third-instar larvae.

To test whether dE2F was required for post-embryonic cell cycles we analyzed clones of *dE2F*-mutant cells that were generated in the eye disc by somatic recombination. *dE2F*-mutant cells generated in first- or second-instar larvae had limited proliferative ability. Unlike the wild-type cells of the twin spot, *dE2F*-mutant clones were very small (typically four cells) and, by the third-larval instar, these cells were cell cycle arrested. These data show that the role of dE2F is not restricted to the embryogenesis but that dE2F is required at multiple stages of development. It is possible therefore that *Drosophila* may use a single gene to replace the extensive family of *E2F* genes found in mammalian cells.

Although dE2F was found in asynchronously dividing cells of the eye disc, a dramatic rise in *dE2F* transcription was observed anterior to the morphogenetic furrow together with a corresponding increase in protein levels. In human cells transcription of the *E2F-1* gene is tied to cell cycle progression and increases as cells enter S phase (Kaelin *et al.*, 1992; Shan *et al.*, 1992; Hsiao *et al.*, 1994; Johnson *et al.*, 1994; Neuman *et al.*, 1994). However, the increase in *dE2F* transcription occurs without synchronous S phase entry and appears to be determined by developmental cues. The expression of many genes is regulated at, or anterior to, the morphogenetic furrow (Carroll and Whyte, 1989; Alphey *et al.*, 1992; Bonini *et al.*, 1993; Heberlein *et al.*, 1993; Ma *et al.*, 1993; Brown *et al.*, 1995) and further work will be necessary to identify elements and signals involved in the regulation of *dE2F* expression.

Surprisingly, elevated levels of dE2F are maintained in post-mitotic cells posterior to the furrow and in cells that are undergoing differentiation. Such expression is unnecessary if E2F is solely required to promote DNA synthesis and suggests that dE2F may have additional functions. This notion is supported by the analysis of dE2F-deficient cells. The observations that many *dE2F*-mutant photoreceptors have abnormal morphology and that photoreceptors are frequently missing from mosaic ommatidia containing wild-type and *dE2F*-mutant cells suggest that dE2F has an important function in these post-mitotic cells. The changes seen in the absence of dE2F are unlikely to be an indirect consequence of the limited proliferation of *dE2F*-mutant cells since previous studies have shown that photoreceptor differentiation proceeds normally in the absence of normal patterned cell division. Analysis of clones homozygous for mutant alleles of replication factor C found that mutant clones were small relative to the wild-type twin spot, but that these cells maintained the normal arrangement and morphology of photoreceptors (Harrison *et al.*, 1995). Furthermore, it was recently shown that expression of transgenic human p21 of the eye disc arrests cells in G_1 and eliminates the second wave of S phases. However, these arrested cells undergo normal differentiation and the photoreceptors that are formed do not have abnormal morphology or decreased survival (de Nooij and Hariharan, 1995). Since photoreceptor differentiation can be uncoupled from cell proliferation, the defects seen following the generation of *dE2F*-mutant clones appear to reflect a specific requirement for dE2F.

It is unlikely that dE2F is required for a specific step in the determination or differentiation of photoreceptor cells since some *dE2F*-mutant photoreceptors were found that appeared normal. Thus it appears that photoreceptor differentiation does not require dE2F, but in the absence of dE2F a significant percentage of these cells degenerate and die. The altered morphology of *dE2F*-mutant cells may be an early event in the degeneration process. Photoreceptor cell bodies were occasionally found that

lacked a rhabdomere (Figure 5A) and may represent a later event in this process. The interpretation that *dE2F*-mutant cells differentiate but fail to survive is supported by the observation of mosaic ommatidia that were normal except for the absence of R5. Since a normal R4 cell was present, this suggests that the R5 cell was initially present but died subsequent to the time of R5 recruitment of R4 (Tomlinson *et al.*, 1988). Similarly seven of the ommatidia with missing cells lacked an R8 cell. Since R8 is the founder cell for each ommatidial cluster and is essential for formation of the ommatidium (reviewed in Dickson and Hafen, 1993), it appears that an R8 cell was initially present, but died subsequent to the non-autonomous requirement for R8. Depending on the time when the R8 cell is lost, the reduced number of photoreceptors in these ommatidia may be due in part to the cell death and in part to the failure of later recruitment events.

These findings suggest that E2F has functions that are separable from its role in cell proliferation. Thus in the eye disc, dE2F is not only necessary for normal cell proliferation but also promotes the survival of fully differentiated cells. One potential explanation for these observations is suggested by the capacity of E2F sites to confer both activation and repression of responsive genes depending on the position of the cell in the cell cycle (Lam and Watson, 1993; Hsiao *et al.*, 1994; Johnson *et al.*, 1994; Neuman *et al.*, 1994; Zhu *et al.*, 1995). Whereas the activation of E2F-regulated genes may be important in promoting cell cycle progression, it may be equally critical that some E2F-regulated genes are effectively silenced in differentiating cells. In the absence of dE2F, the incomplete silencing of certain genes may lead to a conflict between differentiation functions and factors which are normally only expressed during cell proliferation. In the photoreceptor such conflict may cause incomplete or abnormal cell differentiation and result in cell death. If this model is true then a related phenomenon might also be expected to occur in the absence of pRB. Interestingly the central nervous system of RB –/– embryos shows extensive apoptosis (Lee *et al.*, 1994) and defective neuronal differentiation. Furthermore, myotubes formed from RB –/– cells show an unusual propensity to re-enter the cell cycle (Schneider *et al.*, 1994). These defects may also stem from failure to silence E2F-activated genes, a function that would require continued E2F activity in terminally differentiated cells.

Materials and methods

Histochemistry and Western blotting

Mice were immunized with dE2F protein that was expressed in bacteria. The dE2F monoclonal antibody Hao 4 was prepared by fusing splenocytes to NS-1 myeloma cells 4 days after the final boost as described (Harlow and Lane, 1988). Hao 4 was found to be specific for dE2F protein in immunoprecipitation and Western blotting analysis and to supershift E2F complexes detected in E2F gel-shift assays. *Drosophila* extracts were prepared by homogenizing *Drosophila* of the appropriate stage in sample buffer (Laemmli, 1970). For dE2F antibody staining, eye discs were fixed in Brower fixative for 2 min at 4°C. Hao 4 was added as undiluted tissue culture supernatant, and the immunohistochemistry procedure followed was as described (Carroll and Whyte, 1989). For cyclin B/β-gal double staining, primary antibodies were mouse anti-β-galactosidase (Promega), at 1:200, and rabbit anti-cyclin B (D.Glover), at 1:100. For elav/β-galactosidase double staining, the primary antibodies were rat anti-elav (J.Treisman and G.Rubin), at 1:200, and rabbit anti-β-galactosidase, at 1:10 000. X-gal staining of larval tissues was as described (Mlodzik, 1990).

For BrdU/β-gal double staining, third-instar larvae were fed 1 mg/ml BrdU for 5 h at 25°C. Fixation was in Brower fixative (Blair *et al.*, 1994) for 2.5 min at 4°C; primary antibodies were rabbit anti-β-galactosidase (5 prime→3 prime, Inc.), at 1:10 000, and mouse anti-BrdU with nuclease (Amersham), undiluted, for 12 h at 4°C; and the secondary antibodies were rhodamine goat anti-rabbit (Jackson ImmunoResearch), at 1:400, and fluorescein goat anti-mouse, at 1:200, for 90 min at 4°C.

Mosaic analysis

FLP stocks were generated following the scheme of Xu and Harrison (Xu and Rubin, 1993; Xu and Harrison, 1994). Heterozygous flies for adult mosaic analysis were generated by crossing *w*;P(FRT)82B *dE2F*/TM6B males to *whsFLP*;P(FRT)82B P(*w*)90E females. Heterozygous flies for larval mosaic analysis and for gross examination of adult eyes were generated by crossing *w*;P(FRT)82B *dE2F*/TM6B males to *whsFLP*; P(FRT)82B P(*mini–w*;*hs*πM)87E females. Somatic recombination was induced by heat-shocking for 1 h at 37°C at the indicated larval instar.

Plastic sections of adult retinas were prepared as described (Xu and Harrison, 1994). Mosaic analysis was conducted by examination of serial sections of 82 ommatidia; photoreceptors were scored for the presence of pigment and for morphology.

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References

Adnane,J., Shao,Z. and Robbins,P.D. (1995) The retinoblastoma susceptibility gene product represses transcription when directly bound to the promoter. *J. Biol. Chem.*, **270**, 8837–8843.

Alphey,L., Jiminez,J., White-Cooper,H., Dawson,I., Nurse,P. and Glover,D.M. (1992) *twine*, a cdc25 homolog that functions in the male and female germline of *Drosophila*. *Cell*, **69**, 977–988.

Beijersbergen,R.L., Kerkhoven,R.M., Zhu,L., Carlee,L., Voorhoeve,P.M. and Bernards,R. (1994) E2F-4, a new member of the E2F gene family, has oncogenic activity and associates with p107 *in vivo*. *Genes Dev.*, **8**, 2680–2690.

Blair,S.S., Brower,D.L., Thomas,J.B. and Zavortink,M. (1994) The role of *apterous* in the control of dorsoventral compartmentalization and PS integrin gene expression in the developing wing of *Drosophila*. *Development*, **120**, 1805–1815.

Bonini,N.M., Leiserson,W.M. and Benzer,S. (1993) The eyes absent gene: genetic control of cell survival and differentiation in the developing eye. *Cell*, **72**, 379–395.

Brown,N.L., Sattler,C.A., Paddock,S.W. and Carroll,S.P. (1995) Hairy and EMC negatively regulate morphogenetic furrow progression in the *Drosophila* eye. *Cell*, **80**, 879–887.

Carroll,S.B. and Whyte,J.S. (1989) The role of the hairy gene during *Drosophila* morphogenesis: stripes in imaginal discs. *Genes Dev.*, **3**, 905–916.

Chellappan,S., Hiebert,S., Mudryj,M., Horowitz,J. and Nevins,J. (1991) The E2F transcription factor is a cellular target for the RB protein. *Cell*, **65**, 1053–1061.

Dalton,S. (1992) Cell cycle regulation of the human *cdc2* gene. *EMBO J.*, **11**, 1797–1804.

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.

Dickson,B. and Hafen,E. (1993) Genetic dissection of eye development in *Drosophila*. In Bate,M. and Martinez-Arias,A. (eds), *The Development of Drosophila melanogaster*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, Vol. 2, pp. 1327–1362.

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.

Dynlacht,B.D., Brook,A., Dembski,M., Yenush,L. and Dyson,N. (1994) DNA-binding and transactivation properties of *Drosophila* E2F and DP proteins. *Proc. Natl Acad. Sci. USA*, **91**, 6359–6363.

Farnham,P.J. (1995) *Transcriptional Control of Cell Growth: the E2F Gene Family*. Springer-Verlag, New York.

Flemington,E.K., Speck,S.H. and Kaelin,W.G.,Jr (1993) E2F-1 mediated transactivation is inhibited by complex formation with the retinoblastoma susceptibility gene product. *Proc. Natl Acad. Sci. USA*, **90**, 6914–6918.

Golic,K.G. (1991) Site-specific recombination between homologous chromosomes in *Drosophila*. *Science*, **252**, 958–961.

Golic,K.G. and Lindquist,S. (1989) The FLP recombinase of yeast catalyzes site-specific recombination in the *Drosophila* genome. *Cell*, **59**, 499–509.

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

Hamel,P.A., Gill,R.M., Phillips,R.A. and Gallie,B.L. (1992a) Regions controlling hyperphosphorylation and conformation of the retinoblastoma gene product are independent of domains required for transcriptional repression. *Oncogene*, **7**, 693–701.

Hamel,P.A., Gill,R.M., Phillips,R.A. and Gallie,B.L. (1992b) Transcriptional repression of the E2-containing promoters EIIaE, c-myc, and RB1 by the product of the RB1 gene. *Mol. Cell. Biol.*, **12**, 3431–3438.

Harlow,E. and Lane,D. (1988) *Antibodies: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Harrison,S.D., Solomon,N. and Rubin,G.M. (1995) A genetic analysis of the 63E-64A genomic region of *Drosophila melanogaster*: Identification of mutations in a replication factor C subunit. *Genetics*, **139**, 1701–1709.

Heberlein,U., Wolff,T. and Rubin,G.M. (1993) The TGF beta homolog dpp and the segment polarity gene hedgehog are required for propagation of a morphogenetic wave in the *Drosophila* retina. *Cell*, **75**, 913–926.

Helin,K. and Harlow,E. (1993) The retinoblastoma protein as a transcriptional repressor. *Trends Cell Biol.*, **3**, 43–46.

Helin,K., Lees,J.A., Vidal,M., Dyson,N., Harlow,E. and Fattaey,A. (1992) A cDNA encoding a pRB-binding protein with properties of the transcription factor E2F. *Cell*, **70**, 337–350.

Hiebert,S.W., Chellappan,S.P., Horowitz,J.M. and Nevins,J.R. (1992) The interaction of pRb with E2F inhibits the transcriptional activity of E2F. *Genes Dev.*, **6**, 177–185.

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.

Hsiao,K.-M., McMahon,S.L. and Farnham,P.J. (1994) Multiple DNA elements are required for the growth regulation of the mouse E2F1 promoter. *Genes Dev.*, **8**, 1526–1537.

Ivey-Hoyle,M., Conroy,R., Huber,H., Goodhart,P., Oliff,A. and Heinbrook,D.C. (1993) Cloning and characterization of E2F-2, a novel protein with the biochemical properties of transcription factor E2F. *Mol. Cell. Biol.*, **13**, 7802–7812.

Johnson,D.G., Schwartz,J.K., Cress,W.D. and Nevins,J.R. (1993) Expression of transcription factor E2F1 induces quiescent cells to enter S phase. *Nature*, **365**, 349–352.

Johnson,D.G., Ohtani,K. and Nevins,J.R. (1994) Autoregulatory control of E2F-1 expression in response to positive and negative regulators of cell cycle expression. *Genes Dev.*, **8**, 1514–1525.

Kaelin,W.G.,Jr *et al.* (1992) Expression cloning of a cDNA encoding a retinoblastoma-binding protein with E2F-like properties. *Cell*, **70**, 351–64.

Knoblich,J.A. and Lehner,C.F. (1993) Synergistic action of *Drosophila* cyclins A and B during G_2–M transition. *EMBO J.*, **12**, 65–74.

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.

La Thangue,N.B. (1994) DTRF1/E2F: an extending family of heterodimeric factors implicated in cell cycle control. *Trends Biochem. Sci.*, **19**, 108–114.

Laemmli,U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.

Lam,E.W.-F. and Watson,R.J. (1993) An E2F-binding site mediates cell-cycle regulated repression of mouse B-myb transcription. *EMBO J.*, **12**, 2705–2713.

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.

Lees,J.A., Saito,M., Vidal,M., Valentine,M., Look,T., Harlow,E., Dyson,N. and Helin,K. (1993) The retinoblastoma protein binds to a family of E2F transcription factors. *Mol. Cell. Biol.*, **13**, 7813–7825.

Ma,C., Zhou,Y., Beachy,P.A. and Moses,K.A. (1993) The segment polarity gene hedgehog is required for progression of the morphogenetic furrow in the developing *Drosophila* retina. *Cell*, **75**, 927–938.

Mlodzik,M. (1990) β-Galactosidase detection in imaginal discs. In Rubin,L. (ed.), *Rubin Lab Methods Book*. Berkeley, CA, pp. 72–73.

Neuman,E., Flemington,E.K., Sellers,W.R. and Kaelin,W.G.,Jr (1994) Transcription of the E2F1 gene is rendered cell cycle-dependent by E2F DNA binding sites within its promoter. *Mol. Cell. Biol.*, **14**, 6607–6615.

Nevins,J.R. (1992) E2F: A link between the Rb tumor suppressor protein and viral oncoproteins. *Science*, **258**, 424–429.

Ohtani,K. and Nevins,J.R. (1994) Functional properties of a *Drosophila* homolog of the E2F1 gene. *Mol. Cell. Biol.*, **14**, 1603–1612.

Qin,X.-Q., Livingston,D.M., Kaelin,W.G.,Jr 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.

Qin,X.-Q., Livingston,D.M., Ewen,M., Sellers,W.R., Arany,Z. and Kaelin,W.G. (1995) The transcription factor E2F-1 is a downstream target of RB action. *Mol. Cell. Biol.*, **15**, 742–755.

Robinow,S. and White,K. (1988) The locus elav of *Drosophila melanogaster* is expressed in neurons at all developmental stages. *Dev. Biol.*, **126**, 294–303.

Sardet,C., Vidal,M., Cobrinik,D., Geng,Y., Onufryk,C., Chen,A. and Weinberg,R.A. (1995) E2F-4 and E2F-5, two novel members of the E2F family, are expressed in the early phases of the cell cycle. *Proc. Natl Acad. Sci. USA*, **92**, 2403–2407.

Schneider,J.W., Gu,W., Zhu,L., Mahdavi,V. and Nadal-Ginard,B. (1994) Reversal of terminal differentiation mediated by p107 in Rb –/– muscle cells. *Science*, **264**, 1467–1471.

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.

Shan,B., Zhu,X., Chen,P.-L., Durfee,T., Yang,Y., Sharp,D. and Lee,W.-H. (1992) Molecular cloning of cellular genes encoding retinoblastoma-associated proteins: identification of a gene with properties of the transcription factor E2F. *Mol. Cell. Biol.*, **12**, 5620–5631.

Tomlinson,A., Kimmel,B.E. and Rubin,G.M. (1988) *rough*, a *Drosophila* homeobox gene required in photoreceptors R2 and R5 for inductive interactions in the developing eye. *Cell*, **55**, 771–784.

Weintraub,S.J., Prater,C.A. and Dean,D.C. (1992) Retinoblastoma protein switches the E2F site from positive to negative element. *Nature*, **358**, 259–261.

Weintraub,S.J., Chow,K.N.B., Luo,R.X., Zhang,S.H., He,S. and Dean,D.C. (1995) Mechanism of active transcriptional repression by the retinoblastoma protein. *Nature*, **375**, 812–815.

Whitfield,W.G.F., Gonzalez,C., Maldonado-Codina,G. and Glover,D.M. (1990) The A- and B-cyclins of *Drosophila* are accumulated and destroyed in temporally distinct events that define separable phases of the G_2–M transition. *EMBO J.*, **9**, 2563–2572.

Wolff,T. and Ready,D.F. (1993) Pattern formation in the *Drosophila* retina. In Bate,M. and Martinez-Arias,A. (eds), *The Development of Drosophila melanogaster*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, Vol. 2, pp. 1277–1326.

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

Xu,T. and Harrison,S.D. (1994) Mosaic analysis using FLP recombinase. *Methods Cell Biol.*, **44**, 655–681.

Xu,T. and Rubin,G.M. (1993) Analysis of genetic mosaics in developing and adult *Drosophila* tissues. *Development*, **117**, 1223–1237.

Zamanian,M. and La Thangue,N.B. (1992) Adenovirus E1A prevents the retinoblastoma gene product from repressing the activity of a cellular transcription factor. *EMBO J.*, **11**, 2603–2610.

Zhu,L., van den Heuvel,S., Helin,K., Fattaey,A., Ewen,M., Livingston,D., Dyson,N. and Harlow,E. (1993) Inhibition of cell proliferation by p107, a relative of the retinoblastoma protein. *Genes Dev.*, **7**, 1111–1125.

Zhu,L., Zhu,L., Xie,E. and Chang,L.-S. (1995) Differential roles of two tandem E2F sites in repression of the human p107 promoter by retinoblastoma and other proteins. *Mol. Cell. Biol.*, **15**, 3552–3562.

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