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Ectopic expression of dE2F and dDP induces cell proliferation and death in the *Drosophila* eye

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The deregulation of E2F activity is thought to contribute to the uncontrolled proliferation of many tumor cells. While the effects of overexpressing E2F genes have been studied extensively in tissue culture, the consequences of elevating E2F activity *in vivo* are unknown. To address this issue, transgenic lines of *Drosophila* were studied in which ectopic expression of dE2F and dDP was targeted to the developing eye. The co-expression of dDP or dE2F disrupted normal eye development, resulting in abnormal patterns of bristles, cone cells and photoreceptors. dE2F/dDP expression caused ectopic S phases in post-mitotic cells of the eye imaginal disc but did not disrupt the onset of neuronal differentiation. Most S phases were seen in uncommitted cells, although some cells that had initiated photoreceptor differentiation were also driven into the cell cycle. Elevated expression of dE2F and dDP caused apoptosis in the eye disc. The co-expression of baculovirus p35 protein, an inhibitor of cell death, strongly enhanced the dE2F/dDP-dependent phenotype. These results show that, in this *in vivo* system, the elevation of E2F activity caused post-mitotic cells to enter the cell cycle. However, these cells failed to proliferate unless rescued from apoptosis.

Keywords: apoptosis/cell proliferation/E2F transcription factor

Introduction

E2F is a heteromeric factor containing a subunit encoded by a member of the E2F family of genes and a subunit encoded by the DP family of genes. Currently, five E2F genes (E2F-1–5) and two DP genes (DP-1 and DP-2) have been isolated from mammalian cells (Helin *et al.*, 1992; Kaelin *et al.*, 1992; Shan *et al.*, 1992; Girling *et al.*, 1993; Ivey-Hoyle *et al.*, 1993; Lees *et al.*, 1993; Beijersbergen *et al.*, 1994; Ginsberg *et al.*, 1994; Sardet *et al.*, 1995; Wu *et al.*, 1995). In contrast, only a single E2F and a single DP gene have been identified in *Drosophila* (Dynlacht *et al.*, 1994; Ohtani and Nevins, 1994). Like their human counterparts, dDP and dE2F interact to generate a sequence-specific DNA binding factor that activates transcription through E2F sites. In flies, as in human cells, E2F appears to play an important role in the regulation of cell proliferation. In the *Drosophila* embryo, the dE2F gene is required for a program of gene expression that is activated during G_1 to S phase progression and for DNA synthesis (Duronio and O'Farrell, 1994; Duronio *et al.*, 1995). In mammalian cells, E2F sites have been shown to confer cell cycle regulation of the transcription of several genes (reviewed in Nevins, 1992; Helin and Harlow, 1993; La Thangue, 1994; Farnham, 1995). In addition, it has been shown recently that the overexpression of dominant negative forms of DP-1 or DP-2 arrests cells in G_1 phase of the cell cycle (Wu *et al.*, 1996)

In mammalian cells, E2F is regulated, in part, by the retinoblastoma gene product (pRB) and pRB-related proteins (reviewed in Weinberg, 1995). When pRB binds to E2F it inhibits E2F-dependent transcription (Flemington *et al.*, 1993; Helin *et al.*, 1993). This repression is relieved following the phosphorylation of pRB by cyclin-dependent kinases. Mutations in pRB or in the pathway regulating pRB phosphorylation (inactivation of p16, amplification of cyclin D1, mutation or amplification of cdk4; for examples, see He *et al.*, 1994; Otterson *et al.*, 1994; Schmidt *et al.*, 1994; Kamb, 1995; Shapiro *et al.*, 1995; Weinberg, 1995; Wolfel *et al.*, 1995) have been found in a large number of tumor cells, suggesting that the deregulation of E2F-dependent transcription may be a common feature of human tumor cells.

Several groups have investigated the effects of overexpressing E2F genes in tissue culture cells in order to study the effects of raising E2F activity. In a variety of different experimental systems, the overexpression of E2F-1, E2F-4 and DP-1 have all been found to drive cell cycle progression (Johnson *et al.*, 1993; Zhu *et al.*, 1993; Beijersbergen *et al.*, 1994; Qin *et al.*, 1994; Shan and Lee, 1994; Wu and Levine, 1994). However the consequences of overexpression vary considerably between studies. Rat embryo fibroblasts have been reported to be transformed by E2F-1 alone (Johnson *et al.*, 1994; Singh *et al.*, 1994), by DP-1 or DP-2 in co-operation with an activated Ha-*ras* gene (Johnson *et al.*, 1994; Jooss *et al.*, 1995) or by co-expression of E2F-4 and DP-1 together with an activated Ha-*ras* (Beijersbergen *et al.*, 1994). In contrast, several other studies have shown that cells driven into the cell cycle by the high level expression of E2F-1 die by apoptosis (Shan and Lee, 1994; Wu and Levine, 1994; Kowalik *et al.*, 1995). At least in part, the effects of E2F-1 expression appear to depend on the status of p53: in cultured cells that contain wild-type p53, overexpression of E2F-1 caused cells to enter S phase and die; in cells lacking p53 or expressing mutant p53, the high level expression of E2F-1 was tolerated (Qin *et al.*, 1994; Wu and Levine, 1994; Kowalik *et al.*, 1995).

As these previous studies have relied on the overexpression of individual components of E2F in cultured cells, the extent to which these results reflect the outcome of elevating endogenous E2F activity *in vivo* is uncertain. Furthermore, the effects of elevated E2F activity on the proliferation of both differentiated and undifferentiated cells, and on cell differentiation are unknown.

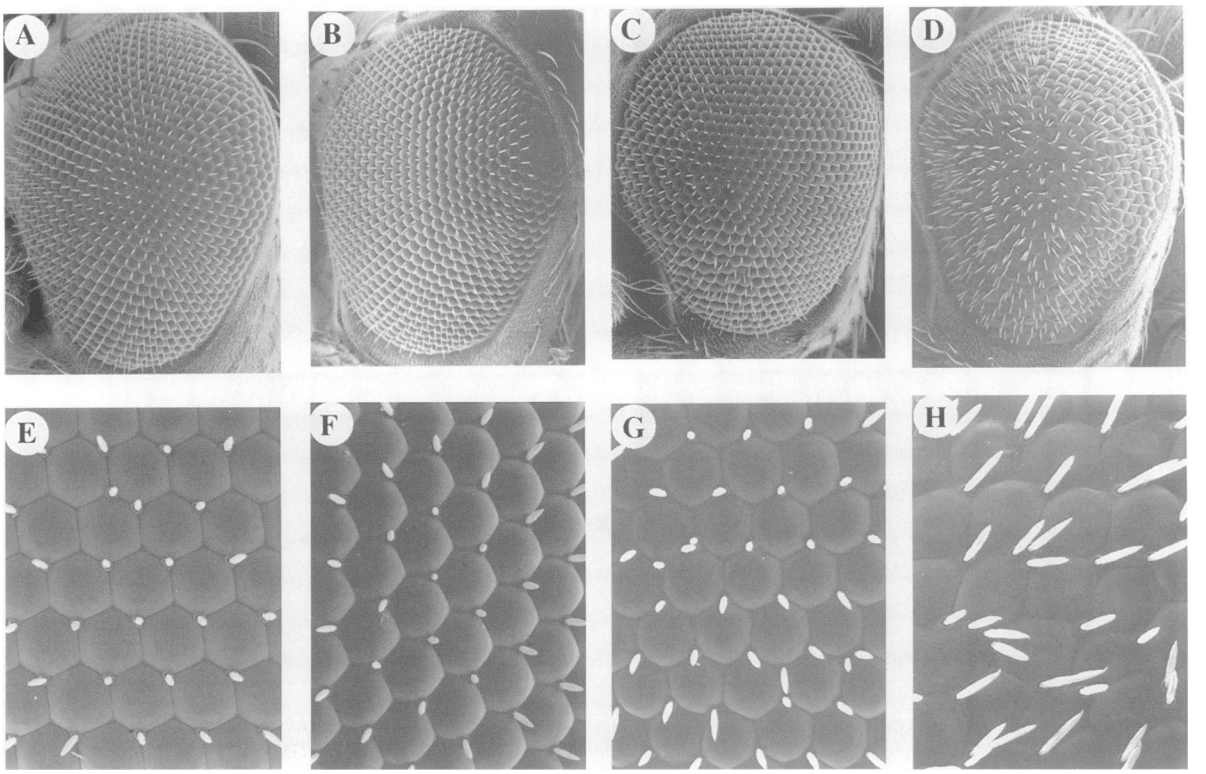

Fig. 1. Scanning electron micrographs of flies carrying GMRdDP and GMRdE2F transgenes. (**A**and **E**) Wild-type eye, (**B** and **F**) GMRdDP/GMRdDP, (**C** and **G**) GMRdE2F/GMRdE2F, (**D** and **H**) GMRdE2F^{2}dDP^2. Magnification is 200× (A–D) or 1000× (E–H).

To address these issues, we investigated the consequences of elevating E2F activity in transgenic animals. Using the pGMR vectors (Hay *et al.*, 1994), expression of the transgenes is directed by the developmentally regulated glass transcription factor to all cells within and posterior to the morphogenetic furrow of the eye imaginal disc (Moses and Rubin, 1991). The patterns of cell proliferation and cell differentiation during eye development have been described in detail (reviewed in Wolff and Ready, 1993) and are summarized briefly here. During the third larval instar, a morphogenetic furrow is initiated at the posterior end of the eye disc and passes through the disc. Anterior to the furrow, cells are undifferentiated and dividing asynchronously. As cells are drawn into the furrow, they enter a synchronous cell cycle arrest. A subset of these cells become committed to differentiate and they begin to express neuronal cell markers. The first cells to be determined will form the R8 photoreceptor cell of the adult ommatidium. Additional cells are recruited sequentially, forming a precluster of cells that are destined to specific fates. Preclusters leave the furrow at the five-cell stage. Cells in the precluster do not re-enter the cell cycle, but all uncommitted cells divide one more time, generating a reservoir of cells for subsequent differentiation events. This division occurs relatively synchronously and produces a band of S phases (the second mitotic wave) behind the furrow. No further S phases are seen in the eye disc in the third larval instar.

Using GMR transgenes, the modest overexpression of dDP or dE2F alone had no dramatic effect on eye development. However, combining GMRdDP and GMRdE2F transgenes and co-expressing dE2F and dDP gave a strong phenotype, indicating that the changes seen reflect the activity of the dDP and dE2F heterodimer. The changes in cell proliferation and differentiation that are caused by this increase in E2F activity are described below.

Results

The combined ectopic expression of dE2F and dDP disrupts normal eye development

We used the pGMR P-element vector (Hay *et al.*, 1994) to provide ectopic expression of dE2F and dDP in the *Drosophila* eye. Three independent transgenic lines P[w+; GMR] were generated for GMRdDP and two lines for GMRdE2F. No phenotypic difference was observed between the different lines. Under scanning electronic microscopy (SEM) the eyes of flies carrying GMRdDP transgenes appeared normal (Figure 1B and F). Similarly, the eyes of flies carrying GMRdE2F transgenes lacked any gross abnormality, although a few additional bristles could be seen (Figure 1C and G). When sectioned, the eyes of transgenic flies carrying two copies of either a GMRdDP transgene or a GMRdE2F transgene were normal, with seven properly oriented photoreceptor cells per ommatidium in cross section (Figure 2A–C).

To test for synergy between dDP and dE2F, GMRdE2F and GMRdDP flies were crossed. Flies carrying one copy of GMRdDP and GMRdE2F showed minor defects (shown later in Figure 8B, E and H) but, interestingly, flies carrying two copies of both GMRdDP and GMRdE2F had severely abnormal eyes. These eyes were rough in appearance, ommatidia lacked their regular hexagonal shape and many more bristles were apparent (Figure 1D and H). GMRdDP and GMRdE2F transgenes were recombined on the second chromosome, and eyes of a

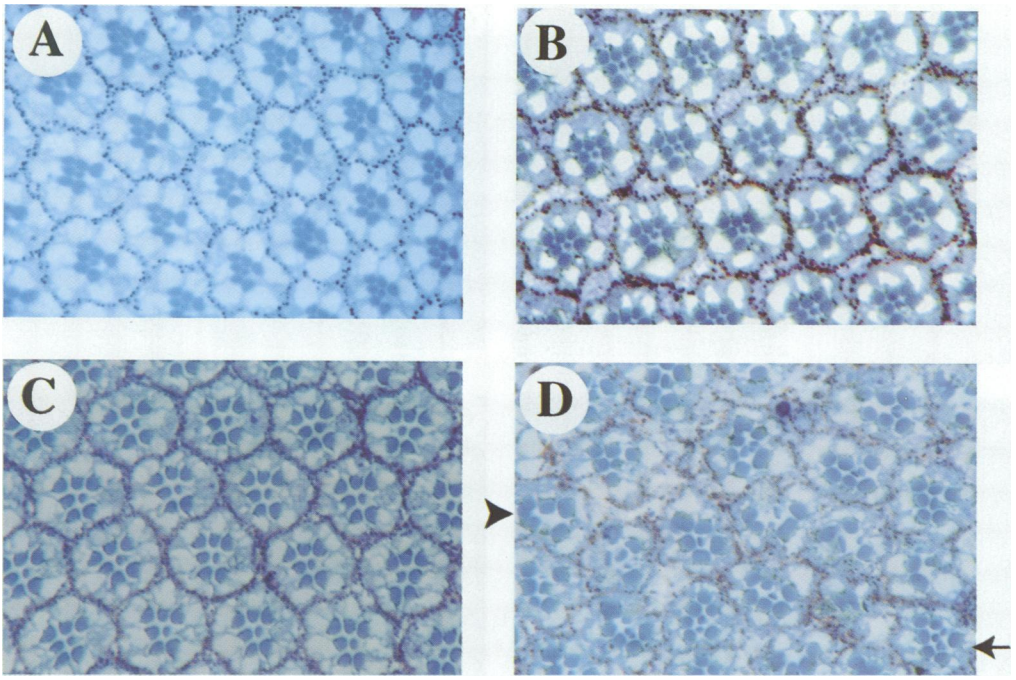

Fig. 2. Eye sections from flies carrying GMRdDP and GMRdE2F transgenes. (**A**) Wild-type eye, (**B**) GMRdDP/GMRdDP, (**C**) GMRdE2F/GMRdE2F, (**D**) GMRdE2F^{2}dDP^2. Examples of ommatidia containing additional or missing photoreceptors in GMRdE2F^{2}dDP^2 eyes are arrowed in (D).

stock homozygous for this chromosome (stock designated GMRdE2F^{2}dDP^2) were examined in greater detail. On sectioning, most ommatidia in GMRdE2F^{2}dDP^2 eyes were abnormal. Strikingly, ~50% of ommatidia had missing photoreceptor cells, and ~25% of the ommatidia had additional photoreceptors (shown in Figure 2D). In addition, the arrangement of ommatidia was disorganized in these eyes and the normal symmetrical pattern of the photoreceptor trapezoids was not apparent.

Pupal retinas were stained with anti-cut antibodies to identify cone cells and bristle precursors (Blochlinger *et al.*, 1993; K.Cadigan, personal communication), two cell types that are determined later in the development of the eye. In the apical focal plane of wild-type retina, cone cells were found in ordered patterns of four cells (Figure 3A). This regular arrangement was perturbed in the retina of GMRdE2F^{2}dDP^2 pupae (Figure 3C). In addition, the cut staining revealed abnormal clusters containing three or five cone cells (arrowed in Figure 3C).

Bristle precursor cell are stained by the anti-cut antibody in the basal plane of the pupal retina (Blochlinger *et al.*, 1993; K.Cadigan, personal communication). In the wild-type eye disc, the clusters of bristle cells were well separated in a regular pattern (Figure 3B). Adult GMRdE2F^{2}dDP^2 eyes had many additional bristles. Consistent with this, the pupal retina showed disorganization of the bristle precursors and many more cut-stained cells were apparent (Figure 3D). Clusters of bristle cells were often unusually close together, and in several places the cut-stained cells appeared as a continuous line.

In summary, the combined overexpression of dDP and dE2F in the *Drosophila* eye was found to cause defects in at least three different cell types: photoreceptors, cone cells and bristle cells. In each case the normal arrangement of these cells was disrupted, and additional differentiated cells were found. In some instances, photoreceptors and cone cells were missing. We sought to investigate the mechanisms underlying these changes. Since the activation of E2F is thought to drive cell proliferation, we considered two alternative explanations. Conceivably, the overexpression of dE2F and dDP might be altering the recruitment of cells into differentiation programs. Alternatively, the overexpression of dE2F and dDP might be forcing differentiating cells to re-enter the cell cycle. To investigate these possibilities, we have further characterized the photoreceptors seen in GMRdE2F^{2}dDP^2.

Neuronal cell differentiation is initiated normally despite the ectopic expression of dE2F and dDP

Given the frequency of photoreceptor abnormalities in the eyes of GMRdE2F^{2}dDP^2 flies, we first investigated whether the ectopic expression of dE2F with dDP had disrupted the initiation of photoreceptor differentiation. The development of the *Drosophila* retina occurs through a stepwise recruitment of cells into preclusters that are determined to form cells of adult ommatidia. This process of cell commitment is initiated in the morphogenetic furrow. Defects in cell cycle control may interfere with this process; the *roughex* mutation, which causes the appearance of S phase cells in the morphogenetic furrow, disrupts normal cell fate determination (Thomas *et al.*, 1994). In GMRdE2F^{2}dDP^2, the ectopic expression of dE2F and dDP commences while cells are in the morphogenetic furrow and is maintained once cells have left the furrow (Figure 4A and B). Immunostaining indicates that dE2F and dDP are synthesized at levels ~5- to 10-fold higher than the endogenous proteins. In wild-type discs, dE2F and dDP are expressed throughout the disc with a modest increase in cells posterior to the morphogenetic furrow (Figure 4C and Brook *et al.*, 1996). To determine whether

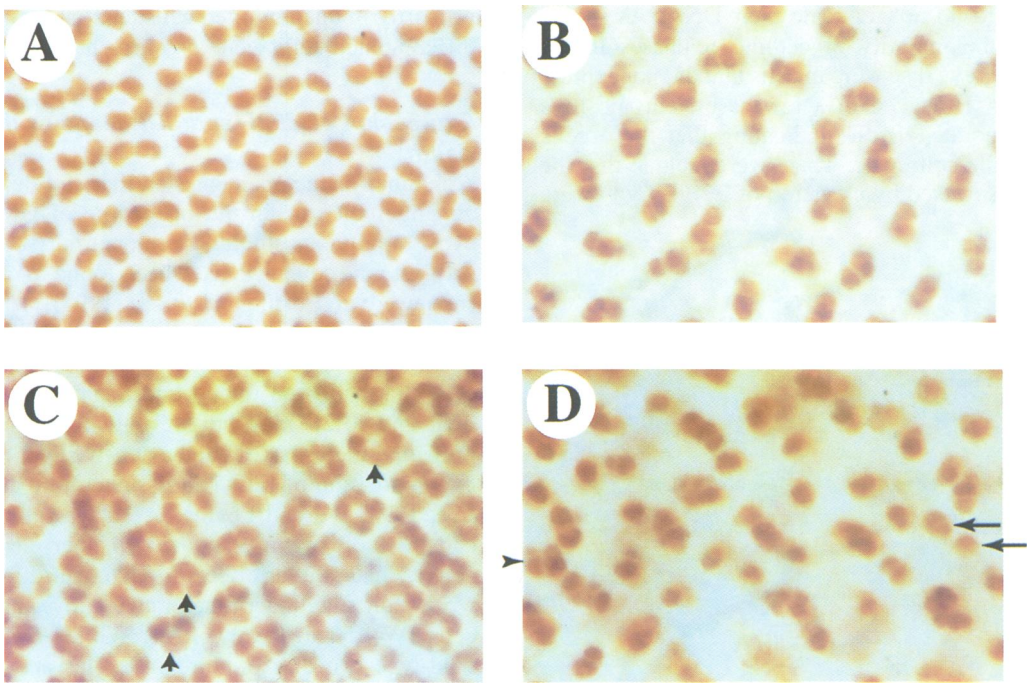

Fig. 3. Staining of pupal eye discs with anti-cut antibody.
(**A** and **B**) Wild-type eye disc, (**C** and **D**) GMRdE2F^{2}dDP^2 eye disc. Cone cells are stained in the apical focal plane (A and C) and bristle precursor cells are stained in basal plane (B and D) of the pupal eye disc. Arrows in (C) indicate examples of abnormal cone cell clusters of three or five cells in the GMRdE2F^{2}dDP^2 discs. In (D) arrows indicate clusters of bristle precursor cells that appear continuous or unusually close together in GMRdE2F^{2}dDP^2.

expression of dDP with dE2F disrupted or delayed the onset of neuronal differentiation, eye discs were incubated with an anti-elav antibody that stains cells that have been recruited into preclusters and are committed to a neuronal fate (Robinow and White, 1988). The elav staining pattern of GMRdE2F^{2}dDP^2 eye discs was indistinguishable from wild-type (Figure 5). As the pattern and position of elav-positive preclusters relative to the morphogenetic furrow is normal, we conclude that ectopic co-expression of dE2F and dDP did not grossly interfere with the initiation of neuronal cell differentiation nor the stepwise recruitment of cells into the preclusters.

Expression of dE2F and dDP leads to ectopic S phases posterior to the furrow and allows neuronal cells to re-enter the cell cycle

Since the expression of dDP with dE2F did not perturb the onset of photoreceptor differentiation, we investigated whether these proteins caused inappropriate cell proliferation. The pattern of cell division in the eye imaginal disc has been described in detail (Wolff and Ready, 1993). In a wild-type eye disc, cells divide asynchronously anterior to the morphogenetic furrow. as cells enter the furrow they are synchronously arrested in a G_0 or G_1 state, and differentiation is initiated. Cells that are recruited into five-cell preclusters do not divide again, but all remaining cells divide once to generate a pool of cells that serves as a reservoir from which all other cell types are recruited. This last round of division occurs relatively synchronously and appears as a stripe (second mitotic wave) on discs stained with 5-bromodeoxyuridine (BrdU) (Figure 6A). To determine the effects of dE2F and dDP co-expression on the pattern of DNA synthesis posterior to the furrow,

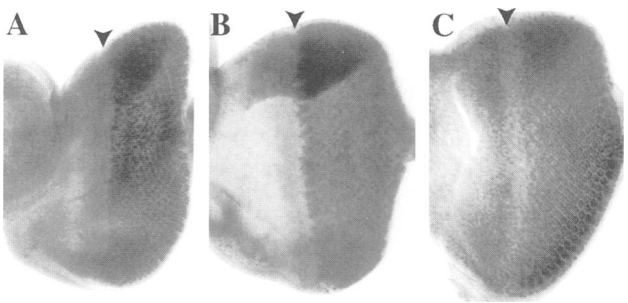

Fig. 4. dE2F and dDP expression patterns in GMRdE2F^{2}dDP^2 eye discs. (**A**) GMRdE2F^{2}dDP^2 eye disc stained for dE2F. (**B**) GMRdE2F^{2}dDP^2 eye disc stained for dDP. (**C**) dDP expression in a wild-type disc. HRP-conjugated goat anti-mouse secondary antibody was used in (A) and (B) for detection, and the signal in (C) was enhanced using Vector's ABC Elite kit (biotin-streptavidin system).

third instar eye imaginal discs were labeled with BrdU and stained with an α-BrdU antibody. As shown in Figure 6B, a significant increase was found in the amount of BrdU incorporated posterior to the furrow in eye discs of GMRdE2F^{2}dDP^2 larvae. In contrast, no significant difference in the amount of BrdU incorporation was observed anterior to the furrow where dE2F and dDP were not expressed. This indicated that expression of dE2F with dDP had disrupted normal cell cycle control, and had driven cells that are normally post-mitotic into the cell cycle.

To determine whether expression of dE2F and dDP was able to cause S phases in differentiating cells, eye discs of GMRdE2F^{2}dDP^2 larvae were labeled with BrdU and double stained with α-BrdU and α-elav antibodies. In normal discs, elav-positive cells are committed to a

neuronal fate and do not replicate their DNA. As shown in Figure 6C, the majority of ectopic S phases in the GMRdE2F^{2}dDP^2 eye disc were in cells that did not express the elav marker. However, in a few cases, elav-positive cells had incorporated BrdU, indicating that these cells had re-entered the cell cycle. Thus, we conclude that the ectopic S phases caused by elevation of E2F activity were primarily in uncommitted cells, and in a minority of cases differentiated cells were also driven into S phase.

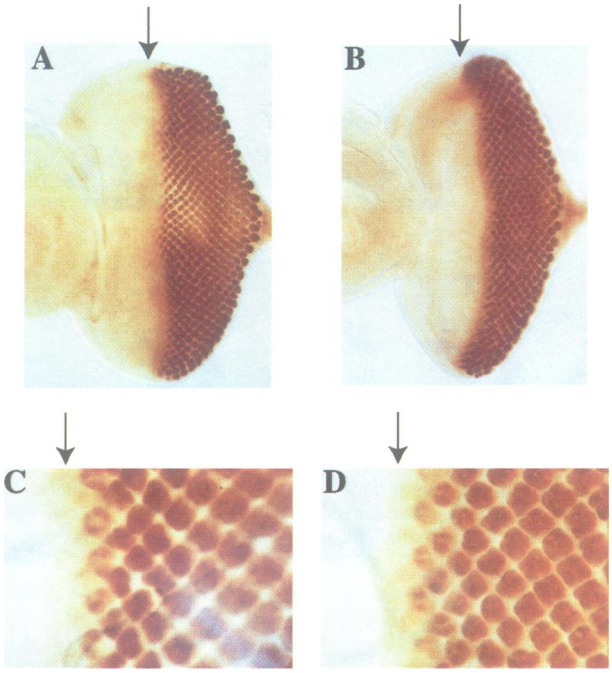

Fig. 5. The onset of neuronal differentiation was not perturbed by ectopic expression of dE2F and dDP in GMRdE2F^{2}dDP^2 eye discs. Anti-elav staining of wild-type (**A** and **C**) and GMRdE2F^{2}dDP^2 eye discs (**B** and **D**). Arrows indicate the position of the morphogenetic furrow. The anterior of the disc is on the left.

Expression of dE2F and dDP leads to increased cell death

A number of factors suggested that cell death might play an important role in the phenotype of GMRdE2F^{2}dDP^2 eyes. In previous studies, attempts to drive differentiating cells into the cell cycle have been found to cause apoptosis (reviewed in Harrington *et al.*, 1994). The observation that many ommatidia in sections of GMRdE2F^{2}dDP^2 eyes had missing photoreceptors suggested that elav-expressing cells that had been forced into S phase by expression of dDP and dE2F may have eventually died. In addition, previous studies have found extensive apoptosis following overexpression of E2F-1 in tissue culture cells (Shan and Lee, 1994; Wu and Levine, 1994; Kowalik *et al.*, 1995), and therefore we investigated whether elevation of E2F activity had similar effects *in vivo*. Third instar eye discs were stained with acridine orange, a dye that stains dying cells (Wolff and Ready, 1991; White *et al.*, 1994). In a wild-type eye disc of third instar larvae, there are very few apoptotic cells (Figure 7A). In contrast, eye discs from GMRdE2F^{2}dDP^2 larvae showed a significant increase in the amount of cell death posterior to the furrow (Figure 7B). Interestingly, extensive acridine orange staining was observed only in the very posterior section of the eye disc despite the fact that ectopic dE2F and dDP expression was constant from the furrow to the posterior end of the disc (data not shown). Rows of preclusters are initiated at intervals of between 60 and 90 min. As apoptosis was only seen at the posterior of the disc, ~10–15 rows behind the furrow, it is clear that there is a significant time delay between the onset of dE2F and dDP expression and the appearance of cell death.

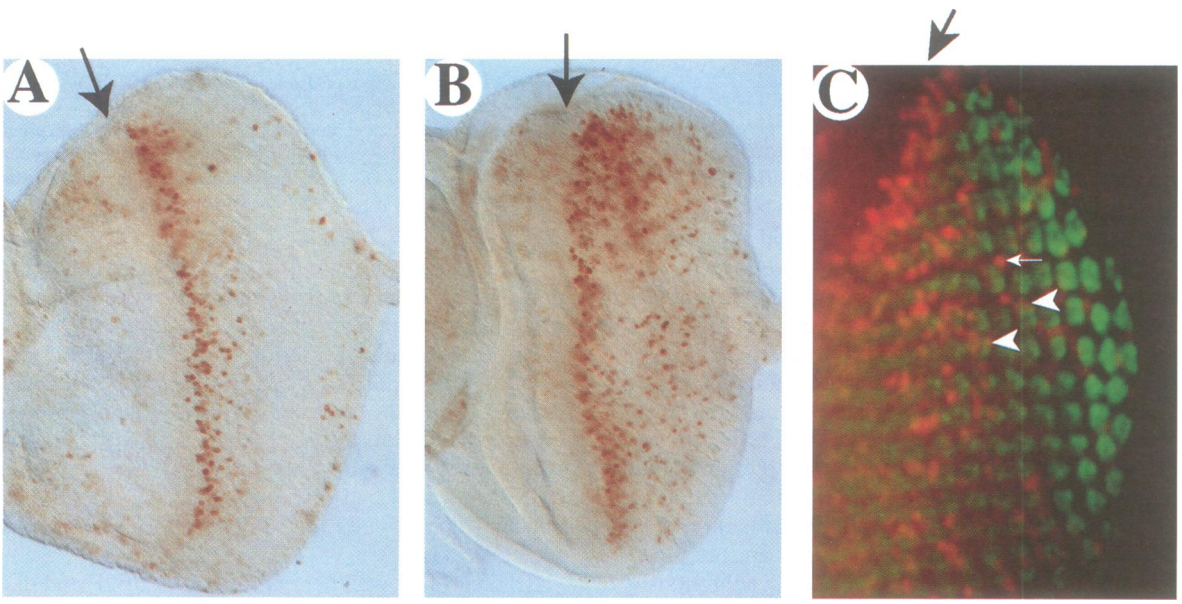

Fig. 6. Ectopic expression of dE2F and dDP causes additional S phase cells. (**A**) The pattern of BrdU incorporation of a wild-type eye disc. (**B**) The pattern of BrdU incorporation of a GMRdE2F^{2}dDP^2 eye disc. (**C**) GMRdE2F^{2}dDP^2 eye disc double stained with anti-elav (green) and anti-BrdU (red) following BrdU incorporation. Arrowheads indicate cells stained with both BrdU and elav antibodies (orange). Arrows indicate the position of the morphogenetic furrow.

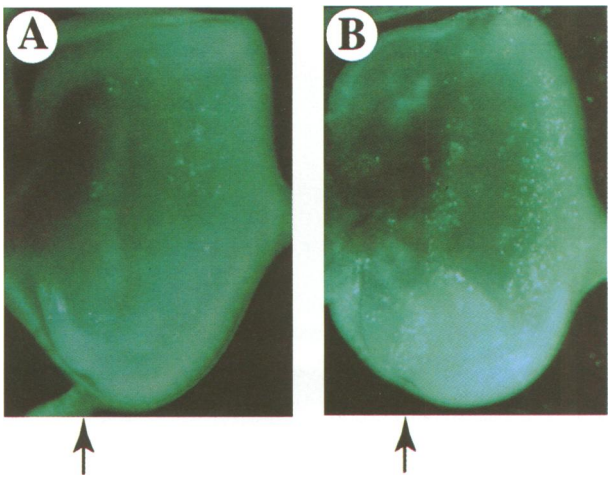

Fig. 7. Ectopic expression of dE2F and dDP leads to increased apoptosis. Apoptotic cells were stained with acridine orange in (**A**) a wild-type eye disc, and (**B**) a GMRdE2F^{2}dDP^2 eye disc. Arrows indicate the position of the morphogenetic furrow.

Prevention of cell death enhances the dE2F and dDP phenotype

Normally there is very little cell death in the larval eye imaginal disc. Extensive apoptosis occurs during the pupal stage when surplus cells that have not been recruited to a specific fate are eliminated. It has been shown that ectopic expression of the baculovirus p35 protein, which has been shown recently to be an inhibitor of ICE family proteases (Bump *et al.*, 1995), inhibits normal and induced apoptosis in the *Drosophila* eye (Hay *et al.*, 1994). Additional cells that are not eliminated in GMRp35 eyes differentiate into pigment cells but cause only a subtle change in the ommatidial spacing pattern. To test further the hypothesis that a large proportion of cells that are driven into S phase by ectopic E2F activity are eliminated by apoptosis, GMRdE2F^{2}dDP^2 flies were crossed with GMRp35 flies. GMRp35 gave a strong enhancement of the rough eye phenotype caused by the overexpression of dE2F and dDP. Enhancement was most striking in flies carrying one copy of the GMRdE2F and GMRdDP transgenes, which had only minor phenotype on their own (Figure 8B, E and H). As shown in Figure 8A–F, the spacing between ommatidia of the GMRdE2FdDP/+;GMRp35/+ eyes was significantly increased compared with GMRdE2FdDP/+ and GMRp35/+ controls. Sections through the GMRdE2 FdDP/+;GMRp35/+ eyes revealed that many additional cells were found between ommatidia (Figure 8I). This indicates that extensive cell proliferation had occurred in the presence of p35, dDP and dE2F, and is consistent with the observation that the majority of the ectopic S phases in the larval eye disc occurred in cells that had not been recruited into preclusters. We conclude that the ectopic expression of dE2F and dDP drives many additional S phases, but cell proliferation is limited by an increase in cell death.

Discussion

The overexpression of individual E2F genes in tissue culture cells has been shown to induce S phase entry and has been found to result in either cell proliferation or cell death. To test the physiological significance of these observations, and to allow the effects of E2F activation to be studied in the context of cell differentiation, we have examined the effects of elevating E2F activity in the *Drosophila* eye. pGMR expression vectors were used to target ectopic expression of dE2F and dDP to cells posterior to the morphogenetic furrow in the developing eye imaginal disc. Several features suggest that this experimental system is appropriate for studies of E2F. First, dE2F and dDP are the only known components of E2F activity in *Drosophila*, and antibodies to each protein eliminate E2F DNA binding activity in cell extracts (Du *et al.*, 1996). Second, dE2F and dDP are both normally expressed in the eye disc and are found in cells posterior to the morphogenetic furrow (Brook *et al.*, 1996; Du *et al.*, 1996). Third, the analysis of clones of *dE2F* mutant cells shows that dE2F is required for cell proliferation in the eye imaginal disc and also for normal photoreceptor development (Brook *et al.*, 1996).

The ectopic expression of either dE2F or dDP alone was found to have very little effect on normal eye development, but strong synergy was seen when GMRdE2F and GMRdDP transgenes were combined. The lack of a phenotype due to expression of dE2F or dDP differs from previous studies where the expression of individual mammalian E2F or DP genes in tissue culture cells was found to drive cell cycle progression. Although there are several possible explanations for this difference, it is likely that the level of overexpression is an important factor. Immunostaining of eye discs indicates the dE2F and dDP are expressed at levels ~5- to 10-fold higher than endogenous proteins in GMRdE2F^{2}dDP^2. In previous studies where mammalian E2F and DP genes have been expressed from cytomegalovirus or Rous sarcoma virus expression constructs following transient transfection (Zhu *et al.*, 1993; Beijersbergen *et al.*, 1994; Johnson *et al.*, 1994; Qin *et al.*, 1994; Jooss *et al.*, 1995), using recombinant adenovirus vectors (Kowalik *et al.*, 1995), or using the TETr vectors (Shan and Lee, 1994), the levels of overexpression are likely to be much greater. The fact that a clear phenotype was seen in the *Drosophila* eye only when both GMRdE2F and GMRdDP transgenes were combined strongly suggested that the changes were due to the bona fide activity of the dDP/dE2F heterodimer. The severity of the phenotype was influenced by the copy number of GMRdE2F and GMRdDP transgenes. We anticipate that these phenotypes may prove a useful tool for the identification of mutants that modify E2F activity.

This experimental system allows the effects of elevating E2F activity to be studied *in vivo* in a variety of contexts: in cells that are proliferating, in cells that have exited the cell cycle but have not committed to a cell fate, and in cells that are post-mitotic and have started to express markers of differentiation.

Ectopic expression of dE2F and dDP led to a marked increase in the number of S phase cells in the eye imaginal disc. No differences were observed in the region containing cells that normally proliferate: the appearance of the second mitotic wave was largely normal, no gross changes were apparent in the onset of S phases or in the number of S phase cells in the wave. However, large numbers of S phase cells were seen posterior to the normal band of S phases and were evenly distributed in a region of the eye disc that normally contains only post-mitotic cells. It

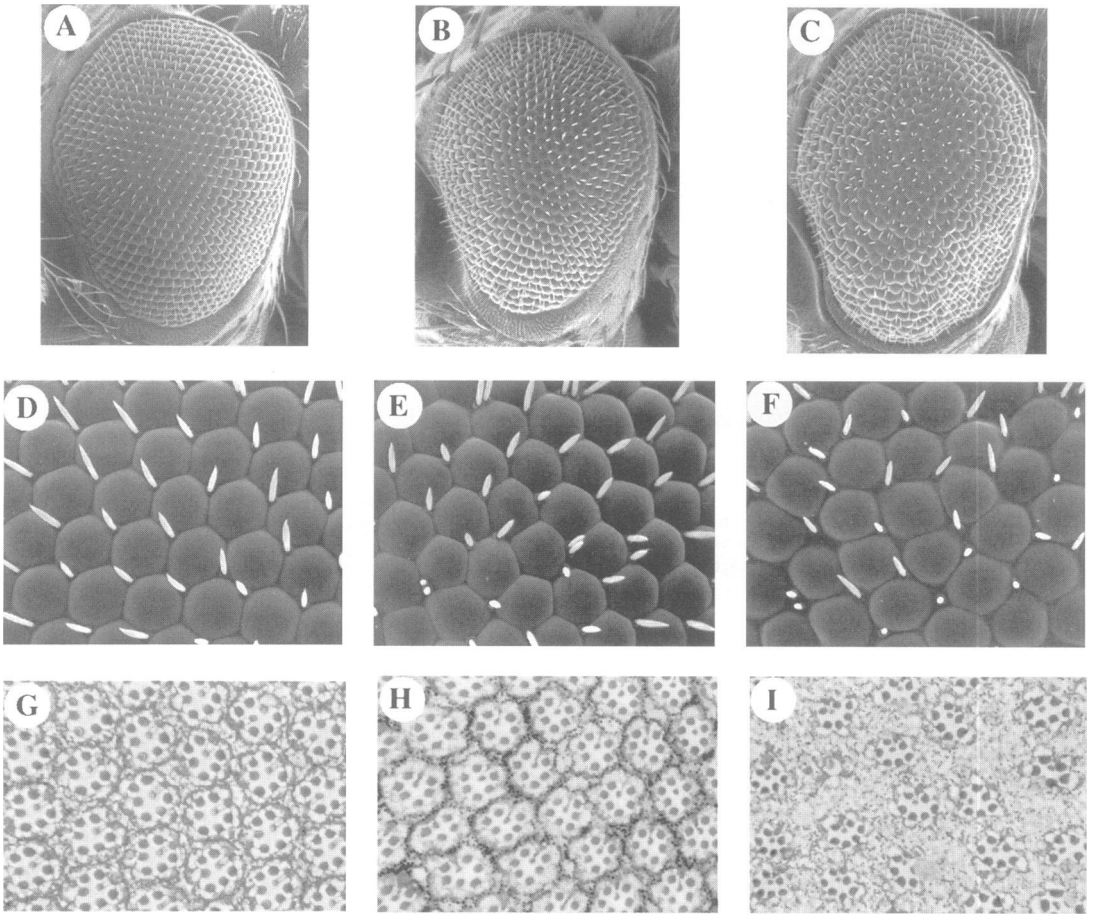

Fig. 8. GMRp35 is an enhancer of the GMRdE2FdDP phenotype. Scanning electron micrographs of (**A** and **D**) GMRp35/+, (**B** and **E**) GMRdDPdE2F/+, (**C** and **F**) GMRdDPdE2F/+;GMRp35/+. Sections of GMRp35/+, GMRdDPdE2F/+ and GMRdDPdE2F/+; GMRp35/+ eyes are shown in (**G**), (**H**) and (**I**) respectively.

is unclear whether these represent post-mitotic cells that have re-entered the cell cycle or proliferating cells that are unable to exit the cell cycle. This region of the eye disc contains cells that are differentiating and cells that have not yet committed to a cell fate. Double staining with an antibody to elav showed that most S phases occurred in uncommitted cells but a few were in cells that expressed elav and had started neuronal differentiation. As judged by the pattern of elav expression, the initiation of photoreceptor differentiation appeared to be normal in GMRdE2F^{2}dDP^2 flies. Thus, the expression of dE2F and dDP was found to drive cell cycle progression primarily in uncommitted cells and did not appear to perturb the onset of neuronal differentiation.

There are several indications that the overexpression of dE2F and dDP forced a minority of differentiating cells to re-enter the cell cycle. In GMRdE2F^{2}dDP^2 discs a small number of cells were found that expressed elav and incorporated BrdU. Furthermore, clusters of cone cells and of bristle precursor cells were observed in pupal discs that contained additional cells. Sections of adult GMRdE2F^{2}dDP^2 eyes revealed that ~25% of adult ommatidia had additional photoreceptors. These findings are consistent with the idea that some cells may have divided after being committed to a specific cell fate. Further observations suggest that the inappropriate proliferation of differentiating cells often resulted in cell death, as has been described in other systems (for a review, see Harrington *et al.*, 1994). Approximately 50% of ommatidia lacked the normal complement of photoreceptors and some cone cells were missing in the pupal disc. This, taken together with the finding of the dramatic increase of apoptotic cells in the eye disc of third instar GMRdE2F^{2}dDP^2 larvae, suggests that dE2F/dDP-driven cell proliferation is associated with cell death in this *in vivo* setting. Recently Richardson *et al.* (1995) have shown that ectopic expression of cyclin E in photoreceptors also led to the appearance of ommatidia with altered numbers of photoreceptors, supporting the idea that the photoreceptor defects in GMRdE^{2}F2dDP^2 could result from abnormal cell cycle control.

It is not clear, however, that inappropriate cell proliferation is the only effect of dE2F and dDP expression. The normal spatial organization of both the cone cell clusters and bristle precursor cells was greatly disrupted in GMRdE2F^{2}dDP^2 eye discs, despite the fact that only a few clusters had abnormal numbers of cells. Clusters of bristle precursors were more densely packed than in wild-type discs, suggesting that more bristle mother cells may have been determined than normal. Another defect seen following dE2F and dDP expression was the observation that the normal symmetrical arrangement of the photoreceptor trapezoid was lost in flies carrying one copy of GMRdE2FdDP. This change was seen although very few ommatidia had abnormal or missing photoreceptors. While these general effects could be an indirect consequence of

the proliferation of surrounding cells, we note that it is also possible that the elevation of E2F activity may have more direct effects on specific differentiation events. For example, the presence of a bristle mother cell will suppress nearby cells from becoming bristle mother cells, and it was shown that this process requires *Notch* function (Cagan and Ready, 1989). It is possible that the increase in bristle number may result from an effect of E2F activity on *Notch* signaling and on determination of bristle mother cells, rather than a change in proliferation of the precursor cells. Further studies will be needed to distinguish between these possibilities.

The patterns of ectopic S phases and apoptosis in the GMRdE2F^{2}dDP^2 larval eye disc are intriguing. Eye discs of GMRdE2F^{2}dDP^2 larva contained many apoptotic cells that stained with acridine orange. Interestingly, apoptosis was seen primarily at the posterior portion of the eye disc. This pattern contrasts with the pattern of ectopic S phases that were seen throughout the region posterior to the second mitotic wave. The difference may reflect simply a time delay between the onset of DNA synthesis and apoptosis. Alternatively, it may indicate that cells driven into the cell cycle by ectopic expression of dE2F and dDP do not die until they receive another signal, or that cells are protected from E2F-induced apoptosis until they migrate a certain distance from the furrow. Importantly, it is clear that DNA replication induced by dE2F and dDP expression is not inherently abnormal. Cells driven to re-enter the cell cycle by dE2F and dDP can proliferate when protected from death by the co-expression of baculovirus p35 protein. Such a situation may parallel aspects of human tumorigenesis and might predict that the elevation of E2F activity would not be expected to provide a growth advantage to tumor cells until cells are saved from apoptosis.

Materials and methods

pGMR alleles

Three independent transgenic lines P[w+; GMR] were generated for GMRdDP and two lines for GMRdE2F. Transgenic lines were isolated following P-element-mediated germline transformation with pGMR (Hay *et al.*, 1994) constructs containing the 4.4 kb dE2F cDNA or the 2.1 kb dDP cDNA (Dynlacht *et al.*, 1994). pGMR constructs was co-injected with the pUChsΔ2-3 helper plasmid into w^{1118} embryos. Injection and transformation were carried out as described in Spradling and Rubin (1982) and Rubin and Spradling (1982). No phenotypic difference was observed among different lines, the phenotype of transgenic lines on the second chromosome was shown.

Scanning electron microscopy

Samples for SEM were prepared as described in Kimmel *et al.* (1990).

Immunohistochemistry

Section of fly heads was carried out as described by Tomlinson and Ready (1987) with the modifications described by Carthew and Rubin (1990) and Hariharan *et al.* (1995). The rat anti-Elav antibody was a gift from J.Treisman and G.Rubin. The anti-cut antibody was from Y.N.Jan. For cut staining, white pre-pupae were collected and cultured at 25°C for 24 h, and pupae retinas were dissected and stained. Monoclonal antibodies Hao4 (Brook *et al.*, 1996) and Yun3 were used for dE2F and dDP staining respectively. Yun3 was prepared by fusing splenocytes taken from mice immunized with full-length dDP protein to NS-1 myeloma cells 4 days after the final boost. Yun3 was found to be specific for dDP in immunoprecipitation and Western blotting analysis and to supershift E2F complexes in E2F gel shift assays.

BrdU labeling

Retinas from late third-instar larvae were dissected and placed in Schneider's medium containing 0.3 mg/ml BrdU for 1 h at room temperature. Tissues were then fixed and stained.

Acridine orange staining

Retinas from late third-instar larvae were dissected in phosphate-buffered saline (PBS) with 2 mg/ml acridine orange, washed with PBS and mounted directly.

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