Functional antagonism between E2F family members

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E2F is a heterogenous transcription factor and its role in cell cycle control results from the integrated activities of many different E2F family members. Unlike mammalian cells, that have a large number of E2F-related genes, the *Drosophila* genome encodes just two E2F genes, *de2f1* and *de2f2*. Here we show that *de2f1* and *de2f2* provide different elements of E2F regulation and that they have opposing functions during *Drosophila* development. dE2F1 and dE2F2 both heterodimerize with dDP and bind to the promoters of E2F-regulated genes in vivo. dE2F1 is a potent activator of transcription, and the loss of *de2f1* results in the reduced expression of E2F-regulated genes. In contrast, dE2F2 represses the transcription of E2F reporters and the loss of *de2f2* function results in increased and expanded patterns of gene expression. The loss of *de2f1* function has previously been reported to compromise cell proliferation. *de2f1* mutant embryos have reduced expression of E2F-regulated genes, low levels of DNA synthesis, and hatch to give slow-growing larvae. We find that these defects are due in large part to the unchecked activity of dE2F2, since they can be suppressed by mutation of *de2f2*. Examination of eye discs from *de2f1*; *de2f2* double-mutant animals reveals that relatively normal patterns of DNA synthesis can occur in the absence of both E2F proteins. This study shows how repressor and activator E2Fs are used to pattern transcription and how the net effect of E2F on cell proliferation results from the interplay between two types of E2F complexes that have antagonistic functions.

[Key Words: E2F; cell cycle; *Drosophila*]

Received April 13, 2001; revised version accepted June 18, 2001.

The E2F transcription factor plays an important role in the control of cell proliferation. E2F-dependent transcription is modulated during cell cycle progression from G₁ to S-phase and results obtained from a variety of different experimental systems show that the levels of E2F are rate-limiting for the G₁/S transition. The overexpression of E2F genes is sufficient to drive quiescent cells into S-phase (Johnson et al. 1993; Asano et al. 1996; Du et al. 1996b; Lukas et al. 1996). In contrast, molecules that inhibit E2F-dependent transcription lead to the accumulation of cells in G₁ (Dobrowolski et al. 1994; Sellers et al. 1995; Ishizaki et al. 1996; Wu et al. 1996). Consistent with these observations, E2F genes behave as oncogenes when overexpressed, and mutations that deregulate E2F activity are found in many tumor cells (for reviews, see Dyson 1998; Helin 1998; Nevins 1998).

E2F-binding sites allow gene transcription to be coupled with cell cycle position. E2F-regulated promoters are repressed in quiescent cells and induced as cells enter S-phase. E2F binding sites are thought to act as

repressor elements in quiescent cells by recruiting pRB (or pRB-related proteins) and pRB-associated proteins (Dyson 1998; Harbour and Dean 2000). pRB has been proposed to block transcription in three general ways: by binding to the transcription activation domain of E2F and blocking its activity (Flemington et al. 1993; Helin et al. 1993), by inhibiting the activity of the basal transcription machinery (Ross et al. 1999), and by recruiting complexes that affect chromatin structure (Sellers et al. 1995; Brehm et al. 1998; Luo et al. 1998; Magnaghi-Jaulin et al. 1998; Meloni et al. 1999; Ross et al. 2001). As cells enter S-phase, these interactions are disrupted and transcriptional repression is relieved. Some studies have suggested that expression of E2F target genes results primarily from de-repression of the promoter; others have suggested that E2F-binding sites also mediate transcriptional activation in S- or G₂-phase (Means et al. 1992; Wade et al. 1992; Lam and Watson 1993; Hsiao et al. 1994; Johnson et al. 1994; Neuman et al. 1994; Lam et al. 1995; Fry et al. 1997; Yamaguchi et al. 1997). The precise number of E2F targets is not known and recent studies suggest that E2F may regulate the expression of many genes that are involved in diverse aspects of cellular function (Muller et al. 2001). The best-known targets of E2F encode proteins that are critical for cell cycle pro-

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Article and publication are at <http://www.genesdev.org/cgi/doi/10.1101/gad.903901>.

gression and DNA synthesis. These include cell-cycle regulators, such as cyclin E (CycE), cyclin A (CycA), *cdc2*, *cdc25*, Myc, B-myb, and products that are required for DNA replication, such as large subunit of DNA polymerase α , ribonucleotide reductase, proliferating cellular nuclear antigen (PCNA), dehydrofolate reductase (DHFR), ORC proteins, and minichromosome maintenance proteins (MCMs) (Helin 1998).

Studies of E2F in mammalian cells are hampered by the complexity of E2F. The term E2F refers to the collective activity of many different proteins. The basic unit of E2F is a heterodimer, composed of an E2F and a DP subunit. Six E2F genes and two DP genes have been studied, and recent surveys of the human genome raise the possibility that several additional family members may also exist (Aravind et al. 2001). Precisely how the functions of all of these proteins are integrated together is unclear. Biochemical experiments show that different types of E2F complexes have similar DNA-binding properties and, even in populations of synchronized tissue culture cells, many different E2F proteins can be found at a given promoter (Takahashi et al. 2000; Wells et al. 2000). E2F-1, E2F-2, E2F-3, E2F-4, and E2F-5 are each capable of activating transcription and can be repressed by pRB-family proteins. Nevertheless, there are several indications that individual E2Fs are tailored for distinct functions. E2F-1, E2F-2, and E2F-3, for example, are more potent transcriptional activators than E2F-4 and E2F-5 when these proteins are overexpressed. The expression of E2F-1, E2F-2, and E2F-3a is cell cycle regulated and the appearance of these proteins at E2F regulated promoters generally correlates with gene expression. Conversely, E2F-4 appears to be the predominant E2F at times when E2F-regulated promoters are repressed (Sardet et al. 1995; Lukas et al. 1996; Moberg et al. 1996; DeGregori et al. 1997; Muller et al. 1997; Verona et al. 1997; Adams et al. 2000; Leone et al. 2000).

The phenotypes resulting from the targeted inactivation of E2F genes in mice confirm that individual E2F genes do have some unique functions. *E2f-1*^{-/-} mice have defects in thymocyte development and are tumor-prone. The loss of *E2f-5* does not cause any defects in cell proliferation (Lindeman et al. 1998). Instead, mice die due to hydrocephalus caused by excessive secretion of cerebral spinal fluid, an alteration caused by abnormalities in cell differentiation. Mice deficient for *E2f-4* display multiple developmental defects and die due to increased susceptibility to opportunistic infections (Humbert et al. 2000a; Rempel et al. 2000). Although E2F-4 accounts for the majority of the endogenous E2F DNA-binding activity in many cell types, E2F-4 is fully dispensable for the cell cycle arrest or proliferation and mutation of E2F-4 gave no discernable effect on transcription of known E2F target genes (Humbert et al. 2000a; Rempel et al. 2000). Unlike the mutant phenotypes described above, *E2f-3*^{-/-} cell lines show reduced rate of proliferation and deregulation in the expression of E2F responsive genes (Humbert et al. 2000b). Interpretation of all of these phenotypes is complicated by the presence of multiple family members with related or overlapping functions, which

might mask or modify the effects of removing an individual form of E2F. For example, studies of *E2f-4*; *E2f-5* double-mutant MEFs show that these forms of E2F have a redundant function that is required for p16-induced cell cycle arrest (Gaubatz et al. 2000). Although E2F is considered to be a critical cell cycle regulator, none of the E2F mouse knockouts that have been reported to date to have resulted in a generalized block to cell proliferation *in vivo*. This discrepancy is thought to be due to functional overlap or compensation between E2F proteins, such that most cell types contain several different E2F complexes that can provide the E2F functions essential for cell proliferation.

Here we describe experiments using *Drosophila* as a model system to study E2F function. The *Drosophila* genome contains two E2F genes and one DP gene (Dynlacht et al. 1994; Ohtani and Nevins 1994; Hao et al. 1995; Sawado et al. 1998b) and hence the issues of functional redundancy and overlap are likely to be less severe. Previous studies have shown that dE2F1 and dDP share many biochemical and functional similarities with their mammalian counterparts. These proteins heterodimerize, bind to consensus E2F sites, and cooperate to activate transcription when overexpressed (Dynlacht et al. 1994). High levels of dE2F1/dDP drive quiescent cells into S-phase and stimulate apoptosis (Asano et al. 1996; Du et al. 1996b). dE2F1 and dDP associate with an RB-related protein (RBF) that represses dE2F1-dependent transcription in tissue culture cells and *in vivo* and blocks dE2F1-induced proliferation (Du et al. 1996a).

de2f1 provides an essential function *in vivo*. *de2f1* mutants are defective during embryogenesis (Duronio et al. 1995), show a significant delay in larval growth, and fail to complete larval development (Royzman et al. 1997; Du 2000). *de2f1* mutant embryos lack a G₁/S transcriptional program that normally accompanies S-phase entry and loss of *de2f1* leads to an almost complete cessation of DNA synthesis by stage 13 of embryogenesis (Duronio et al. 1995; Royzman et al. 1997). Analysis of *de2f1* mutant clones in imaginal discs confirmed that dE2F1 is required for normal cell proliferation (Brook et al. 1996; Neufeld et al. 1998) and suggested that E2F also acts in postmitotic cells (Brook et al. 1996). Studies of partial loss-of-function alleles in the ovary have implicated E2F in the shut off of DNA synthesis in follicle cells and have shown that *de2f1* is required in this cell type for amplification of chorion gene clusters (Royzman et al. 1997).

dDP mutant embryos resemble *de2f1* mutants in lacking a G₁/S transcriptional program, but the effects of *dDP* mutation on the expression of genes that are normally expressed at G₁/S varies and depends on the target gene examined (Royzman et al. 1997; Duronio et al. 1998). Examination of *dDP* mutant clones and specific alleles of *dDP* shows that dDP is required during oogenesis (Myster et al. 2000) and that it is required for the shut off of DNA synthesis in follicle cells (Royzman et al. 1999). However, the patterns of DNA synthesis and cell proliferation are not severely affected in *dDP* mutant embryos or *dDP* mutant larvae, indicating that the functions of dE2F1 and dDP are not equivalent.

The differences between the *de2f1* and *dDP* mutant phenotypes have led to speculation that dE2F1 might have functions that are independent of dDP, or alternatively that dDP might have functions that are independent of dE2F1 (Royzman et al. 1997; Duronio et al. 1998). One gene that is likely to have an impact on these phenotypes is *de2f2*, a *de2f1*-related gene that was uncovered by direct sequencing of transcription units within the 39B-D cytological region (D. Huen et al., unpubl.), in a two-hybrid screen using RBF1 as bait (W. Du and N. Dyson, unpubl.), and in the *Drosophila* genome project (Sawado et al. 1998b; Rubin et al. 2000). Biochemical experiments have shown that dE2F2 can cooperate with dDP to generate specific DNA-binding activity on a consensus E2F-binding site and that a dE2F2 expression plasmid weakly repressed the transcription of an E2F-reporter construct when transfected into the *Drosophila* Kc cell line (Sawado et al. 1998b). Nothing is known about the normal function of dE2F2. Here we show that dE2F2 is a physiological partner for dDP and RBF and that dE2F2 acts antagonistically to dE2F1 during *Drosophila* development. We find that mutations in *de2f2* relieve the block to DNA synthesis and larval development that is caused by mutation of *de2f1*. These results show that E2F-control of cell proliferation results from the interplay between two types of E2F complexes that have opposing activities and antagonistic functions.

Results

The endogenous dE2F2 protein associates with dDP and RBF

As an initial step in the characterization of dE2F2 we generated dE2F2-specific antibodies and determined whether dE2F2 associates with dDP and RBF in vivo. SL2 cell extracts were immunoprecipitated in parallel using anti-dDP and anti-dE2F2 mouse monoclonal antibodies coupled to beads. After washing, the immune complexes were separated by SDS-PAGE and Western blots were probed with anti-RBF, anti-dE2F2, and anti-dDP antibodies (Fig. 1). Both RBF and dDP were detected in the anti-dE2F2 immunoprecipitates. No signal was observed using a negative control antibody or without cell extracts. In the same way, dE2F2 was readily detected in anti-dDP immune complexes. Immunoprecipitation experiments were repeated using embryo extracts and gave similar results (Fig. 1). We conclude that dE2F2 associates with dDP and RBF under normal physiological conditions.

dE2F2 represses transcription in Drosophila cell culture

To compare the transcriptional properties of dE2F2 and dE2F1, both cDNAs were cloned into an expression vector and tagged with a *myc* epitope on the N terminus. The use of a common epitope enabled us to examine both the activities and the levels of these proteins in transient transfection experiments. Expression plasmids were cotransfected into *Drosophila* SL2 cells together

Figure 1. dE2F2 associates with dDP and RBF in vivo. Bead-coupled anti-dE2F2, anti-dDP, or anti-E1A antibodies were used to immunoprecipitate complexes from SL2 cell or embryonic extracts (+). Associated proteins were detected by Western blot analysis with antibodies specific for RBF, dE2F2, or dDP. Because dE2F2 and dDP migrate in the vicinity of IgG heavy chain, additional controls were included in which the coupled beads were incubated in lysis buffer alone (minus sign). RBF migrates as a doublet in embryonic extracts.

with a reporter plasmid in which the luciferase gene is fused to the promoter region from -168 to $+23$ of *PCNA* gene containing two canonical E2F sites (Yamaguchi et al. 1995). The contribution of endogenous E2F activity on the luciferase reporter was estimated when the cells were transfected with an empty vector.

Equal amounts of dE2F1 and dE2F2 plasmids were separately transfected into *Drosophila* SL2 cells and the luciferase levels produced from the reporter plasmid were measured in triplicate samples (Fig. 2A). Luciferase activity was stimulated upon transfection of dE2F1, in a manner that was dependent on the E2F-binding sites in the promoter, as described previously (Dynlacht et al. 1994). In contrast, transfection of the dE2F2 plasmid repressed the reporter construct by up to 10-fold (Fig. 2A). The repressive activity of dE2F2 is much greater than the twofold effect reported previously (Sawado et al. 1998b). The greater level of dE2F2-mediated repression described here may be due to the use of different cell lines or to higher levels of expression provided by the *ie4* baculovirus promoter. Because dE2F1 and dE2F2 had opposite effects on transcription we performed competition experiments in which a constant amount of dE2F1 was challenged with increasing amounts of dE2F2 and a constant amount of dE2F2 was challenged with increasing amounts of dE2F1. As might be expected, the degree of activation or repression depended on the ratio of the expression constructs. Interestingly however, Western blot analysis of the transfected cells showed that a barely detectable quantity of dE2F1 protein was sufficient to overcome the repression by dE2F2; whereas even a 20-fold excess of dE2F2 could only partly block transcriptional activation by dE2F1 (Fig. 2B,C).

Although these results suggest that dE2F1 and dE2F2 have opposing effects on the *PCNA* promoter, an alternative, albeit trivial, explanation for these results might

Figure 2. dE2F2 represses transcription in transient transfection assays. (A) SL2 cells were cotransfected with 2 μg of pIE4 alone, pIE4-myc-dE2F1 or pIE4-myc-dE2F2 together with 2 μg of pIE4-lacZ internal control plasmid and 2 μg of the luciferase reporter constructs containing either the wild-type *PCNA* promoter or the *PCNA* promoter with mutated E2F sites. Solid bars, wild-type PCNA; checked bars, mutant PCNA. (B) Increasing amounts of pIE4-myc-dE2F1 were cotransfected with 1 μg of pIE4-myc-dE2F2. (C) Increasing amounts of pIE4-myc-dE2F2 were cotransfected with 1 μg of pIE4-myc-dE2F1. Expression of the transfected proteins was monitored by Western blotting using an anti-myc antibody. In each panel, luciferase activity was normalized to the β-galactosidase control. Activation observed in transfection with the vector alone is due to an endogenous E2F activity. The means and the standard deviations of three experiments are shown. (D) dE2F2 and dE2F1 occupy the *PCNA* and *DNA polα* promoters in SL2 cells in vivo. Following formaldehyde cross-linking, proteins were immunoprecipitated with anti-dE2F2 (α-E2F2 IP), with anti-dE2F1 (α-E2F1 IP), or nonspecific (NS IP) antibodies, and the associated chromatin fragments were used as a template for PCR amplification with primers specific for *DNA polα*, *PCNA*, and *rp49* promoter sequences. Three dilutions of total chromatin (triangle) were amplified with the same set of primers.

arise if *PCNA* were an exclusive target for dE2F1. If this were the case, the failure of dE2F2 to activate the reporter might be misleading and a consequence using a promoter that is not normally regulated by dE2F2. To test this possibility, we used a chromatin immunoprecipitation assay to enrich for DNA fragments occupied by dE2F1 or dE2F2 in vivo. Proteins bound to DNA in SL2 cells were cross-linked using formaldehyde, chromatin was isolated, and E2F proteins was immunoprecipitated using antibodies specific for either dE2F1 or dE2F2 antibodies. The presence of E2F-regulated promoters was detected by PCR using gene-specific primers. As shown in Figure 2D, *PCNA* promoter sequences were selectively enriched in the anti-dE2F1 and anti-dE2F2 immunoprecipitates relative to control fragments. No enrichment was observed using unrelated antibodies to control for nonspecific DNA-binding activity, and a similar enrichment was seen using primers to a second dE2F1-inducible promoter, *DNApolα*.

We conclude that dE2F2 is normally present at promoters that have previously been shown to be induced by dE2F1. Whereas dE2F1 expression activates transcrip-

tion from these promoters, dE2F2 expression represses transcription. However, low levels of dE2F1 readily overcome the repressor activity of dE2F2.

Interactions between *de2f1* and *de2f2* in transgenic animals

To test the physiological relevance of these observations we asked whether similar interactions between dE2F1 and dE2F2 could be detected in vivo. Genetic interactions between dE2F1, dDP, and RBF have been examined previously using pGMR transgenes that allow ectopic expression to be targeted specifically to developing eye. Unfortunately, GMR-*de2f2* transgenes gave no discernable eye phenotype. Immunostaining and Western blot analysis of eye discs showed that, unlike the previously described transgenes, expressing dE2F1, dDP, and RBF the GMR-*de2f2* transgenes were insufficient to increase dE2F2 levels above the endogenous protein. This suggests that endogenous dE2F2 is expressed at high level in the eye disc. An affinity-purified rabbit polyclonal antibody gave a strong ubiquitous staining throughout the

disc (Fig. 3A). The levels of dE2F2 do not appear to vary greatly within the disc, in contrast to dE2F1 that has a dynamic pattern of expression (see Asano et al. 1996; Brook et al. 1996).

The use of UAS-*de2f2* and GMR-GAL4 transgenes provided a higher level of dE2F2-expression. Immunostaining of eye discs from UAS-*de2f2*; GMR-GAL4 larvae confirmed that the ectopic expression of dE2F2 begins as cells are entering the morphogenetic furrow and is maintained posterior to the furrow in differentiating cells (Fig. 3B). Western blot analysis showed that dE2F2 is produced at 5- to 10-fold higher levels than the endogenous protein (Fig. 3B). Under scanning electron microscopy (SEM) the eyes of flies carrying one copy of UAS-*de2f2* and GMR-GAL4 show severely abnormal eyes compared to the GMR-GAL4 control (Fig. 3C,D). These eyes were rough in appearance and ommatidia lacked their regular pattern. This phenotype was generated with three independent UAS-*de2f2* transgenes, suggesting that the effects are not due to the particular site of the transgene insertion. The phenotype was enhanced when two copies of the UAS-*de2f2* transgene were present indicating that the effects of dE2F2 were not saturated (Fig. 3E). When the levels of dE2F1 were manipulated, we observed that eye phenotype induced by the *de2f2* transgene was almost completely suppressed by elevated levels of dE2F1 (Fig. 3F). Conversely, the roughness of the eye was slightly enhanced in flies heterozygous for *de2f1*

and carrying either the *de2f1^{rm729}* or *de2f1⁷¹⁷²* mutant alleles (Fig. 3G).

These eye interactions are consistent with results of the transfection assays; both types of experiments raise the possibility that dE2F1 and dE2F2 might have opposing activities. However, both sets of results are drawn from settings in which dE2F1 and/or dE2F2 are expressed at abnormal levels and it is uncertain that these effects accurately reflect the activities of the endogenous proteins. To test this we generated mutant alleles of *de2f2* and compared the phenotypes of *de2f2* mutant and *de2f1*; *de2f2* double-mutant animals.

Generation of *de2f2* mutant allele

The *de2f2* gene is located at 39B2-3 on the left arm of the chromosome 2 and, to date, no mutations in *de2f2* have been reported. An orphan *P*-element insertion line, *l(2)k16403*, was found in which the *P* element is inserted into the adjacent gene, *MPP6*, 667 bp upstream of the 5' end of *de2f2*. To generate a deletion of *de2f2*, which does not disrupt *MPP6*, a two-step male recombination approach was utilized (Preston et al. 1996; for details and a map, see Materials and Methods). Briefly, in the first round, two deletions, *de2f2^{G5.1}* and *de2f2^{Q3.1}*, were generated, which extend distally from the *P*-element insertion site. The *de2f2^{Q3.1}* chromosome, carrying the smaller of the two deletions, was used in a second round

Figure 3. Ectopic expression of dE2F2 can induce a rough-eye phenotype that is suppressed by overexpression of dE2F1. (A) dE2F2 is ubiquitously expressed in wild-type imaginal disc, with no overt change in G₁-phase cells of the morphogenetic furrow (arrow) or in S-phase cells. Eye discs were stained with anti-dE2F2 (red) and anti-BrdU (green) to identify the first and second mitotic waves. (B) dE2F2 expression is induced posterior to the morphogenetic furrow in GMR-GAL4/+; UAS-*de2f2*/+ animals. (C–G) Scanning electron microscopy micrographs of *Drosophila* eyes. The ommatidial pattern of control flies, GMR-GAL4/+ (C), is disrupted followed overexpression of dE2F2 in GMR-GAL4/+; UAS-*de2f2*/+ (D), and this effect is enhanced in GMR-GAL4/+; UAS-*de2f2*/UAS-*de2f2* (E). This phenotype is strongly suppressed by overexpression of dE2F1 in GMR-GAL4/GMR-*de2f1*; UAS-*de2f2*/+ (F) and slightly enhanced in flies heterozygous for *de2f1^{rm729}* allele, GMR-GAL4/+; UAS-*de2f2*/*de2f1^{rm729}* (G).

of male recombination to generate a duplication event that restored the sequence of *MPP6* but not *de2f2* (*de2f2*^{76Q1}).

The following data strongly argue that *de2f2*^{76Q1} represents a null allele for *de2f2* and that the neighboring *MPP6* gene is functional. Sequencing the *de2f2* genomic region from the *de2f2*^{76Q1} chromosome revealed that the *de2f2*^{76Q1} deletion extends from a position 378 bp upstream of the *de2f2* transcription start site to amino acid 5 of the coding sequence (for more details, see Materials and Methods). Hence this deletion removes *de2f2* promoter sequences, the 5' untranslated region, and the 5' end of the dE2F2 ORF, including the presumptive start codon. The *de2f2*^{76Q1} chromosome contains 273 bp upstream of the *MPP6* transcription start site. This is sufficient for *MPP6* function since *de2f2*^{76Q1} fully complements a lethal point mutation in *MPP6*, *MPP6*^{U39}, in which the sixth amino acid is converted into a stop codon and, presumably, represents a null allele (D.S. Huen, unpubl.). In contrast, neither *de2f2*^{Q3.1} nor *de2f2*^{G5.1} can complement *MPP6*^{U39}. Taken together, these data show that *MPP6* is functional in the *de2f2*^{76Q1} allele.

To confirm that the *de2f2*^{76Q1} allele represents a null allele for *de2f2*, total RNA or protein extracts were prepared from second instar larvae trans-heterozygous for *de2f2*^{76Q1} and *de2f2*^{G5.1} alleles and compared to wild-type controls (for details of crosses, see Materials and Methods). Northern blot analysis using the full length *de2f2* cDNA showed that *de2f2*^{76Q1}/*de2f2*^{G5.1} trans-heterozygous mutant larvae contained no detectable *de2f2* transcripts (data not shown), and Western blot analysis with anti-dE2F2 antibodies showed that these animals contained no detectable dE2F2 protein (Fig. 4A).

Flies carrying the *de2f2*^{76Q1} allele were crossed to various deletions removing *de2f2*. The *de2f2*^{76Q1} allele was found to be semiviable over both deletions, *de2f2*^{G5.1} and *de2f2*^{Q3.1}, as well as over the larger deficiencies *Df(2L)TW1* and *Df(2L)prA-14*, that also remove *de2f2*. The number of trans-heterozygous escapers was variable for each combination of alleles and, moreover, was strongly affected by the genetic background. We observed trans-heterozygotes at frequencies that varied between 2% and 50% of the expected number. This variation suggests that animals deficient for dE2F2 activity are highly sensitive to heterozygosity at other loci in the genome.

de2f2^{76Q1} homozygotes survived until adult stages at frequencies between 20% and 35% of the expected numbers. In addition to reduced viability, *de2f2* null flies exhibit reduced fertility. Only 50% of eggs laid by *de2f2* null females hatched compared to 95% for wild-type females. About 5% of the eggs deposited by *de2f2* null females have fragile, thin chorions (data not shown; P. Cayirlioglu and B. Duronio, pers. comm.). *de2f2* null males have significantly reduced fertility but did give progeny at low frequency when crossed to wild-type females. Curiously, normal progeny were recovered from crosses of *de2f2* null males and females, indicating that animals can survive without any paternal, maternal, or

Figure 4. The loss of *de2f2* suppresses larval growth defects caused by loss of *de2f1*. (A) Western blot analysis using polyclonal anti-dE2F2 antisera on extracts of *de2f2*^{G5.1}/*de2f2*^{76Q1} and *de2f2*^{G5.1}/*de2f2*^{76Q1}; *de2f1*⁹¹/*de2f1*^{rm729} larvae illustrates that no detectable amount of dE2F2 protein is synthesized from the *de2f2*^{76Q1} allele. (B) Larvae on the fifth day after egg laying of the following genotype Canton S (wild-type), *de2f1*⁹¹/*de2f1*^{rm729} (*de2f1*⁻), *de2f2*^{76Q1}/*de2f2*^{G5.1} (*de2f2*⁻), and *de2f2*^{76Q1}/*de2f2*^{G5.1}; *de2f1*⁹¹/*de2f1*^{rm729} (*de2f2*⁻; *de2f1*⁻).

zygotic source of dE2F2. Thus, the loss of *de2f2* results in reduced fertility and viability, but dE2F2 is not essential for animal development.

Loss of de2f2 suppresses phenotypes caused by loss of de2f1

Studies in mammalian cells strongly indicate that several E2F family members share overlapping functions. If the *Drosophila* E2F proteins perform functions in vivo that are similar and overlapping, then *de2f1*; *de2f2* double mutants would be predicted to be more severely affected than either the *de2f1* or *de2f2* single mutants. However, if dE2F1 and dE2F2 act antagonistically, as

suggested above, then this result is unlikely. Although several outcomes are possible, the simplest possibility to consider is that the double mutant might be less severely affected than the single *de2f1* or *de2f2* mutants.

To answer this question, we generated animals trans-heterozygous for null alleles of dE2F2 (*de2f2^{76Q1}* and *de2f2^{G5.1}*) and dE2F1 (*de2f1⁹¹* and *de2f1^{rm729}*). Both *de2f1* alleles have been characterized previously and are considered to act as null alleles (Duronio et al. 1995; Royzman et al. 1997). *Trans*-heterozygous combinations of alleles were used to minimize the possible effects of any additional mutations that might be linked in *cis*. Embryos *trans*-heterozygous for *de2f1* mutations lack the G₁/S transcriptional program, show a dramatic reduction in DNA synthesis after stage 13, and hatch to give severely abnormal larvae (Duronio et al. 1995; Royzman et al. 1997; Du 2000). Five days after egg laying (AEL) *de2f1* mutant larvae were much smaller than wild type (Fig. 4B). These animals failed to develop to the third larval instar and, as previously reported, contained severely underdeveloped imaginal discs and CNS.

Strikingly, the slow growth and abnormal larval development of the *de2f1* mutant are almost completely suppressed by removing the dE2F2 activity. *Trans*-heterozygous double mutants, *de2f2^{76Q1}/de2f2^{G5.1}*, *de2f1⁹¹/de2f1^{rm729}*, developed normally without any significant delay in larval growth, reached pupal stage, and finally died as mid- or late pupae. The imaginal discs and CNS of *de2f1*; *de2f2* double-mutant larvae were similar in development to wild-type primordia. The suppression of the *de2f1* mutant phenotype is not restricted to a particular *de2f1* allelic combination. Similar results were obtained when *de2f1^{rm729}* or *de2f1⁹¹* were placed over deficiency *Df(3R)e-BS2* that uncovers *de2f1*.

de2f1 has previously been shown to be required for normal cell proliferation and DNA replication at several stages of *Drosophila* development. *de2f1* mutant clones fail to proliferate in eye and wing imaginal discs (Brook et al. 1996; Neufeld et al. 1998). Surprisingly, in eye discs that lack both *de2f1* and *de2f2* the pattern of DNA synthesis is largely normal (Fig. 5). In wild-type or in mutant discs BrdU staining is localized primarily to the large area of asynchronously dividing cells in the first mitotic wave and in the synchronous second mitotic wave (Fig. 5A,B). Similar patterns were observed in *de2f2* mutant discs (Fig. 5C). No BrdU incorporation was detected in the morphogenetic furrow, and even relatively long pulses of BrdU incorporation (2 h) failed to reveal a significant number of ectopic S-phases posterior to the second mitotic wave in the double-mutant discs (Fig. 5D,E). Staining for ELAV, a marker of committed neuronal precursors, showed that the onset of neuronal differentiation was not severely perturbed by the absence of E2F proteins (Fig. 5F–H).

In examining large numbers of eye discs for these genotypes we observed that the intensity of label incorporated into the most intensely labeled nuclei during long-term BrdU pulses tended to be slightly reduced in *de2f1*; *de2f2* double mutant relative to wild-type discs (Fig. 5D,E). However this reduction is considerably less

Figure 5. The patterns of DNA synthesis in eye discs lacking either dE2F2 or both dE2F1 and dE2F2 are similar to wild-type discs. Eye discs from third instar larvae are shown from wild-type (A,D,F,I), or *de2f2^{76Q1}/de2f2^{G5.1}*; *de2f1⁹¹/de2f1^{rm729}* (B,E,G,I) or *de2f2^{76Q1}/de2f2^{G5.1}* (C,H) animals. (A–E) shows eye discs labeled with BrdU for 20 min (A–C) or 2 h (D–E). (F–H) The onset of neuronal differentiation as revealed by staining for ELAV. (I,J) The staining patterns for cyclin A (green), a marker of G₂ cells, and phosH3 (red), a marker of mitotic cells. Note that the patterns of each of these markers are similar in discs of wild-type and double-mutant animals.

than the normal variation that occurs between discs of a single genotype. To further examine cell cycle progression in the absence of E2F regulation, *de2f2*; *de2f1* double-mutant discs were double stained with anti-cyclin A and anti-phosphorylated histone H3 (phosH3) antibodies. Cyclin A is commonly used as a G₂ marker because it is expressed in cells that have passed the G₁/S transition and is degraded when cells enter mitosis (Knoblich and Lehner 1993). The phos H3 antibody preferentially stains M-phase cells (de Nooij et al. 1996). The patterns of Cyclin A and phosH3 staining in *de2f2*; *de2f1* double-mutant eye discs (Fig. 5J) were indistinguishable from wild type (Fig. 5I). Thus, cells of the eye imaginal disc can proliferate asynchronously in the first mitotic wave, arrest synchronously in the morphogenetic furrow, re-enter the cell cycle synchronously in the second mitotic wave, permanently exit the cell cycle, and differentiate all in the absence of both dE2F1 and dE2F2.

We conclude that the loss of *de2f2* suppresses the larval growth, cell proliferation, and DNA-replication defects that are caused by the mutation of *de2f1*. This suppression confirms that dE2F1 and dE2F2 have antagonistic functions, at least during larval development. In theory, the effects of dE2F2 that are evident in the *de2f1* mutant could be caused by an unusual dE2F2 activity that is independent of dDP. However, the loss of dDP function behaves in a similar way to loss of *de2f2* and suppresses the developmental defects of *de2f1* mutant larvae (Fig. 6). Thus it is most likely that the severe defects seen in *de2f1* mutant larvae require the action of dE2F2/dDP heterodimers.

Deregulation of the expression of E2F target genes in the de2f2 single-mutant and de2f2; de2f1 double-mutant eye discs

The relatively normal patterns of cell proliferation in *de2f1; de2f2* mutants are, at first glance, difficult to reconcile with the idea that E2F is a critical regulator of gene expression and cell proliferation. We therefore examined the expression of *de2f1*-regulated genes in *de2f2* and *de2f1; de2f2*-mutant animals. Third instar eye discs were chosen for this analysis to avoid the possible contribution of maternally supplied products. Maternally supplied dE2F1 is rapidly depleted and zygotic *de2f1* expression becomes limiting during embryogenesis. Although dE2F2 is abundant in embryo extracts, dE2F2 protein and mRNA was undetectable in Western and Northern blot analyses of *de2f2* or *de2f1; de2f2* mutant larvae collected early in the second instar (Fig. 4A; data not shown).

Initially we monitored expression of *PCNA*, one of the best-characterized E2F-regulated genes, by in situ hy-

bridization as described in previous reports. In wild-type eye discs, the pattern of *PCNA* expression is tightly coupled to the pattern of DNA replication. High levels of *PCNA* transcripts are present anterior to the furrow and within a narrow stripe that overlaps with the second mitotic wave. No *PCNA* expression is present in the furrow and in the cells just anterior to the furrow (Fig. 7A). In *de2f1; de2f2* double-mutant eye discs, *PCNA* expression was abnormal. In these animals, a weak staining was observed in mutant eye discs without any specific pattern (Fig. 7B). *PCNA* transcripts appeared to be present at a low level in the anterior portion of the disc, including the morphogenetic furrow and the second mitotic wave. In *de2f2* mutant animals, *PCNA* expression was not downregulated as cells enter the furrow, and this resulted in an expanded region of *PCNA* transcription that encompassed both the first and second mitotic waves and the morphogenetic furrow (Fig. 7C).

Although in situ hybridization is an excellent method for monitoring expression patterns, without a suitable internal control it is a poor way for comparing expression levels. To compare the overall levels of E2F-target genes expression, we performed Northern blot analysis using total RNA from third instar eye imaginal discs dissected from *de2f2* single and *de2f1; de2f2* double-mutant larvae. As shown in Figure 7G, the steady state level of *PCNA* transcripts was elevated in the *de2f2* mutant and declined in the double mutant to a level that was lower than the wild-type control. These results suggest that dE2F2 represses *PCNA* expression and that the elevated level of *PCNA* expression in the *de2f2* mutant is due to the activity of dE2F1. The expression of *MCM3*, another proposed target of dE2F1 (Duronio et al. 1998), increased in the *de2f2* single mutant and this effect was suppressed in the double mutant, but in this case the total amount of *MCM3* RNA in the double-mutant discs was indistinguishable from wild type. Even though the pattern of *RNR2* expression has been shown to be dependent on dE2F1 (Duronio et al. 1995), we saw little change in the total level of *RNR2* transcripts in *de2f1* mutant larvae (data not shown) or in *de2f2*, or *de2f1; de2f2* mutant eye discs (Fig. 7G).

We conclude that both *de2f1* and *de2f2* are required for the normal pattern of *PCNA* expression. *de2f2* is needed to repress *PCNA* expression in the morphogenetic furrow; whereas *de2f1* is required for the high levels of expression in the first and second mitotic waves. Although the pattern of *PCNA*, *MCM3*, and *RNR2* transcripts depends on *de2f1* and/or *de2f2*, significant levels of each of these transcripts can be detected in the absence of both *de2f1* and *de2f2*.

Because *Cyclin E* is one of the best-known targets of E2F and is rate limiting for S-phase entry we examined the pattern of *Cyclin E* transcription in the *de2f2; de2f1* double-mutant animals. The normal pattern of *Cyclin E* expression was not altered in *de2f2* mutant discs and a similar pattern was also evident in *de2f2; de2f1* double mutants. However in the absence of both dE2F1 and dE2F2, the variations in *Cyclin E* expression were reduced and the pattern of expression was less distinct (Fig.

Figure 6. Loss of dDP function rescues the developmental defects of *de2f1* mutant larvae. Larvae are shown on the fifth day after egg laying for the following genotypes: Canton S (wild-type), *de2f1⁹¹/de2f1^{rm729}* (*de2f1⁻*), *dDP^{a2}/Df(2R)vg-B(dDP⁻)*, and *dDP^{a2}/Df(2R)vg-B; de2f1⁹¹/de2f1^{rm729}* (*dDP⁻; de2f1⁻*).

Figure 7. Changes in the expression of E2F-regulated genes in animals lacking *de2f1* and *de2f2*. Data is shown with material from wild type (Canton S), *de2f2*^{76Q1}/*de2f2*^{G5.1}, *de2f1*⁹¹/*de2f1*^{rm729} and *de2f2*^{76Q1}/*de2f2*^{G5.1} animals. (A–F) In situ hybridization of the third instar eye discs with probes to *PCNA* (A–C) or *Cyclin E* (D–F). Note that *PCNA* expression is not downregulated anterior to, or within, the morphogenetic furrow (arrow) in *de2f2* deficient discs (C) and that *PCNA* is expressed at low levels without any distinguishable pattern in the absence of both dE2F1 and dE2F2 (B). The pattern of *Cyclin E* expression is normal in *de2f2* deficient discs (F) and is also detectable in double-mutant discs, although the variations in *Cyclin E* expression are less dramatic in the absence of both dE2F1 and dE2F2 (E). (G) Northern blot analysis using

total RNA from dissected eye discs, and probes to several E2F regulated genes. Note that the levels of *Cyclin E*, *MCM3*, and *RNR2* transcripts are similar in wild-type and double-mutant larvae; whereas the levels of *PCNA* are reduced in the absence of both dE2F1 and dE2F2.

7E). Northern analysis showed that the steady-state level of *Cyclin E* transcripts was not decreased in the absence of E2F proteins (Fig. 7G). These results are consistent with evidence that *de2f1* contributes to the pattern of *Cyclin E* expression but is not required for *Cyclin E* transcription (Duronio and O'Farrell 1995). The finding that E2F target genes are expressed in *de2f2*; *de2f1* double mutants may explain, at least in part, why normal cell proliferation is possible in the absence of E2F proteins.

Discussion

Drosophila contains only two E2F family members and the results described above show that their gene products have opposite activities and antagonistic effects on cell proliferation during the larval stages of development. We find that dE2F1 and dE2F2 both heterodimerize with dDP, bind to E2F sequences, and interact with RBF. However dE2F1 is a potent activator of transcription, and the loss of *de2f1* results in the loss of expression of E2F-regulated genes (Dymlacht et al. 1994; Duronio et al. 1995; Royzman et al. 1997). Conversely, the overexpression of dE2F2 repressed transcription of E2F reporters in transfection experiments and the loss of *de2f2* function resulted in increased and expanded patterns of gene expression in vivo. The mechanism of dE2F2-mediated repression is not known. Given that dE2F2 and RBF physically interact and both proteins are required for the repression of the *PCNA* promoter it seems likely that dE2F2 acts in conjunction with a pocket protein.

The most definitive evidence that dE2F1 and dE2F2 have opposing functions is the finding that the loss of *de2f2* suppresses the proliferation defects in *de2f1* mutant larvae. This suppression leads to the surprising conclusion that the strong phenotypes seen in *de2f1* mu-

nants are not simply due to the absence of dE2F1 but also to the unchecked activity of dE2F2. In the absence of both proteins, larval cell proliferation is relatively normal. This result explains why *dDP* mutants have a much milder phenotype than *de2f1* mutants (Royzman et al. 1997). Based on this set of observations, one could argue that the primary function of dE2F1 at this stage of development is to relieve the effects of dE2F2.

Chromatin immunoprecipitation experiments show that dE2F2 is normally present at promoters that are induced by dE2F1. Thus, the simplest interpretation of this data is that the antagonistic functions of dE2F1 and dE2F2, result from their opposing effects on the same set of target genes (Fig. 8). In this model, we suggest that E2F-mediated repression limits the rate of cell proliferation in the imaginal disc, and that the burst of E2F-regulated gene expression that precedes and parallels S-phase entry results from the ability of dE2F1-mediated activation to override dE2F2-mediated repression. This idea fits with the observation that relatively low levels of dE2F1 are able to overcome much higher levels of dE2F2 in transient transfection experiments and in transgenic animals, and with the patterns of dE2F1 and dE2F2 expression in the eye disc. Whereas dE2F2 appears to be expressed throughout the eye disc, high levels dE2F1 are restricted to areas containing asynchronously dividing cells, and are seen transiently in the second mitotic wave (Asano et al. 1996; Brook et al. 1996). The tight regulation of dE2F1 expression, and the idea that dE2F1 normally overrides dE2F2, may explain why the loss of dE2F2 does not give rise to widespread inappropriate proliferation.

In the absence of both dE2F1 and dE2F2, gene expression is presumably dependent on other transcription factors that bind to these promoters and the outcome is expected to vary depending on the factors that are pres-

Figure 8. Model for the regulation of E2F-dependent transcription during larval development. Two functionally different dE2F complexes can occupy E2F-regulated promoters. One complex contains dE2F2 and represses transcription, most likely via interaction with RBF and RBF-associated proteins (A). As dE2F2 is ubiquitously expressed at high levels, we suggest that it is present on most vacant promoters. During the G₁/S transition, newly synthesized dE2F1 binds to the target promoters and overrides the activity of dE2F2 (B), allowing a burst of gene expression as cells enter S-phase. The *de2f1* mutant phenotype is caused by the failure of dE2F1 to override dE2F2 and the unchecked activity of dE2F2. In double-mutant animals (C) the loss of dE2F1-mediated activation is compensated by the loss of dE2F2-mediated repression, and the expression of target genes is regulated by other transcription factors (TF) that bind to these promoters.

ent and the net contribution of the E2F-binding sites to activation or repression. We note that several factors have been found to bind to the *PCNA* promoter, in addition to E2F (Yamaguchi et al. 1996; Hayashi et al. 1997; Sawado et al. 1998a; Hayashi et al. 1999), and that mutation of all three E2F-binding sites in *DNApolα* promoter reduces, but does not abolish, transcription in transgenic animals (Yamaguchi et al. 1997). Moreover most studies of E2F-regulated promoters in mammalian cells show that the mutation of E2F-binding sites alters the timing of gene expression but does not block transcription (Means et al. 1992; Johnson et al. 1994). Clearly, the level of E2F-independent transcription is sufficient for relatively normal rates of growth and cell proliferation within the imaginal discs.

Precisely how dE2F1 normally overrides dE2F2 is unclear. It is possible that dE2F1 simply replaces dE2F2 on key regulatory sites. However many E2F-regulated promoters contain multiple E2F-binding sites and it is possible that dE2F1 and dE2F2 act through distinct sites on the same set of promoters. To date, no experiments have unequivocally identified all of the transcriptional targets of E2F that are most important for cell proliferation. Thus it is possible that, although dE2F1 and dE2F2 have opposite effects on the expression of some common targets, they exert their antagonistic effects on cell proliferation by regulating different subsets of target genes. It is also possible that dE2F2 has a gain-of-function effect in the absence of dE2F1, and blocks the expression of a

subset of genes that it would never normally regulate. Finally, it is conceivable that either dE2F1 or dE2F2, or both, act directly at origins of replication (Royzman et al. 1999). However, that the relatively normal patterns of BrdU incorporation in *de2f1*, *de2f2* mutant larvae indicates that neither of these proteins have an essential function in DNA replication at this stage of development.

The rescue of cell proliferation defects in *de2f1* mutant larvae by mutation of *de2f2* does not imply that E2F-regulation is nonessential in *Drosophila*. *de2f1*, *de2f2* double mutants die during pupation and we have not yet observed any adult escapers that completely lack both dE2F1 and dE2F2. At present it is uncertain whether the E2F function needed during the pupal stage is unique to *de2f1* or is redundant between *de2f1* and *de2f2*. Conceivably, the pupal lethality of *de2f1*, *de2f2* mutants could result from a defect in either the differentiation, or proliferation, or survival of a critical cell type. Because dE2F1 and dE2F2 both form complexes with RBF in vivo, it is feasible that both proteins may mediate transcriptional repression in some cell types. It is also evident that the individual dE2F proteins do have unique functions that are essential at specific stages of *Drosophila* development. For example, the work of Cayirlioglu and coworkers suggests that dE2F2 has a unique role in the shut-off of DNA endoreplication in follicle cells (Cayirlioglu et al., in prep.), and Orr-Weaver and colleagues have shown that dE2F1 is required for the control of chorion gene amplification in this same cell type (Royzman et al. 1999). Thus the functional antagonism apparent between *de2f1* and *de2f2* may be a characteristic of proliferating cells and may not apply to all cell types and all stages of development.

Thus far, studies of the E2F/RB pathway in *Drosophila* have given results that closely parallel studies in mammalian cells. Although E2F-1, E2F-2, E2F-3, E2F-4, and E2F-5 are all able to activate transcription and be repressed by pRB-family members, E2F-1, E2F-2, and E2F-3 are generally more tightly linked to activation of transcription; whereas E2F-4 and E2F-5 are the family members most strongly linked to repression. We speculate that the function of dE2F1 may resemble the role played by the strong activator E2Fs, while the role of dE2F2 may resemble the functions of the corepressor E2Fs. Intriguingly, *E2f-3*-deficient MEFs show a proliferation defect that resembles the *de2f1* mutant phenotype; conversely *E2f-4*, *E2f-5* double-mutant MEFs have defects in cell cycle arrest (Bruce et al. 2000; Gaubatz et al. 2000). By analogy with the *Drosophila* E2Fs, it will be interesting to determine whether any defects in cell proliferation caused by the mutation of E2F-3, E2F-2, or E2F-1 (either singly or in combination) can be suppressed by inactivation of E2F-4 or E2F-5.

These results highlight two general issues. One implication of this work concerns the role of E2F in cell cycle control. Many studies have shown that E2F proteins are potent regulators of the cell cycle; either increasing or decreasing E2F activity can have striking effects on cell cycle progression. However, we find that fairly normal

patterns of cell proliferation are possible in the absence of both *de2f1* and *de2f2*. This indicates that, while the activities of individual E2F proteins do affect cell cycle progression, E2F-regulation is not an obligate component of the cell cycle machinery.

Second, this study shows one of the many complications that can arise when one member of a family of related proteins is specifically removed. In the case of E2F, several different family members can target the same promoter elements. Thus, the phenotype that is seen when one E2F family member is removed reflects a combination of changes that involves both the absence of the targeted gene product and the altered importance of the E2F complexes that remain in the cell. To some extent, the effect of removing activator E2Fs could be regarded as an increase in the activity of repressor E2Fs or vice-versa. In normal cells, E2F-mediated control of cell proliferation results from the balanced interplay between E2Fs with different, even opposing, activities.

Materials and methods

Generation of the *de2f2* mutant

de2f2 maps to the base of the left arm of chromosome 2 at position 39B2-3. A semilethal *P* element line, *l(2)k16403*, containing an insertion 667 bp upstream of the 5' end of *de2f2* was used to generate mutant alleles of *de2f2*. The insertion is responsible for the lethality, because excision of the *P* element reverts the lethal phenotype.

Analysis of the genomic sequence revealed a putative ORF in close proximity of the *P*-element insertion in *l(2)k16403*. A corresponding cDNA was cloned using a Rapid Amplification of cDNA Ends (RACE) protocol and the transcription map of this region was established (Fig. 9). The *P* element in *l(2)k16403* is

Figure 9. Molecular map of the *de2f2* region. Position of the *P*-element insertion in *l(2)k16403* is designated by a triangle. *de2f2* alleles *de2f2*^{Q3.1} (*Q3.1*) and *de2f2*^{G5.1} (*G5.1*) were generated in the first round of male recombination; *de2f2*^{76Q1} (*76Q1*) was made in the second round of male recombination from the *de2f2*^{Q3.1} chromosome. Open boxes show sequences deleted in the respective alleles. Exon-intron structure of transcripts synthesized in the region. *MPP6* unit produces two mRNAs (*MPP6s* and *MPP6l*) with alternative polyadenylation sites.

inserted in the 5' UTR of the *MPP6* gene, 16 bp downstream of the transcription start site (GenBank accession no. AY029612). *MPP6* encodes a *Drosophila* homolog of a human M-phase phosphoprotein 6 (Matsumoto-Taniura et al. 1996).

A standard approach to make a null allele by using a preexisting *P*-element insertion is to generate imprecise excisions of the *P* element. Deletions generated in this way from *l(2)k16403* would automatically remove *MPP6* sequences. To overcome this problem we employed a different approach based on the phenomenon of male recombination. It is known that mobilization of *P* element results in significant increase in frequency of crossing over in the male germline, with the breakpoint being within several kilobases of an existing *P* element. Moreover, most of the recombinants carry either a deletion or a duplication of the genomic sequences immediately flanking the *P*-element insertion site (Preston et al. 1996). The major advantage of this approach is that the generated aberrations retain a mobile *P* element. Hence, this chromosome could be subsequently used in following rounds of recombination. The recombinants can be identified with the distant flanking chromosomal markers. The strategy for the generation of the *de2f2* mutant is briefly outlined below. In the first round, rearranged chromosomes carrying deletions extending distally from the *P*-element insertion into *de2f2* were selected. In the next step, these deletions were used to generate small duplications to restore the sequences of *MPP6* but not *de2f2*.

For the first round of male recombination, jump-start males *l(2)k16403/dp b cn bw*; $\Delta 2-3$ *Dr/+* were crossed to *dp b cn bw* females. Potential recombination events in *l(2)k16403/dp b cn bw*; $\Delta 2-3$ *Dr/+* males were revealed by segregation among the second-chromosome distal markers and found to be at frequency ~0.2%. Males that are *dp b cn bw*⁺ presumably carry either a distal deletion or proximal duplication. A total of 18 males were crossed individually to *Sp/CyO*, *dp cn bw* to establish stocks. As revealed by PCR analysis, in two stocks, *de2f2*^{G5.1} and *de2f2*^{Q3.1}, the recombination event resulted in a distal deletion that removed *de2f2*; their precise position was determined by sequencing. In *de2f2*^{Q3.1} allele 870 bp distal to the *P*-element insertion site are removed, while in *de2f2*^{G5.1} the deletion extends 5621 bp.

To restore the integrity of the *MPP6* promoter, both alleles were used for a second round of recombination. First, *dp*⁺ and *b*⁺ markers were recombined on the *de2f2*^{Q3.1} and *de2f2*^{G5.1} chromosomes. Then, jump-start males, *de2f2*^{Q3.1}/*dp b cn bw*; $\Delta 2-3$ *Dr/+* or *de2f2*^{G5.1}/*dp b cn bw*; $\Delta 2-3$ *Dr/+*, were crossed to *dp b cn bw* females. Seventy-five *dp*⁺ *b*⁺ *cn bw* recombinants were isolated and analyzed by PCR and sequencing. In 53 cases, recombination was accompanied by the precise excision of the *P*-element and 12 chromosomes were identical to parental; in three cases proximal deletions were generated and in four cases the structure was not determined. Three chromosomes carried distal duplications and two of them originated from the *de2f2*^{G5.1} chromosome were large and restore *de2f2*. In one case a duplication event from the *de2f2*^{Q3.1} chromosome was recovered. In the generated duplication, *de2f2*^{76Q1}, 273 bp upstream of *MPP6* were restored.

Fly stocks

The following alleles were used: *de2f1*⁹¹ and *de2f1*^{rm729} are null alleles for *de2f1* (Duronio et al. 1995), *dDP*^{a2} is a null allele of *dDP* (Royzman et al. 1997), *Df(2R)vg-B* is a deficiency uncovering the *dDP* gene (Royzman et al. 1997), *MPP6*^{U39} is a point mutation in *MPP6*, which converts the sixth amino acid into a stop codon and will be described elsewhere (D.S. Huen, unpubl.).

To analyze various mutant combinations, the mutant alleles were balanced over *T(2;3)CyO TM6B, Cy Tb* translocation. All mutations were analyzed in trans-heterozygous conditions. Trans-heterozygous mutant larvae were identified by the absence of a dominant marker *Tb*. Trans-heterozygous *de2f2* mutant larvae were collected from the cross of *de2f2^{76Q1}/T(2;3)CyO TM6B, Cy Tb* and *de2f2^{G5.1}/T(2;3)CyO TM6B, Cy Tb*. *de2f1; de2f2* double mutants were collected by crossing *de2f2^{76Q1}; de2f1^{rm729}/T(2;3)CyO TM6B, Cy Tb* to *de2f2^{G5.1}; de2f1⁹¹/T(2;3)CyO TM6B, Cy Tb*. To make *de2f1; dDP* null larvae, *dDP^{ad2}; de2f1⁹¹/T(2;3)CyO TM6B, Cy Tb* flies were crossed to *Df(2R)vg-B; de2f1^{rm729}/T(2;3)CyO TM6B, Cy Tb*.

For *P*-element transformation, the *de2f2* cDNA was cloned into the pUAST vector and injected with the $\Delta 2-3$ helper plasmid into y^w embryos. Several independent lines on each chromosome were established.

DNA and RNA manipulations

All standard DNA manipulations were performed as described (Sambrook et al. 1989). Total RNA was isolated from third instar eye discs using Trizol (GIBCO BRL). Approximately 5 μ g of RNA isolated from 50 discs were loaded per lane. The RNA was fractionated on 1% agarose gels containing 2.2M formaldehyde. The RNA was transferred by capillary blotting in 10 $\times$ SSC from the gel to neutral nylon membranes (S&S) and then hybridized to radio-labeled antisense RNA probes prepared by in vitro transcription with T3 or T7 RNA polymerase (Promega). The pre-hybridization and hybridization solutions were 0.5 M NaCl, 7% dextran sulfate, 0.2 mg/mL heparin, 1% sarkosyl, 0.1 M sodium phosphate (pH 7.0), 0.1% ficoll, 0.1% polyvinylpyrrolidone, 0.1% sodium pyrophosphate, and 50% (v/v) formamide. The filters were washed three times in a solution, containing 5mM sodium phosphate, 0.2% SDS, and 1mM EDTA for 1 h at 75 °C.

To obtain the 5' and 3' ends of the transcripts synthesized with the *de2f2* region, the RACE protocol was performed using the Marathon cDNA Amplification Kit (Clontech) according to the manufacturer's recommendations. As a template for cDNA synthesis, the poly(A)⁺ RNA was isolated from Canton S adult flies using a polyAT track kit (Promega).

In situ hybridization with the eye discs was performed as described (Du 2000). *PCNA* and *Cyclin E* riboprobe were prepared by in vitro transcription with DIG RNA labeling mix and detected with alkaline phosphatase conjugated anti-DIG antibody (Boehringer-Mannheim).

Antibodies, Western blots, and immunoprecipitations

Mice were immunized with dE2F2 protein expressed in *Escherichia coli*. The dE2F2 monoclonal antibodies mei3 and mei8 were prepared by fusing splenocytes to NS-1 myeloma cells as described (Harlow and Lane 1988). mei8 was found to be specific for dE2F2 in Western blotting; however, mei3 was specific in immunoprecipitation experiments. A rabbit antiserum to dE2F2 was raised by injecting a rabbit with the recombinant full-length dE2F2 protein. dE2F2 polyclonal antibodies were affinity purified over a GST-dE2F2 column.

Mouse monoclonal anti-dDP (Yun mix) and mouse monoclonal anti-RBF (DX-3) were described previously (Du et al. 1996a,b). IP-Westerns on SL2 cells were performed by standard methods (Du et al. 1996a). The interactions between dE2F2, dDP, and RBF were not disrupted by EtBr treatment. For immunoprecipitations, anti-dE2F2 mei3 antibodies were used at 1:1 and anti-dDP at 1:1 dilution. For Western blot analysis, anti-dE2F2 mei8 antibody were used at 1:2, anti-RBF at 1:2 and anti-dDP at 1:3. Peroxidase-conjugated anti-rabbit and anti-mouse

secondary antibodies were from Amersham. For Western blot analysis of trans-heterozygous *de2f2* mutant animals, non-*Tb* second instar larva were collected from the cross between *de2f2^{76Q1}/T(2;3)CyO TM6B, Cy Tb* and *de2f2^{G5.1}/T(2;3)CyO TM6B, Cy Tb* and the extracts were prepared following the protocol for SL2 cells.

Transient transfections

For transfections, the dE2F1 and dE2F2 ORFs were cloned into pIE4 vector (Novagen) under the control of baculovirus *ie1* promoter and the constructs were verified by sequencing. *Drosophila* SL2 cells were transfected with CellFectin (GIBCO BRL) with 10 μ g of plasmid DNA. The reporter constructs consists of the luciferase gene fused to either the wild-type *PCNA* promoter, p5'-168DPCNA_{luc} or the *PCNA* promoter with mutated E2F sites, p5'-168DPCNA_{mutI&mutII}_{luc} (Sawado et al. 1998b). To determine the efficiency of transfection and to normalize the luciferase read-outs, the β -galactosidase expressing construct, pIE4-*lacZ*, was included in each experiment. Cells were harvested 36 h after transfection and assayed for β -galactosidase and luciferase activity by standard methods (Dynalacht et al. 1994).

Chromatin immunoprecipitations

Chromatin immunoprecipitations were performed using modified methods published previously (Dimova et al. 1999; Meluh and Broach 1999). *Drosophila* SL2 cells were grown at 25 °C in spinner flasks in Schneider's *Drosophila* medium (GIBCO BRL) supplemented with 10% fetal calf serum. Formaldehyde was added to the tissue culture medium to a final concentration of 1% and cross-linking was performed for 15 min at room temperature and terminated with glycine (final concentration 0.125 M). The equivalent of $\sim 10^8$ cells were used per immunoprecipitation reaction and incubated with either 2 μ L of anti-dE2F2 polyclonal rabbit antibody, with 2 μ L of anti-dE2F1 polyclonal guinea pig antibody, with rabbit anti-mouse whole serum (non-specific antibody), or without an antibody. Analysis of DNA was performed using multiplex PCR. The following primer sets were used: *PCNA*, 5'-GCAGAGCTGCGTGGAATGATTAAA GC-3' and 5'-TTGACCCAGGCGTGCTCGAACATAT-3'; *DNA pol α* , 5'-CAGGTCGGATTTCCCGCCAAAATA-3' and 5'-GTGACCAGGGATGGAGGATGATCA-3'; *rp49*, 5'-TCCG GCAAGGTATGTGCGTGATTT-3' and 5'-ATATCGATCCG ACTGGTGCGGAT-3'. PCR products were resolved on 6% polyacrylamide gels.

Immunofluorescence

For immunostaining, rabbit affinity purified anti-dE2F2 antibodies were used at a 1:100 dilution, anti-Cyclin A (Knoblich and Lehner 1993) at 1:100, anti-phospho-Histone H3 (Upstate Biotechnology) at 1:200, and rat anti-elav (O'Neill et al. 1994) at 1:200. For BrdU labeling experiments, eye discs from third instar larvae were dissected in Schneider's medium for 10 min and labeled with 0.2 mg/mL BrdU (Sigma) at room temperature. Eye discs were fixed and BrdU was detected with mouse anti-BrdU antibody (Becton Dickinson) at 1:50. Secondary antibodies were Cy5 donkey anti-rabbit, Cy3 donkey anti-mouse (Jackson Immunolaboratories), and Alexa Fluor 488 anti-rat (Molecular Probes). Images were collected on a Zeiss LSM510 confocal microscope and processed with Adobe Photoshop 5.5.

Acknowledgments

We thank Adelaide Carpenter, John Roote, and Yian-Rong Chen for expert technical assistance, Bloomington *Drosophila* Stock

Center for the provision of stocks, and our colleagues for valuable discussions. We thank Dr. Terry Orr-Weaver for the kind gift of dE2F1 antisera; Drs. Masamitsu Yamaguchi and Peter Bangs for the reporter constructs; Dr. Bob Duronio for exchanging information prior to publication; Dr. Nick Tapon for help with confocal microscopy; and Drs. Iswar Hariharan, Ken Moberg, and Ed Harlow for insightful comments on the manuscript. D.D. was supported by an NIH training grant. D.S.H. was supported by grants from the Cancer Research Campaign and Medical Research Council. The provision of laboratory facilities and technical support by B.A.J. Ponder and M. Ashburner is gratefully acknowledged by D.S.H. This work was supported by NIH grant number GM53203 (N.D.), a CRC program grant and an MRC program grant to M. Ashburner, S. Russell, and D. Gubb.

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Genes Dev. 2001, **15**:

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