Development 128, 5085-5098 (2001)
Printed in Great Britain © The Company of Biologists Limited 2001
DEV5954

Drosophila E2f2 promotes the conversion from genomic DNA replication to gene amplification in ovarian follicle cells

Pelin Cayirlioglu[1,2], Peter C. Bonnette[1,3], M. Ryan Dickson[1] and Robert J. Duronio[1,2,3,4,*]

[1]Department of Biology, University of North Carolina, Chapel Hill, NC 27599, USA
[2]Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC 27599, USA
[3]Program in Molecular Biology and Biotechnology, University of North Carolina, Chapel Hill, NC 27599, USA
[4]Curriculum in Genetics and Molecular Biology, University of North Carolina, Chapel Hill, NC 27599, USA
*Author for correspondence (e-mail: duronio@med.unc.edu)

Accepted 24 September 2001

SUMMARY

***Drosophila* contains two members of the E2F transcription factor family (*E2f* and *E2f2*), which controls the expression of genes that regulate the G1-S transition of the cell cycle. Previous genetic analyses have indicated that *E2f* is an essential gene that stimulates DNA replication. We show that loss of *E2f2* is viable, but causes partial female sterility associated with changes in the mode of DNA replication in the follicle cells that surround the developing oocyte. Late in wild-type oogenesis, polyploid follicle cells terminate a program of asynchronous endocycles in which the euchromatin is entirely replicated, and then confine DNA synthesis to the synchronous amplification of specific loci, including two clusters of chorion genes that encode eggshell proteins. *E2f2* mutant follicle cells terminate endocycles on schedule, but then fail to confine DNA synthesis to sites of gene amplification and inappropriately begin genomic DNA replication. This ectopic DNA synthesis does not represent a continuation of the endocycle program, as the cells do not complete an entire additional S phase. *E2f2* mutant females display a 50% reduction in chorion gene amplification, and lay poorly viable eggs with a defective chorion. The replication proteins ORC2, CDC45L and ORC5, which in wild-type follicle cell nuclei localize to sites of gene amplification, are distributed throughout the entire follicle cell nucleus in *E2f2* mutants, consistent with their use at many genomic replication origins rather than only at sites of gene amplification. RT-PCR analyses of RNA purified from *E2f2* mutant follicle cells indicate an increase in the level of *Orc5* mRNA relative to wild type. These data indicate that *E2f2* functions to inhibit widespread genomic DNA synthesis in late stage follicle cells, and may do so by repressing the expression of specific components of the replication machinery.**

Key words: E2F2, E2F, *Drosophila*, Oogenesis, ORC, Cell cycle, Replication

INTRODUCTION

The E2F family of transcription factors is thought to impart both positive and negative influences on the cell cycle (Dyson, 1998; Harbour and Dean, 2000). The current model suggests that in the positive role E2F activates the transcription of a broad spectrum of genes required for cell cycle progression and DNA synthesis (Ishida et al., 2001; Muller et al., 2001). In the negative role, E2F is converted into a transcriptional repressor of the same set of genes by binding to a member of the pRB family of tumor suppressors (i.e. pRB, p107 and p130). In spite of the widespread acceptance of this model of E2F function, the relative contributions of the transcriptional repressor and activator roles of E2F to cell cycle control during animal development remain unclear (Muller and Helin, 2000). Functional 'E2F' is a heterodimer composed of subunits encoded by the E2F and DP family of genes, with six and two members currently described, respectively, in mammals. The existence of an E2F gene family suggests some specialization of function, both molecularly and developmentally. One possibility is that specific E2F proteins primarily activate cell cycle progression by stimulating transcription, while others primarily inhibit the cell cycle by repressing transcription.

Mouse knockout experiments have provided some evidence for this hypothesis in mammals (Bruce et al., 2000; Field et al., 1996; Gaubatz et al., 2000; Humbert et al., 2000a; Humbert et al., 2000b; Lindeman et al., 1998; Pan et al., 1998; Rempel et al., 2000; Tsai et al., 1998; Yamasaki et al., 1998; Yamasaki et al., 1996; Ziebold et al., 2001). However, much of this evidence comes from the analysis of embryonic fibroblasts in culture, and less is known about how the E2F family affects cell cycle progression in various tissues in the intact animal. *Drosophila* provides a simpler genetic system to examine the contribution of E2F function to cell cycle control directly in a developing animal. The *Drosophila* genome contains two E2F (*E2f* and *E2f2*), a single *Dp* and two pRB (*Rbf* and *Rbf2*) genes (Du et al., 1996; Dynlacht et al., 1994; Frolov et al., 2001; Hao et al., 1995; Ohtani and Nevins, 1994; Sawado et al., 1998). *E2f*, *Dp* and *Rbf* are each essential (Du and Dyson, 1999; Duronio et al., 1998; Duronio et al., 1995; Royzman et al., 1997). Loss of

either *E2f* or *Dp* function abrogates the expression of replication genes, and compromises DNA synthesis and cell proliferation in several different developmental contexts, suggesting that E2F/DP action stimulates progression through the cell cycle (Brook et al., 1996; Duronio et al., 1998; Duronio et al., 1995; Neufeld et al., 1998; Royzman et al., 1997). By contrast, there is no evidence that E2F plays a negative role in either transcription or cell cycle progression. However, there is evidence that DP acts to repress transcription of at least one gene (i.e. *Mcm3*) in the embryonic CNS (Duronio et al., 1998). Moreover, loss of RBF function in embryos causes transcriptional de-repression of E2F targets and allows cells to inappropriately transit from G1 into S phase (Du and Dyson, 1999). One hypothesis is that E2F2, and not E2F, acts as part of transcriptional repressor complex containing DP and RBF that actively inhibits cell cycle progression. In order to test this, we engineered null mutations of *E2f2*. Our phenotypic analyses of these mutants indicate that E2F2 is not essential for fly development, but is necessary for fertility of females where it plays a role in controlling DNA replication in follicle cells during oogenesis.

A *Drosophila* ovary contains 14-16 ovarioles, which are egg assembly lines consisting of a series of connected egg chambers at successive stages of oogenesis (Spradling, 1993). Each egg chamber contains a cyst of 16 germ cells descended from a single founding stem cell. One of these cells becomes the oocyte, while the remaining 15 differentiate into polyploid nurse cells that support oocyte growth. The germ cell cyst is encapsulated within a single epithelial layer of somatic follicle cells. The follicle cells arise from two stem cells within the germarium, a specialized structure at the anterior end of each ovariole where egg chambers are first formed. After an early period of cell proliferation, follicle cells switch to endoreduplication or endocycles, through which most cells achieve a 16C DNA content by stage 10 of oogenesis. At this stage, the follicle cells make another developmental switch and begin to amplify synchronously and selectively a small number of distinct loci, the two most prominent of which contain clusters of chorion genes that encode the eggshell proteins (Calvi and Spradling, 1999; Orr-Weaver, 1991). This process occurs by repeated firing of one or more replication origins located within the gene clusters. DNA sequences that define replicator and origin elements required for high level chorion gene amplification (e.g. ~60fold) have been defined molecularly (Austin et al., 1999; Lu et al., 2001).

Many of the molecules required for genomic DNA replication during a typical S phase also appear to control chorion gene amplification (Calvi and Spradling, 1999; Spradling, 1999). This includes members of the pre-replicative complex (pre-RC), such as the origin recognition complex (ORC), DUP/CDT1 and CDC45L, which may play a role in both initiation and elongation, and the S phase kinases Cyclin E/CDK2 and CDC7/DBF4 (Asano and Wharton, 1999; Austin et al., 1999; Calvi et al., 1998; Landis et al., 1997; Landis and Tower, 1999; Loebel et al., 2000; Whittaker et al., 2000). E2F complexes are also required for normal chorion gene amplification. Viable, hypomorphic alleles of *E2f* and *Dp* cause decreased chorion gene amplification, resulting in production of eggs with a thin shell (Royzman et al., 1999). Conversely, *Rbf* mutant follicle cells overamplify chorion genes (Bosco et al., 2001). Although it is well established in both flies and mammals that E2F can control the transcription of genes encoding components of the pre-RC, recent data suggest that E2F complexes may regulate chorion gene amplification more directly. E2F and RBF co-immunoprecipitate with ORC1 and ORC2 from ovary extracts, and chromatin immunoprecipitations show that this complex is present at the chorion locus in vivo (Bosco et al., 2001). These physical interactions raise the possibility that E2F/DP/RBF complexes regulate gene amplification directly at the replication origin, rather than, or in addition to, a mechanism involving changes in gene expression (Cayirlioglu and Duronio, 2001).

The developmental and cellular mechanisms by which follicle cells first change from mitotic cycles to endocycles, and then from endocycles to gene amplification, are not known. pRB-containing complexes appear to contribute to the latter transition. In a subset of follicle cells within individual egg chambers isolated from either *Dp* or *Rbf* female sterile mutants, replication is not restricted to sites of gene amplification, but rather occurs throughout the entire nucleus (Bosco et al., 2001; Royzman et al., 1999). One interpretation of these data is that follicle cells continue endocycles inappropriately, rather than switching to gene amplification. *E2f* female sterile mutants do not display this phenotype, suggesting the hypothesis that a E2F2/DP/RBF complex regulates the transition from endocycles to gene amplification in follicle cells. Our results indicate that deletion of *E2f2* causes ectopic follicle cell genomic DNA replication at a stage when these cells should have terminated endocycles and begun gene amplification. As a consequence, gene amplification at the chorion loci is reduced, causing *E2f2* mutant females to produce poorly viable eggs with a thin eggshell. However, in contrast to *Rbf* mutants, which fail to terminate endocycles and execute one additional endo S phase (Bosco et al., 2001), *E2f2* mutant follicle cells exit the endocycle program on schedule and do not fully complete an additional endocycle S phase. This suggests that E2F2 acts during the gene amplification phase to restrict DNA synthesis to particular loci.

MATERIALS AND METHODS

Generation of yeast two hybrid and UAS constructs

Two hybrid analysis was carried out in *S. cerevisiae* strain PJ69-4A (James et al., 1996). The entire *Dp* open reading frame was cloned into the GAL4-based pGBT8 bait vector (a derivative of pGBT9 from Clontech) after engineering an *Eco*RI restriction site by PCR at the initiator Met. An *Nco*I site was PCR engineered at the initiating codon of *E2f* and *E2f2* cDNA for subcloning into the activation domain vector pACT2 (Clontech). E2F2$^{1\text{-}218}$ and E2F2$^{1\text{-}62}$ were made by removing sequences from pACT2-*E2f2* with convenient *Eco*RV and *Eco*RI restriction sites, respectively. A clone that will produce E2F2$^{1\text{-}188}$ was made by Klenow fill in of the *Nde*I site within *E2f2* cDNA, before subcloning into pACT2-*E2f2*.

E2F2 genetics and molecular biology

Df(2L)DS8 (Sinclair et al., 1980) was provided by Minx Fuller. *Df(2L)TW161* (Wright et al., 1976) was provided by the BDGP. The *Rbf* alleles were provided by Terry Orr-Weaver (Bosco et al., 2001; Du and Dyson, 1999). The P-element insertion line *l(2)16402* was obtained from the Szeged *Drosophila melanogaster* P Insertion Mutant Stock Centre. This line contained three P-element insertions, two on the second chromosome and one on the third chromosome. The insertion located just upstream of *E2f2*, designated *l(2)16402a*, was detected by PCR using primers from the *E2f2* region and a primer,

ITR, that hybridizes to P-element ends. A 600 bp fragment obtained from PCR using a *E2f2* primer (BDO78, 5′ TTCATGGCATGCGGACTA 3′) and ITR (5′ CGACGGGACCACCTTATGTTATT 3′) was subcloned using the TOPO™ TA CLONING® KIT (Invitrogen) and sequenced. The other two P-element insertions in the original *l(2)16402* line were separated from *l(2)16402a* by meiotic recombination.

P-element excision was begun by crossing yw^{67} *l(2)16402a*/*CyO* and yw^{67}; *Sco/CyO;* Δ*2,3 Sb/TM6* flies. Single progeny males of genotype *l(2)16402a*/*CyO*; Δ*2,3 Sb*/+ were crossed to yw^{67}; Pin^{88k}/*CyO*, and single, balanced w^- male progeny were backcrossed to yw^{67}; Pin^{88k}/*CyO* females to recover excision events. After mating, these same males were subjected to PCR using primers spanning the *E2f2* locus. One excision line, $Df(2L)E2f2^{329}$, produced a 650 nucleotide PCR product with PCB15 (5′ GTTGCAGTGTAGCTATAGTCCTAA 3′) and BDO78. This fragment was subcloned and sequenced to identify deletion breakpoints. *Df(2L)1129* was generated by mobilizing P-element line *l(2)07215* (Fig. 3). P-element mobilization events were generated as described above, with the exception that w^+ individuals were selected instead of w^-. *Df(2L)1129* was among 11 unidirectional deletions towards *E2f2* that were identified as PCR negative with primers BDO95 (5′ CAGTGAACAAGGTACATG 3′) and ITR but PCR positive with BDO96 (5′ TTAAGGTCCAGTAGCTTC 3′) and ITR. BDO95 and BDO96 bind distal and proximal, respectively, to *l(2)07215*. Genomic DNA adjacent to the P-element was rescued from these 11 lines by inverse PCR and sequenced to identify deletion breakpoints.

The P element transgenes shown in Fig. 3 were each built using pCaSpeR4 and the following inserts recovered from cosmids obtained from the BDGP: P[$E2f2^+$; $Mpp6^-$], a 4.1 kb *Hin*dIII-*Sac*II genomic fragment containing only *E2f2*; P[$E2f2^+$; $Mpp6^+$], a 6.4 kb *Hin*dIII genomic fragment containing *E2f2, Mpp6* and CG9249; P[$E2f2^-$; $Mpp6^+$], a 4 kb *Rsr*II-*Hin*dIII genomic fragment containing only *Mpp6* and CG9249; P[$E2f2^{1\text{-}188}$; $Mpp6^+$], the same genomic fragment as P[$E2f2^+$; $Mpp6^+$], except with a 14 bp deletion in *E2f2* that was randomly generated during the cloning process. This results in a frameshift after amino acid 188 followed by a translation stop two codons later.

E2f2 null flies were of genotype yw^{67}; $Df(2L)E2f2^{329}$/$Df(2L)E2f2^{329}$; P[$E2f2^-$; $Mpp6^+$]/+. Rescued *E2f2* null flies were generated similarly with the P[$E2f2^+$; $Mpp6^+$] transgene instead. Experiments involving the $E2f2^{1\text{-}188}$ allele used a recombinant chromosome containing *Df(2L)DS8* and the transgene P[$E2f2^{1\text{-}188}$; $Mpp\text{-}6^+$] in *trans* to either *l(2)16402a* or $Df(2L)E2f2^{329}$.

Quantitative Southern hybridization

DNA isolated from 110 stage 13 egg chambers dissected from wild-type or *E2f2* null females was digested with *Sal*I and subjected to Southern blot analysis using a 3.8 kb *Sal*I fragment from the third chromosome chorion gene cluster cloned into pT2 (kindly provided by Brian Calvi). An 8.3 kb *Hin*dIII genomic fragment containing the *rosy* gene (kindly provided by Jeff Sekelsky) was used as an unamplified control. Signal intensities were determined using a PhosphoImager. The relative intensity of the chorion locus signal between wild-type and mutant was compared by normalizing to the *rosy* signal.

BrdU labeling and antibody staining

Ovaries were dissected and labeled with BrdU as described previously (Lilly and Spradling, 1996). Anti-BrdU antibodies (Becton Dickinson) were detected in situ using Cy3- or rhodamine-conjugated goat anti-mouse secondary antibodies (Jackson). The DNA was stained with DAPI at a final concentration of 1μg/ml for 1 minute. For antibody staining, the ovaries were dissected in Schneider's medium, fixed in 6% formaldehyde (Sigma) for 15 minutes, washed twice with PBT and incubated with 10% normal goat serum in PBT for 30 minutes. Affinity-purified rabbit anti-ORC2 antibodies (kindly provided by Mike Botchan) (Pak et al., 1997), rabbit anti-ORC5 and anti-CDC45L serums (kindly provided by Sue Cotterill) (Loebel et al., 2000) were used at a dilution of 1:100. Rhodamine-conjugated goat anti-rabbit secondary antibodies (Jackson) were used to detect the primaries.

Nuclear isolation, flow cytometry and cell sorting

Ovarian nuclei were isolated from 40-50 females dissected in Schneider's medium as described previously (Lilly and Spradling, 1996), except that 100 ng/μl propidium iodide was used to detect DNA. Nuclei were stored on ice before analysis of ploidy using a Becton Dickinson FACScan. For analysis of intact follicle cells, ~100 females/per experiment of genotype c323/+; Df(2L)$E2f2^{329}$/$E2f2^{1\text{-}188}$; UAS-GFP/+ or c323/+; Df(2L)$E2f2^{329}$/+; UAS-GFP/+ were dissected. Cell suspensions were prepared as described previously (Bryant et al., 1999), except that a 125 μm mesh was used to filter trypsinized tissue. Isolated follicle cells were stained with 2 μg/ml Hoechst 33342 for 45 minutes and stored on ice before FACS analysis and sorting using a MoFlo high-speed molecular flow cytometer. Ploidy was determined by excitation at 364 nm, and GFP-positive cells were sorted by excitation at 488 nm.

RT-PCR analysis

RNA was isolated from total follicle cell preparations using Trizol Reagent (GibcoBRL). For the detection of mRNA, equal amounts of total RNA were used in 50μl reverse transcription reactions using the Sigma Enhanced RT-PCR Kit, according to the manufacturer's instructions. All PCR reactions took place over 40 cycles, except those for *rp49* (*RpL32* – FlyBase) which lasted for 20 cycles. For quantification, a 10 μl sample was collected from each reaction at cycles 5, 10 and 15 for *rp49* and at cycles 22, 25, and 30 for all other genes, and then subjected to electrophoresis through a 1.5% agarose gel. The following primers and annealing temperatures were used: ORC1 forward, 5′ AAATTCACTTGGAGCAGCCGG 3′ and ORC1 reverse, 5′ TGGGTTCTTGTACGGCCTC 3′ at 60°C; ORC2 forward, 5′ TGGGCTCCAAGCACCAGCTG 3′ and ORC2 reverse, 5′ AGCAGCGAGTTCTCGAACGC 3′ at 58°C; ORC5 forward, 5′ TGGAACAGTTCGCCCAGG 3′ and ORC5 reverse, 5′ GTGCACTGCAGCCTAGCG 3′ at 58°C; RNR2 (RNRS – FlyBase) forward, 5′ GTACCTGTTTAACGCTATCG 3′ and RNR2 reverse, 5′ AAACGGTCCGCTACGAAC 3′ at 55°C; PCNA (MUS209 – FlyBase) forward, 5′ GGCATGAATCTGGGCAG 3′ and PCNA reverse, 5′ AGCGGAACATCTGCGCA 3′ at 55°C; RP49 primers were purchased from OriGene Technologies and used at 55°C.

RESULTS

E2F2 binds DP in a manner similar to other E2F proteins

E2f2 encodes a protein of 370 amino acids containing an N-terminal DNA-binding domain, followed by a leucine zipper dimerization domain and a C-terminal pRB-interacting domain, each of which are similar to the corresponding region of other members of the E2F family (Fig. 1) (Sawado et al., 1998). A yeast two-hybrid assay was used to determine which regions of E2F2 are required for interaction with DP (Fig. 1). Both full-length E2F2 and $E2F2^{1\text{-}218}$, which contains only the DNA-binding and leucine zipper dimerization domains, were able to interact with DP as well as or better than E2F. A mutant capable of expressing the first 188 residues of E2F2 could also bind DP. This mutation was tested because $E2f2^{1\text{-}188}$ was used as an allele in our genetic studies. The phenotypes caused by this mutation in flies are described in the following sections. As expected, a mutant capable of expressing only the first 62

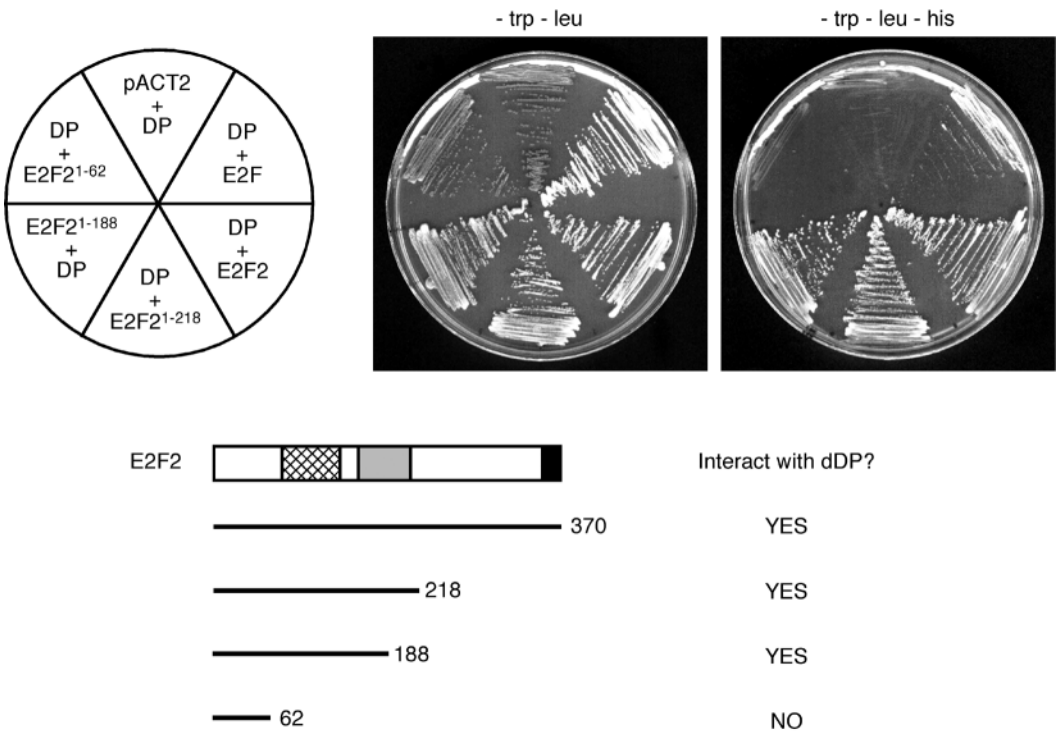

Fig. 1. E2F2 interacts with DP in a yeast two hybrid assay. DP was used as bait to investigate its ability to interact with full-length E2F2 and various C-terminal deletions of E2F2 constructed in the pACT2 activation domain vector. The DNA binding, dimerization and pRB interaction domains of E2F2 are indicated by hatched, shaded and black boxes, respectively. An interaction between DP and different E2F2 constructs was scored as the ability to grow on minimal media lacking adenine.

amino acids of E2F2 (i.e. truncated before the DNA-binding domain) was not able to interact with DP in this assay. Thus, the basic architecture of E2F2 is similar to other members of the E2F protein family.

Generation of E2F2 mutations

Mutation of either *E2f* or *Dp* inhibits the embryonic expression of several well established target genes (e.g. *RNR2*, *Cyclin E*, or *Mcm3*) that are used as indicators of 'E2F' function in vivo. We began our genetic analysis of *E2f2* by examining embryos homozygous for a deficiency (*Df(2L)DS8*) (Sinclair et al., 1980) that should remove the *E2f2* locus, located at cytological position 39B2-3 on chromosome 2 (Adams et al., 2000). In situ hybridization experiments were used to confirmed that *Df(2L)DS8* deletes *E2f2*. *E2f2* is expressed throughout embryonic development, with the highest levels detected in cycling cells (Fig. 2). This includes mitotically active cells during germband extended stages (Fig. 2C), as well as proliferating cells of the CNS and endoreduplicating cells of tissues such as the midgut at later embryonic stages (Fig. 2D,E). By contrast, *E2f2* mRNA was not detected in *Df(2L)DS8* homozygous mutant embryos at any stage after the onset of zygotic transcription (Fig. 2F). In spite of the disruption of *E2f2* gene expression in homozygous deficiency embryos, there was no alteration to the pattern of expression of *RNR2*, *Cyclin E* or *Mcm3* (not shown; stage 14 was the latest stage examined). This indicates either that E2F2 does not regulate these genes in the embryo, or that a maternal pool of E2F2 is sufficient to provide function during embryogenesis. Indeed, maternal *E2f2* mRNA can be detected either by

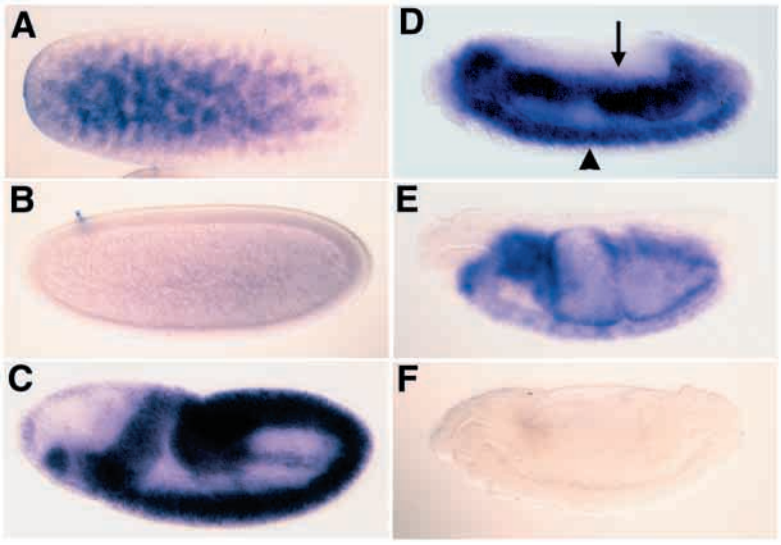

Fig. 2. Embryonic expression of *E2f2*. The expression of *E2f2* was analyzed by whole-mount in situ hybridization of wild-type embryos of different developmental stages using an antisense E2F2 cDNA probe. Embryos are oriented with anterior towards the left and dorsal towards the top. (A) Maternal *E2f2* mRNA can be detected in syncytial stage embryos. (B) A cellularizing blastoderm embryo, at which time maternal message has been destroyed. (C) A stage 10 germ band extended embryo. *E2f2* is expressed throughout the embryo during the post-blastoderm divisions, but its expression is not coupled to the cell cycle. (D) A stage 13 germband retracted embryo. *E2f2* expression is confined to dividing cells of the CNS (arrowhead) and endoreduplicating cells such as the gut (arrow). (E) A stage 15 germband retracted embryo. *E2f2* expression is still confined to dividing and endocycling cells, and its expression is still not coupled to a particular cell cycle phase. (F) A stage 14 *Df(2L)DS8/Df(2L)DS8* embryo. Zygotic *E2f2* expression is lacking, indicating that this deletion removes the *E2f2* gene.

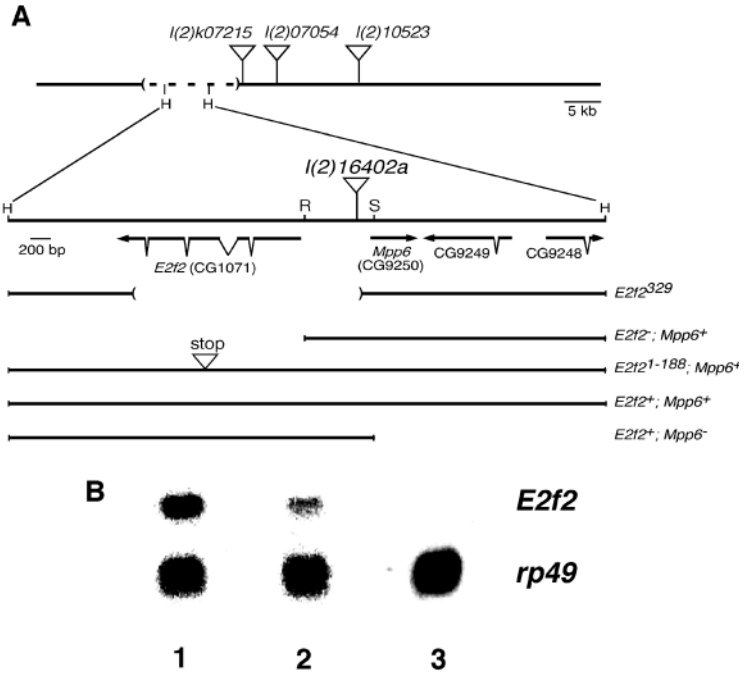

Fig. 3. The *E2f2* locus and construction of *E2f2* mutations. (A) The top line is a low resolution schematic of the *E2f2* locus located at 39B2-3 on the left arm of chromosome 2. Triangles represent the location of lethal P-element insertions in the region. The broken line indicates the extent of *Df(2L)1129*, a 12 kb deletion generated by transposase-mediated excision of *l(2)k07215*. This deletion uncovers the genes CG9246, CG9247, CG9248, CG9249, CG9250 (*Mpp6*) and CG1071 (*E2f2*). The second line is a higher resolution schematic of a 6.4 kb *Hin*dIII (H) restriction fragment isolated from a cosmid clone containing the *E2f2* locus. The intron-exon structure of *E2f2* and three other genes identified within this restriction fragment are shown beneath this line. *Mpp6* (CG9250) is divergently transcribed from *E2f2*, and its translational start site lies 900 bp upstream of the *E2f2* translational start site. We have not recovered *Mpp6* cDNAs, and the arrow represents a 450 bp open reading frame that predicts a 148 residue protein highly similar to mammalian M-phase phosphoprotein-6 (Matsumoto-Taniura et al., 1996). Only the first exon and intron of CG9248 are contained in the *Hin*dIII fragment shown. The next line indicates the extent of *Df(2L)E2f2*^{329}, which was generated by excision of *l(2)16402a*. The next series of lines each indicate a region of genomic DNA included in a P-element transgene used for constructing *E2f2* mutant flies (*E2f2*$^{-}$; *Mpp6*$^{+}$ and *E2f2*$^{1\text{-}188}$; *Mpp6*$^{+}$), or for rescuing *E2f2* mutant phenotypes (*E2f2*$^{+}$; *Mpp6*$^{+}$ and *E2f2*$^{+}$; *Mpp6*$^{-}$). R and S indicate the *Rsr*II and *Sac*II restriction sites used for constructing the P[*E2f2*$^{+}$; *Mpp6*$^{-}$] and P[*E2f2*$^{-}$; *Mpp6*$^{+}$] transgenes, respectively. (B) Northern blot hybridization of total RNA extracted from dissected ovaries and simultaneously hybridized with *E2f2* and *rp49* probes. *rp49* encodes a ribosomal protein and is used as a loading control. Lane 1, *yw*^{67} wild type; lane 2, *Df(2L)E2f2*^{329}/*E2f2*$^{1\text{-}188}$; Lane 3, *Df(2L)E2f2*^{329}/*Df(2L)E2f2*^{329}.

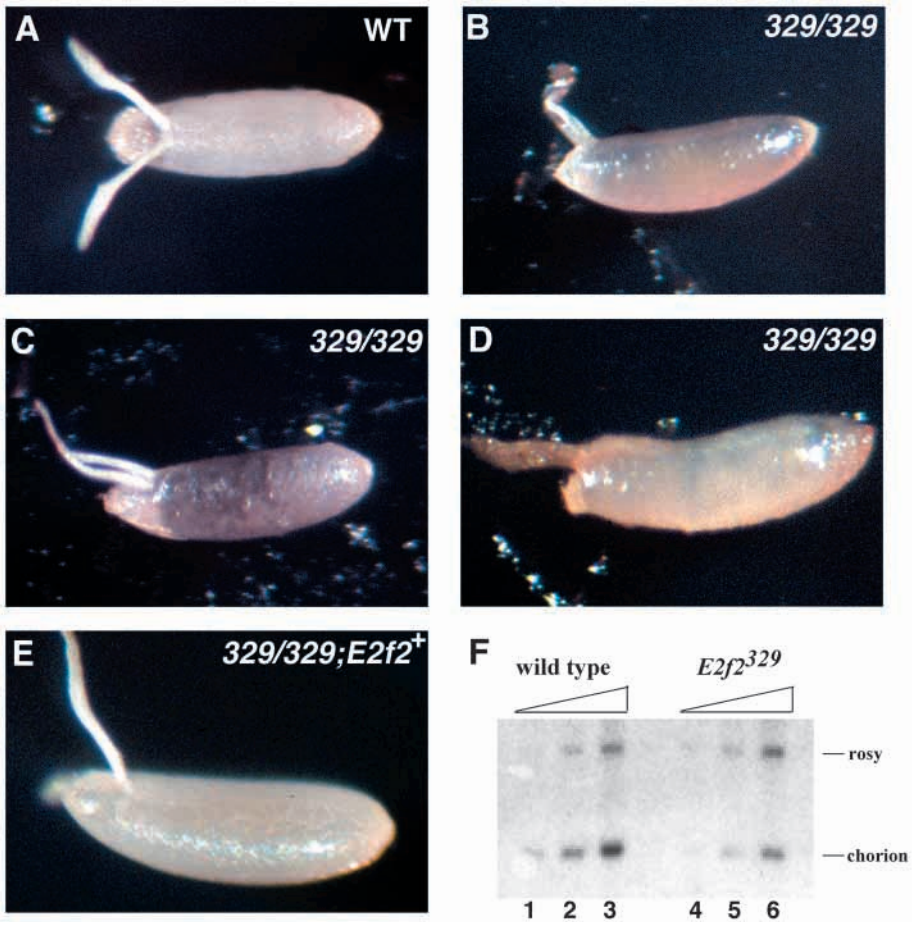

Fig. 4. Decreased chorion gene amplification and thin eggshells caused by loss of *E2f2*. (A) Photomicrograph of a *yw*^{67} wild-type egg from the dorsal perspective. All other panels show anterior towards the left and dorsal towards top. (B-D) Eggs laid by a *Df(2L)E2f2*^{329}/*Df(2L)E2f2*^{329} mutant females. Note that the chorions are more translucent compared with wild type. (E) Egg laid by a *Df(2L)E2f2*^{329}/*Df(2L)E2f2*^{329}; P[*E2f2*$^{+}$; *Mpp6*$^{+}$] female. (F) Southern hybridization was used to measure chorion gene amplification in stage 13 egg chambers dissected from *yw*^{67} wild type (lanes 1, 2, 3) and *Df(2L)E2f2*^{329}/*Df(2L)E2f2*^{329}; P[*E2f2*$^{-}$; *Mpp6*$^{+}$]/+ (lanes 4, 5, 6) females. Genomic DNA (1 μg, lanes 1 and 4; 2.5 μg, lanes 2 and 5; 5 μg, lanes 3 and 6) was simultaneously hybridized with a *rosy* gene probe and a third chromosome chorion gene cluster probe. The intensity of the 7.8 kb *rosy* fragment and the 3.8 kb chorion fragment were compared for each lane using a PhosphoImager. A 50% decrease in chorion gene amplification is reproducibly observed in *E2f2* mutants.

northern analysis (Sawado et al., 1998) or by in situ hybridization (Fig. 2A). This maternal mRNA is destroyed by the cellular blastoderm stage (Fig. 2B), indicating that any E2F2 activity in homozygous *Df(2L)DS8* deletion embryos would have to be provided by perdurance of maternal E2F2 protein.

In order to generate *E2f2* mutations, we identified a P-element insertion, *l(2)16402a*, located 856 and ~650 nucleotides upstream of the *E2f2* translation and transcription start sites, respectively (Fig. 3A). Transposase-mediated excision of *l(2)16402a* was used to recover a small deletion (*Df(2L)E2f2*^{329}) containing one breakpoint at the P-element insertion point and the other 11 bp downstream of the *E2f2* stop codon near the end of the transcription unit (Fig. 3A), thereby removing all *E2f2* coding sequence. Both *l(2)16402a* and)*Df(2L)E2f2*^{329} are 100% lethal in *trans* to each other, or in *trans* to deletions *Df(2L)DS8*, *Df(2L)TW161* (Wright et al., 1976) and *Df(2L)1129* (see Materials and Methods; Fig. 3A). However, the location of the *l(2)16402a* P-element insertion and the proximal breakpoint of the *Df(2L)E2f2*^{329} deletion made it uncertain whether the lethality in these situations was due to mutation of *E2f2*, or the divergently transcribed gene, *Mpp6* (Fig. 3A). Complementation analyses using P-element transgenes containing various fragments of genomic DNA from the region was used to distinguish between these possibilities (Fig. 3A). Both a transgene containing *E2f2* and *Mpp6* (P[*E2f2*$^{+}$; *Mpp6*$^{+}$]) as well as a transgene containing *Mpp6* but lacking *E2f2* (P[*E2f2*$^{-}$; *Mpp6*$^{+}$]) rescued the lethality of *l(2)16402a* and *Df(2L)E2f2*^{329}, whereas a transgene containing only *E2f2* (P[*E2f2*$^{+}$; *Mpp6*$^{-}$]) did not. Therefore, the lethality of both *l(2)16402a* and *Df(2L)E2f2*^{329} is due to mutation of *Mpp6* and not *E2f2*. The P-element insertion site and the *Df(2L)E2f2*^{329} breakpoint are very close to the transcription start site of *Mpp6* (Frolov et al., 2001), and therefore probably disrupts expression of the gene.

In combination with the *l(2)16402a* and *Df(2L)E2f2*^{329} mutations, the genomic transgenes containing *Mpp6* provided a way of engineering *E2f2* mutant strains. Flies were generated that were homozygous for mutations of the *E2f2* locus (e.g. *Df(2L)E2f2*^{329}/*Df(2L)E2f2*^{329}) and that also contained a transgene providing *Mpp6* function. Two such transgenes were used for this purpose (Fig. 3A). The first, P[*E2f2*$^{-}$; *Mpp6*$^{+}$], lacks *E2f2* entirely and provided the null situation. This was confirmed by demonstrating that the single *E2f2* mRNA species observed in wild-type ovary RNA preparations is not detected in the null mutant (Fig. 3B). The second transgene, P[*E2f2*$^{1\text{-}188}$; *Mpp6*$^{+}$], contains a small internal deletion within exon 3 of the *E2f2* gene that arose spontaneously during propagation of a plasmid in *E. coli*. This deletion causes a frameshift predicted to produce a truncated protein lacking the pRB binding domain located at the C terminus. As described above, E2F2$^{1\text{-}188}$ is capable of binding DP in a two hybrid assay (Fig. 1). However, the amount of mRNA expressed from *E2f2*$^{1\text{-}188}$ is substantially reduced relative to wild type (Fig. 3B). Together, these data suggest that *E2f2*$^{1\text{-}188}$ produces low levels of a protein capable of binding DP, but incapable of binding RBF.

Null mutants of *E2f2* cause a severe reduction in female fertility

Flies that completely lack *E2f2* or are hemizygous for the *E2f2*$^{1\text{-}188}$ allele eclose at near wild-type Mendelian frequency with no overt morphological defects, indicating that zygotic expression of *E2f2* is not essential for development. While adult *E2f2* mutant males are fully fertile, mutant females have significantly reduced fertility, suggesting that oogenesis is perturbed by loss of *E2f2* function. The ovaries of *E2f2* mutant females do not mature as rapidly as wild type after adult eclosion: late stage egg chambers are not visible in dissected mutant ovaries until ~5 days after eclosion, compared with ~1.5 days for wild type. After 6 days, the mutant females lay eggs at a rate similar to wild type. However, only 10% and 8% of eggs laid by *Df(2L)E2f2*^{329}/*Df(2L)E2f2*^{329} and *Df(2L)E2f2*^{329}/*E2f2*$^{1\text{-}188}$ females hatch, respectively. The latter phenotype is substantially rescued by a *E2f2* transgene (Table 1). The eggs that do hatch are able to develop and produce viable adult flies. More than 90% of the eggs laid by *E2f2*$^{1\text{-}188}$/*Df(2L)E2f2*^{329} and *Df(2L)E2f2*^{329}/*Df(2L)E2f2*^{329} females have a defective eggshell, or chorion (Fig. 4A-D; Table 2), and the null phenotype is rescued by a *E2f2* transgene (Fig. 4E; Table 2). The mutant chorions appear far more translucent than the normally opaque wild-type chorion (Fig. 4A-D), which is indicative of a thin eggshell. The eggs are more fragile than wild type, and many appear collapsed.

These phenotypes caused us to begin examining oogenesis in *E2f2* mutant females, focussing in particular on follicle cells, because of the chorion defects. The *Drosophila* eggshell is a crosslinked protein matrix composed of several chorion proteins produced by a single epithelial follicle cell layer that surrounds the developing oocyte (Spradling, 1993). As described above, the chorion protein biosynthetic capacity of the follicle cells relies on a developmentally controlled cell cycle program that includes both polyploidization and amplification of two clusters of chorion genes, one on the X chromosome and the other on chromosome 3. The 60- to 80-fold amplification of chorion genes assures that sufficient amounts of chorion proteins are rapidly synthesized during eggshell formation. Southern hybridization experiments were carried out to investigate whether *E2f2* mutants had a reduced level of chorion gene amplification (Fig. 4F). DNA isolated from stage 13 egg chambers dissected from wild-type and

Table 1. Hatch rates of eggs laid by *E2f2* mutant females

Genotype	% of eggs that hatch	Number of eggs scored
+/Df(2L)E2f2^{329}	99	749
Df(2L)E2f2^{329}/*Df(2L)E2f2*^{329}	10	712
E2f2$^{1\text{-}188}$/*Df(2L)E2f2*^{329}	8	1275
E2f2$^{1\text{-}188}$/*Df(2L)E2f2*^{329}*; P[E2f2]*	72	397
E2f2$^{1\text{-}188}$/*E2f2*^{16402a}	44	1062

Table 2. Eggshell phenotype of *E2f2* mutant females

Genotype	% eggs with thin chorion*	Number of eggs scored
+/Df(2L)E2f2^{329}	10	749
E2f2$^{1\text{-}188}$/*Df(2L)E2f2*^{329}	93	1176
Df(2L)E2f2^{329}/*Df(2L)E2f2*^{329}	97	714
Df(2L)E2f2^{329}/*Df(2L)E2f2*^{329}*; P[E2f2]*	6	594
E2f2$^{1\text{-}188}$/*E2f2*^{16402a}	84	673

*Any egg displaying any one of the following phenotypes was included in this category: (1) lucid chorion, (2) collapsed egg and (3) irregularities in the chorion (e.g. lucid or 'thin' patches). See Fig. 4.

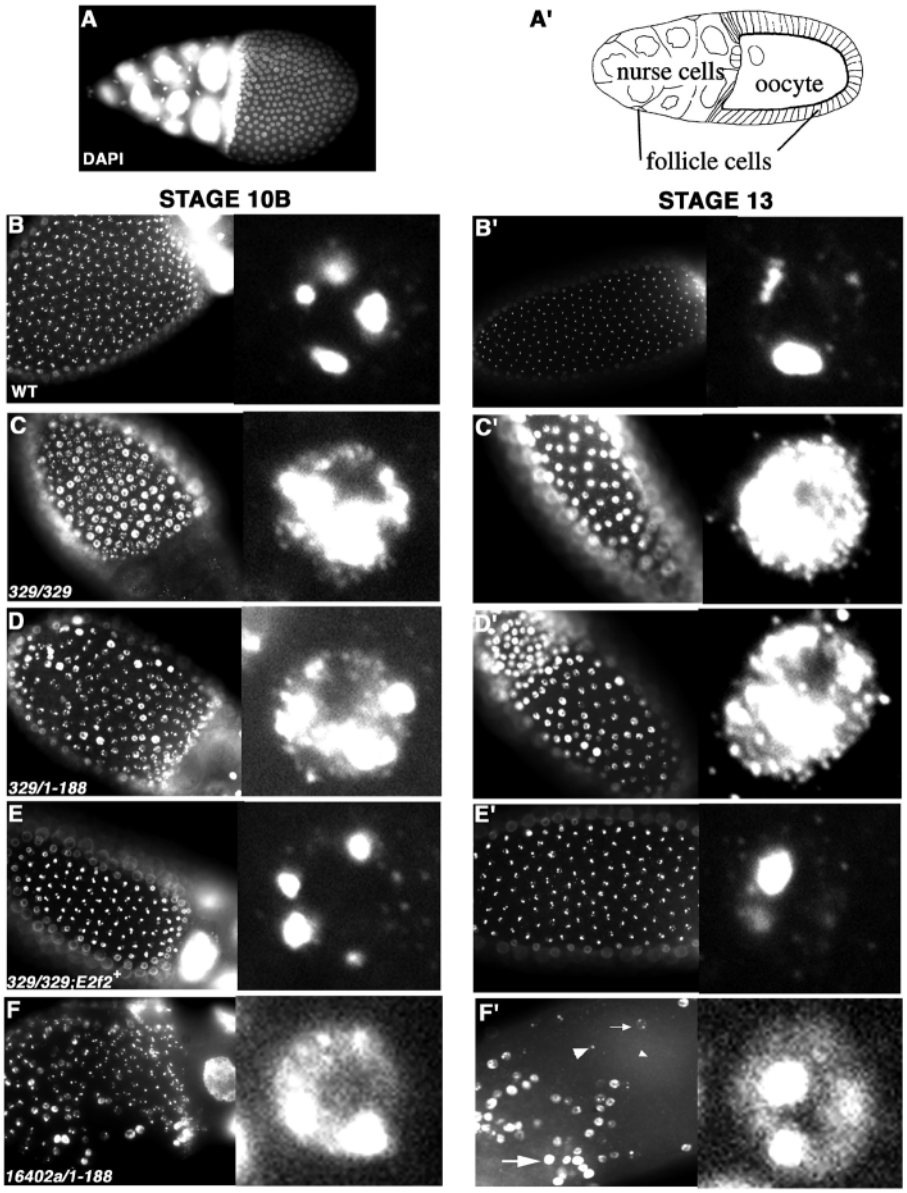

Fig. 5. *E2f2* restricts follicle cell DNA replication to gene amplification foci. (A) A wild-type stage 10B egg chambers stained with DAPI. (A′) Schematic of a wild-type stage 10B egg chamber. At this stage, the columnar follicle cells that execute chorion gene amplification and that will eventually secrete the eggshell surround the developing oocyte in a single epithelial layer. Dissected ovaries were pulse labeled with BrdU for 1 hour and then immediately fixed. Incorporated BrdU was detected by indirect immunofluorescence. (B-F) Stage 10B egg chambers; (B′-F′) stage 13 egg chambers. The left image in each panel is a surface view of the follicle cell epithelium located over the oocyte. The right image in each panel is a high magnification view of a single follicle cell nucleus. (B) yw^{67} wild type. Distinct foci of BrdU incorporation corresponding with sites of gene amplification are observed in wild type. (C) $Df(2L)E2f2^{329}/Df(2L)E2f2^{329}$; P[$E2f2^{-}$; $Mpp6^{+}$]/+. BrdU incorporation is detected throughout *E2f2* null mutant follicle cells. (D) $Df(2L)E2f2^{329}/Df(2L)DS8$, P[$E2f2^{1\text{-}188}$; $Mpp6^{+}$]. This phenotype is similar to null at stage 13, but slightly weaker at stage 10B. (E) $Df(2L)E2f2^{329}/Df(2L)E2f2^{329}$; P[$E2f2^{+}$; $Mpp6^{+}$]/+. A wild type *E2f2* transgene rescues the null mutant phenotype. (F) $E2F2^{16402a}/Df(2L)DS8$, P[$E2f2^{1\text{-}188}$; $Mpp6^{+}$]. In this hypomorphic situation, different classes of replication patterns are apparent (best seen in F′): replication throughout the entire nucleus (large arrow), normal gene amplification foci (large arrowhead), absent replication (small arrowhead), and both amplification foci and genomic replication (small arrow).

$Df(2L)E2f2^{329}/Df(2L)E2f2^{329}$ mutant ovaries was probed with a 3.8 kb chromosome 3 chorion gene cluster probe. A probe from the *rosy* gene was used as a non-amplified control. In four independent experiments, a comparison of signal intensities between *rosy* and chorion genes indicated that chorion gene amplification was reduced two to three times by mutation of *E2f2* when compared with wild type (Fig. 4F).

E2f2 mutants display inappropriate genomic replication in late stage follicle cells

The reduced chorion gene amplification in *E2f2* mutant females suggests that E2F2 plays a role in the developmentally regulated cell cycle program in follicle cells. Moreover, female sterile alleles of *Dp*, *E2f* and *Rbf*, each cause follicle cell replication defects (Bosco et al., 2001; Royzman et al., 1999). DNA synthesis occurring during follicle cell endocycles and chorion gene amplification can be visualized in situ by BrdU pulse labeling of dissected ovaries (Calvi et al., 1998; Calvi and Spradling, 1999). By stage 10B of oogenesis in wild type (Fig. 5A,A′), all follicle cells have exited endoreduplication cycles and have begun chorion gene amplification. While follicle cell endocycles within each egg chamber are asynchronous, gene amplification occurs simultaneously throughout the epithelium (Calvi et al., 1998). Consequently, at stage 10B, BrdU incorporation is detected within every follicle cell in at least four subnuclear foci, the two largest of which correspond to the chorion gene clusters on chromosomes X and 3 (the others remain unidentified) (Calvi et al., 1998) (Fig. 5B). Gene amplification continues through stage 13 (7 hours older than stage 10B), at which point BrdU incorporation at the chromosome 3 chorion cluster predominates (Fig. 5B′). *E2f2* mutant follicle cells have a very different profile of BrdU incorporation. In stage 10B and later $Df(2L)E2f2^{329}/Df(2L)E2f2^{329}$ or $Df(2L)E2f2^{329}/E2f2^{1\text{-}188}$ egg chambers, BrdU incorporation was observed throughout the entire nucleus, rather than in the characteristic subnuclear foci (Fig. 5C,D, respectively). There was cell to cell variability in the intensity of ectopic genomic BrdU labeling at stage 10B, with some nuclei having quite little or no ectopic

replication. This variability was slightly more pronounced in the $E2f2^{1\text{-}188}$ hemizygote nuclei compared with the $Df(2L)E2f2^{329}$ deletion, suggesting that $E2f2^{1\text{-}188}$ is not null (compare Fig. 5C with 5D). By stage 13 every follicle cell nucleus in the null situation displayed intense genomic replication (Fig. 5C′). Similarly, the severity of the $Df(2L)E2f2^{329}/E2f2^{1\text{-}188}$ phenotype increased by stage 13, but again there was more cell to cell variability in the intensity of labeling compared to *E2f2* null cells (Fig. 5D′). Inclusion of a wild-type *E2f2* transgene restores the aberrant BrdU incorporation pattern in $Df(2L)E2f2^{329}/Df(2L)E2f2^{329}$ egg chambers to normal (Fig. 5E,E′; Table 3). These data suggest that at the time DNA synthesis is normally restricted to chorion gene amplification, replication is instead occurring throughout the entire genome in the *E2f2* mutant follicle cells.

The penetrance of the ectopic genomic replication phenotype is virtually 100% in either $Df(2L)E2f2^{329}/Df(2L)E2f2^{329}$ or $Df(2L)E2f2^{329}/E2f2^{1\text{-}188}$ mutants, in that all egg chambers contain many cells with inappropriate genomic replication (Table 3). By contrast, egg chambers of the genotype $E2f2^{16402a}/E2f2^{1\text{-}188}$ had a strong but less penetrant phenotype: 88% of the egg chambers scored had some follicle cells with inappropriate genomic replication at or after stage 10B (Table 3). In addition, of those $E2f2^{16402a}/E2f2^{1\text{-}188}$ egg chambers that scored as mutant, the number of follicle cells that were undergoing genomic replication was clearly less than 100%. That is, some of the cells displayed genomic replication (large arrow, Fig. 5F′) while other cells displayed normal amplification foci (large arrowhead, Fig. 5F′). In addition, two other classes of aberrant BrdU incorporation patterns were observed in $E2f2^{16402a}/E2f2^{1\text{-}188}$ follicle cells. In some nuclei, both gene amplification foci and genomic BrdU incorporation were apparent in the same nucleus (small arrow Fig. 5′), while in others no BrdU incorporation was detected, suggesting that neither gene amplification or genomic replication was occurring (small arrowhead, Fig. 5F′). This variable penetrance and expressivity suggests that the *l(2)16402a* mutation is hypomorphic for *E2f2* function, perhaps because the P-element insertion reduces *E2f2* gene expression (as it likely does for *Mpp6* as well). Consistent with this interpretation, eggs laid by $E2f2^{16402a}/E2f2^{1\text{-}188}$ mothers have a less severe phenotype compared to those from null allele combinations when scoring hatching frequency (Table 1) and the proportion of eggs with a defective chorion (Table 2).

Table 3. Penetrance of the follicle cell replication phenotype in *E2f2* mutant ovaries

Genotype	% egg chambers with mutant follicle cell replication pattern*	Number of egg chambers scored†
$E2f2^{1\text{-}188}/Df(2L)E2f2^{329}$	100	76
$E2f2^{1\text{-}188}/Df(2L)E2f2^{329}$; P[E2f2]	49	62
$Df(2L)E2f2^{329}/Df(2L)E2f2^{329}$	99	148
$Df(2L)E2f2^{329}/Df(2L)E2f2^{329}$; P[E2f2]	5	106
$E2f2^{16402a}/E2f2^{1\text{-}188}$	88	107

*Defined as egg chambers with at least ~10 nuclei displaying ectopic genomic replication.
†Includes only stage 10B through stage 13 egg chambers.

E2f2 mutant follicle cells do not complete an additional endo S phase

One possible cause of the ectopic genomic replication in stage 10B-13 *E2f2* mutant egg chambers is that the cells inappropriately enter an additional endocycle in the later developmental stages. In this case, follicle cell ploidy should increase from 16C to 32C. This was observed in an *Rbf* female sterile mutant, which cytologically causes a similar BrdU incorporation phenotype to *E2f2* mutants (Bosco et al., 2001) (Fig. 6E,F). FACS profiles of DAPI- or PI-stained nuclei isolated from whole ovaries have been previously used to determine follicle cell ploidy (Asano and Wharton, 1999; Bosco et al., 2001; Lilly and Spradling, 1996). Using this method, we were unable to distinguish a significant difference in the FACS profile between $Df(2L)E2f2^{329}/Df(2L)E2f2^{329}$ or $Df(2L)E2f2^{329}/E2f2^{1\text{-}188}$ mutant nuclei and wild-type controls (Fig. 6A). Each profile contained four prominent peaks representing 2C-16C follicle cell nuclei (Lilly and Spradling, 1996). While a small 32C peak was occasionally observed in the mutants (Fig. 6B), it was not reproducible. Moreover, other cell types can lead to the appearance of a small 32C peak in some wild-type preparations (Fig. 6A). To circumvent these problems, we analyzed pure populations of follicle cells at stage 9 and beyond. This was achieved by FACS analysis of trypsin dissociated ovaries (Bryant et al., 1999) using a follicle cell-specific GAL4 driver (c323) (Manseau et al., 1997) to induce a UAS-GFP transgene. The driver is activated initially at stage 9 in wild-type egg chambers, and persists until the end of oogenesis (Calvi et al., 1998). Importantly, the expression of c323-induced GFP expression in *E2f2* mutant egg chambers occurs during the same developmental stages as wild type (not shown). This indicates that mutation of *E2f2* does not affect developmental control of the c323 driver, allowing us to compare directly ploidy values between the same population of wild-type and mutant follicle cells. Using this technique, wild-type 8C and 16C GFP-positive follicle cells were readily distinguished as a subset of the entire FACS profile obtained by staining the cells with the DNA binding dye Hoechst 33342 (Fig. 6C). Interestingly, in the $Df(2L)E2f2^{329}/E2f2^{1\text{-}188}$ mutant profile there was no indication of a 32C cell population (Fig. 6D). By contrast, follicle cells isolated from Rbf^{120}/Rbf^{14} mutant egg chambers clearly contain a 32C population that is not detected in wild type preparations (Fig. 6E,F). Taken together, these data indicate that the ectopic genomic BrdU incorporation seen in *E2f2* mutant follicle cells does not result from an additional, complete endocycle S phase.

The conversion from asynchronous follicle cell endocycles to synchronous gene amplification is under developmental control. This can be seen at successive stages of wild-type egg chamber development, where the proportion of BrdU-positive cells within the follicle cell epithelium decreases until very few cells are replicating at stage 10A, immediately before the onset of gene amplification (Calvi et al., 1998). To determine whether *E2f2* mutant follicle cells are responding to the developmental cues that terminate endocycle DNA synthesis, the number of BrdU positive nuclei in pre-stage 10B wild-type and *E2f2* mutant egg chambers was determined (Fig. 7). The number of BrdU positive follicle cell nuclei in pre-stage 10B $Df(2L)E2f2^{329}/Df(2L)E2f2^{329}$ mutant egg chambers was similar to wild type. Importantly, stage 10A mutant egg chambers contained very few BrdU-positive cells, just as in wild type (Fig. 7). This observation suggests that *E2f2* mutant

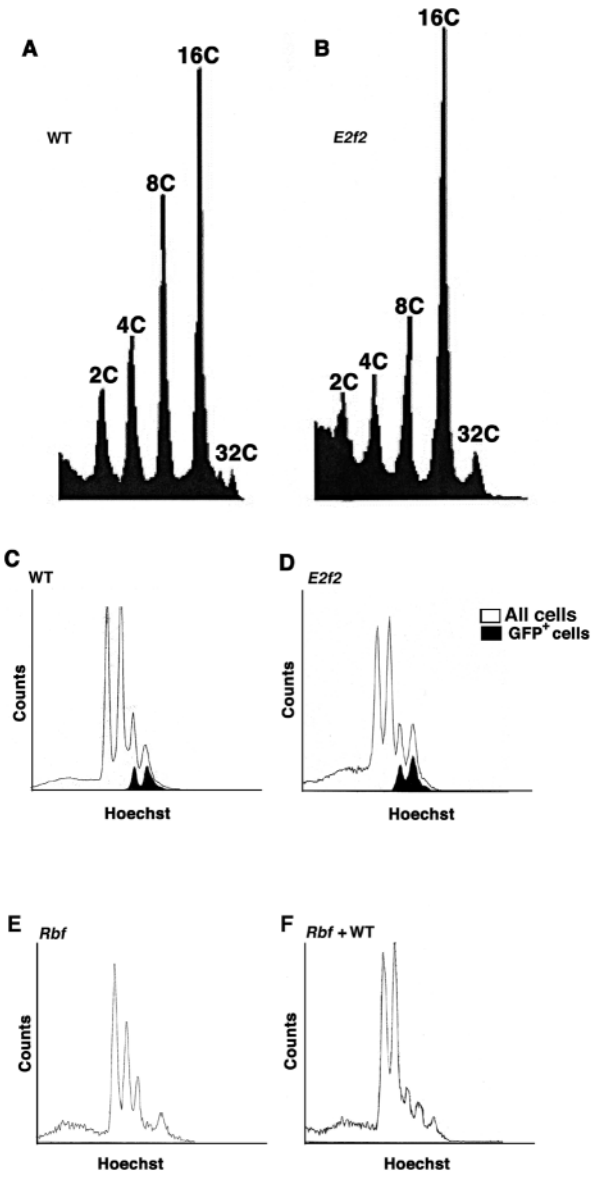

Fig. 6. DNA content of *E2f2* mutant follicle cells determined by FACS analysis. (A) Nuclei preparations from yw^{67} wild-type ovaries were stained with propidium iodide and subjected to FACS analysis. Nuclei with 2C DNA content are from mitotically active follicle cells, and the three follicle cell endocycles give rise to the 4C, 8C and 16C nuclei. A small 32C peak is always observed in wild type, probably from other less abundant cell types in the ovary (e.g. nurse cells). (B) FACS profile of nuclei prepared from $Df(2L)E2f2^{329}/Df(2L)DS8$, P[$E2f2^{1\text{-}188}$; $Mpp6^{+}$] ovaries. The size of the 32C peak varies between preparations, with this particular profile containing the largest peak obtained. (C) Intact follicle cells were prepared from c323:GAL4/+; $Df(2L)E2f2^{329}$/+; UAS-GFP/+ ovaries and subjected to FACS analysis. The open profile represents signal from the DNA-binding dye Hoechst 33342, and the shaded profile represents GFP-positive cells. The GAL4 driver begins expression during stage 9 and continues until the end of oogenesis. By stage 9, all follicle cells have completed the first endocycle S phase, and therefore GFP-positive cells are only found in the 8C and 16C populations. (D) FACS profile of follicle cell preparations from c323:GAL4/+; $Df(2L)E2f2^{329}/Df(2L)DS8$, P[$E2f2^{1\text{-}188}$; $Mpp6^{+}$]; UAS-GFP/+ ovaries. Note that the profile is similar to wild type, indicating that *E2f2* mutant follicle cells do not achieve a 32C ploidy value. (E) FACS profile of follicle cell preparations from Rbf^{120}/Rbf^{14} ovaries. Note the population of cells at 32C. (F) Equal amounts of follicle cells prepared from yw^{67} and Rbf^{120}/Rbf^{14} ovaries were mixed prior to FACS analysis, because the 16C peak was reproducibly small in the *Rbf* mutant preparations. Note that five peaks are clearly visible when compared with wild type (C).

follicle cells terminate the endocycles at the normal developmental time. From these data and the FACS analysis, we interpret the inappropriate BrdU incorporation in *E2f2* mutant follicle cells as a failure to adequately restrict DNA synthesis to sites of gene amplification, rather than as a continuation of endocycles (see Discussion). Thus, E2F2 appears to facilitate the conversion to the gene amplification phase of follicle cell development by preventing the cells from initiating genomic DNA replication in response to signals that trigger the onset of synchronous gene amplification.

Localization of replication factors to amplification foci is disrupted in *E2f2* mutants

Proteins of the ORC complex assemble at origins of DNA replication and recruit factors (e.g. CDC45L) required to initiate bi-directional DNA synthesis (Takisawa et al., 2000). During oogenesis, localization of different ORC proteins within the follicle cell nuclei is dynamically regulated, coincident with changing patterns of DNA replication. It has been shown previously by immunodetection that ORC1, ORC2, ORC5, and CDC45L are distributed throughout the entire follicle cell nucleus when these cells are performing genomic replication during endocycle S phase, and that after stage 10, these proteins are detected in foci that correlate with sites of chorion gene amplification (Asano and Wharton, 1999; Loebel et al., 2000; Royzman et al., 1999). As *E2f2* mutant follicle cells fail to restrict DNA replication to gene amplification foci, we determined the localization of replication factors in follicle cells. Anti-ORC2, -ORC5 and CDC45L antibodies each label sites of chorion gene amplification in wild-type stage 10B egg chambers (Fig. 8A), presumably because these are the major sites of active DNA

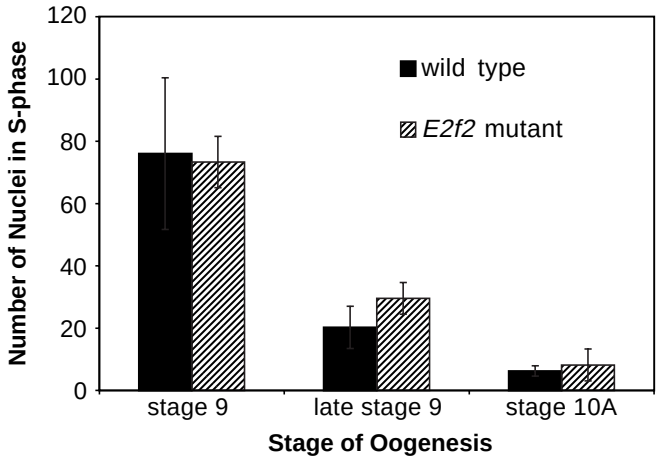

Fig. 7. *E2f2* mutant follicle cells terminate endocycles on schedule. BrdU-positive nuclei were counted in photomicrographs of stage 9 and stage 10 egg chambers dissected from wild-type and $Df(2L)E2f2^{329}/Df(2L)E2f2^{329}$ mutant females. Each measurement represents an average of counts from 8-10 egg chambers.

synthesis. This is consistent with the known role of these proteins in replication origin firing, and indeed female sterile mutants of *Orc2* have reduced chorion gene amplification (Landis et al., 1997). In *E2f2* mutant egg chambers, the distinct localization pattern of these replication proteins is lost, resulting in the detection of all three proteins throughout the entire nucleus (Fig. 8B). This phenotype is rescued by a *E2f2* transgene (Fig. 8C). These data are consistent with the firing of origins in addition to those at the chorion loci causing inappropriate genomic DNA synthesis. Whether the mis-localization of ORC components and CDC45 in *E2f2* mutants is a direct cause or a consequence of the ongoing ectopic replication is not known.

Expression of E2F target genes in *E2f2* mutant follicle cells

One possible mechanism by which E2F2 could inhibit DNA replication is to act as a transcription factor to modulate the expression of at least one crucial replication factor. For example, if E2F2 was part of a repressor complex, loss of *E2f2* function could lead to increases in replication gene expression that might trigger widespread DNA synthesis. In order to test this idea, we examined the abundance of several mRNAs encoded by genes either known to be (e.g. *Orc1*, *RNR2*, *PCNA*) (Asano and Wharton, 1999; Duronio and O'Farrell, 1995) or possibly (e.g. *Orc2* and *Orc5*) regulated by E2F. RNA was extracted from total follicle cell preparations and subjected to RT-PCR (see Materials and Methods). Relative to *rp49* controls, more *Orc5* mRNA was reproducibly (n=4) detected in $Df(2L)E2f2^{329}/Df(2L)E2f2^{329}$ or $Df(2L)E2f2^{329}/E2f^{1\text{-}188}$ mutant samples compared with wild type (Fig. 9). An increased *Orc2* mRNA level was also detected in some experiments (two out of four). For *Orc1*, *RNR2* and *PCNA* there was no substantial difference in the amount of mRNA detected between wild type and *E2f2* mutants (Fig. 9). These data suggest (1) that E2F target genes are expressed at or above wild-type levels after loss of *E2f2* function and (2) that de-repression of specific target genes, such as those encoding members of the ORC complex, could contribute to the inappropriate DNA synthesis seen in *E2f2* mutant follicle cells.

DISCUSSION

In the *Drosophila* ovary, both polyploidization and gene amplification are required for follicle cells to acquire the biosynthetic capability needed to produce the eggshell. We show that E2F2 acts in follicle cells to restrict DNA synthesis to sites of chorion gene amplification by preventing genomic replication.

Chorion gene amplification is characterized by the repeated firing of an origin of DNA replication within the chorion locus. The mechanisms by which chorion replication origins are repeatedly used while other origins are not, is unclear. Two general possibilities exist: either the assembly of the pre-RC required for initiating DNA synthesis is confined to sites of gene amplification, or pre-RC assembly occurs throughout the genome and initiation is confined to amplification sites. Two distinct phases of follicle cell gene amplification have been detected. The first phase occurs during endocycles, where at least the third chromosome chorion locus amplifies to low levels during each endo S phase (Calvi et al., 1998; Royzman et al., 1999). The second phase occurs synchronously during stage 10B at several loci, including both of the two chorion gene clusters, after the termination of endocycles (Calvi et al., 1998). *E2f2* mutant egg chambers display defects in this second phase, failing to restrict DNA synthesis to amplification foci. This cellular phenotype is associated with an approximate halving in chorion gene copy number and production of eggs with a thin chorion. However, this apparent amplification defect is much less severe than other mutations (e.g. *Orc2* and *chiffon/dbf4*), which virtually eliminate chorion gene amplification and cause a thin eggshell phenotype (Landis et al., 1997; Landis and Tower, 1999). Consequently, while a small reduction in chorion gene copy number caused by mutation of *E2f2* could contribute somewhat to defective chorion biosynthesis, this may not be the sole cause of the observed chorion defects. Similarly, the reduced fertility of *E2f2* mutant females may not result entirely from desiccation caused by a thin eggshell, owing to follicle cell defects. Although it is highly likely that the follicle cell replication defects are an indication that *E2f2* activity is required in this cell type, *E2f2* may play additional roles in the germline. Indeed, *Dp* mutants have germline defects that disrupt

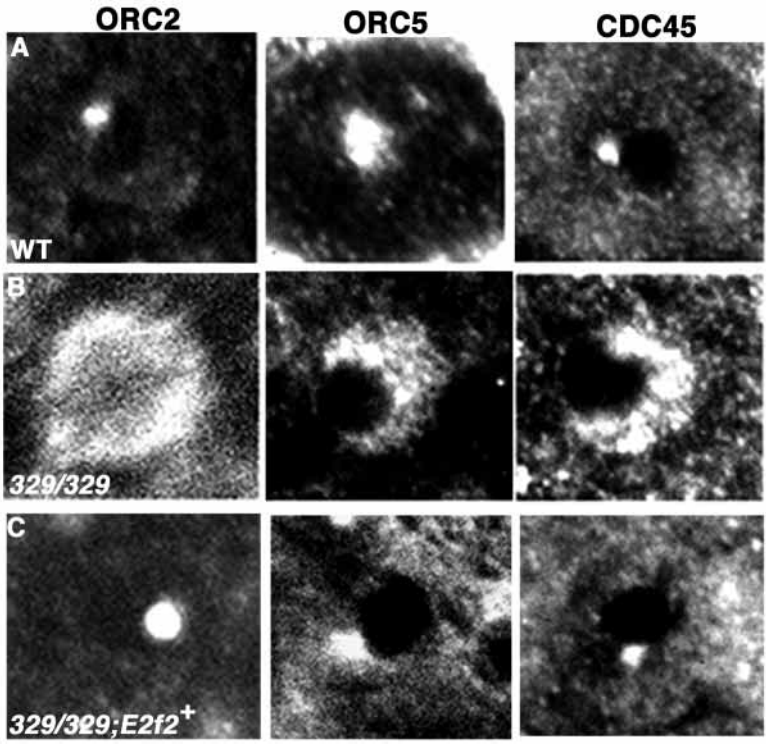

Fig. 8. Replication initiation proteins are mis-localized in *E2f2* mutant follicle cell nuclei. Ovaries were dissected, fixed and treated with anti-ORC2 (left panels), anti-ORC5 (middle panels) or anti-CDC45L (right panels) antibodies. An image of a single nucleus from a stage 10B egg chamber is shown in each panel. (A) yw^{67} wild type. These three proteins are detected in a focus coincident with the third chromosome chorion cluster.
(B) $Df(2L)E2f2^{329}/Df(2L)E2f2^{329}$; P[$E2f2^{-}$; $Mpp6^{+}$]/+. In the mutant, these replication proteins are not localized. (C) $Df(2L)E2f2^{329}/Df(2L)E2f2^{329}$; P[$E2f2^{+}$; $Mpp6^{+}$]/+. Mis-localization in the mutant is rescued by a wild-type *E2f2* transgene.

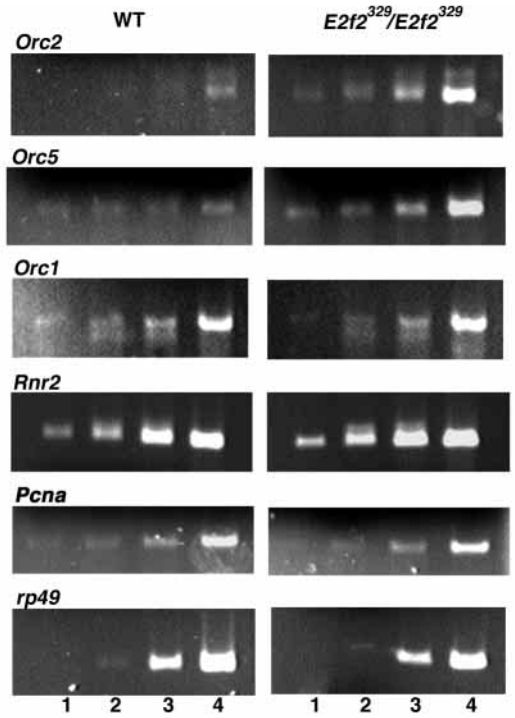

Fig. 9. Gene expression analysis in *E2f2* mutant follicle cells. Total RNA was isolated from intact follicle cells prepared by trypsin dissociation of dissected ovaries and subjected to RT-PCR analysis with gene specific primers. Left panels are from yw^{67} wild-type RNA, and right panels are from *Df(2L)E2f2*^{329}/*Df(2L)DS8*, P[$E2f2^{1\text{-}188}$; $Mpp6^{+}$] RNA. RT-PCR reactions were sampled at cycle 22 (lane 1), cycle 25 (lane 2), cycle 30 (lane 3) and cycle 40 (lane 4) for ORC2, ORC5, ORC1, RNR2 and PCNA, and cycles 5, 10, 15 and 20, for rp49 (lanes 1-4, respectively).

oogenesis (Myster et al., 2000; Royzman et al., 1999). Thus, determining the cellular basis for the reduced fertility of *E2f2* mutant females will require an analysis of genetically mosaic ovaries.

Possible interpretations of the DNA synthesis phenotype depend on when during follicle cell development *E2f2* primarily functions. If, for example, *E2f2* acts during the endocycles, which in wild type generate cells with a 16C DNA content, then the ectopic replication in *E2f2* mutants could represent the inappropriate continuation of endocycle S phase into late stages of oogenesis. Our data do not support this model, as we were unable to observe a mutant follicle cell population with a 32C or greater DNA content. In addition, the number and pattern of BrdU-positive nuclei in pre-stage 10B egg chambers is the same in mutant and wild type, including a cessation of endocycles just before the onset of gene amplification. This suggests that the early program of endocycles terminates on schedule in the *E2f2* mutants. An alternative explanation for the *E2f2* mutant phenotype is that endocycles are actually *delayed* relative to egg chamber stage. In this scenario, genomic DNA synthesis occurring at stages 10B and later would represent an endo S phase of cells with less than a 16C DNA content. Consequently, if genomic replication was delayed relative to morphological development, then when compared with wild type, a larger fraction of the GFP-positive cell population in *E2f2* mutants should contain cells with less than a 16C DNA content, possibly including 4C cells. This is true because only those cells within stage 9 and older egg chambers in both mutant and wild type are GFP positive. We could find no evidence by FACS analysis of a GFP-positive 4C follicle cell population in *E2f2* mutants, nor a reproducible increase in the number of cells with less than 16C DNA content compared with wild type. While we cannot rule out a minor delay in late endocycles, we currently favor the interpretation that the consequences of E2F2 function are manifested specifically during the synchronous phase of gene amplification either to prevent pre-RC assembly at ectopic locations or to confine firing to sites of amplification.

How might loss of *E2f2* cause this? One possibility is that E2F2 is part of a transcription repressor complex, and that loss of *E2f2* function leads to an increase in the expression of genes encoding limiting replication components. An increase in the level of *Orc2* and *Orc5* mRNA was detected by RT-PCR, suggesting that inappropriate DNA synthesis could be triggered by increased accumulation of pre-RC components. Consistent with this hypothesis, a two- to threefold increase in the expression of ORC1 using a heat shock promoter stimulates an ectopic genomic replication phenotype in follicle cells similar to that caused by mutation of *E2f2* (Asano and Wharton, 1999). How an increased abundance of ORC proteins could trigger ectopic replication is not clear, but the observation fits a model in which limited assembly of pre-RC helps confine DNA synthesis to sites of gene amplification. Alternatively, *E2f2* could positively regulate the expression of a ‘specificity factor’ that acts to confine replication to sites of gene amplification. These questions could be addressed through comprehensive analyses of mRNA abundance between mutant and wild-type follicle cells.

There are alternatives to gene expression based models for modulation of DNA synthesis in follicle cells. E2F/DP/RBF complexes control the extent of chorion gene amplification (Bosco et al., 2001; Royzman et al., 1999), and may do so via a direct interaction with the pre-RC. E2F, DP and RBF co-immunoprecipitate with ORC2 (Bosco et al., 2001), and both E2F and ORC2 associate with chorion DNA in chromatin IP assays (Austin et al., 1999; Bosco et al., 2001). In addition, direct regulation of replication origins by chromatin bound pRB/E2F complexes may be conserved: in cultured primary mammalian cells, pRB, p107 and p130 are localized to sites of DNA synthesis early in S phase and may help control the nuclear organization of replication (Kennedy et al., 2000). Perhaps DNA-bound E2F2 controls the localization and/or assembly of replication factors at origins throughout the genome. Indeed, *E2f2* is required for the proper localization of ORC2, ORC5 and CDC45L to amplification foci. However, it is difficult to distinguish whether mislocalization of replication factors is the cause, or simply a consequence, of the ectopic genomic replication seen in *E2f2* mutant follicle cells. More definitive answers to these questions await the characterization of the location of the E2F2 protein within follicle cells, and whether it directly associates with replication factors.

Two pieces of evidence suggest that disruption of E2F2/DP/RBF complexes contributes to the phenotypes we report. First, $E2f2^{1\text{-}188}$ appears to be a loss of function allele, as it causes a phenotype very similar to complete deletion of *E2f2*. $E2f2^{1\text{-}188}$ produces a truncated protein capable of binding

DP (Fig. 1) but lacking the RBF interaction domain, suggesting the possibility that much of the functions of E2F2 require association with an pRb family member. Second, *Dp* and *Rbf* female sterile alleles each cause a similar cytological phenotype in BrdU-labeled follicle cells to that seen by mutation of *E2f2* (Bosco et al., 2001; Royzman et al., 1999). In each case ,genomic replication was observed at a stage when only gene amplification should be occurring. There are, however, notable differences between the *E2f2*, *Rbf* and *Dp* mutant phenotypes. The *E2f2* null phenotype is 100% penetrant, whereas the *Rbf* and *Dp* follicle cell replication phenotypes are not fully penetrant (Bosco et al., 2001; Royzman et al., 1999). The difference in penetrance could be explained by partial loss of Dp and RBF function, as the *Dp* and *Rbf* alleles used in these experiments are hypomorphic (null alleles are zygotically lethal). But more significantly, the *E2f2* and *Rbf* mutant FACS profiles are different. Bosco et al. (Bosco et al., 2001) detected a 32C population in nuclear preparations from *Rbf* mutant ovaries, which we have confirmed using analyses of isolated, intact follicle cells. We could not detect a 32C population of similar size relative to wild type in either nuclear or intact follicle cell preparations of *E2f2* mutant ovaries. These data suggest that the BrdU labeling observed cytologically in *E2f2* mutants does not represent an additional endocycle S phase, which is expected to generate a 32C peak. It is possible that the ectopic replication in the *E2f2* mutants is actually much less than in the *Rbf* mutants, such that by the completion of oogenesis and egg laying a 32C ploidy value is never reached. As RBF can bind either E2F2 or E2F (Bosco et al., 2001; Du et al., 1996; Frolov et al., 2001), mutation of *Rbf* and *E2f2* could have overlapping but distinct phenotypes, perhaps because each mutation would affect the distribution of cellular pRB/E2F complexes differently.

E2F, DP and RBF also play a role in establishing the extent of chorion gene amplification. Viable, hypomorphic mutations of *E2f* and *Dp* that encode proteins predicted to bind DNA poorly result in a decreased level of chorion gene amplification. By contrast, both a viable, hypomorphic allele of *Rbf* and the $E2f^{i2}$ nonsense allele, which produces a truncated protein lacking the RBF-binding domain, cause an increase in chorion gene amplification (Bosco et al., 2001). These data suggest that E2F/DP stimulates gene amplification, whereas the E2F/DP/RBF complex attenuates it. Both *E2f2* null and $E2f2^{1\text{-}188}$ (lacking the RBF binding domain) alleles cause similar phenotypes, including reduced chorion gene amplification. This could be because E2F2 function is mediated mostly, or even exclusively, via complexes with a pRb protein, in contrast to E2F. Moreover, the opposite amplification phenotypes caused by $E2f^{i2}$ and $E2f2^{1\text{-}188}$ suggest a different role in amplification for the two wild-type proteins. One possibility is that loss of *E2f2* indirectly affects gene amplification, perhaps because a limiting replication factor is used at multiple, ectopic replication origins, thereby reducing its availability for use at the chorion loci and interfering with efficient gene amplification. Alternatively, the lack of E2F2 could make more RBF available to bind E2F, thus driving the formation of excess E2F/DP/RBF complexes, which would limit the extent of gene amplification (Bosco et al., 2001).

Irrespective of the mechanism by which E2F2 regulates DNA replication in follicle cells, *E2f2* is not absolutely required for much of *Drosophila* development. This is despite prominent *E2f2* gene expression beginning at embryonic stages in cycling cells (both dividing and endocycling) and continuing throughout development (Sawado et al., 1998). *E2f2* and *E2f* are the only two E2F-like genes in *Drosophila* (Adams et al., 2000), and the lack of an obvious phenotype in *E2f2* single mutants is not due to redundancy with *E2f*. *E2f* is an essential gene (Duronio et al., 1995), with homozygous embryos hatching into slow growing larvae that die before pupation (Du, 2000; Royzman et al., 1997). The *E2f* lethal phase is due at least in part to E2F2 activity, as *E2f E2f2* double mutant progeny grow at a normal rate and survive until mid- to late-pupal development (Frolov et al., 2001). This indicates that E2F and E2F2 perform opposing roles during development, and suggests that E2F2 acts to inhibit growth and cell cycle progression, but only when E2F is limiting or absent. Nevertheless, the ability of E2F2 to antagonize E2F is not absolutely essential. While E2F2 does in fact inhibit replication and cell cycle progression in other contexts, this function would be nonessential if it is redundant with other mechanisms that inhibit cell cycle progression during *Drosophila* development, such as the activation of CDK inhibitors (de Nooij et al., 1996; Foley and Sprenger, 2001; Lane et al., 1996; Sprenger et al., 1997; Thomas et al., 1994; Thomas et al., 1997), transcriptional downregulation (Du and Dyson, 1999; Li and Vaessin, 2000) or an increased rate of protein degradation (Reed and Orr-Weaver, 1997; Sigrist and Lehner, 1997). It follows from this model that the follicle cells specifically rely more heavily on E2F2 than other mechanisms to inhibit genomic DNA replication.

We thank Gio Bosco, Mike Botchan, Brian Calvi, Sue Cotterill, Minx Fuller, Jeff Sekelsky, Alan Spradling and Terry Orr-Weaver for reagents, the Bloomington and Szeged stock centers for fly lines, Larry Arnold for assistance with FACS analysis, Tim Donaldson for help with yeast transformation, Mary Lilly, Brian Calvi and Jackie Lees for helpful discussions, Nick Dyson for communicating unpublished results, and Mark Peifer, Steve Crews, Denise Myster and Jeff Sekelsky for comments on the manuscript. This work was supported by NIH grant GM57859 and the Cancer Research Fund of the Damon Runyon-Walter Winchell Foundation Award, DRS-10, to R. J. D.

REFERENCES

Adams, M. D., Celniker, S. E., Holt, R. A., Evans, C. A., Gocayne, J. D., Amanatides, P. G., Scherer, S. E., Li, P. W., Hoskins, R. A., Galle, R. F. et al. (2000). The genome sequence of Drosophila melanogaster. *Science* **287**, 2185-2195.

Asano, M. and Wharton, R. P. (1999). E2F mediates developmental and cell cycle regulation of ORC1 in Drosophila. *EMBO J.* **18**, 2435-2448.

Austin, R. J., Orr-Weaver, T. L. and Bell, S. P. (1999). Drosophila ORC specifically binds to ACE3, an origin of DNA replication control element. *Genes Dev.* **13**, 2639-2649.

Bosco, G., Du, W. and Orr-Weaver, T. L. (2001). DNA replication control through interaction of E2F-RB and the origin recognition complex. *Nat. Cell Biol.* **3**, 289-295.

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

Bruce, J. L., Hurford, R. K., Jr, Classon, M., Koh, J. and Dyson, N. (2000). Requirements for cell cycle arrest by p16INK4a. *Mol Cell* **6**, 737-742.

Bryant, Z., Subrahmanyan, L., Tworoger, M., LaTray, L., Liu, C. R., Li, M. J., van den Engh, G. and Ruohola-Baker, H. (1999). Characterization of differentially expressed genes in purified Drosophila follicle cells: toward a general strategy for cell type- specific developmental analysis. *Proc. Natl. Acad. Sci. USA* **96**, 5559-5564.

Calvi, B. R. and Spradling, A. C. (1999). Chorion gene amplification in Drosophila: a model for metazoan origins of DNA replication and S-phase control. *Methods* **18**, 407-417.

Calvi, B. R., Lilly, M. A. and Spradling, A. C. (1998). Cell cycle control of chorion gene amplification. *Genes Dev.* **12**, 734-744.

Cayirlioglu, P. and Duronio, R. J. (2001). Cell cycle: Flies teach an old dogma new tricks. *Curr. Biol.* **11**, R178-R181.

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

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

Du, W. and Dyson, N. (1999). The role of RBF in the introduction of G1 regulation during Drosophila embryogenesis. *EMBO J.* **18**, 916-925.

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

Duronio, R. J. and O'Farrell, P. H. (1995). Developmental control of the G1 to S transition in Drosophila: cyclin E is a limiting downstream target of E2F. *Genes Dev.* **9**, 1456-1468.

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

Duronio, R. J., Bonnette, P. C. and O'Farrell, P. H. (1998). Mutations of the Drosophila dDP, dE2F, and cyclin E genes reveal distinct roles for the E2F-DP transcription factor and cyclin E during the G1-S transition. *Mol. Cell Biol.* **18**, 141-151.

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

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

Field, S. J., Tsai, F. Y., Kuo, F., Zubiaga, A. M., Kaelin, W. G., Jr, Livingston, D. M., Orkin, S. H. and Greenberg, M. E. (1996). E2F-1 functions in mice to promote apoptosis and suppress proliferation. *Cell* **85**, 549-561.

Foley, E. and Sprenger, F. (2001). The cyclin-dependent kinase inhibitor Roughex is involved in mitotic exit in Drosophila. *Curr. Biol.* **11**, 151-160.

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

Gaubatz, S., Lindeman, G. J., Ishida, S., Jakoi, L., Nevins, J. R., Livingston, D. M. and Rempel, R. E. (2000). E2F4 and E2F5 play an essential role in pocket protein-mediated G1 control. *Mol. Cell* **6**, 729-735.

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

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

Humbert, P. O., Rogers, C., Ganiatsas, S., Landsberg, R. L., Trimarchi, J. M., Dandapani, S., Brugnara, C., Erdman, S., Schrenzel, M., Bronson, R. T. et al. (2000a). E2F4 is essential for normal erythrocyte maturation and neonatal viability. *Mol. Cell* **6**, 281-291.

Humbert, P. O., Verona, R., Trimarchi, J. M., Rogers, C., Dandapani, S. and Lees, J. A. (2000b). E2f3 is critical for normal cellular proliferation. *Genes Dev.* **14**, 690-703.

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

James, P., Halladay, J. and Craig, E. A. (1996). Genomic libraries and a host strain designed for highly efficient two- hybrid selection in yeast. *Genetics* **144**, 1425-1436.

Kennedy, B. K., Barbie, D. A., Classon, M., Dyson, N. and Harlow, E. (2000). Nuclear organization of DNA replication in primary mammalian cells. *Genes Dev.* **14**, 2855-2868.

Landis, G. and Tower, J. (1999). The Drosophila chiffon gene is required for chorion gene amplification, and is related to the yeast Dbf4 regulator of DNA replication and cell cycle. *Development* **126**, 4281-4293.

Landis, G., Kelley, R., Spradling, A. C. and Tower, J. (1997). The k43 gene, required for chorion gene amplification and diploid cell chromosome replication, encodes the Drosophila homolog of yeast origin recognition complex subunit 2. *Proc. Natl. Acad. Sci. USA* **94**, 3888-3892.

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

Li, L. and Vaessin, H. (2000). Pan-neural prospero terminates cell proliferation during drosophila neurogenesis. *Genes Dev.* **14**, 147-151.

Lilly, M. A. and Spradling, A. C. (1996). The Drosophila endocycle is controlled by Cyclin E and lacks a checkpoint ensuring S-phase completion. *Genes Dev.* **10**, 2514-2526.

Lindeman, G. J., Dagnino, L., Gaubatz, S., Xu, Y., Bronson, R. T., Warren, H. B. and Livingston, D. M. (1998). A specific, nonproliferative role for E2F-5 in choroid plexus function revealed by gene targeting. *Genes Dev.* **12**, 1092-1098.

Loebel, D., Huikeshoven, H. and Cotterill, S. (2000). Localisation of the DmCdc45 DNA replication factor in the mitotic cycle and during chorion gene amplification. *Nucleic Acids Res.* **28**, 3897-3903.

Lu, L., Zhang, H. and Tower, J. (2001). Functionally distinct, sequence-specific replicator and origin elements are required for Drosophila chorion gene amplification. *Genes Dev.* **15**, 134-146.

Manseau, L., Baradaran, A., Brower, D., Budhu, A., Elefant, F., Phan, H., Philp, A. V., Yang, M., Glover, D., Kaiser, K. et al. (1997). GAL4 enhancer traps expressed in the embryo, larval brain, imaginal discs, and ovary of Drosophila. *Dev. Dyn.* **209**, 310-322.

Matsumoto-Taniura, N., Pirollet, F., Monroe, R., Gerace, L. and Westendorf, J. M. (1996). Identification of novel M phase phosphoproteins by expression cloning. *Mol. Biol. Cell* **7**, 1455-1469.

Muller, H. and Helin, K. (2000). The E2F transcription factors: key regulators of cell proliferation. *Biochim. Biophys. Acta* **1470**, M1-M12.

Muller, H., Bracken, A. P., Vernell, R., Moroni, M. C., Christians, F., Grassilli, E., Prosperini, E., Vigo, E., Oliner, J. D. and Helin, K. (2001). E2Fs regulate the expression of genes involved in differentiation, development, proliferation, and apoptosis. *Genes Dev.* **15**, 267-285.

Myster, D. L., Bonnette, P. C. and Duronio, R. J. (2000). A role for the DP subunit of the E2F transcription factor in axis determination during Drosophila oogenesis. *Development* **127**, 3249-3261.

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

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

Orr-Weaver, T. L. (1991). Drosophila chorion genes: cracking the eggshell's secrets. *BioEssays* **13**, 97-105.

Pak, D. T., Pflumm, M., Chesnokov, I., Huang, D. W., Kellum, R., Marr, J., Romanowski, P. and Botchan, M. R. (1997). Association of the origin recognition complex with heterochromatin and HP1 in higher eukaryotes. *Cell* **91**, 311-323.

Pan, H., Yin, C., Dyson, N. J., Harlow, E., Yamasaki, L. and Van Dyke, T. (1998). Key roles for E2F1 in signaling p53-dependent apoptosis and in cell division within developing tumors. *Mol. Cell* **2**, 283-292.

Reed, B. H. and Orr-Weaver, T. L. (1997). The Drosophila gene morula inhibits mitotic functions in the endo cell cycle and the mitotic cell cycle. *Development* **124**, 3543-3553.

Rempel, R. E., Saenz-Robles, M. T., Storms, R., Morham, S., Ishida, S., Engel, A., Jakoi, L., Melhem, M. F., Pipas, J. M., Smith, C. et al. (2000). Loss of E2F4 activity leads to abnormal development of multiple cellular lineages. *Mol. Cell* **6**, 293-306.

Royzman, I., Whittaker, A. J. and Orr-Weaver, T. L. (1997). Mutations in Drosophila DP and E2F distinguish G1-S progression from an associated transcriptional program. *Genes Dev.* **11**, 1999-2011.

Royzman, I., Austin, R. J., Bosco, G., Bell, S. P. and Orr-Weaver, T. L. (1999). ORC localization in Drosophila follicle cells and the effects of mutations in dE2F and dDP. *Genes Dev.* **13**, 827-840.

Sawado, T., Yamaguchi, M., Nishimoto, Y., Ohno, K., Sakaguchi, K. and Matsukage, A. (1998). dE2F2, a novel E2F-family transcription factor in Drosophila melanogaster. *Biochem. Biophys. Res. Commun.* **251**, 409-415.

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

Sinclair, D. A. R., Moore, G. D. and Grigliatti, T. A. (1980). Isolation and preliminary characterization of putative histone gene deficiencies in Drosophila melanogaster. *Genetics* **94**, s96-s97.

Spradling, A. C. (1993). Developmental genetics of oogenesis. In *The Development of Drosophila melanogaster*. Vol. 1 (ed. M. Bate and A. Martinez-Arias), pp. 1-70. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Spradling, A. C. (1999). ORC binding, gene amplification, and the nature of metazoan replication origins. *Genes Dev.* **13**, 2619-2623.

Sprenger, F., Yakubovich, N. and O'Farrell, P. H. (1997). S-phase function of Drosophila cyclin A and its downregulation in G1 phase. *Curr. Biol.* **7**, 488-499.

Takisawa, H., Mimura, S. and Kubota, Y. (2000). Eukaryotic DNA replication: from pre-replication complex to initiation complex. *Curr Opin. Cell Biol.* **12**, 690-696.

Thomas, B. J., Gunning, D. A., Cho, J. and Zipursky, L. (1994). Cell cycle progression in the developing Drosophila eye: roughex encodes a novel protein required for the establishment of G1. *Cell* **77**, 1003-1014.

Thomas, B. J., Zavitz, K. H., Dong, X., Lane, M. E., Weigmann, K., Finley, R. L., Jr, Brent, R., Lehner, C. F. and Zipursky, S. L. (1997). roughex down-regulates G2 cyclins in G1. *Genes Dev.* **11**, 1289-1298.

Tsai, K. Y., Hu, Y., Macleod, K. F., Crowley, D., Yamasaki, L. and Jacks, T. (1998). Mutation of E2f-1 suppresses apoptosis and inappropriate S phase entry and extends survival of Rb-deficient mouse embryos. *Mol. Cell* **2**, 293-304.

Whittaker, A. J., Royzman, I. and Orr-Weaver, T. L. (2000). Drosophila double parked: a conserved, essential replication protein that colocalizes with the origin recognition complex and links DNA replication with mitosis and the down-regulation of S phase transcripts. *Genes Dev.* **14**, 1765-1776.

Wright, T. R., Hodgetts, R. B. and Sherald, A. F. (1976). The genetics of dopa decarboxylase in Drosophila melanogaster. I. Isolation and characterization of deficiencies that delete the dopa- decarboxylase-dosage-sensitive region and the alpha-methyl-dopa- hypersensitive locus. *Genetics* **84**, 267-285.

Yamasaki, L., Jacks, T., Bronson, R., Goillot, E., Harlow, E. and Dyson, N. J. (1996). Tumor induction and tissue atrophy in mice lacking E2F-1. *Cell* **85**, 537-548.

Yamasaki, L., Bronson, R., Williams, B. O., Dyson, N. J., Harlow, E. and Jacks, T. (1998). Loss of E2F-1 reduces tumorigenesis and extends the lifespan of $Rb1^{+/-}$ mice. *Nat. Genet.* **18**, 360-364.

Ziebold, U., Reza, T., Caron, A. and Lees, J. A. (2001). E2F3 contributes both to the inappropriate proliferation and to the apoptosis arising in Rb mutant embryos. *Genes Dev.* **15**, 386-391.