

ORC localization in *Drosophila* follicle cells and the effects of mutations in *dE2F* and *dDP*

Irena Royzman,^{1,2} Richard J. Austin,¹ Giovanni Bosco,^{1,2} Stephen P. Bell,¹ and Terry L. Orr-Weaver¹⁻³

¹Department of Biology, Massachusetts Institute of Technology and ²Whitehead Institute, Cambridge, Massachusetts 02142 USA

We isolated mutations in *Drosophila* *E2F* and *DP* that affect chorion gene amplification and ORC2 localization in the follicle cells. In the follicle cells of the ovary, the ORC2 protein is localized throughout the follicle cell nuclei when they are undergoing polyploid genomic replication, and its levels appear constant in both S and G phases. In contrast, when genomic replication ceases and specific regions amplify, ORC2 is present solely at the amplifying loci. Mutations in the DNA-binding domains of *dE2F* or *dDP* reduce amplification, and in these mutants specific localization of ORC2 to amplification loci is lost. Interestingly, a *dE2F* mutant predicted to lack the carboxy-terminal transcriptional activation and RB-binding domain does not abolish ORC2 localization and shows premature chorion amplification. The effect of the mutations in the heterodimer subunits suggests that E2F controls not only the onset of S phase but also origin activity within S phase.

[Key Words: DNA replication; ORC; E2F; DP; *Drosophila*; oogenesis]

Received January 13, 1999; revised version accepted February 16, 1999.

The E2F transcription factor plays a pivotal role in the control of S-phase entry. This transcription factor is a heterodimer of E2F and DP proteins, and there are several forms of both proteins in mammalian cells (Weinberg 1995; Dyson 1998). Ectopic expression of the best-characterized E2F protein, E2F-1, is sufficient to drive serum-starved, quiescent fibroblasts through S phase, suggesting a positive role for E2F in S-phase entry (Johnson et al. 1993; Qin et al. 1994). E2F activates the transcription of genes whose products regulate the initiation of replication (Ohtani et al. 1996). Its activity is also needed for a normal rate of replication, because E2F activates the transcription of components of the DNA replication machinery such as DNA polymerase α (Nevins 1992). E2F has not been demonstrated to have a role in controlling replication once S phase has initiated. It has been shown, however, that inactivation of E2F by cyclin A is needed for the completion of DNA replication and S phase (Krek et al. 1995).

E2F can repress transcription of genes needed for S phase when complexed with a pocket protein such as the retinoblastoma protein (RB) (Weintraub et al. 1992). The E2F/DP heterodimer tethers RB to E2F-dependent promoters through a small RB-binding region embedded in the transactivation domain of the E2F protein (Helin et al. 1993). In addition to occluding the E2F activation do-

main, RB represses transcription actively at these promoters and is thought to affect the state of chromatin accessibility through an interaction with a histone deacetylase (Weintraub et al. 1995; Brehm et al. 1998; Luo et al. 1998; Magnaghi-Jaulin et al. 1998). Late in G₁, phosphorylation of RB by cyclin D/CDK4,6 and cyclin E/CDK2 frees E2F/DP and triggers the expression of E2F-dependent genes including cyclin E (Ewen et al. 1993; Kato et al. 1993; Ohtani et al. 1995; Botz et al. 1996; Geng et al. 1996). The cyclin E/CDK2 complex then creates a positive feedback loop that is thought to result in an irrevocable commitment to S phase (Sherr 1996).

Drosophila homologs to E2F, DP, and an RB-like protein (RBF) have been identified (Dymlacht et al. 1994; Ohtani and Nevins 1994; Hao et al. 1995; Du et al. 1996a). We will refer to the individual subunits of E2F as *dE2F* and *dDP* and will use the term E2F to designate the heterodimer transcription factor. As in mammalian cells, ectopic *dE2F* expression is sufficient to drive quiescent cells into S phase (Asano et al. 1996; Du et al. 1996b). We have shown that null mutations in *dE2F* and *dDP* cause lethality late in development with some tissues being underdeveloped or absent (Royzman et al. 1997). A positive role for the E2F heterodimer in cell cycle progression was inferred from these defects. This role has been confirmed by analysis of E2F function in developing wing discs (Neufeld et al. 1998). In addition, G₁-S transcription of *cyclin E* and genes encoding rep-

³Corresponding author.
E-MAIL weaver@wi.mit.edu; FAX (617) 258-9872.

lication functions such as *proliferating cell nuclear antigen* (*PCNA*) and *ribonucleotide reductase 2* (*RNR2*), observed in late embryogenesis normally, is missing in the *dE2F* and *dDP* mutants (Duronio et al. 1995, 1998; Royzman et al. 1997). Despite the pronounced effect on E2F-dependent G₁-S transcription, replication in the mutants was only slowed and a block to replication was not observed.

Drosophila oogenesis makes it possible to examine aspects of DNA replication that are not readily apparent during embryogenesis (for reviews, see Spradling 1993; Royzman and Orr-Weaver 1998). Ovarian follicle cells undergo a set of mitotic divisions before switching to an endo cycle (a cycle consisting of only S phase and a gap phase) and becoming polyploid. Genomic replication ceases after four endo cycles, but two genomic regions that contain clusters of chorion genes continue to replicate so that the chorion genes are amplified as much as 80-fold relative to genomic DNA. The chorion genes encode the eggshell proteins. Amplification of the chorion genes is needed to produce sufficient chorion protein for a normal eggshell, and amplification occurs by repeated rounds of initiation of DNA replication and fork movement to produce a gradient of amplified DNA extending ~100 kb (for reviews, see Orr-Weaver 1991; Calvi and Spradling 1999). Mutants with reduced amplification have a phenotype of thin eggshells and female sterility.

Chorion gene amplification appears to use components that are required normally for initiating DNA replication. Origin recognition complex (ORC) is a complex of six subunits and is required for initiation of replication (for review, see Dutta and Bell 1997). Mutations in the *Drosophila orc2* gene disrupt amplification (Landis et al. 1997). Overexpression and inhibition studies indicate that *cyclin E* is needed for amplification also (Calvi et al. 1998). Because the levels of Cyclin E protein oscillate with genomic replication but remain constant in follicle cells undergoing amplification, Calvi et al. (1998) postulated that the high Cyclin E activity blocks genomic replication and that some mechanism permits the amplicons to escape this block to rereplication.

Here we report the identification and analysis of new mutations in *Drosophila dE2F* that cause cell-cycle defects in oogenesis. These mutations, in addition to a female-sterile allele of *dDP* isolated previously (Royzman et al. 1997), allowed us to analyze the role of E2F in DNA replication in follicle cells. We find that E2F influences chorion amplification and affects localization of ORC within the nucleus.

Results

Female-sterile mutations in the dE2F gene

We recovered two viable mutations in the gene for the *dE2F* subunit of the E2F transcription factor, *dE2F¹* and *dE2F²*. These mutants were identified in a screen of mutagenized third chromosome lines (Moore et al. 1998) that affected *PCNA* expression in embryos. When placed in *trans* to a deficiency or a null *dE2F* allele, the *dE2F¹*

mutation caused reduced viability, and the recovered adults were female sterile and had eye and bristle defects. The mutant females were able to lay eggs, but the eggs failed to develop. These phenotypes were fully recessive and not seen in *dE2F¹/TM3* control siblings (*TM3* is a balancer chromosome that carries a wild-type copy of the *dE2F* gene). These phenotypes resembled closely those observed for a weak allele of *dDP*, *dDP^{a1}* (Royzman et al. 1997). The *dE2F²* mutation was fully viable in *trans* to a deficiency or null *dE2F* allele; these adults had slightly rough eyes and the females had reduced fertility. These phenotypes were fully recessive. Unexpectedly, the two alleles were viable and fertile in *trans* to each other.

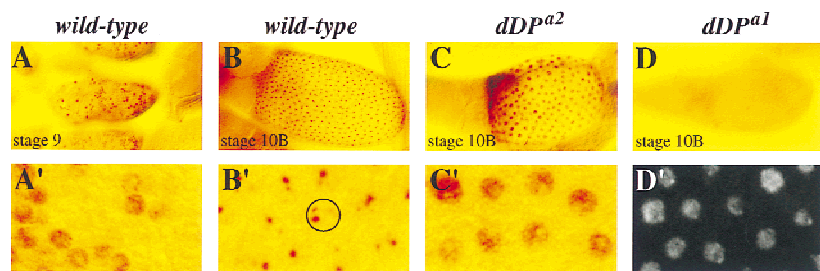
We sequenced the *dE2F* coding regions from the mutants; both mutants had changes in the *dE2F* open reading frame (ORF) (Fig. 1). The *dE2F¹* mutation is a G → A nucleotide transition that converts amino acid Asp-296 of the *dE2F* DNA-binding domain to Asn. This residue lies in a region that is highly similar between the E2F and DP families of proteins, and this Asp is conserved in all known *Drosophila* and vertebrate E2F and DP proteins (Fig. 1B) (Hao et al. 1995; Bandara et al. 1997). The finding that the *dE2F¹* mutation is analogous to the missense change in the DNA-binding region in the *dDP^{a1}* allele may explain the similarity of the phenotypes exhibited by the two mutants (see Fig. 1B). The mutation in *dE2F²* is a C → T change that converts Gln-527 to a stop codon. The resultant E2F protein is predicted to lack the carboxy-terminal RB-binding and transcriptional activation region shown to be both necessary and sufficient for transcriptional activation in *Drosophila* SL2 cells (Ohtani and Nevins 1994).

Phenotypic analysis of *Drosophila E2F⁹¹*, a null allele that lacks all the recognized functional domains of the E2F family of proteins, established that *dE2F* is essential for viability and normal growth (Duronio et al. 1995; Royzman et al. 1997). Viability and growth are not affected in the *dE2F²* mutant, and thus the *dE2F²* mutant appears to retain significant physiological function. We also tested whether reducing by half the dosage of the *cyclin E* gene, a critical target of E2F in *Drosophila* (Duronio et al. 1996), would effect the viability of *dE2F²/dE2F⁹¹* flies and found that it had no effect. In contrast, the same reduction of *cyclin E* enhanced the lethality of the *dE2F¹/dE2F⁹¹* mutant organisms, as we were not able to recover *dE2F¹/dE2F⁹¹* adults that were heterozygous for a strong but completely recessive *cyclin E* mutation.

The role of dE2F and dDP in chorion gene amplification

In follicle cells, chorion gene clusters escape restrictions on rereplication, and amplification of these regions continues after the cessation of general genomic replication (Calvi et al. 1998). The amplification of chorion genes in follicle cells is required for the rapid synthesis of the eggshell (chorion), thus mutations that disrupt this process cause female sterility and thin eggshells. Consistent

Figure 2. *dDP* mutants affect amplification and the shutoff of genomic replication. (A–D) DNA replication was assayed by BrdU labeling observed by antibody staining detected by the HRP reaction. BrdU incorporation is indicated by the red staining. (A–D) The same magnification, and shown below (A'–D') at a higher magnification. (A,A') In stage 9 control *dDP^{a1}/+* egg chambers genomic replication is asynchronous among follicle cell nuclei. The egg chamber contains follicle cells that are labeled (S phase) with BrdU and those that are not labeled (G phase). The same pattern of nuclear BrdU incorporation is observed in the female-sterile *dE2F* and *dDP* mutants. (B,B') In stage-10B control egg chambers, polyploidization of the follicle cell genome is no longer observed. Instead, synchronous subnuclear BrdU incorporation is seen. The subnuclear dots correspond to amplifying chorion clusters. The nucleus of a single follicle cell is indicated by a circle. The different intensity and size of foci (one large, one medium, and two small) reflect the extent of gene amplification (Calvi et al. 1998). (C,C') A stage-10B *dDP^{a2}/Df; hs-dDP/+* mutant egg chamber is shown. Genomic replication is occurring synchronously in the follicle cells of this mutant egg chamber. The same phenotypes were observed for the *dDP^{a1}/Df* mutant. Note that the nuclei are much larger than at stage 9, indicating higher ploidy due to inappropriate genomic replication. (D) A stage-10B *dDP^{a1}/Df* egg chamber is shown. Genomic replication has been shutoff, but amplification is not observed. The same replication defect was observed for *dDP^{a2}/Df; hs-dDP/+*. (D') A magnified DAPI image of *D* showing that the follicle cell nuclei are present and polyploid.



striking that the mutant stage 10B genomic replication is synchronous in all the follicle cells, a property associated with amplification but not genomic replication (Fig. 2, cf. C' to A' and B'). Thus the genomic replication is not likely to be the consequence of follicle-cell genomic replication being delayed relative to egg chamber development and persisting into stage 10B. Second, it is unlikely that this genomic replication results from a slower S phase, with replicating follicle cells accumulating until replicating cells are continuous across the cell layer. We saw either stage-10B egg chambers with no replicating follicle cells or all of the follicle cells replicating, no evidence of a gradual increase in follicle cells in S phase. Third, these egg chambers are at stage 10B by morphological criteria, the oocyte and nurse cell size, and centripetal migration of the follicle cells. Fourth, we see the stage-appropriate change in Cyclin E protein distribution in the mutant stage-10B nurse cells (see below and Fig. 6, below). We postulate that the difference between the phenotypes shown in Figure 2, C and D is from variation in the levels of active dDP among egg chambers. Taken

together, the phenotypes suggest that *dDP* plays a dual role in the regulation of replication in follicle cells. It is needed to activate chorion gene amplification, but it also may be required to inhibit follicle-cell genomic replication. Importantly, the results of the BrdU analysis correlate directly with the severe eggshell defects observed for the *dDP* mutants.

BrdU labeling of *dE2Fⁱ¹/Df* mutant egg chambers showed that *dE2F* also is required for amplification; subnuclear BrdU incorporation was either absent or reduced greatly in these mutant egg chambers (Fig. 3, cf. A and B). The *dE2Fⁱ²* mutation, however, had a different effect on amplification. BrdU labeling of *dE2Fⁱ²* mutant ovaries appeared to have significantly larger labeled foci (stage 10B and in the later stages) than those observed in wild type (Fig. 3, cf. C and B). In addition to the over-amplifying follicle cells, some *dE2Fⁱ²* egg chambers had small patches that failed to incorporate BrdU (data not shown). The increased amount of BrdU incorporation in the *dE2Fⁱ²* mutant could explain the thicker eggshell, if additional copies of the chorion genes were amplified. The

Table 1. Replication phenotypes of *dE2F* and *dDP* mutants in follicle cells

Genotype	Inappropriate genomic replication (%) ^a (Fig. 2C)	Chorion amplification foci (%) ^b				Total no. of 10B–11 egg chambers
		absent (fig. 2D)	reduced (Fig. 3A)	normal (Fig. 2B)	increased (Fig. 3C)	
<i>dDP^{a1}/+</i>	0	16	8	76	0	38
<i>dDP^{a1}/Df</i>	18 ^c	82	0	0	0	51
<i>dE2Fⁱ¹/dE2F^{o1}</i> or <i>dE2Fⁱ¹⁷² d</i>	5 ^c	43	30	21	0	61
<i>dE2Fⁱ²/dE2F^{o1}</i> or <i>Df^f</i>	0	10	11	19	60	42

^aGenomic follicle cell replication is inappropriate at stages 10B–11.

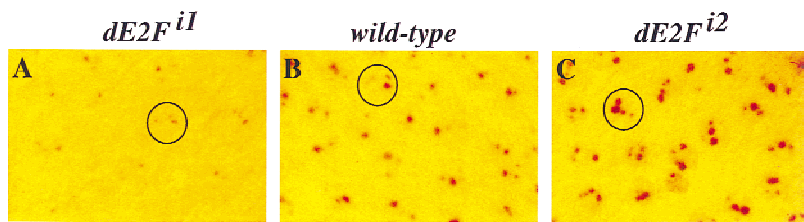
^bThe percent of egg chambers with given phenotype.

^cGenomic replication occurred in synchrony in all of the follicle cells.

^dThe data are combined for genotypes *dE2Fⁱ¹/dE2F^{o1}* and *dE2Fⁱ¹/dE2Fⁱ¹⁷²*.

^eGenomic replication persisted in a few follicle cells.

^fThe data are combined for genotypes *dE2Fⁱ²/dE2F^{o1}* and *dE2Fⁱ²/Df*.



sities. It is sometimes difficult to see them in the same focal plane. In *dE2Fⁱ²/Df* mutant egg chambers (C) the size and intensity of the BrdU foci are increased greatly. The mutant phenotype makes it easier to see the four subnuclear dots within each follicle cell nucleus (circle).

dE2Fⁱ² mutant phenotype also indicates that the carboxy-terminal transcriptional activation and RB-binding domains of *dE2F* are not required for formation of amplification foci. Both of the *dE2F* mutations are recessive for these amplification effects.

We quantitated the timing and level of amplification in the *dDP* and *dE2F* mutants. The *dDP* and *dE2F* mutations were isolated in stocks that had been isogenized for a single second or third chromosome, respectively. Consequently we determined the developmental pattern of amplification in these strain backgrounds by using *dDP^{a1}/CyO* and *dE2Fⁱ¹/TM3* control females, as these alleles are fully recessive. Because the *dDP^{a1}* and *dE2Fⁱ¹* mutant flies have reduced viability, we developed a PCR assay that requires fewer egg chambers than Southern blots (see Materials and Methods) to compare the levels of amplification of the chorion clusters to the unamplified *actin 5C* gene (Fig. 4) or the *rosy* gene (data not shown). An essential 320-bp control element from the maximally amplified region of the third chromosome, *ACE3*, was assayed (Orr-Weaver et al. 1989). A 252-bp control fragment from within the *ACE1* element of the X chromosome chorion cluster was also tested to confirm

that the PCR assay was accurate (Spradling et al. 1987). To test the linearity and accuracy of the PCR assay, the amplification levels of *dDP^{a1}/CyO* control egg chambers were quantified both by the PCR assay (Fig. 4) and by a quantitative Southern blot (data not shown). The developmental timing of amplification was identical in the two experiments and the levels of amplification were comparable.

In agreement with previous results of Calvi et al. (1998), we found that in our strain backgrounds *ACE1* did not amplify until stage 10B (Fig. 4). *ACE1* was amplified fourfold in stage-10B egg chambers and sevenfold in stage-13 egg chambers. In contrast, we detected low levels, ~1.5-fold, of amplification of *ACE3* in stage 10A (Fig. 4). Calvi et al. (1998) previously demonstrated amplification of the third chromosome cluster in stage 10A, prior to amplification of the χ cluster. We found that *ACE3* was amplified 8-fold in stage-10B egg chambers and 16-fold in stage-13 egg chambers (Fig. 4). Using the PCR assay, significant amplification of *ACE3* and *ACE1* occurs when BrdU incorporation is first evident at the amplifying loci, stage 10B.

Quantitative analysis of the *dDP^{a1}/Df* mutant egg

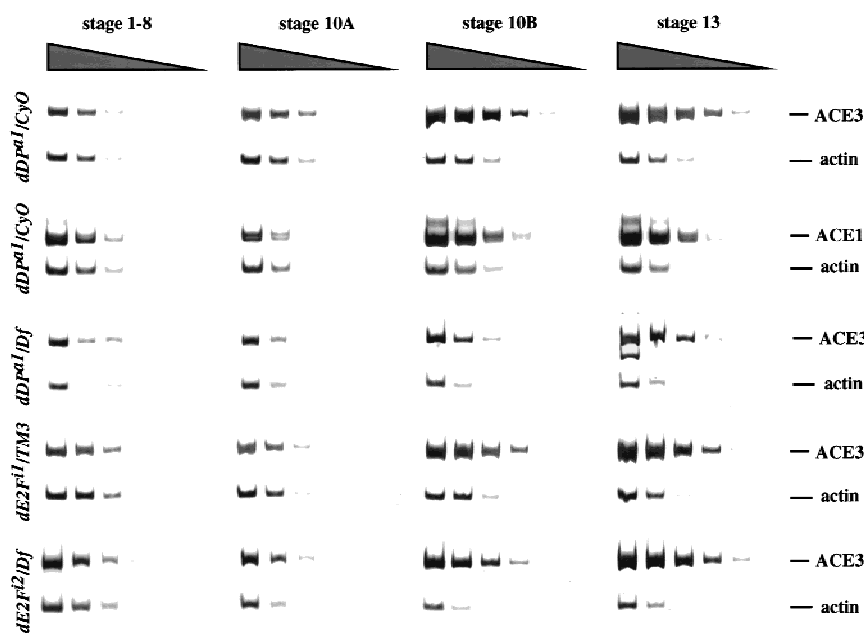


Figure 4. Quantitation of chorion gene amplification. Each row shows gels of the PCR products from egg chambers of stage 1–8, 10A, 10B, and 13. Within each egg chamber sample, a series of fourfold serial dilutions of egg chamber DNA were used for independent PCR reactions, and these PCR products are shown from left to right. The sizes of the *ACE3*, *actin*, and *ACE1* products are shown on the right. The levels of amplification can be visualized by the intensity of the *ACE3* or *ACE1* products relative to the unamplified *actin* control. The genotype for each row is shown at left. In the *dDP^{a1}/CyO* and *dE2Fⁱ¹/TM3* controls amplification is observed readily in stage 10B, and *ACE3* amplifies to higher levels than *ACE1*. In the *dDP^{a1}/Df* mutant egg chambers, amplification occurs with correct developmental timing, but the levels are reduced. In contrast, in the *dE2Fⁱ²/Df* mutant *ACE3* is already amplified fourfold in stage 10A, and levels are elevated in later stages.

chambers showed that amplification occurred with correct developmental timing, but the levels of *ACE3* amplification were reduced to twofold in stage-10B egg chambers and fourfold in stage-13 egg chambers (Fig. 4, *dDP^{a1}/Df* row). This is consistent with the thin eggshells present in this mutant, and undetectable BrdU foci in the follicle cells. Although we did not detect BrdU incorporation in these follicle cells cytologically, the PCR assay is more sensitive and shows that amplification is not completely blocked.

In contrast to the reduced amplification in the *dDP^{a1}/Df* mutant, in the *dE2Fⁱ²/Df* mutant amplification initiated earlier and was amplified fourfold at stage 10A (Fig. 4, *dE2Fⁱ²/Df* row). *ACE3* was amplified 16-fold in stage-10B egg chambers from this mutant, whereas 32-fold amplification occurred at stage 13. The increased copy number of chorion genes in earlier egg chamber stages in the mutant can explain the thickened eggshells if these extra copies lead to increased levels of chorion proteins.

Expression of dE2F in the ovary

Because E2F is required for oogenesis, we examined the expression of *dE2F* and *dDP* in wild-type ovaries. The *dDP* transcript was constitutively high at all stages of oogenesis (data not shown). To examine *dE2F* transcription, we used a *dE2F* enhancer trap line, *dE2F^{rm729}/+* (Duronio et al. 1995; Brook et al. 1996). β -Galactosidase staining of ovaries from *dE2F^{rm729}/+* females revealed that there are high levels of the reporter protein in follicle and nurse-cell nuclei starting at stage 10A (Fig. 5A). To analyze the expression of dE2F protein in wild-type and mutant ovaries, we used antibodies against dE2F (Asano et al. 1996). These antibodies detect the dE2F protein specifically, because we did not see staining above background in the ovaries of *dE2F⁹¹/Df* females that were rescued to adulthood by transient expression of *hs-dE2F* (data not shown). In wild-type ovaries an increase in the levels of dE2F protein in follicle and nurse cell nuclei was observed at stage 10B, reflecting the induction of *dE2F* transcript (Fig. 5B,C). dE2F protein was also detected in ovaries from the *dDP^{a1}*, *dE2Fⁱ¹*, and *dE2Fⁱ²* mutants, so neither the mutations in *dE2F* itself or in *dDP* affect dE2F protein stability significantly (Fig. 5D; data not shown). This is true even for the *dE2Fⁱ²* mutation that predicts a truncated protein. We conclude that the replication defects in the mutant follicle cells are not caused by loss of induction of expression or instability of the dE2F protein.

The dDP and dE2F mutations do not affect cyclin E levels or activity

Given that *cyclin E* is an important target of the E2F transcription factor (Duronio and O'Farrell 1995), we sought to determine whether the effects of the *dDP* and *dE2F* mutants on chorion gene amplification result from

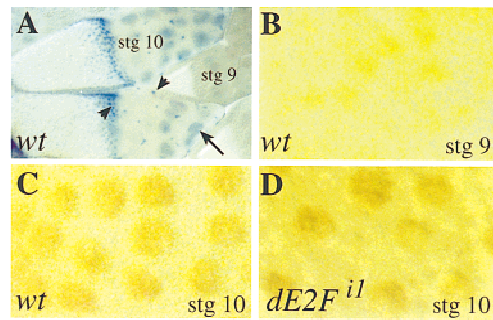


Figure 5. *dE2F* expression correlates with E2F activity. *A* is at a lower magnification than panels *B–D*. (*A*) β -Galactosidase staining of egg chambers from *dE2F^{rm729}/+*, a *dE2F* enhancer trap line in which the expression of β -galactosidase profiles the level of *dE2F* transcripts (Duronio et al. 1995; Brook et al. 1996). A stage-9 egg chamber is embedded between two stage-10 egg chambers. The β -galactosidase reporter is induced at stage 10 in all of the follicle cells of the egg chamber. This induction correlates with the transition to amplification in the follicle cells. The level of β -galactosidase is highest in the follicle cells covering the anterior region of the oocyte, correlating with a gradient of BrdU incorporation from anterior to posterior in the follicle cells of the egg chamber (data not shown). The small blue cells are the follicle cells (short arrow); the larger cells are nurse cells (large arrow). (*B–D*) dE2F protein levels in wild-type and mutant follicle cells following staining with a polyclonal antibody (Asano et al. 1996). The antibody staining was detected by a HRP reaction, and a field of follicle cells is shown in each panel at the same magnification. (*B*) In a stage-9 (and earlier stages) wild-type egg chambers some follicle cells are positive for dE2F staining and others are not. Note that even in the follicle cells that show dE2F expression, the levels of dE2F protein are low. (*C*) In wild-type stage-10 follicle cells there is an increase in the levels of dE2F protein in all the follicle cells. (*D*) dE2F protein is also detected in *dE2Fⁱ¹* mutant follicle cells. The same staining pattern was observed for the *dE2Fⁱ²* and *dDP^{a1}* mutant.

a change in the levels or activity of Cyclin E. We did not observe a change in the levels of *cyclin E* transcripts in stage 10A or 10B follicle cells from either wild-type or the *dDP* and *dE2F* mutant ovaries (data not shown). We tested further for an effect of E2F on Cyclin E in follicle cells by monitoring Cyclin E protein in wild-type and mutant ovaries with a monoclonal antibody against Cyclin E (Richardson et al. 1995). Surprisingly, in *dDP^{a1}*, *dDP^{a2}/Df*; *hs-dDP*, *dE2Fⁱ¹*, and *dE2Fⁱ²* mutants the levels of Cyclin E protein were normal in follicle cells at all stages, including stage 10B (Fig. 6A–C; data not shown). It is difficult to measure the pool of active Cyclin E, but Calvi et al. (1998) have shown that mAb MPM2 staining responds to Cyclin E and is a marker for Cyclin E activity in follicle cells. Overproduction or inhibition of Cyclin E results in a corresponding change in MPM2 staining. In the *dDP* and *dE2F* mutants there was no effect on MPM2 staining in follicle cells at any stage (shown for stage 10B; Fig. 6D–F). Therefore, the *dDP* and *dE2F* mutant effects on chorion gene amplification appear not to occur via Cyclin E. We also note that two other expected transcriptional targets of E2F, *PCNA* and *RNR2*, were not

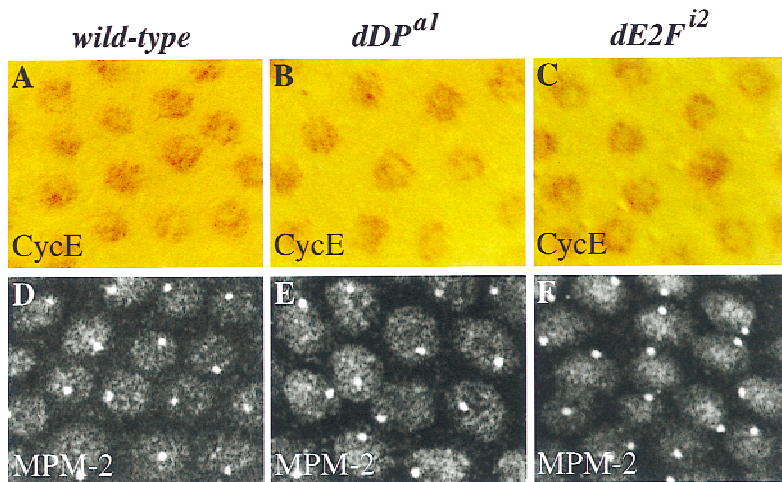


Figure 6. Cyclin E levels and activity are not affected in the *dDP* and *dE2F* mutants. A field of follicle cells from a stage-10B egg chamber is shown in each panel. (A–C) Cyclin E protein levels in wild-type and mutant follicle cells following staining with a monoclonal antibody (Richardson et al. 1995). The antibody staining is detected by the HRP reaction. (A) In wild-type follicle cells cyclin E is present at uniformly high levels in all the nuclei. (B,C) The levels of Cyclin E protein appear normal in mutant *dDP^{a1}/Df* and *dE2Fⁱ²/Df* follicle cells. (D–F) MPM2 staining reflects cyclin E activity in follicle cells (Calvi et al. 1998). MPM2 staining is visualized by confocal immunofluorescence. (D) In wild-type follicle cells a bright nuclear sphere is seen in all the follicle cell nuclei. (E,F) An identical MPM2 sphere is observed in the mutant *dDP^{a1}/Df* and *dE2Fⁱ²/Df* follicle cells.

induced in the follicle cells of either wild-type or the *dDP* and *dE2F* mutants (data not shown). This suggests that a G₁–S transcriptional program is not driving amplification in the follicle cells.

Localization of ORC and MCMs in follicle cells

Given the replication defects in the *dDP* and *dE2F* mutants and because these mutations did not affect Cyclin E activity or *PCNA* and *RNR2* expression in the follicle cells, we determined whether these mutations affected two other replication proteins, ORC and MCM. The ORC and MCM proteins are required to form the prereplication complex that is needed for the initiation of replication (for review, see Dutta and Bell 1997). *Drosophila* ORC2 was an especially good candidate, because in the *orc2* female-sterile mutant the eggshells are thin and amplification of chorion gene clusters is defective (Landis et al. 1997; Calvi et al. 1998). The *orc2* transcripts in follicle cells were not induced at stage 10 when the transition to amplification occurs (data not shown). Thus, the burst of E2F expression at this time does not trigger a detectable change in *orc2* transcription. We then examined ORC2 protein levels in follicle cells using an antibody against *Drosophila* ORC2 that recognizes a single band on an immunoblot of *Drosophila* protein extracts (data not shown). During follicle cell mitotic divisions as well as subsequent follicle cell genomic polyploidization, the levels of ORC2 were uniformly nuclear and constant in all the follicle cells (Fig. 7A). This is in contrast to the pattern of BrdU incorporation at these stages (refer to Fig. 2A,A'). Thus, prior to stage 10, ORC2 expression is the same in follicle cells that are undergoing S phase and those that are in a gap phase of the mitotic or endo cell cycle. This is consistent with the observations in other organisms that ORC is bound to chromatin throughout the cell cycle (Dutta and Bell 1997).

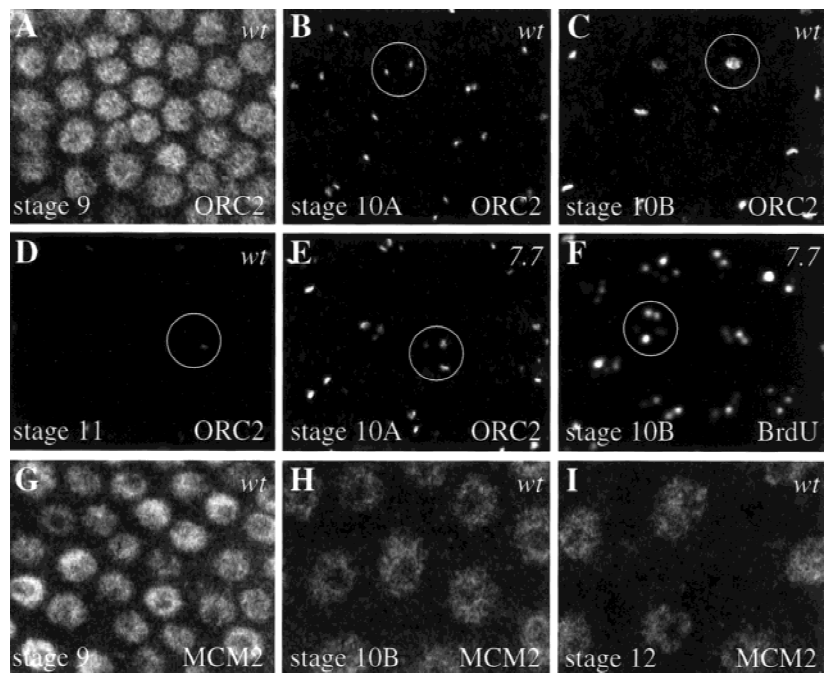
At stage 10A we observed a striking change in the localization of ORC2. ORC2 was no longer present uniformly throughout the follicle cell nucleus. Instead we saw two foci of the same size in all follicle cells (Fig. 7,

cf. B and A). In some follicle cells we were able to see three or four foci (data not shown). The ORC2 staining at stage 10A is in the pattern of BrdU incorporation observed later, at stage 10B. To determine the relationship between the ORC2 and BrdU foci, we performed double-labeling experiments (Fig. 8). The ORC foci were present prior to BrdU incorporation in stage 10A (Fig. 8A). ORC localization overlapped sites of BrdU incorporation in small, early stage-10B egg chambers (Fig. 8B). In larger (later) stage-10B egg chambers there was only a single focus of ORC labeling (Fig. 7C), and it colocalized with one of the sites of BrdU incorporation (Fig. 8C). By stage 11, the intensity of the single remaining ORC focus was reduced significantly (Fig. 7D), and ORC2 was no longer detectable by stage 12 (data not shown).

To demonstrate that ORC2 binds to amplifying regions specifically, we examined ORC2 localization in a *Drosophila* transformant strain with a 7.7-kb chorion fragment from the third chromosome cluster that is inserted onto the second chromosome and undergoes amplification (Orr-Weaver and Spradling 1986). In this strain we observed consistently an additional third ORC2 signal at stage 10A in all the follicle cells (Fig. 7, cf. E and B). The intensity of BrdU foci in this transformant line reflects the known levels of amplification with one large dot (presumably the endogenous third chromosome cluster), an intermediate dot (the amplified transposon), a smaller dot (the less amplified X cluster), and two faint dots (Fig. 7F). Thus both a new focus of ORC2 localization and BrdU labeling are produced by the transposon with the 7.7-kb chorion fragment, supporting the conclusion that ORC2 localizes specifically to the amplifying regions.

Because the MCMs associate with chromatin in an ORC-dependent manner, we also examined the localization of the MCMs in follicle cells. MCM2 was located throughout the nucleus and showed staining similar to that seen with MCM proteins in human and *Xenopus* replication systems (for review, see Chong et al. 1996; Dutta and Bell 1997) (Fig. 7). We observed similar staining with *Drosophila* MCMs 4 and 5 (data not shown).

Figure 7. ORC and MCM localization during amplification in follicle cells. The ORC and MCM proteins were visualized by confocal immunofluorescence, and a field of follicle cells is shown in each panel. (A–D) The developmental sequence of changes in ORC2 localization in wild-type follicle cells. (A) ORC2 localization was uniform and constant in all the follicle cell nuclei of early egg chambers (shown for stage 9). Note that ORC localization does not reflect the pattern of BrdU incorporation at this stage (see Fig. 2A'). (B) At stage 10A, ORC2 protein (just prior to the onset of amplification as detected by BrdU) changes from uniform nuclear staining to specific localization at the amplifying loci. Two signals of approximately equal size and intensity were seen clearly and consistently in every follicle cell nucleus. A single follicle cell nucleus is designated by a circle. (C) At stage 10B only one ORC2 focus is observed. (D) By stage 11, ORC2 signal was greatly diminished, and it was no longer detectable at stage 12. (E) Three ORC2 signals were seen consistently at stage 10A in the fly strain with an additional 7.7-kb chorion fragment. Note that the third dot is smaller than the other two ORC2 foci. (F) Amplification is detected by BrdU incorporation in the fly strain carrying the amplifying 7.7-kb transposon. Five BrdU foci were observed consistently in all the follicle cell nuclei (one large dot, two intermediate, and two small). This is in contrast to BrdU detection in wild-type follicle cells in which only four dots were observed (one large, one intermediate, and two small) (see Fig. 2B'). (G,H) MCM2 localization in wild-type follicle cells is shown using a polyclonal antibody (Su et al. 1996; Su and O'Farrell 1998). (G) In stage-9 follicle cells MCM2 expression had a mosaic pattern with some follicle cell nuclei staining stronger than others. (H,I) MCM2 expression was lower in all the follicle cells at the onset of amplification (stage 10B). Similar staining was observed with MCM4 and MCM5.



During the mitotic divisions and subsequent follicle-cell genomic polyploidization, MCM staining appeared bright in some follicle cells and faint in others (Fig. 7G). This staining pattern has been reported previously for embryonic and larval tissues, and the bright MCM signal may correlate with binding of MCM to chromatin prior to replication (Su and O'Farrell 1997, 1998). In contrast to ORC, MCM protein remained nuclear at stage 10 and discrete subnuclear foci were not observed (Fig. 7, cf. H and B). Note that MCM staining is faint and diffuse in all the follicle cell nuclei of stage-10 egg chambers and all subsequent stages.

dDP and dE2F affect ORC localization

We determined whether the levels or localization of MCMs and ORC2 were affected in the *dDP* and *dE2F* mutants. Immunostaining with anti-MCM2 and MCM5 showed that there was no effect in all of the mutants examined (*dDP^{a1}*, *dE2Fⁱ¹*, and *dE2Fⁱ²*) (Fig. 9B; data not shown). During the mitotic divisions and follicle cell genomic polyploidization (prior to stage 10A), immunostaining with anti-ORC2 revealed that the levels of ORC2 protein in all mutants were identical to wild type (data not shown).

Interestingly, we detected significant differences in the localization of ORC in the *dDP* and *dE2F* mutants

during amplification (stages 10 and later). In the *dE2Fⁱ¹* mutant, ORC2 localization was not detectable in follicle cells at stage 10A and all subsequent stages (Fig. 9, cf. D and C). Thus, the greatly diminished or failed localization of ORC2 can explain the significant reduction in BrdU incorporation at the amplifying foci in the *dE2Fⁱ¹* mutant. Although we cannot detect ORC localization, it is possible that an amount of ORC insufficient to observe by immunofluorescence is localized, and this accounts for the weak BrdU incorporation seen in the mutant. In the *dDP^{a1}* mutant nuclear ORC2 staining persisted inappropriately in stage 10 and later stages (Fig. 9E). It is possible that ORC2 is localized to the amplifying loci in these mutant egg chambers but that this localization cannot be distinguished above the background of total genomic staining. The lack of specific ORC2 localization could explain the reduced amplification and inappropriate genomic replication phenotypes observed for this mutant. Our inability to detect specific localization of ORC2 to the amplicons in the *dDP^{a1}* and *dE2Fⁱ¹* mutants is not because of the inability to visualize ORC2 immunofluorescence on DNA amplified to lower levels. By PCR analysis of our wild-type strains, *ACE1* is not amplified in stage-10A egg chambers and *ACE3* is amplified only 1.5-fold (Fig. 4), but ORC2 localization is readily visible. Thus, both the *dDP* and the *dE2F* DNA-binding mutations have a pronounced effect on ORC2 localization.

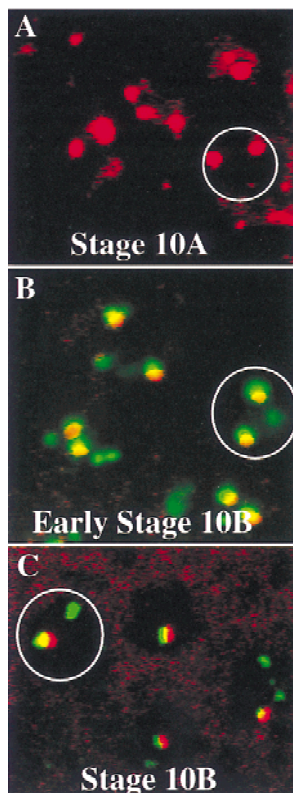


Figure 8. ORC2 and BrdU double labeling. Egg chambers were double labeled with anti-ORC2 and BrdU and staged according to Spradling (1993). ORC2 staining is shown in red and BrdU labeling in green. A field of follicle cells is shown in each panel. (A) In stage-10A egg chambers two large foci of ORC2 staining are present in each follicle cell nucleus (designated by a circle), but there is no BrdU incorporation. (B) Early stage-10B egg chambers are small, only slightly larger than stage 10A, and in these the two large ORC2 foci coincide with sites of BrdU incorporation. (C) In stage-10B egg chambers that are large and developmentally later, only a single focus of ORC2 is present and it corresponds to the largest dot of incorporated BrdU.

We analyzed the effect on ORC2 localization in the *dE2Fⁱ²* mutant that is predicted to have a truncated dE2F protein. In this mutant amplification was increased at stages 10A and B (Figs. 3C and 4), and the eggshell was thicker. Consistent with the continued amplification observed in this mutant, no effect on the localization of ORC2 protein was observed and the levels of staining were not dramatically different from wild type (Fig. 9F). Therefore, ORC2 immunostaining in all three *dDP* and *dE2F* mutants shows a correlation between the BrdU foci, amplification levels, and ORC2 localization.

Discussion

In the follicle cells ORC2 is present throughout the nucleus when the endo cycle is occurring, whereas it is restricted to sites of amplification once genomic replication ceases. Mutations in *dDP* or *dE2F* affect levels of

amplification and ORC2 localization in corresponding ways. These results indicate that E2F influences ORC localization either via an E2F transcriptional target(s) or possibly via a more direct mechanism. The replication phenotypes of the mutants and their effects on ORC localization suggest that E2F is not only important for the onset of S phase but the control of origin activity within S phase.

ORC localization and amplification

At developmental stages when the follicle cells are in the S-G endo cycle and genomic replication is occurring, prior to stage 10A, ORC2 is present throughout the nucleus. ORC2 is detected in all of the follicle cells of each egg chamber, yet the endo cycle occurs across the follicle cell layer asynchronously, with only some groups of follicle cells incorporating BrdU in any given egg chamber. Thus it appears that in the endo cycle, as in the archetypal cycle, ORC is present in the nuclei whether they are in S or G phase. In *Drosophila* the six ORC proteins form a stable complex (Gossen et al. 1995); thus, ORC2 localization is likely to reflect the presence of the ORC complex.

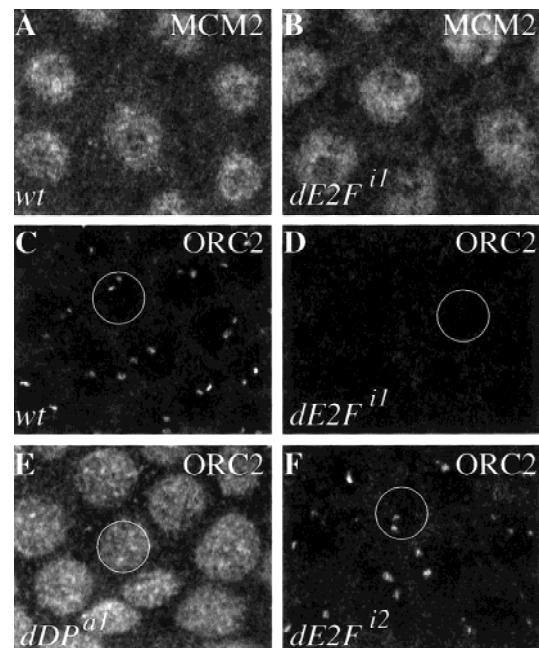


Figure 9. The *dDP* and *dE2F* mutants affect the localization of ORC but not MCMs. The ORC and MCM proteins were visualized by confocal immunofluorescence, and a field of follicle cells is shown in each panel. (A,B) The localization of MCM-2 is identical in wild-type (A) and *dE2Fⁱ¹* mutant stage-10 (B) follicle cells. Similar staining was seen with *dE2Fⁱ²* and *dDP^{a1}*. (C) ORC2 localization in wild-type stage-10A follicle cells. A single follicle cell nucleus is designated by a circle. (D) In the *dE2Fⁱ¹* mutant ORC2 localization was not detected in follicle cells at stage 10A or subsequent stages. (E) In the *dDP^{a1}* mutant ORC2 localization persists inappropriately throughout the follicle cell nucleus (cf. Fig. 7A). (F) The *dE2Fⁱ²* mutant does not appear to affect ORC2 localization.

After the endo cycle and genomic replication cease in the follicle cells, ORC2 is present at specific nuclear foci in all of the follicle cells (stage 10A). We used an additional transposon copy of chorion DNA to show that at least two of the sites of ORC2 localization correspond to chorion amplicons. In stage 10B the ORC2 foci overlap sites of BrdU incorporation. In *Drosophila* mitotic cells and salivary gland polytene chromosomes ORC2 has been observed localized preferentially to the centric heterochromatin, but we did not observe this in the follicle cells (Pak et al. 1997).

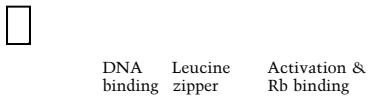
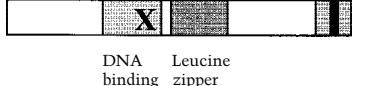


The developmental pattern of ORC2 localization provides new insights into the mechanism of chorion gene amplification. First, the specific localization of ORC2 precedes detectable BrdU labeling or amplification of *ACE1* by quantitative assays. It also precedes most of *ACE3* amplification. This suggests that binding of ORC to the amplification origins is a prerequisite for repeated initiation and amplification. Second, the disappearance of both genomic ORC2 staining and amplicon localization are very striking. Between stages 9 and 10A ORC2 changes abruptly from uniform distribution in the nucleus to specific localization to amplifying foci. By late stage 10B a single site of ORC2 localization is detectable; this overlaps the largest focus of BrdU incorporation, the site of the third chromosome chorion gene cluster (Calvi et al. 1998). By stage 11 this focus of ORC2 has nearly disappeared. In contrast, the two large and two small foci of BrdU incorporation persist until at least cycle 13. Because a 1-hr pulse of BrdU labeling was done, and development from stage 10B to stage 13 takes 7.5 hr (Spradling 1993), the BrdU seen in stage 12 and 13 egg chambers is not because of development of stage 10B egg chambers labeled during the pulse. Thus nucleotide incorporation persists after ORC2 dissociation, and perhaps reflects an elongation rather than an initiation event.

In summary, these observations raise the possibility that delocalization of ORC from the genome is linked to the cessation of genomic replication. Our results indicate that ORC localization to the amplicons in stage 10A permits the repeated initiation of amplification. BrdU incorporation begins in stage 10B, and in early 10B egg chambers ORC2 localization is coincident with sites of BrdU incorporation. Later in stage 10B, ORC2 appears to be present solely at the third chromosome chorion cluster, the one that amplifies to highest levels. By quantitative analysis we find that at stage 10B *ACE3*, a fragment known to be near the preferred amplification origin (Delidakis and Kafatos 1989; Heck and Spradling 1990), has already undergone several rounds of amplification. After stage 11, ORC2 is no longer detectable. Thus we propose that ORC localizes to amplicons in stage 10A, permits repeated initiation, and that by stage 10B nearly all of the initiation events have occurred. This is marked by the dissociation of ORC. Because the third chromosome chorion cluster amplifies to higher levels it undergoes a larger number of initiation events, and ORC persists the longest at this site. Our model proposes that during stages 11–13 the BrdU incorporation observed results from elongation of previously initiated replication forks, producing the 100-kb gradient of amplified DNA found in stage-13 egg chambers (Spradling 1981).

E2F and ORC localization

We found that mutations in *dDP* or *dE2F* affect both amplification and ORC2 localization (Table 2). In contrast to the striking effects on ORC2, the *dE2F* and *dDP* mutations do not affect either the MCM proteins or Cyclin E. Cyclin E appears to be necessary for amplification (Calvi et al. 1998). The fact that the levels and activity of

Table 2. Summary of *dE2F*⁻ and *dDP*⁻ defects

Genotype	Mutant protein	Viability	Viability with reduced <i>cyclin E</i> dose	Eggshell morphology	Inappropriate genomic replication	Amplification (BrdU foci)	ORC2 foci
<i>dE2F</i> ⁹¹		-	- ^a	N.D.	N.D.	N.D.	N.D.
<i>dE2F</i> ¹¹		+	-	thin	no	-/+ ^b	-
<i>dE2F</i> ¹²		+	+	thick	no	++ ^c	+
<i>dDP</i> ^{a1}		+	N.D.	very thin	yes	-	- ^d

^aThese animals die earlier in development with a twofold reduction of the zygotic *cyclin E* contribution.

^b(-/+) Reduced, but not absent, levels of amplification.

^c(++) Increased levels of amplification relative to wild type.

^dUniform nuclear ORC2 localization persists inappropriately.

Cyclin E are not affected in *dDP* or *dE2F* mutant follicle cells suggests that the role of Cyclin E in amplification is either parallel to or upstream of E2F.

In evaluating the mechanism by which *dDP* and *dE2F* affect ORC localization and DNA replication it is useful to consider each of the three alleles and the distinct effects separately. There are two aspects of ORC localization: clearing of ORC uniformly present in the follicle cell nuclei and subsequent specific localization of ORC to the amplicons. The *dDP^{a1}* mutation has the most severe effect in reducing BrdU incorporation and produces eggs with the thinnest shells. In addition, in some egg chambers continued follicle cell polyploidization occurs in place of amplification. The fact that in all the *dDP* mutant egg chambers nuclear localization of ORC2 persists, and ORC2 is not detectable specifically at amplifying foci could indicate that amplification requires that ORC be cleared from genomic chromatin and assembled at amplification origins. There are two outcomes from persistence of genomic ORC localization. It either blocks amplification or in a minority of egg chambers permits continued genomic replication. The clearing of ORC from genomic origins may be linked to a global change that permits rereplication and amplification of those loci that retain ORC binding.

The *dE2Fⁱ¹* mutants have less severe phenotypic effects. ORC is cleared from genomic origins but is not localized to amplification origins. The outcome of this appears to be that genomic polyploidization appropriately stops, but amplification is reduced. These effects also support the idea that ORC concentration at amplifying foci is needed for rereplication. We propose that the *dE2Fⁱ¹* defect is less severe than that of *dDP^{a1}* because a second *dE2F* gene exists (identified by D. Huen and N. Dyson, pers. comm.; Sawado et al. 1998) that is able to compensate partially for the *dE2F* mutant protein.

The absence of an effect of the *dE2Fⁱ²* mutation on ORC localization is consistent with the fact that in this mutant genomic replication ceases and amplification occurs. It is striking that amplification occurs earlier and has increased levels in mutant flies with a predicted truncated form of *dE2F* lacking the RB-binding domain. Thus restriction of the onset and extent of origin amplification may be regulated by E2F complexed with RB. It has been demonstrated that RB, when complexed to E2F, is capable of recruiting histone deacetylase and thereby converting chromatin to a compacted state (Brehm et al. 1998; Luo et al. 1998; Magnaghi-Jaulin et al. 1998). This state is correlated with inaccessibility to transcription factors, and it is reasonable to propose that it would also hinder binding of replication factors. Thus in this model, E2F in complex with RB would cause histone deacetylation in the vicinity of replication origins, leading to inhibition of amplification until stage 10B. The inability of *dE2Fⁱ²* protein to bind RB would prevent inhibition and result in premature amplification.

The differences between the three mutations in the E2F subunits provides insights into the mechanism by which E2F may influence ORC localization. This effect could be direct or indirect. Both the *dDP^{a1}* and *dE2Fⁱ¹*

mutations are predicted to weaken E2F DNA binding. Thus the known E2F activities should be present but at reduced levels. For example, these two mutant proteins should retain transactivation activity and the ability to bind RB, repress transcription, and alter chromatin structure. Despite these activities, ORC foci are not detected, implying that the ability of E2F to bind DNA is crucial for its ability to influence ORC localization. This conclusion is supported by the fact that ORC is localized properly in the *dE2Fⁱ²* mutant in which the protein has a normal DNA-binding motif and is predicted to lack the transactivation and RB-binding domains.

The suggestion that the critical activity of E2F in controlling ORC localization is DNA binding makes it possible that E2F has a direct interaction with ORC to localize it to amplification origins. There are candidate E2F-binding sites within the amplification control region for the third chromosome cluster. Using polyclonal antibodies against *dE2F* (Asano et al. 1996), we were unable to detect *dE2F* at discrete nuclear foci when amplification is occurring (I. Royzman and T. Orr-Weaver, unpubl.); however, *dE2F* may be more difficult to visualize in situ than ORC.

Another alternative is that E2F influences ORC by one of its transcriptional targets. There may be an E2F transcriptional target whose gene product affects ORC localization. Alternatively, the key target might be another subunit of ORC. In human cells ORC1, but not ORC2, is transcriptionally regulated by E2F (Ohtani et al. 1996). The observation that the truncated form of *dE2F* (*dE2Fⁱ²*) is sufficient for ORC2 localization would then suggest that *dE2F* normally activates the transcription of the critical target gene by recruiting another positive regulator to the promoter or by displacing a negative regulator. Asano and Wharton found recently that the pattern of ORC1 localization in the follicle cells parallels that seen with ORC2 (Asano and Wharton 1999). Furthermore, overexpression of ORC1 in late-stage follicle cells results in its localization throughout the nucleus and a restoration of genomic replication. Because ORC1 is transcriptionally regulated by E2F, they propose that E2F influences ORC localization and origin activity via effects on the level of ORC1.

The mutations in *dDP* and *dE2F* reveal a previously unrecognized role for E2F in controlling replication origin activity within S phase by affecting ORC localization. These results both define a new cell cycle function for E2F and suggest that it affects replication complex assembly directly or via one of its targets. Defining this mechanism will greatly enhance our understanding of the regulation and developmental control of replication initiation.

Materials and methods

Fly strains

dDP^{a1} and *dDP^{a2}* alleles were described previously (Royzman et al. 1997). The deficiency uncovering *dDP*, *Df(2R)vg56*, was provided by R. Duronio and colleagues (1998). *dE2Fⁱ¹*, *dE2Fⁱ²*, *dE2Fⁱ¹*,

dE2F^{mm729} alleles (Duronio et al. 1995), and the transgenic lines *P[w⁺, hsp70-dE2F]*, *P[w⁺, hsp70-dDP]* were provided by N. Dyson (Duronio et al. 1996). The deficiency uncovering *dE2F*, *Df(3R)e-BS2* was obtained from the Bloomington Stock Center. *cyclin E^{PZ5}* was provided by J. Roote (University of Cambridge, UK). The R7.7-6 chorion transformant has been described previously (Orr-Weaver and Spradling 1986).

New mutations in *dE2F*

dE2Fⁱ¹ and *dE2Fⁱ²* were isolated from third chromosome mutant lines established in the laboratory of R. Lehmann (Skirball Institute, New York University Medical Center, NY). The EMS mutagenesis and crosses to establish balanced stocks have been described (Moore et al. 1998). We screened these lines for mutations that disrupt *G₁-S* transcription by the strategy previously described (Rozzman et al. 1997).

To demonstrate that *dE2Fⁱ¹* and *dE2Fⁱ²* are mutations in the *dE2F* gene, both mutant lines were sequenced. Genomic DNA was isolated from adults transheterozygous for the mutagenized chromosome and *Df(3R)e-BS2*. The *dE2F* ORF was amplified from the mutant genomic DNA, and the PCR products were sequenced directly by Research Genetics.

PCR amplification assay

Ovaries were dissected in EBR (Ephrussi and Beadle 1936) to isolate staged egg chambers. Approximately 15–30 egg chambers were used. Excess EBR was removed and was replaced with 500 μ l of lysis buffer [50 mM HEPES (K⁺) at pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% deoxycholate]. The egg chambers were disrupted by brief sonication, and the insoluble material was removed by centrifugation. The supernatant was digested with proteinase K, extracted with phenol/chloroform, and ethanol precipitated. The resulting pellet was dissolved in TE buffer at a volume of 2 μ l per egg chamber. Fourfold serial dilutions were made of the dissolved pellet, and each dilution was analyzed by PCR. PCR reactions (50 μ l), containing 5 μ l of egg chamber DNA, *Vent* buffer (New England Biolabs), 200 μ M dNTPs, 1 μ M each primer, and *Taq* DNA polymerase, were subjected to 28 rounds of thermocycling. Gel electrophoresis, photography, densitometry, and quantitation, were as described previously (Aparicio et al. 1997). Levels of amplification were quantified for stage 10A, 10B, or 13 egg chambers by taking the ratio of the *ACE3* or *ACE1* product to that of the actin product and dividing by the chorion/actin ratio from control, preamplification stage 1–8 egg chambers. The dilutions used for quantitation were those from the dilution series that were in the linear range of the film and densitometer.

Primers used for PCR were CACTACCGTTTGAGTTCTTGTGCTG and GGAAGGGGAAAGCTACTTACATTTGG for *actin*; GGTAACCTGAGCCTGGCCAACATC and CCGCATAGTTTCGATCAGTATTGC for *ACE3*; TCTTCACTGGCTATCGCAGGAATGTTATC and CACCAAAAGCCATCGAGATCTCCGCCAC for *rosy*; GGAGCAACTATAATTTTACGGCCTC and CTCTAGTTGCAAAGAGATTTGAAGATG for *ACE1*.

Preparation of the ORC2 antibodies

The *Drosophila* ORC2 coding sequence was PCR amplified from a 4- to 8-hr embryonic cDNA library (Brown and Kafatos 1988). The PCR product was subcloned into pCYB2 (New England Biolabs) using *NdeI* and *SmaI* sites engineered into the termini of the PCR product. DmORC2 protein was expressed in bacteria and was purified using the Impact system (New En-

gland Biolabs) following the manufacturer's instructions. The purified protein was used to raise antibodies in rabbits.

Cytological analysis and microscopy

In situ hybridizations (Tautz and Pfeifle 1989) were carried out with digoxigenin-labeled RNA probes exactly as described in Rozzman et al. (1997). For whole-mount in situ hybridization to ovaries, ovaries were prepared as described by Ephrussi et al. (1991) and treated for 35 min in proteinase K. Subsequent steps were carried out as for embryos. The β -galactosidase expression pattern in *dE2F^{mm729}* ovaries was determined according to a standard protocol (Montell et al. 1992).

Ovaries were labeled with BrdU as described previously (Lilly and Spradling 1996). BrdU labeling was visualized using a goat anti-mouse antibody conjugated to horseradish peroxidase (HRP) (Bio-Rad) at a dilution of 1:200, or a DTAF goat anti-mouse secondary antibody (Jackson) at a dilution of 1:150. Nuclei were counterstained with DAPI as above.

For antibody stainings, ovaries were fixed in 8% EM-grade formaldehyde (Ted Pella, Inc., Irvine, CA) for 5 min, and extracted for 2 hr in 1% Triton X-100 in PBS, and then blocked for 1 hr in 1 \times PBS, 1% BSA, 0.3% Triton X-100, and 2% normal goat serum. For detection of Cyclin E, mouse mAb 8B10 was provided by H. Richardson and used at a dilution of 1:5 (Richardson et al. 1995; Lilly and Spradling 1996). The HRP goat anti-mouse secondary antibody (Bio-Rad) was used at 1:200. MPM-2 antibody (Davis et al. 1983) was purchased from DAKO Corporation. MPM-2 was diluted 1:100 (Calvi et al. 1998) and detected with a Cy3-conjugated donkey anti-mouse secondary antibody (Jackson Immunoresearch Laboratories) at a dilution of 1:150. For E2F detection, a rabbit polyclonal antibody was provided by R. Wharton (Asano et al. 1996) and used at a dilution of 1:100. We showed that this antibody specifically recognizes dE2F in the ovary, because *dE2Fⁱ¹/Df* females rescued to the adult stage by *hsE2F* did not show protein in the ovary. An HRP-conjugated goat anti-rabbit secondary antibody (Jackson) was used at a dilution of 1:100. The MCM antibodies (affinity-purified MCM2 and MCM5 and MCM4 sera) were provided by T. T. Su and P. O'Farrell (Su et al. 1996). MCM2 and MCM5 were used at 1:50 and MCM4 was used at 1:500. The ORC2 sera were used at 1:2500. ORC2 and the MCM proteins were detected using a Cy3-conjugated donkey anti-rabbit secondary antibody (Jackson) at a dilution of 1:150.

For double labeling with BrdU and anti-ORC2 antibodies, the ovaries were labeled with BrdU, fixed, bound to anti-ORC2 antibodies and secondary antibodies as described above. Fixation was performed a second time for 20 min with 4:1:1 of 16% formaldehyde/buffer B/H₂O. Buffer B is (100 mM KH₂PO₄/K₂HPO₄ at pH 6.8, 450 mM KCl, 150 mM NaCl, 20 mM MgCl₂). Our standard protocol was then followed for detection of BrdU.

A Zeiss Axiophot microscope equipped with Nomarski optics and fluorescence was used to examine and photograph the ovaries. Plan-Neofluar 10 \times , 20 \times , and 40 \times objectives were used. The images shown in Figures 6, D–F, and 7–9 were captured with a MRC 600 Bio-Rad confocal laser scanning head equipped with a krypton/argon laser, mounted on a Zeiss Axioskop microscope. A 40 \times Plan Neofluar objective was used.

Acknowledgments

We are grateful to M. Asano and R. Wharton for the antibodies against dE2F and for sharing information prior to publication, N. Dyson for providing stocks and sharing unpublished information, H. Richardson for the cyclin E antibodies, T. Su and P.

O'Farrell for the MCM antibodies, and B. Calvi and A. Spradling for communicating results prior to publication. We are grateful to the laboratory of R. Lehmann for sharing mutagenized lines. D. Fenger assisted with the confocal microscopy. J. Lees and D. Lee provided critical comments on the manuscript, and A. Amon, N. Dyson, M. Meyerson, I. Rebay, and D. Fenger gave helpful comments on earlier versions. We also thank J. Lees and A. Whittaker for many helpful discussions. I.R. was supported by a National Institutes of Health (NIH) predoctoral training grant. R.J.A. was supported by a postdoctoral fellowship from the NIH (GM18170).

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked 'advertisement' in accordance with 18 USC section 1734 solely to indicate this fact.

References

- Aparicio, O.M., D.M. Weinstein, and S.P. Bell. 1997. Components and dynamics of DNA replication complexes in *S. cerevisiae*: Redistribution of MCM proteins and Cdc45p during S phase. *Cell* **91**: 59–69.
- Asano, M. and R.P. Wharton. 1999. E2F mediates developmental and cell cycle regulation of ORC1 in *Drosophila*. *EMBO J.* (in press).
- Asano, M., J.R. Nevins, and R.P. Wharton. 1996. Ectopic E2F expression induces S phase and apoptosis in *Drosophila* imaginal discs. *Genes & Dev.* **10**: 1422–1432.
- Bandara, L.R., V.M. Buck, M. Zamanian, L.H. Johnston, and N.B. La Thangue. 1993. Functional synergy between DP-1 and E2F-1 in the cell cycle-regulating transcription factor DRTF1/E2F. *EMBO J.* **12**: 4317–4324.
- Bandara, L.R., R. Girling, and N.B. LaThangue. 1997. Apoptosis induced in mammalian cells by small peptides that functionally antagonize the Rb-regulated E2F transcription factor. *Nat. Biotechnol.* **15**: 896–901.
- Botz, J., K. Zerfass-Thome, D. Spitkovsky, H. Delius, B. Vogt, M. Eilers, A. Hatzigeorgiou, and P. Jansen-Durr. 1996. Cell cycle regulation of the murine *cyclin E* gene depends on an E2F binding site in the promoter. *Mol. Cell. Biol.* **16**: 3401–3409.
- Brehm, A., E.A. Miska, D.J. McCance, J.L. Reid, A.J. Bannister, and T. Kouzarides. 1998. Retinoblastoma protein recruits histone deacetylase to repress transcription. *Nature* **391**: 597–601.
- Brook, A., J.-E. Xie, W. Du, and N. Dyson. 1996. Requirements for dE2F function in proliferating cells and in post-mitotic differentiating cells. *EMBO J.* **15**: 3676–3683.
- Brown, N.H. and F. Kafatos. 1988. Functional cDNA libraries from *Drosophila* embryos. *J. Mol. Biol.* **203**: 425–437.
- Calvi, B.R. and A.C. Spradling. 1999. Chorion gene amplification in *Drosophila*: A model for origins of DNA replication and S phase control. In *Genetic approaches to eukaryotic replication and repair* (ed. P. Fisher) Academic Press, New York, NY (In press.)
- Calvi, B.R., M.A. Lilly, and A.C. Spradling. 1998. Cell cycle control of chorion gene amplification. *Genes & Dev.* **12**: 734–744.
- Chong, J.P., J.P. Thommes, and J.J. Blow. 1996. The role of MCM/P1 proteins in the licensing of DNA replication. *Trends Biochem. Sci.* **21**: 102–106.
- Davis, F.M., T.Y. Tsao, S.K. Fowler, and P.N. Rao. 1983. Monoclonal antibodies to mitotic cells. *Proc. Natl. Acad. Sci.* **80**: 2926–2930.
- Delidakis, C. and F.C. Kafatos. 1989. Amplification enhancers and replication origins in the autosomal chorion gene cluster of *Drosophila*. *EMBO J.* **8**: 891–901.
- Du, W., M. Vidal, J.-E. Xie, and N. Dyson. 1996a. RBF, a novel RB-related gene that regulates E2F activity and interacts with *cyclin E* in *Drosophila*. *Genes & Dev.* **10**: 1206–1218.
- Du, W., J.-E. Xie, and N. Dyson. 1996b. Ectopic expression of dE2F and dDP induces cell proliferation and death in the *Drosophila* eye. *EMBO J.* **15**: 3684–3692.
- Duronio, R.J. and P.H. O'Farrell. 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., P.H. O'Farrell, J.-E. Xie, A. Brook, and N. Dyson. 1995. The transcription factor E2F is required for S phase during *Drosophila* embryogenesis. *Genes & Dev.* **9**: 1445–1455.
- Duronio, R.J., A. Brook, N. Dyson, and P.H. O'Farrell. 1996. E2F-induced S phase requires *cyclin E*. *Genes & Dev.* **10**: 2505–2513.
- Duronio, R.J., P.C. Bonnette, and P.H. O'Farrell. 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.
- Dutta, A. and S.P. Bell. 1997. Initiation of DNA replication in eukaryotic cells. *Annu. Rev. Cell Dev. Biol.* **13**: 293–332.
- Dynlacht, B.D., A. Brook, M. Dembski, L. Yenush, and N. Dyson. 1994. DNA-binding and *trans*-activation properties of *Drosophila* E2F and DP proteins. *Proc. Natl. Acad. Sci.* **91**: 6359–6363.
- Dyson, N. 1998. The regulation of E2F by pRB-family proteins. *Genes & Dev.* **12**: 2245–2262.
- Ephrussi, A., L.K. Dickinson, and R. Lehmann. 1991. *oskar* organizes the germ plasm and directs localization of the posterior determinant *nanos*. *Cell* **66**: 37–50.
- Ephrussi, B. and G. Beadle. 1936. A technique of transplantation for *Drosophila*. *Am. Nat.* **70**: 218–225.
- Ewen, M.E., H.K. Sluss, C.J. Sherr, H. Matsushime, J. Kato, and D.M. Livingston. 1993. Functional interactions of the retinoblastoma protein with mammalian D-type cyclins. *Cell* **73**: 487–497.
- Geng, Y., E.N. Eaton, M. Picon, J.M. Roberts, A.S. Lundberg, A. Gifford, C. Sardet, and R.A. Weinberg. 1996. Regulation of *cyclin E* transcription by E2Fs and retinoblastoma protein. *Oncogene* **12**: 1173–1180.
- Gossen, M., D.T.S. Pak, S.K. Hansen, J.K. Acharya, and M.R. Botchan. 1995. A *Drosophila* homolog of the yeast origin recognition complex. *Science* **270**: 1674–1677.
- Hao, X.F., L. Alphey, L.R. Bandara, E. Lam, D. Glover, and N.B. LaThangue. 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.
- Heck, M. and A. Spradling. 1990. Multiple replication origins are used during *Drosophila* chorion gene amplification. *J. Cell Biol.* **110**: 903–914.
- Helin, K., C.-L. Wu, A.R. Fattaey, J.A. Lees, B.D. Dynlacht, C. Ngwu, and E. Harlow. 1993. Heterodimerization of the transcription factors E2F-1 and DP-1 leads to cooperative *trans*-activation. *Genes & Dev.* **7**: 1850–1861.
- Johnson, D.G., J.K. Schwarz, W.D. Cress, and J.R. Nevins. 1993. Expression of transcription factor E2F1 induces quiescent cells to enter S phase. *Nature* **365**: 349–352.
- Kato, J., H. Matsushime, H. Hiebert, M.E. Ewen, and C.J. Sherr. 1993. Direct binding of *cyclin D* to the retinoblastoma gene product (pRb) and pRb phosphorylation by the *cyclin D*-dependent kinase CDK4. *Genes & Dev.* **7**: 331–342.
- Krek, W., G. Xu, and D.M. Livingston. 1995. *Cyclin A*-kinase regulation of E2F-1 DNA binding function underlies sup-

- pression of an S phase checkpoint. *Cell* **83**: 1149–1158.
- Landis, G., R. Kelley, A.C. Spradling, and J. Tower. 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.* **94**: 3888–3892.
- Lilly, M.A. and A.C. Spradling. 1996. The *Drosophila* endocycle is controlled by Cyclin E and lacks a checkpoint ensuring S-phase completion. *Genes & Dev.* **10**: 2514–2526.
- Luo, R.X., A.A. Postigo, and D.C. Dean. 1998. Rb interacts with histone deacetylase to repress transcription. *Cell* **92**: 463–473.
- Magnaghi-Jaulin, L., R. Groisman, I. Naguibneva, P. Robin, S. Lorain, J.P. Le Villain, F. Troalen, D. Trouche, and A. Harel-Bellan. 1998. Retinoblastoma protein represses transcription by recruiting a histone deacetylase. *Nature* **391**: 601–605.
- Montell, D.J., P. Rorth, and A.C. Spradling. 1992. slow border cells, a locus required for a developmentally regulated cell migration during oogenesis, encodes a *Drosophila* C/EBP. *Cell* **71**: 51–62.
- Moore, L.A., H.T. Broihier, M. Van Doren, L.B. Lunsford, and R. Lehmann. 1998. Identification of genes controlling germ cell migration and embryonic gonad formation in *Drosophila*. *Development* **125**: 667–678.
- Morkel, M., J. Wenkel, A.J. Bannister, T. Kouzarides, and C. Hagemeier. 1997. An E2F-like repressor of transcription. *Nature* **390**: 567–568.
- Neufeld, T.P., A.F. de la Cruz, L.A. Johnston, and B.A. Edgar. 1998. Coordination of growth and cell division in the *Drosophila* wing. *Cell* **93**: 1183–1193.
- Nevins, J.R. 1992. E2F: A link between the Rb tumor suppressor protein and viral oncoproteins. *Science* **258**: 424–429.
- Ohtani, K. and J.R. Nevins. 1994. Functional properties of a *Drosophila* homolog of the E2F1 gene. *Mol. Cell. Biol.* **14**: 1603–1612.
- Ohtani, K., J. DeGregori, and J.R. Nevins. 1995. Regulation of the cyclin E gene by transcription factor E2F1. *Proc. Natl. Acad. Sci.* **92**: 12146–12150.
- Ohtani, K., J. DeGregori, G. Leone, D.R. Herendeen, T.J. Kelley, and J.R. Nevins. 1996. Expression of the *HsOrc1* gene, a human *ORC1* homolog, is regulated by cell proliferation via the E2F transcription factor. *Mol. Cell. Biol.* **16**: 6977–6984.
- Orr-Weaver, T.L. 1991. *Drosophila* chorion genes: Cracking the eggshell's secrets. *BioEssays* **13**: 97–105.
- Orr-Weaver, T. and A. Spradling. 1986. *Drosophila* chorion gene amplification requires an upstream region regulating *s18* transcription. *Mol. Cell Biol.* **6**: 4624–4633.
- Orr-Weaver, T.L., C.G. Johnston, and A.C. Spradling. 1989. The role of ACE3 in *Drosophila* chorion gene amplification. *EMBO J.* **8**: 4153–4162.
- Pak, D.T.S., M. Pflumm, I. Chesnokov, D.W. Huang, R. Kellum, J. Marr, P. Romanowski, and M.R. Botchan. 1997. Association of the origin recognition complex with heterochromatin and HP1 in higher eukaryotes. *Cell* **91**: 311–323.
- Qin, X.-Q., D.M. Livingston, W.G. Kaelin, and P.D. Adams. 1994. Deregulated E2F1 expression leads to S-phase entry and p53-mediated apoptosis. *Proc. Natl. Acad. Sci.* **91**: 10918–10922.
- Richardson, H., L.V. O'Keefe, T. Marty, and R. Saint. 1995. Ectopic cyclin E expression induces premature entry into S phase and disrupts pattern formation in the *Drosophila* eye imaginal disc. *Development* **121**: 3371–3379.
- Royzman, I. and T.L. Orr-Weaver. 1998. S phase and differential DNA replication during *Drosophila* oogenesis. *Genes Cells* **3**: 767–776.
- Royzman, I., A.J. Whittaker, and T.L. Orr-Weaver. 1997. Mutations in *Drosophila DP* and *E2F* distinguish G₁-S progression from an associated transcriptional program. *Genes & Dev.* **11**: 1999–2011.
- Sawado, T., M. Yamaguchi, Y. Nishimoto, K. Ohno, K. Sakaguchi, and A. Matsukage. 1998. dE2F2, a novel E2F-family transcription factor in *Drosophila melanogaster*. *Biochem. Biophys. Res. Commun.* **251**: 409–415.
- Sherr, C.J. 1996. Cancer cell cycles. *Science* **274**: 1672–1677.
- Spradling, A. 1981. The organization and amplification of two clusters of *Drosophila* chorion genes. *Cell* **27**: 193–202.
- Spradling, A.C. 1993. Developmental genetics of oogenesis. In *The development of Drosophila melanogaster* (ed. M. Bate and A. Martinez-Arias), pp. 1–70. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Spradling, A.C., D.V. de Cicco, B.T. Wakimoto, J.F. Levine, L.J. Kalfayan, and L. Cooley. 1987. Amplification of the X-linked *Drosophila* chorion gene cluster requires a region upstream from the *s38* chorion gene. *EMBO J.* **6**: 1045–1053.
- Su, T.T. and P.H. O'Farrell. 1997. Chromosome association of minichromosome maintenance proteins in *Drosophila* mitotic cycles. *J. Cell Biol.* **139**: 13–22.
- . 1998. Chromosome association of minichromosome maintenance proteins in *Drosophila* endoreplication cycles. *J. Cell Biol.* **140**: 451–460.
- Su, T.T., G. Feger, and P.H. O'Farrell. 1996. *Drosophila* MCM protein complexes. *Mol. Biol. Cell* **7**: 319–329.
- Tautz, D. and C. Pfeifle. 1989. A non-radioactive in situ hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene *hunchback*. *Chromosoma* **98**: 81–85.
- Trimarchi, J.M., B. Fairchild, R. Verona, K. Moberg, N. Andon, and J.A. Lees. 1998. E2F-6, a member of the E2F family that can behave as a transcriptional repressor. *Proc. Natl. Acad. Sci.* **95**: 2850–2855.
- Weinberg, R.A. 1995. The retinoblastoma protein and cell cycle control. *Cell* **81**: 323–330.
- Weintraub, S.J., C.A. Prater, and D.C. Dean. 1992. Retinoblastoma protein switches the E2F site from positive to negative element. *Nature* **358**: 259–261.
- Weintraub, S.J., K.N.B. Chow, R.X. Luo, S.H. Zhang, S. He, and D.C. Dean. 1995. Mechanism of active transcriptional repression by the retinoblastoma protein. *Nature* **375**: 812–815.