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The E2F cell cycle regulator is required for *Drosophila* nurse cell DNA replication and apoptosis

Irena Royzman[a,b], Aki Hayashi-Hagihara[a,1], Kimberley J. Dej[a], Giovanni Bosco[a,2], Janice Y. Lee[a,b], Terry L. Orr-Weaver[a,b,*]

[a]*Whitehead Institute, Nine Cambridge Center, Cambridge, MA 02142, USA*
[b]*Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02142, USA*

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Abstract

During *Drosophila* oogenesis nurse cells become polyploid, enabling them to provide the developing oocyte with vast amounts of maternal messages and products. The nurse cells then die by apoptosis. In nurse cells, as in many other polyploid or polytene tissues, replication is differentially controlled and the heterochromatin is underreplicated. The nurse cell chromosomes also undergo developmentally induced morphological changes from being polytene, with tightly associated sister chromatids, to polyploid, with dispersed sister chromatids. We used female-sterile *dE2F1* and *dDP* mutants to assess the role of the E2F cell cycle regulator in oogenesis and the relative contributions of transcriptional activation versus repression during nurse cell development. We report here that E2F1 transcriptional activity in nurse cells is essential for the robust synthesis of S-phase transcripts that are deposited into the oocyte. *dE2F1* and *dDP* are needed to limit the replication of heterochromatin in nurse cells. In *dE2F1* mutants the nurse cell chromosomes do not properly undergo the transition from polyteny to polyploidy. We also find that *dDP* and *dE2F1* are needed for nurse cell apoptosis, implicating transcriptional activation of E2F target genes in this process.

Keywords: DNA replication; *Drosophila*; Nurse cell; Cell cycle regulation; E2F

1. Introduction

The E2F transcription factor plays crucial roles in controlling cell proliferation, apoptosis, and differentiation in metazoans. E2F is a heterodimer of two subunits, E2F and DP. In mammals there are six members of the E2F family and two DP proteins (for review see Trimarchi and Lees, 2002). E2F can activate or repress transcription, and it appears that three family members, E2F1, E2F2, and E2F3 function primarily as activators. In contrast E2F4, E2F5, and E2F6 act to repress gene expression. For E2F4 and E2F5 repression is mediated by association with members of the retinoblastoma protein family (Rb). Rb, or the p107 or p130 family members, can form a trimeric complex with E2F that represses transcription both by blocking the transactivation domain of the E2F subunit and by recruiting chromatin remodeling proteins such as histone deacetylases (for review see Harbour and Dean, 2000). Rb is released from the E2F/DP heterodimer after hyperphosphorylation by the cyclin dependent kinases, CDK4/Cyclin D and CDK2/Cyclin E. Association with Rb family members also serves to keep the activating E2Fs inactive in G0 or G1 of the cell cycle, becoming active only after release of Rb.

Drosophila provides the opportunity to decipher the function of E2F during development because there are a smaller number of E2F family members, thus simplifying genetic analysis. In addition, the ability to recover a range of alleles in genes by forward genetic screens permits analysis of requirements for the genes in different developmental contexts. Mutations have been characterized for the two *Drosophila* E2F subunits, the single DP subunit, and one of the two identified Rb homologs (*Rbf*) (Cayirlioglu et al., 2001; Du and Dyson, 1999; Duronio et al., 1995, 1998; Frolov et al., 2001; Neufeld et al., 1998; Royzman et al., 1997, 1999). Analysis of these mutants shows that E2F regulates cell division, cell growth, and in some contexts differentiation.

* Corresponding author. Tel.: +1-617-258-5425; fax: +1-617-258-9872.
E-mail address: weaver@wi.mit.edu (T.L. Orr-Weaver).
[1] Present address: Kansai Advanced Research Center, Communications Research Laboratory, 588-2, Iwaoka-cho, Nishi-ku, Kobe, 651-2492, Japan.
[2] Present address: Department of Molecular and Cellular Biology, University of Arizona Cancer Center, Tucson, AZ 85724-5024, USA.

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Several lines of evidence show that the *Drosophila* E2F1 subunit is needed for activation of cell cycle gene transcription, whereas E2F2 appears to act as a repressor. This conclusion arises from the mutant phenotype and double mutant phenotypes. In *Drosophila* embryos a defined set of cell divisions occur and for many regulators maternal stockpiles are sufficient for embryogenesis. In *Drosophila* the larval tissues are polytene, and thus during larval development growth is due to increasing cell size from increased ploidy produced by an S–G endo cell cycle (for review see Edgar and Orr-Weaver, 2001). The only dividing cells during larval development are in the nervous system and in the imaginal discs which will produce adult structures during pupation. Because the imaginal tissues are dispensible until pupation, mitotic defects do not affect larval viability. In contrast, defects in DNA replication affect the endo cycle and larval viability. In particular, larval size is affected because increases in ploidy contributes significantly to the growth seen from the first instar larval stage.

Although transcription of E2F target genes such as *cyclin E* (needed for the G1–S transition) and *RNR2* and *PCNA* (needed for DNA replication) is reduced in null *dE2F1* mutants (Duronio et al., 1995; Royzman et al., 1997), these mutations do not cause lethality early in development, rather are late larval/early pupal lethal (Royzman et al., 1997). It is notable that the *dE2F1* mutant larvae grow slowly and have delayed development, at least in part due to a reduction in DNA replication rates (Royzman et al., 1997). In contrast, *dDP* mutants are pupal lethal, but they do not show slow larval development (Duronio et al., 1998; Royzman et al., 1997). Because dDP is the partner for both dE2F1 and dE2F2, this distinction in phenotype raised the possibility that in *dE2F1* mutants increased repression of E2F targets resulted from dE2F2 acting on responsive promoters (Frolov et al., 2001). This is supported by the phenotype of *dE2F2* mutants. These mutants are viable and have defects only during oogenesis (Cayirlioglu et al., 2001; Frolov et al., 2001). Strikingly, *dE2F2* mutants suppress the slow larval development of *dE2F1* mutants, implying that E2F2 represses transcription (Frolov et al., 2001). In the absence of *dE2F1* and *dE2F2*, or in *dDP* mutants, DNA replication and endo cycles are run at nearly normal rates off basal levels of transcription. Further support for the conclusion that dE2F1 activates transcription whereas dE2F2 represses it via an association with Rbf comes from the observation that *dE2F1* mutations can suppress *Rbf* mutant phenotypes (Du, 2000).

Drosophila oogenesis serves as a model to analyze E2F in many biological contexts including the archetypal division cycle, the endo cycle leading to increased ploidy, apoptosis, and differentiation. Development occurs within egg chambers composed of somatic follicle cells, germline-derived nurse cells, and the oocyte (for review see Spradling, 1993). The follicle cells also undergo a series of cell cycle changes (for reviews see Bosco and Orr-Weaver, 2002; Calvi and Spradling, 1999). These cells proliferate until stage 7 of egg chamber development. From stages 7 to 9 the follicle cells undergo three rounds of endo cycles to reach a ploidy of 16C before genomic replication is shut off. The heterochromatin is not replicated during these cycles (Hammond and Laird, 1985). At stage 10B four intervals in the genome continue to replicate, amplifying these regions above the overall ploidy of the genome (Calvi et al., 1998). Two of these intervals contain eggshell protein genes, and the increased gene copy number produced by this amplification is necessary for adequate levels of gene expression (for reviews see Calvi and Spradling, 1999; Orr-Weaver, 1991).

The role of E2F in the follicle cells has been determined from the phenotypes of female-sterile mutations in *dDP*, *dE2F1*, *dE2F2*, and *Rbf*. These studies showed that Rbf and dDP are needed to shut off the endo cycle in the follicle cell (Bosco et al., 2001; Royzman et al., 1999). Rbf, dDP, and dE2F1 limit the number of origin firings during amplification; there is a complex between Rbf/dDP/dE2F1 and the Origin Recognition Complex bound at amplification origins, so this effect of Rbf may be a direct action on the origin complex (Bosco et al., 2001). During amplification dE2F2 is needed to prevent replication at ectopic genomic sites (Cayirlioglu et al., 2001).

The nurse cells are sister cells to the oocyte, derived from four mitotic divisions. While the oocyte enters meiosis, the nurse cells enter the endo cycle. The function of the *Drosophila* nurse cells is to synthesize and deposit into the oocyte the maternal components necessary for early embryonic development. Polyploidization facilitates the high levels of gene expression needed for this. During the first five endo cycles the chromosomes are polytene, i.e. the replicated sister chromatids are tightly aligned (Dej and Spradling, 1999). In these cycles the entire genome is replicated. At cycle six (stage 5 of egg chamber development) nurse cell chromosomes compact and the sister chromatids disperse (Dej and Spradling, 1999). In subsequent cycles the sister chromatids of the nurse cells have an interphase appearance, but FISH analysis shows they remain attached at particular sites (Dej and Spradling, 1999). In addition, after cycle six there is a change in the parameters of S phase of the endo cycle, such that the heterochromatin is no longer replicated (Dej and Spradling, 1999). The underrepresented heterochromatin lies in the centromere region of each large chromosome and comprises the majority of the fourth chromosome (Dej, 1999; Dej and Spradling, 1999). The block to replication of heterochromatin in the endo cycle could be due either to a direct inactivation of replication origins or to an abbreviated S phase in which late replication of the heterochromatin does not occur (Lilly and Spradling, 1996).

In order to deposit the synthesized transcripts and proteins into the oocyte, the nurse cells undergo developmentally-induced apoptosis (Foley and Cooley, 1998; McCall and Steller, 1998). At stage 11 of egg chamber development the nurse cells initiate apoptosis, triggering a

cascade of events including nuclear envelope breakdown and transfer of nurse cell cytoplasmic and nuclear contents into the developing oocyte. Nurse cells mutant for a null allele or a weak allele of *dDP* were generated by germ line clones (Myster et al., 2000). The *dDP* mutant nurse cells failed to undergo apoptosis and did not deposit their contents into the oocytes, a dumpless phenotype. Given the distinct roles of dE2F1 and dE2F2 in transcriptional activation and repression it was of interest to determine whether the effect of dDP on apoptosis was mediated via one of the E2F subunits. We analyzed the nurse cell phenotypes of female-sterile alleles of *dE2F1* and *dDP* and found that dE2F1 is required for nurse cell apoptosis together with dDP. In addition, the *dDP* and *dE2F1* mutations exerted striking effects on nurse cell DNA replication and chromosome morphology.

2. Results

2.1. Female-sterile alleles of dDP and dE2F1

We previously isolated three alleles of E2F that are female sterile, dDP^{a1}, $dE2F1^{i1}$ and $dE2F1^{i2}$. These mutants were identified in a screen to recover mutations that disrupted the pattern of G1-S transcription in late embryogenesis, and embryonic transcription of E2F-responsive genes is reduced in all three mutants (Royzman et al., 1997, 1999). The $dDP^{a1}$and $dE2F1^{i1}$ mutations cause amino acid substitutions in the DNA binding domains of each protein, and the $dE2F1^{i2}$ mutation results in a protein lacking the transactivation and RB-binding domains of dE2F1 (Royzman et al., 1997, 1999).

The molecular differences between these mutant proteins provides insights into the roles of the dE2F1 and dE2F2 proteins. Because these distinctions are relevant for interpreting the phenotypes described below we review them here. We showed that the truncated $dE2F1^{i2}$ mutant protein binds to dDP but not to Rbf and that it can still bind to DNA (Bosco et al., 2001). The dDP^{a1} and $dE2F1^{i1}$ mutant proteins would not bind DNA. Thus in the $dE2F1^{i1}$ mutants the dE2F2 protein should bind to all E2F sites, causing enhanced repression as it does in *dE2F1* null mutants. In contrast in the $dE2F1^{i2}$ mutants neither dE2F1 mediated transcriptional activation or repression could occur, but ectopic repression by dE2F2 is not expected. In the dDP^{a1} mutants neither the activation activity of *dE2F1* or the repression activity of dE2F2 should be present. Consistent with this dDP^{a1} and $dE2F1^{i1}$ have reduced viability in trans to their respective deficiencies, but the $dE2F1^{i2}$ mutation does not have a marked effect on viability. In addition, the $dE2F1^{i2}$ mutation has been shown to rescue *Rbf* null mutants, implying it does block ectopic repression from dE2F2 (Du, 2000).

In this study, we used these three female-sterile mutations to evaluate the role of E2F in nurse cell endoreplication and oocyte development. Females bearing dDP^{a1}, $dE2F^{i1}$, and $dE2F^{i2}$ mutations in trans to deficiencies were initially characterized by staining their ovaries with DAPI to reveal the DNA. The mutant egg chambers contained 16 germline cells, 15 nurse cells and an oocyte, indicating the proper completion of the four mitotic divisions that produce the egg chamber cyst. In addition, the nurse and follicle cells became polyploid. Thus, although the ovaries were smaller than wild-type, the cell cycle appeared normal overall in the *dDP* and *dE2F1* mutants. The predominant defects we observed were a failure of nurse cells to deposit their contents into the oocyte and degenerate and an increased amount of heterochromatic DNA in the nurse cell nuclei. These phenotypes are described in detail below. Occasional degenerating early egg chambers (stage 8 and earlier) were observed in the mutants.

2.2. Heterochromatin is inappropriately replicated in dDP and dE2F1 mutants

Although the nurse cells became polyploid in the mutants, the parameters of the endo cycle S phase were affected in all three mutants. The properties of the nurse cell endo cycle have been analyzed by Dej and Spradling and the associated chromosome morphologies described (Dej and Spradling, 1999). Normally, after the fifth endo cycle genomic replication in the nurse cells is incomplete, with sequences residing in heterochromatic regions of the genome being underrepresented (Fig. 1A,B for schematic). In wild-type egg chambers, staining with DAPI gives one predominant and a few small, intensely labeled regions of brightly-staining heterochromatin (Fig. 1C, arrow). FISH studies demonstrated that the predominant DAPI-staining focus is the fourth chromosome and the smaller clusters are in the heterochromatin on 3L and 3R (Dej and Spradling, 1999). DAPI staining of $dE2F1^{i1}$ and $dE2F1^{i2}$ in trans either to a deficiency or a null *dE2F1* allele ($dE2F1^{91}$) and dDP^{a1}/*Df* showed a dramatic increase in the amount of nurse cell heterochromatin (Fig. 1D and data not shown).

To establish that this apparent over-replication of heterochromatin in the mutants was due to reduced E2F function and not a novel E2F activity caused by these mutations, we generated adult female flies that were mutant for null alleles of *dE2F1* and *dDP*. This was accomplished by rescuing the lethality of null $dE2F1^{91}$and dDP^{a2} mutants by crossing in *hsp70-dE2F1* and *hsp70-dDP* transgenes (Duronio et al., 1996). We achieved a partial rescue of lethality with basal expression of the wild-type genes from the *hsp70* promoter. The rescued flies were expected to have reduced E2F activity relative to wild-type but to retain enough function to be viable. Indeed, the recovered *hs-dE2F1*; $dE2F1^{91}$/*Df* and dDP^{a2}/*Df*; *hs-dDP* mutant adults had rough eyes and were female sterile. DAPI staining revealed that the nurse cell heterochromatin in both mutants was increased in size and staining intensity as compared to heterozygous sibling controls (Fig. 1E and data not shown). These results confirm

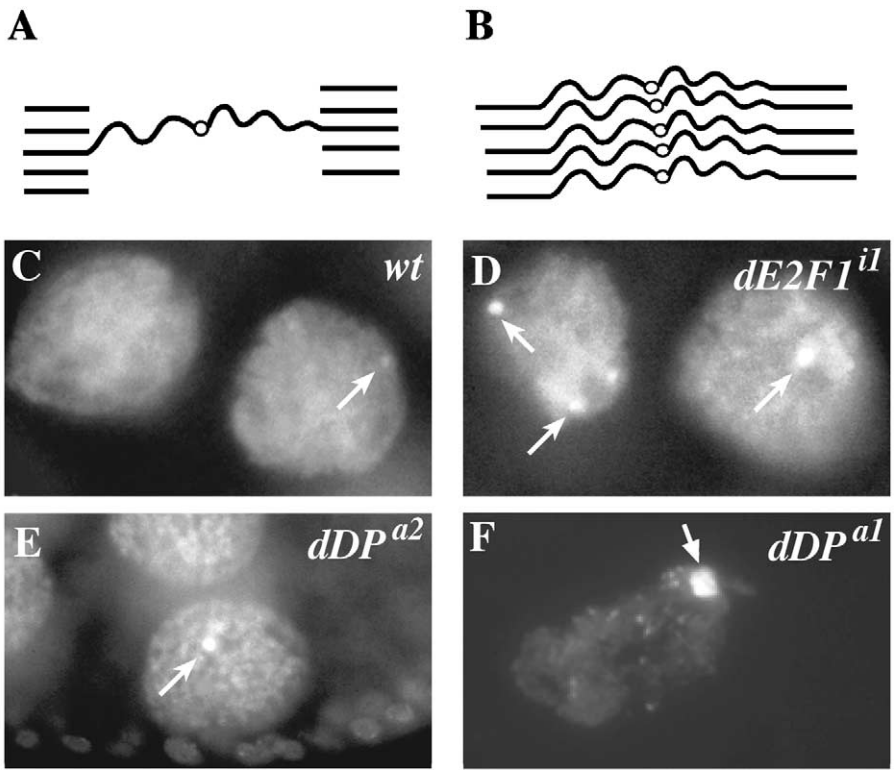

Fig. 1. E2F mutants show increased levels of heterochromatic staining. (A) Schematic of replication in polyploid cells: sequences residing in heterochromatic regions (curved lines) of the genome are not completely duplicated. (B) Schematic showing replication of heterochromatin in the mutants. DAPI was used to monitor the degree of underrepresentation of heterochromatic sequences, because heterochromatin stains intensely with DAPI. (C) In control $dE2F1^{i2}$/+ stage 10 nurse cells (wt), underreplication was normal and the heterochromatin is scarcely visible (arrow). (D) In mutant $dE2F1^{i1}/dE2F1^{91}$ and $dE2F1^{i2}/Df$ stage 10 nurse cells a large spot of intense DAPI staining is seen together with several smaller foci (arrows), indicative of the increased levels of heterochromatic sequences. (E) In the *dDP* null mutant that was rescued to adulthood, $hsDP;dDP^{a2}/Df$, large intensely staining blocks of heterochromatin were observed in each nurse cell nucleus (arrow). The same over-replication of heterochromatin relative to wild-type was seen in $hsE2F1;dE2F1^{91}/Df$ mutant nurse cells (data not shown). (F) A squashed nucleus from a dDP^{a1}/Df female reveals chromosome morphology and shows that the intensely stained DAPI focus localizes to the periphery of the nucleus, as does the fourth chromosome (Dej, 1999). Several other intense DAPI foci are visible in the remainder of the chromosome mass.

that the increased presence of nurse cell heterochromatin in the dDP^{a1}, $dE2F1^{i1}$, and $dE2F1^{i2}$ female-sterile mutants is due to reduced dE2F1 function, as opposed to a novel activity caused by these mutations. The observation of this phenotype in all three mutants implies that it is not due to inappropriate repression by dE2F2.

The fourth chromosome can be identified in nurse cells if the chromatin is spread by squashing the nuclei; FISH experiments using both a satellite DNA probe and a unique fourth chromosome euchromatic sequence demonstrate that the chromosome lies on the edge of the nucleus (Dej, 1999) (see Fig. 3A,B). The morphology of this chromosome as an intense DAPI spot to one side or slightly removed from the remainder of the chromatin makes its identity unmistakable. To test whether the large DAPI focus in the *dDP* and *dE2F1* mutants corresponded to the fourth chromosome and thus an increased level of heterochromatin, we squashed mutant nurse cells and examined the morphology. These experiments showed a DAPI-stained focus on the edge that indicates a very large fourth chromosome, as well as scattered foci of intense DAPI staining that may correspond to the heterochromatin on chromosome *3* (Fig. 1F and data not shown). A similar morphology to that of the *dDP* and *dE2F1* mutants was observed on squashed nuclei from $cyclin\ E^{01672}$ mutants in which the fourth chromosome was demonstrated to be overreplicated (Lilly and Spradling, 1996) (data not shown).

To test more directly whether inappropriate DNA replication in the heterochromatin occurs in these mutants we pulse labeled ovaries from one of the mutants, $dE2F1^{i2}/Df$, with BrdU, squashed the chromosomes and detected BrdU incorporation by antibody labeling. The fourth chromosome was identified by its characteristic nuclear position and DAPI morphology (Dej, 1999; Dej and Spradling, 1999). In the $dE2F1^{i2}/TM3$ sibling controls we observed BrdU incorporation on only small regions of the fourth chromosome after the polytene to polyploid transition and the onset of underreplication of the heterochromatin in the nurse cells (Fig. 2A–C, asterisk). Presumably this limited labeling of the fourth chromosome corresponds to euchromatic regions within the overall heterochromatic fourth chromosome. In contrast, in $dE2F1^{i2}/Df$ mutants BrdU was incorporated throughout the fourth chromosome (Fig. 2D–F, asterisk).

The incorporation of BrdU into the fourth chromosome in

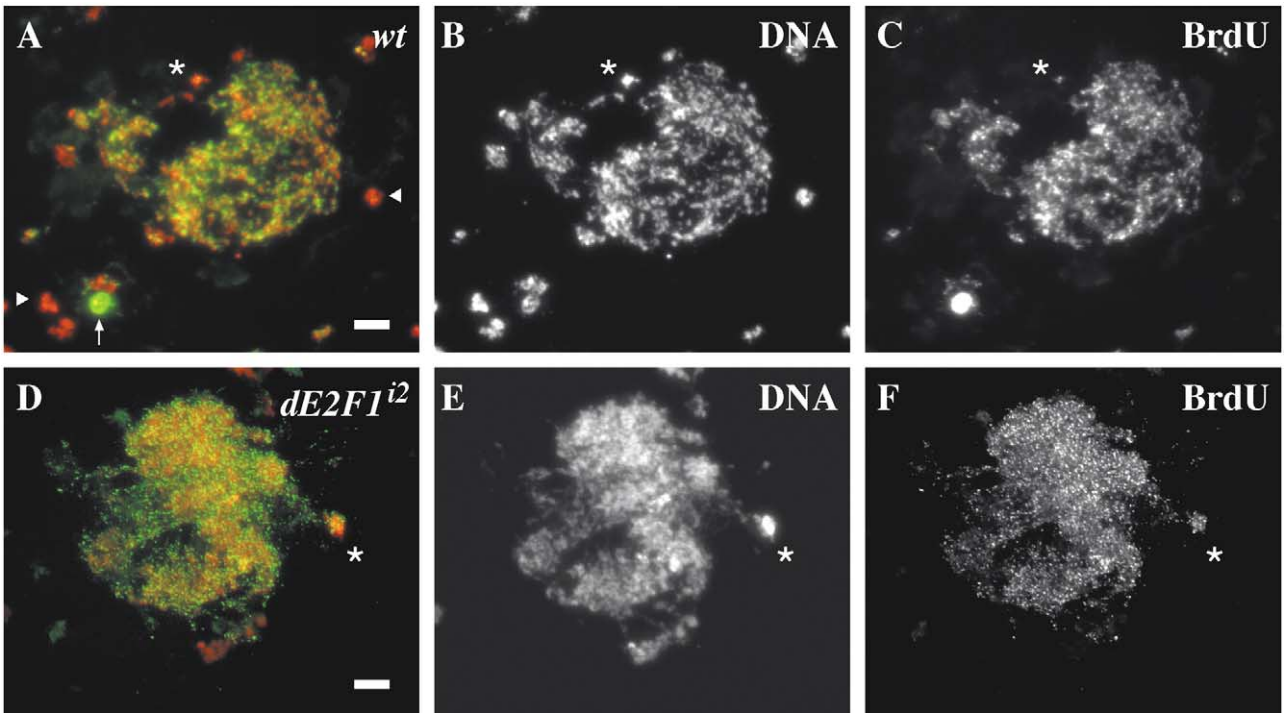

Fig. 2. Inappropriate DNA replication in the fourth chromosome of *dE2F1*^{i2} mutants. Dissected ovaries were pulse labeled with BrdU prior to squashing and staining with anti-BrdU antibodies. In the merged images (A,D) DNA is shown in red and BrdU in green. The two stained channels are split on the right. (A) In *dE2F1*^{i2}*/TM3* controls (wt) a nurse cell nucleus undergoing DNA replication is shown. The fourth chromosome is shown by the asterisk. Only part of the chromosome is labeled with BrdU, presumably the euchromatic portion. Some follicle cells are in S phase (arrow) whereas others did not undergo DNA replication during the pulse (arrowheads). (B) The DNA channel. (C) The BrdU channel. (D) In this *dE2F1*^{i2}*/Df* nucleus the fourth chromosome, very enlarged and brightly stained compared to controls, is stretched out to the right (asterisk). The fourth chromosome has incorporated BrdU throughout the chromosome. (E) The DNA channel. (F) The BrdU channel. In both (A) and (D) the scale bar is 10 μm.

dE2F1^{i2}*/Df* mutants and the increase in fourth chromosome size and DAPI staining in all of the mutants show that the heterochromatin continues to be replicated rather than being blocked for replication when E2F1 activity is reduced.

2.3. Polytene chromosome morphology is affected in dE2F1 mutants

The experiments to examine the cytology of the fourth chromosome in the *dDP* and *dE2F1* mutants also permitted us to analyze the requirements for E2F activity during the morphological changes in nurse cell chromosomes from polytene to polyploid states. During the first five endo cycles when nurse cell chromosomes are polytene the two homologous copies of each chromosomes are associated and have a banded DAPI staining pattern (Fig. 3A–C) (Dej and Spradling, 1999). Prior to dispersal of the sister chromatids the large chromosome arms contract and become bulbous, whereas the fourth chromosome persists as a brightly staining sphere (Fig. 3D) (Dej and Spradling, 1999). Once the sister chromatids are dispersed, the nurse cell chromatin takes on a more interphase-like appearance (Fig. 3E), although occasionally some degree of oligoteny can be detected in squashes (data not shown) (Dej and Spradling, 1999).

In *dE2F1*^{i2}*/Df* mutants two striking effects on nurse cell chromosome morphology were observed. Although the mutant chromosomes went through the polytene and bulbous states, the homologous copies of each of the large chromosomes frequently were separated rather than associated as in wild-type (Fig. 3F). The fourth chromosome was always a single sphere. In the bulbous stage the separation of the homologs was very apparent, because ten large chromosome arms were present rather than five in each nucleus (Fig. 3G). In the *dE2F1*^{i2}*/Df* mutants the sister chromatids often did not disperse after the bulbous stage. In contrast they became more condensed, having a polytene rather than interphase appearance. Individual chromosome arms were visible, and the chromosomes were clustered as if the homologous sister chromatids remained in proximity (Fig. 3H). Thus after the bulbous stage it seemed that the nurse cells went through a phase that was endo mitotic with condensed chromosomes rather than into an interphase state. Although we did find mutant nurse cells in which the polytene chromosomes dispersed as in Fig. 2D, it was common to see later nurse cells with persistent polytene chromosomes. This was observed in about half of the nurse cell squashes. The grouping of replicated sister chromatids became less pronounced with further endo cycles and increased ploidy.

We were unable to analyze the cytology of the *dDP*^{a1}*/Df* mutant nurse cells with the same precision because oogen-

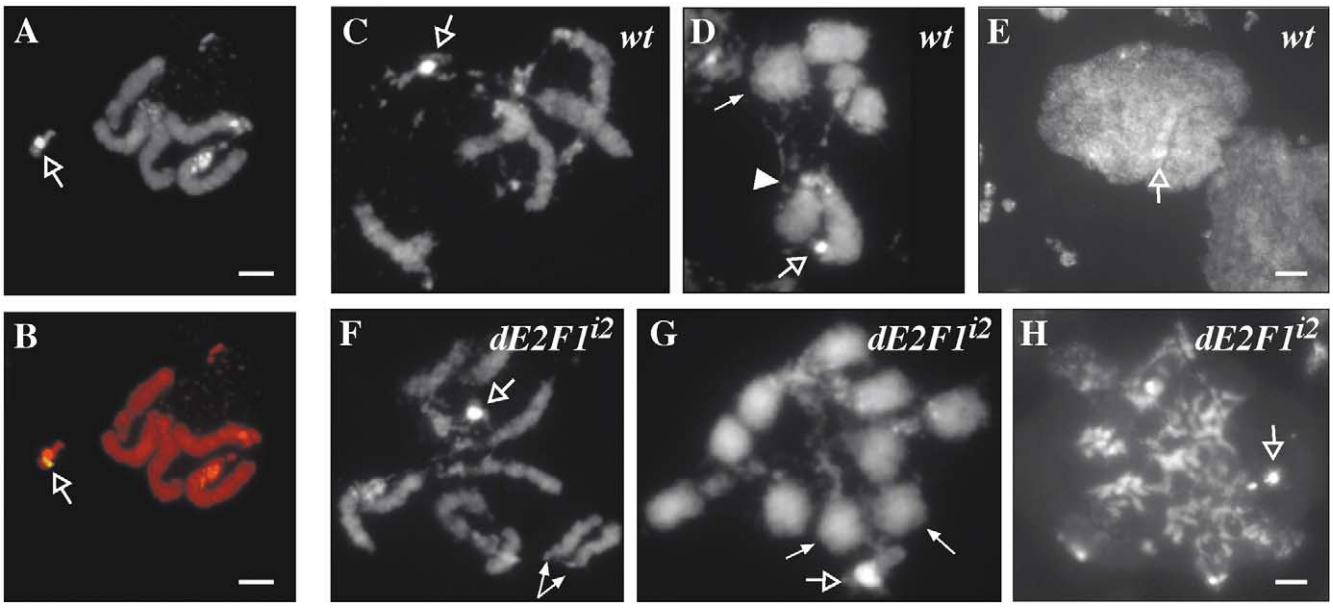

Fig. 3. The *dE2F1*^{i2} mutation affects nurse cell chromosome morphology. Nurse cell chromosomes normally progress through three morphological states, as shown by the *dE2F1*^{i2}*/TM3* controls (wt). (A–C) For the first five endo cycles the chromosomes are polytene, with the homologous copies of each chromosome associated. The fourth chromosome is the brightly stained dot (open arrow). In panels A and B the fourth chromosome is identified by in situ hybridization with the P1 clone DS08057 from 102B1–B2 in the euchromatin of the fourth chromosome. (A,C) DAPI stain of the DNA; (B) The in situ hybridization with a FITC probe is shown in yellow and the DNA in red. Panels (A) and (B) are reproduced with permission from Dej (1999). (D) During endo cycle five the chromosomes condense into bulbous shapes. The two homologous copies of each chromosome arm are together, as highlighted by the thin arrow. The fourth chromosome remains brightly stained (open arrow), and the heterochromatin of the third chromosome also stains more intensely with DAPI (arrowhead). (E) From cycle 6 on, the replicated chromatids are dispersed and the chromosomes have a more interphase-like appearance. Note that the fourth chromosome (open arrow) does not enlarge any further and is slightly larger than the size of the heterochromatic dot in surrounding follicle cells. (F) In *dE2F1*^{i2}*/Df* mutants the chromosomes are polytene for the initial endo cycles, but homologous copies of the chromosomes frequently are separated. Two separated homologs are indicated by the thin arrows. The fourth chromosome is shown by the open arrow. (G) The bulbous stage occurs in *dE2F1*^{i2}*/Df* mutants but often with separated homologs, resulting in 10 blobs plus the intensely stained fourth chromosome (open arrow). Two of the separated homologs are shown by the thin arrows. (H) Polytene chromosomes and distinct chromosome arms frequently persist in later endo cycles in *dE2F1*^{i2}*/Df* mutants. This nucleus is about 128C, so each visible chromosome is polytene and contains approximately 16 sister chromatids. Note the large size and intense staining of the fourth chromosome (open arrow), consistent with its inappropriate continued replication. The scale bars are 5 μm.

esis was delayed in these mutants. Thus it was difficult to obtain mutant nurse cells in endo cycles 4–8. In the later egg chambers, as shown in Fig. 1F, we did not observe persistent polytene chromosomes as in the *dE2F1*^{i2} mutants. The reduced viability of the *dE2F1*^{i1} mutants precluded a cytological analysis of these nurse cells. Although the *cyclinE*^{01672} allele shows increased heterochromatic DNA replication, it does not exhibit the perturbations in nurse cell chromosome structure seen in the *dE2F1*^{i2} mutants (data not shown).

2.4. dDP and dE2F1 are needed for transcription of some target genes in the nurse cells

We tested whether the activity of the E2F transcription factor was needed for transcription of known E2F target genes in the nurse cells by examining transcript levels of *PCNA*, *ORC1*, and *RNR2* by in situ hybridization (Asano and Wharton, 1999; Duronio et al., 1995, 1998). All three of these transcripts are present at background levels until stage 9 of egg chamber development. The transcripts are induced at high levels in stage 10 (Fig. 4A,C, and data not shown for *RNR2*). Staining of egg chambers with antibodies against dE2F1 protein showed that the levels of protein increase at the same time that E2F targets are expressed (Fig. 4G). The *dE2F1*^{i1}, *dE2F1*^{i2}, and *dDP*^{a1} mutations all caused a significant reduction, although not elimination, of these transcripts (Fig. 4B,D and data not shown). To compare the transcript levels more accurately in the mutants we did the in situ hybridizations and histochemical staining for *ORC1* with *dDP*^{a1}*/CyO* controls and *dDP*^{a1}*/Df* mutant ovaries in the same tube. The mutant ovaries were distinguished from sibling controls by their smaller size and by the dumpless phenotype (see below). In this experiment it was clear that the levels of *ORC1* transcript were significantly reduced in *dDP*^{a1} mutants. The mutations in *dDP* and *dE2F1* did not generally block transcription at stages 9 and 10 of egg chamber development, because the levels of *cyclin E* transcripts were not detectably altered by these mutations (Fig. 4E,F, and data not shown for *dDP*^{a1} and *dE2F1*^{i2}). A reduction in the levels of *RNR2* transcripts was observed by Myster et al. in germline clones of *dDP* mutants (Myster et al., 2000).

Reduction of the levels of Cyclin E protein can result in

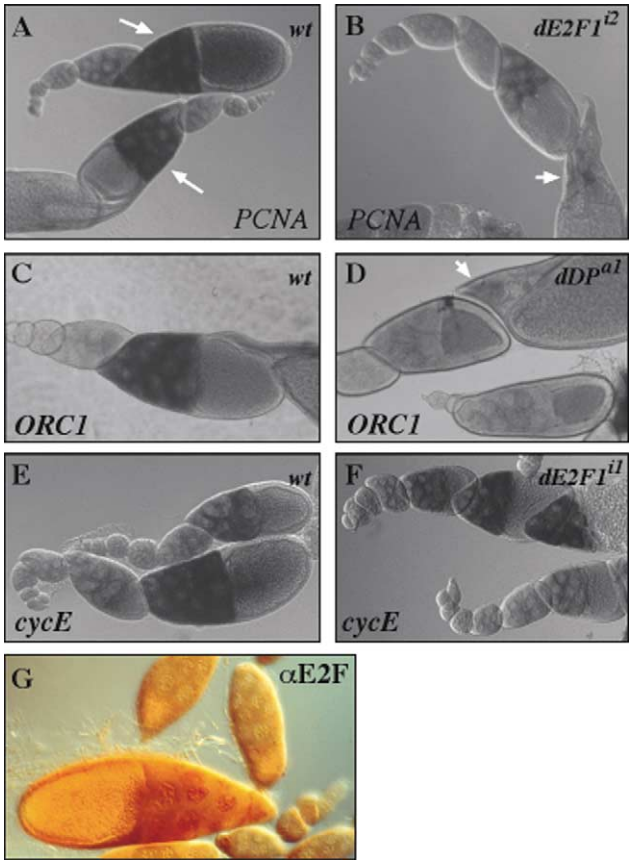

Fig. 4. dE2F1/DP is needed for some, but not all, nurse cell transcription. (A) *PCNA* expression in a continuum of developing control $dE2F1^{i2}/TM3$ egg chambers. *PCNA* transcripts were detected by whole-mount in situ hybridization. *PCNA* expression is low in early egg chambers, and it is highly induced at stage10B (arrow). (B) In mutant $dE2F1^{i2}/Df$ egg chambers *PCNA* expression is diminished compared to wild type. In the mutant egg chambers the nurse cell nuclei do not break down and transcripts persist in stage 14 egg chambers (arrow). The same effect on *PCNA* transcription was observed in the $dE2F1^{i1}$ and dDP^{a2} mutants (data not shown). (C,D) To standardize the hybridization and staining reactions to compare transcript levels, ovaries from dDP^{a1}/CyO *and* dDP^{a1}/Df mutant females were hybridized to an *ORC1* probe and stained in the same tubes. The mutant ovaries were recognized by the persistence of nurse cell nuclei in late egg chambers (arrow in D). The levels of *ORC1* transcripts are reduced in the dDP^{a1}/Df mutants. The levels of *ORC1* transcripts also were reduced in the $dE2F1^{i1}$ and $dE2F1^{i2}$ mutants (data not shown). (E,F) In contrast to the effects of *dE2F1* and *dDP* mutations on the levels of *PCNA*, *ORC1* and *RNR2* (data not shown) transcripts, in these mutants there was not a detectable reduction in the levels of *cyclin E* transcripts (data not shown for $dE2F1^{i2}/Df$ and dDP^{a1}/Df). (G) Egg chambers stained with anti-E2F1 antibodies. The E2F1 protein is detectable in the nurse cells of the stage 10 egg chamber but is at background levels in the earlier egg chambers.

inappropriate replication of the heterochromatin in nurse cells (Lilly and Spradling, 1996). Given that there was no effect of the *dDP* and *dE2F1* mutations on *cyclin E* transcript levels, it seemed unlikely that the E2F1 mutant effects were solely due to decreased Cyclin E. To examine this possibility further, we analyzed Cyclin E protein levels in the nurse cells by antibody staining.

Consistent with the findings of Lilly and Spradling (1996), we observed varying levels of Cyclin E protein in wild-type nurse cells within an individual egg chamber (Fig. 5A). Nurse cell nuclei were observed with high and low levels of Cyclin E protein (Fig. 5A, arrows). The levels of Cyclin E protein in the 16th cell of each egg chamber, the developing oocyte, were always high (Fig. 5A, arrowhead). In the *dDP* and *dE2F1* mutant nurse cell nuclei and oocyte, Cyclin E protein was readily observed and the levels of Cyclin E protein were similar to wild-type (Fig. 5B–D). Thus, the induction of *cyclin E* appears not to be affected by the *dDP* and *dE2F1* mutations in this developmental context, and the E2F mutant defects are not likely to be solely the consequence of reduced Cyclin E. In addition, *dDP* and *dE2F1* mutant nurse cell nuclei with high and low levels of Cyclin E were observed (Fig. 5B–D). It is likely, therefore, that the levels of Cyclin E properly oscillate with the S–G endo cycle in the mutants.

2.5. Nurse cell apoptosis and dumping is affected by dDP and dE2F1 mutations

The function of nurse cells is to synthesize maternal components and deposit them into the oocyte. This is accomplished by high levels of transcription in the nurse cells in stage 10 and subsequent dumping of the nurse cell nuclear and cytoplasmic contents into the oocyte. The initiating event for nurse cell dumping appears to be the onset of apoptosis in stage 11 which leads to nuclear envelope breakdown, actin bundle

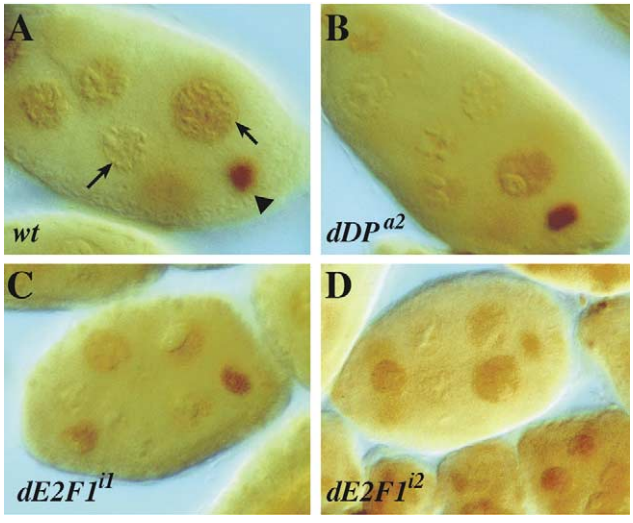

Fig. 5. Cyclin E protein oscillates in mutant *dDP* and *dE2F1* nurse cells. (A–D) Cyclin E protein levels in wild-type and mutant nurse cells following staining with a monoclonal antibody (Richardson et al., 1995). The antibody staining was detected by horseradish peroxidase (HRP) reaction, and a single early egg chamber is shown in each panel in the same orientation. (A) In wild-type nurse cell nuclei the levels of Cyclin E oscillate (arrows). Some nurse cell nuclei have low or undetectable levels of Cyclin E protein while others have high levels of Cyclin E. Note that the level of Cyclin E protein is always high in the 16th cell, the oocyte (arrowhead). (B–D) The oscillations in Cyclin E protein levels in nurse cells from the *dDP* null that was rescued to adulthood, dDP^{a2}, and from the female-sterile $dE2F1^{i1}/dE2F1^{9l}$ and $dE2F1^{i2}/Df$ mutants appear similar to wild-type.

assembly, and cortical contraction (Foley and Cooley, 1998; Matova et al., 1999). The cell death machinery has been shown to be required for nurse cell apoptosis and dumping (McCall and Steller, 1998). E2F is necessary in mammals for cell death arising from cellular defects, and the activating family members E2F1 and E2F3 are critical (Tsai et al., 1998; Ziebold et al., 2001). The nurse cells in *Drosophila* provide the opportunity to define the role of E2F in developmentally-induced apoptosis. Myster et al. (2000) showed that *dDP* is required for nurse cell apoptosis. The *dE2F1* female-sterile alleles permitted us to test whether this action was via an activating E2F, as in mammals.

We observed in the mutants that nurse cells persisted inappropriately until the final stages of egg chamber development (Fig. 4B,D). The oocytes were small, indicating a possible block to the transport of large amounts of RNA and protein from the nurse cells. We examined this in more detail using two assays for apoptosis; nuclear envelope integrity was monitored by anti-lamin staining and DNA fragmentation was measured by TUNEL labeling which measures the presence of nicked DNA.

In early stage egg chambers the pattern of lamin staining and the structure of the nuclear envelope was the same in wild-type and the mutants (Fig. 6A,B). In stage 11 of wild-type egg chamber development anti-lamin staining showed that the nurse cell nuclear envelope had broken down, nuclear and cytoplasmic components were transferred to the oocyte, and in stage 12 the nurse cell nuclei labeled with TUNEL (Figs. 6C and 7A). In the dDP^{a1}, $dE2F1^{i1}$, and $dE2F1^{i2}$ mutants, however, the lamin staining persisted beyond the stage when the envelope is broken down in controls (Fig. 6D–F). In addition we observed that *PCNA* and *ORC1* transcripts were retained in the nurse cells until stage 14 rather than being transferred to the oocytes (Figs. 4B,D and 6G). Myster et al. observed that *RNR2* transcripts were retained in nurse cells of germline clones of *dDP* mutants (Myster et al., 2000). Furthermore, Cyclin E protein was not released from the nurse cell nuclei and was present in mature stage 14 egg chambers (Fig. 6H).

In wild-type and heterozygous control ovaries, all 15 nurse cell nuclei stained positively for TUNEL during stages 12–13 of oogenesis (Fig. 7A). By stage 14, the nurse cells degenerated, and TUNEL staining was no longer observed (Fig. 7C). In the majority of *dDP* and *dE2F1* mutant egg chambers, however, a cluster of nurse cells remained attached to the stage 14 oocyte and completely failed to label with TUNEL (Fig. 7B and data not shown). Consistent with a failure to undergo DNA fragmentation, a DAPI counterstain revealed that the nurse cell nuclei retained their DNA, and it was not highly condensed or in clumps (Fig. 7B′). Thus, both by morphological and biochemical criteria the majority of *dDP* and *dE2F1* mutant nurse cells do not show the features of apoptosis. In some of the *dE2F1* and *dDP* mutant egg chambers, one or a few persisting nurse cell nuclei did stain with TUNEL, but not until stage 14 (Fig. 7D,D′). The effects on apoptosis were similar for all the female-sterile mutants: dDP^{a1}, dDP^{a2}/Df; *hsDP*, $dE2F1^{i1}$, and $dE2F1^{i2}$. Staining with the vital dye acridine orange (a marker for apoptotic cells) yielded results that were consistent with those from the TUNEL analysis (data not shown). We conclude that dE2F1 activity promotes normal apoptosis. Moreover, the C-terminal region of the dE2F1 protein is important for this function, because the $dE2F1^{i2}$ truncation mutant is defective in apoptosis.

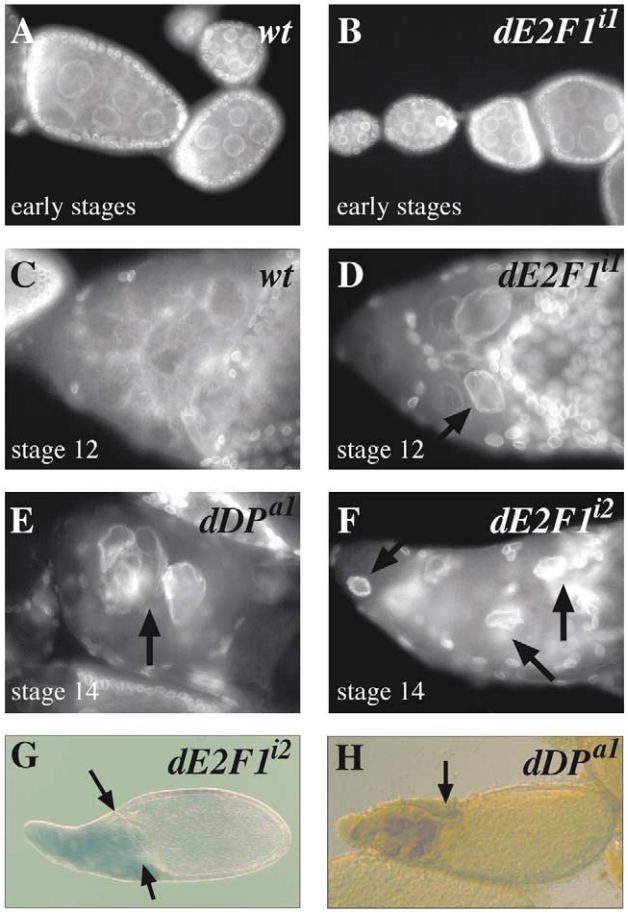

Fig. 6. dE2F1 is required for nuclear envelope breakdown. Early and late egg chambers were stained with a monoclonal antibody to nuclear lamin Dm_0. The large rings surround the highly polyploid nurse cell nuclei and the small rings surround the follicle cells. In the early stages, nuclear envelope staining is identical for the sibling control (A) and the $dE2F1^{i2}/Df$ mutant egg chambers (B). (C) At stage 12, nurse cell nuclear envelope staining becomes diffuse in the controls, but the nuclear envelopes (arrow) persist in the $dE2F1^{i2}/Df$ mutant (D). Nuclear lamin continues to be visible in dDP^{a1}/Df (E) and $dE2F1^{i2}/Df$ (F) mutant nurse cells even at stage 14 (arrows). The same phenotype was observed for the $dE2F1^{i1}/dE2F1^{91}$ mutant (data not shown). (G) The nurse cell cytoplasm is not dumped into the oocyte in these mutants, as evidenced by the persistence of *PCNA* transcripts in the nurse cell region of mutant $dE2F1^{i2}/Df$ stage14 egg chambers. This egg chamber was hybridized in situ to a probe for the *PCNA* transcripts. The dorsal appendages that define this as a stage 14 egg chamber are shown by arrows. (H) The persistence of Cyclin E protein is the mutant nurse cell nuclei is consistent with a failure of nuclear breakdown. This dDP^{a1}/Df mutant egg chamber was bound to the Cyclin E antibody. Although it is a stage 14 egg chamber (see dorsal appendages marked by arrow), intense staining for Cyclin E persists in the nurse cell nuclei.

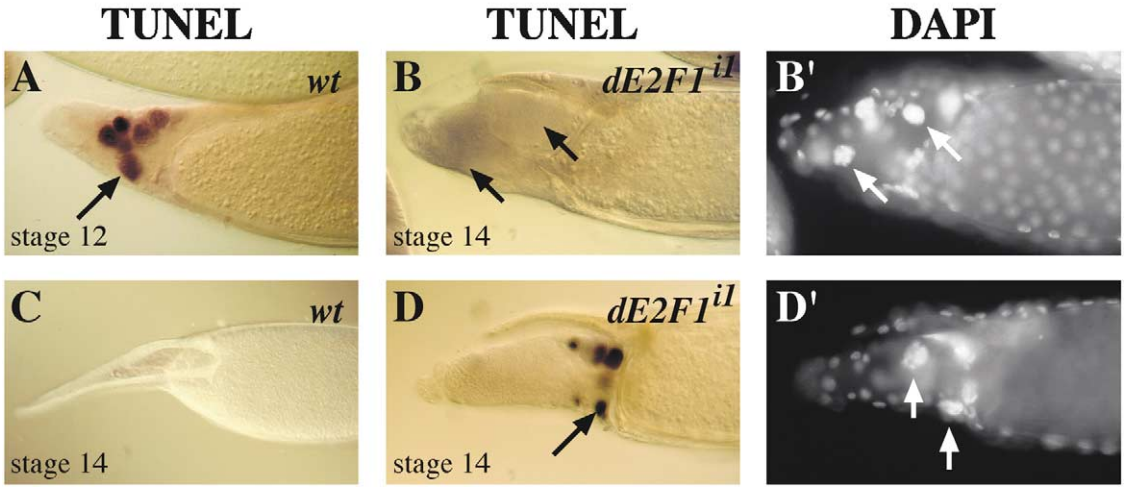

Fig. 7. E2F is necessary for normal apoptosis. The TUNEL method was used for in situ detection of DNA fragmentation in dying nuclei. A DAPI counterstain was used to detect all nuclei. (A) In wild-type ovaries nurse cell nuclei stain positively for TUNEL (purple staining and arrow) at stage 12. By stage 14 (C) the nurse cells are completely eliminated. In stage 14 $dE2F1^{i1}/dE2F1^{7172}$ mutant egg chambers (B,D) the nurse cell cluster remains attached to the oocyte. In most egg chambers, DNA fragmentation as evidenced by TUNEL staining does not occur (B, arrows). In a minority of egg chambers a few nurse cell nuclei label with TUNEL in the last stage of oogenesis (D, arrows). Failure to initiate apoptosis at the normal developmental time is also evidenced by the presence of nurse cell nuclei (arrows) in (B′) and (D′), the DAPI counterparts to (B) and (D), respectively. The arrows in (D′) highlight nurse cell nuclei that do not label with TUNEL. The same apoptotic defects were observed for dDP^{a1}/Df and $dE2F1^{i2}/Df$ mutants (data not shown).

2.6. Embryos from dDP and dE2F1 mutant mothers do not undergo embryonic divisions

The phenotype of embryos produced by *dE2F1* and *dDP* mutant mothers was consistent with the E2F-induced transcription of cell cycle genes being essential for the early embryonic divisions. The dDP^{a1}, $dE2F1^{i1}$, and $dE2F1^{i2}$ mutant females laid few eggs, presumably because of the failure of the nurse cells to dump their contents into the oocyte and undergo apoptosis. In the rare cases in which dumping occurred and mature eggs were laid, most were unfertilized, apparently due to defects in the chorion structure as a consequence of amplification failures (Royzman et al., 1999) (Table 1). The few fertilized embryos arrested in early division cycles (Table 1). In addition, the morphology of the polar bodies was affected (Fig. 8). The polar bodies are the unused meiotic products, and they arrest in a metaphase-like state with the chromosomes arranged in a characteristic rosette structure (Fig. 8A). In embryos from dDP^{a1}/Df mutant mothers in which DNA was visible, meiosis was completed but the polar body chromosomes were either condensed (Fig. 8B), improperly arranged (Fig. 8C), or dispersed in the embryo (data not shown). Occasionally the polar body structure was normal (Table 1). In embryos collected from $dE2F1^{i2}/Df$ mutant mothers 125 appeared unfertilized, and some had normal polar body rosettes whereas others showed aberrant morphology as in Fig. 8B,C. Only nine embryos showed some nuclear divisions, but with aberrant chromosome condensation. Thus the requirement for E2F for the induction of E2F-responsive genes at stages 9 and 10 of egg chamber development and the early-arrest phenotype of embryos from mutant mothers indicate that E2F plays an important role in the accumulation of messages in the developing oocyte necessary for the embryonic divisions.

3. Discussion

This analysis of the *dE2F1* and *dDP* female-sterile mutations reveals a critical role for the E2F cell cycle regulator in controlling the parameters of the endo cycle in the nurse cells that lead to underreplication of heterochromatin and

Table 1
Phenotypes of embryos from dDP^{al}/Df mutant mothers

No development: unfertilized (total $n = 242$)					1–2 Embryonic divisions
No DNA visible	Condensed P.B. chromosomes[a]	Aberrant P.B. morphology[b]	Dispersed chromosomes[c]	Rosette[d]	
95	41	55	29	22	38

[a] Polar bodies (P.B.) had overcondensed chromosomes such that individual arms were not visible, as in Fig. 8B.
[b] Although individual chromosomes were visible they were not properly arranged in a rosette structure, as in Fig. 8C.
[c] Individual chromosomes were dispersed rather than gathered together in a rosette.
[d] These eggs contained polar bodies with a normal rosette structure.

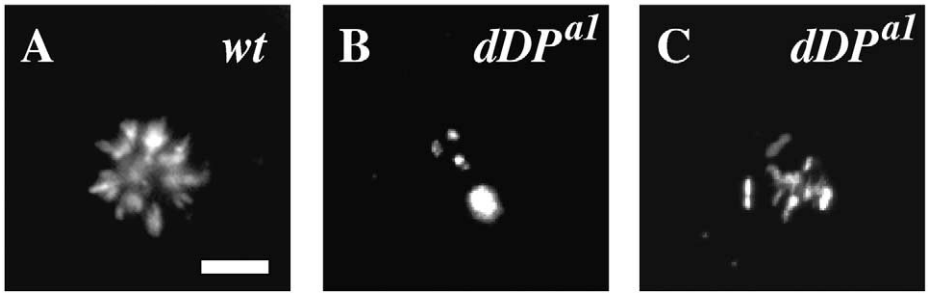

Fig. 8. Early embryonic divisions are disrupted by mutations in *dE2F1* and *dDP*. (A) After the completion of meiosis in wild-type eggs the chromosomes of the three unused meiotic products, the polar bodies, condense into a characteristic rosette structure. The scale bar is 5 μm. (B) In some embryos from dDP^{a1}/Df mutant mothers (Table 1) the polar body chromosomes are highly condensed and do not form a rosette. (C) In other embryos from mutant mothers (Table 1) the chromosomes are condensed normally but their arrangement is abnormal. Similar defects were seen in embryos from $dE2F1^{i2}/Df$ mutant mothers (data not shown).

changes in polytene chromosome morphology. These studies also demonstrate that *dE2F1* is necessary for nurse cell apoptosis, implicating the activation function of E2F in promoting developmentally-induced apoptosis.

If the dE2F1/dDP complex has a direct role in repressing heterochromatin replication, it could act either by affecting S-phase replication patterns or by altering the chromatin in heterochromatic domains. A weak allele of *cyclin E* has been shown to permit replication of the heterochromatin, and it was proposed that reduction in the oscillations of Cyclin E led to longer S phases in the nurse cells that permitted heterochromatin to be replicated (Lilly and Spradling, 1996). Such a mechanism could account for the *dDP* and *dE2F1* mutant phenotypes. Although we did not detect a reduction of Cyclin E protein or transcripts in the mutants, it is possible that levels are reduced enough to affect late replication in S phase. Because overexpression of Cyclin E also causes inappropriate heterochromatin replication in the nurse cells it is difficult to test whether the E2F1 effects are the consequence of reductions in Cyclin E (Lilly and Spradling, 1996). The abundant induction of *cyclin E* transcripts in *dE2F1* and *dDP* mutant stage 10 egg chambers shows that in the nurse cells there is an E2F1-independent mechanism of activating *cyclin E* transcription. Evidence has been obtained previously for such a mechanism in the embryonic nervous system (Duronio et al., 1996), and the *cyclin E* promoter has been shown to be complex, with many regulatory elements responding to developmental control (Jones et al., 2000).

An alternative to E2F1 affecting the properties of S phase is that the heterodimer is responsible for conferring the proper chromatin configuration onto heterochromatin that blocks its replication after endo cycle six. The mammalian Rb/E2F complexes have been shown to bind to several types of proteins that affect chromatin structure, including histone deacetylases, histone methyltransferases, and chromatin remodeling complexes (Dahiya et al., 2001; Harbour and Dean, 2000; Nielsen et al., 2001). One of these associated proteins, HP1, is known to control heterochromatin in *Drosophila* (Kellum et al., 1995). Furthermore, mutations in *dE2F1* have been shown to affect position effect variegation, a property of heterochromatin structure (Seum et al., 1996). In follicle cells the RBF/E2F1/DP complex binds at origins with the Origin Recognition Complex and limits origin firing (Bosco et al., 2001), so it is conceivable that a similar complex could block origin activity in the heterochromatin in later nurse cell endo cycles.

Mutations in *Rbf* do not reveal a role for this protein in inhibiting replication of heterochromatin or controlling chromosome morphology. Germline clones of *Rbf* lay normal mature eggs that develop until late in embryogenesis (Du and Dyson, 1999), and a female-sterile allele with reduced levels of Rbf does not visibly affect nurse cell chromosomes (Bosco, G. and Orr-Weaver, T., unpublished results). However, the Rbf protein could be stable or present at sufficient levels in these mutants. In addition, a second Rb homolog exists in *Drosophila*, and this protein could play essential repressive roles in nurse cell differentiation.

The mechanism by which dE2F1 controls nurse cell chromosome morphology remains to be elucidated. There are three possibilities. First, dE2F1 may not be directly involved in the polyteny-polyploidy switch, and the defects observed in the mutants could be a relatively nonspecific effect of disrupting nurse cell differentiation. Second, the failure to properly regulate heterochromatin replication could alter chromosome structure and affect the transition to polyploidy. Third, dE2F1 could have a direct role in triggering the change in chromosome morphology, either itself affecting chromatin structure or via one of its transcriptional targets. The transition from polytene to polyploid morphology appears to involve the induction of mitotic activities and must be precisely controlled by activity of the Anaphase Promoting Complex or Cyclosome (Kashevsky et al., 2002). Given the myriad of cell cycle genes that can be regulated both negatively and positively by mammalian E2F proteins (Muller et al., 2001; Ren et al., 2002), E2F targets could control these transitions in chromosome structure. Further investigation will be required to distinguish these possibilities.

These experiments extend the previous observations on

the role of dDP in nurse cell apoptosis by uncovering a requirement for dE2F1. This is relevant to mammalian work in which the activating E2F1 and E2F3 proteins have been shown to contribute to both p53-dependent and p53-independent apoptosis (Tsai et al., 1998; Ziebold et al., 2001). It is significant that both dE2F1^{i1}, which blocks DNA binding, and dE2F1^{i2}, which binds DNA normally but cannot transactivate or bind Rbf, prevent apoptosis. In mammalian cells overexpression of transactivation defective *E2F-1* mutants but not *E2F-1* DNA-binding mutants can induce apoptosis in cell culture (Hsieh et al., 1997; Phillips et al., 1997). Further, RB overexpression inhibits apoptosis induced by wild-type *E2F-1* and RB-binding competent *E2F-1* mutants (Hsieh et al., 1997). These observations led to the notion that DNA binding rather than the transactivation activity of E2F-1 is necessary for its apoptotic function, and that apoptosis is the result of alleviation of RB-E2F-1 repression of apoptotic target genes. The phenotypes exhibited by the *dE2F* and *dDP* mutants do not support this model. Rather, the *Drosophila* mutants strongly implicate transcriptional activation of E2F targets as being crucial for developmentally-induced apoptosis. If repression were critical, then apoptosis should have occurred in the *dE2F1^{i1}* mutants in which dE2F2 is expected to repress all E2F targets. The cell death activators *reaper* and *hid* are not required for nurse cell apoptosis, so it is not yet clear which E2F targets could activate apoptosis in these cells (Foley and Cooley, 1998).

Previous work on *dDP* identified two other effects on oogenesis not observed in our mutants (Myster et al., 2000). Defects in dorsal-ventral patterning of the oocyte and in oocyte determination were observed in the germline clones of *dDP* mutants (Myster et al., 2000). In the *dDP* mutants the GURKEN mRNA is not properly localized, levels of GURKEN protein are reduced, and as a consequence the eggs have abnormal dorsal appendages. In addition, egg chambers were present in which an extra germ line mitotic division occurred, as were egg chambers with extra oocytes. These defects are clearly the consequence of loss of *dDP* function in the germ line clones because they are rescued by the presence of a *dDP* transgene (Myster et al., 2000). We did not observe dorsal appendage patterning defects in the *dE2F1^{i1}* and *dE2F1^{i2}* mutants or the defects in cystoblast divisions or oocyte determination. While it is tempting to speculate that dE2F2 may regulate these biological processes, we also did not see these defects in the *dDP* female-sterile allele or the *dDP* null rescued to adulthood. Therefore in our experiments sufficient dDP and dE2F1 activity may have been present for dorsal-ventral patterning and oocyte determination.

The intriguing phenotypes arising from female-sterile mutations in *dDP* and *dE2F1* reveal new biological functions for the E2F cell cycle regulator not uncovered by experiments in mammalian cell culture and knock-out mice. This highlights the utility of partial loss-of-function alleles that uncover phenotypes in distinct developmental contexts and developmental models in which such phenotypes can be analyzed precisely.

4. Experimental procedures

4.1. Fly strains

dDP^{a1}, *dDP^{a2}*, *dE2F1^{i1}*and *dE2F1^{i2}* mutants were described previously (Royzman et al., 1997, 1999). The deficiency uncovering *dDP*, *Df(2R)vg56*, was provided by R. Duronio (Duronio et al., 1998). A null *dE2F1* allele, *dE2F1^{91}* and the strong *dE2F1^{7172}* allele (Duronio et al., 1995) and the transgenic lines *P*[w^+, *hsp70-dE2F1*], *P*[w^+, *hsp70-dDP*] were provided by N. Dyson (Duronio et al., 1996). The deficiency uncovering *dE2F1*, *Df(3R)e-BS2*, was obtained from the Bloomington Stock Center. The *cyclin E^{01672}* allele was provided by A. Spradling (Lilly and Spradling, 1996).

4.2. In situ hybridization and antibody staining

In situ hybridizations (Tautz and Pfeifle, 1989) to whole-mount ovaries were carried out with digoxigenin-labeled RNA probes as described previously by Royzman et al. (1997). Ovaries were prepared as described in Ephrussi et al. (1991) and treated for 35 min in proteinase K. Subsequent steps were carried out as for embryos. TUNEL and acridine orange staining were used to detect apoptotic cells in ovaries. The ovaries were processed as described in McCall and Steller (1998). Ovaries assayed for cell death by TUNEL were counter-stained for 20 min with 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI; Sigma).

In preparation for antibody stainings, ovaries were fixed in 8% EM grade formaldehyde (Ted Pella) in PBS for 5 min, extracted for 2 h in 1% Triton X-100 in PBS, and then blocked for 1 h in 1× PBS, 1% BSA, 0.3% Triton X-100, and 2% normal goat serum. A mouse monoclonal antibody 8B10 was provided by H. Richardson for detection of cyclin E and used at a dilution of 1:5 (Lilly and Spradling, 1996; Richardson et al., 1995). A goat anti-mouse secondary antibody conjugated to horseradish peroxidase (Bio-Rad) was used at 1:200. To detect dE2F1 protein, a rabbit polyclonal antibody provided by M. Asano and R. Wharton (Asano et al., 1996) was used at a dilution of 1:100. An HRP-conjugated goat anti-rabbit secondary antibody (Jackson Immuno Research Laboratories) was used at a dilution of 1:100. Nuclear lamin Dm_0 staining was carried out using Ab101, an antibody provided by K. McCall and H. Steller (Smith et al., 1987). Ab101 was diluted 1:1.5 and detected with a Cy3-conjugated donkey anti-mouse secondary antibody (Jackson) at a dilution of 1:150. The staining pattern with this antibody has been described (McCall and Steller, 1998) and is similar to that seen with a polyclonal antibody to nuclear lamin Dm_0 (Smith and Fisher, 1989).

4.3. Chromosome cytology in nurse cells and embryos and BrdU labeling

To label nurse cells with BrdU the ovaries were dissected in Grace's medium and the ovarioles teased apart. They were then incubated in Grace's medium with 10 μM BrdU for 1 h at room temperature. For squashing the ovaries were fixed in acetic acid/lactic acid and squashed as described (Dej and Spradling, 1999). Embryos were fixed and strained with DAPI as described (Kashevsky et al., 2002).

4.4. Microscopy

Egg chambers were examined on a Zeiss Axiophot microscope equipped with Nomarski optics and fluorescence and were photographed either with a 35-mm camera or a SPOT RT CCD camera and software. Plan-Neofluar 20× and 40× objectives were used. Squashed nurse cell chromosomes were photographed either on a Zeiss Axiophot microscope with Plan-Neofluar 40× or 100× objectives with a SPOT RT CCD camera and software, or on a Nikon TE200 Inverted microscope with Plan-Neofluar 40× and Planapo 100× objectives with a Princeton Instruments CCD and Metamorph software from Universal Imaging. Polar bodies were imaged on a Bio-Rad MRC600 confocal laser system mounted on a Zeiss inverted microscope with Plan-Neofluar 40× and 100× objectives.

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