Development 134, 467-478 (2007) doi:10.1242/dev.02738

Rbf1-independent termination of E2f1-target gene expression during early *Drosophila* embryogenesis

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The initiation and maintenance of G1 cell cycle arrest is a key feature of animal development. In the *Drosophila* ectoderm, G1 arrest first appears during the seventeenth embryonic cell cycle. The initiation of $G1_{17}$ arrest requires the developmentally-induced expression of Dacapo, a p27-like Cyclin E-Cdk2 inhibitor. The maintenance of $G1_{17}$ arrest requires Rbf1-dependent repression of E2f1-regulated replication factor genes, which are expressed continuously during cycles 1-16 when S phase immediately follows mitosis. The mechanisms that trigger Rbf1 repressor function and mediate $G1_{17}$ maintenance are unknown. Here we show that the initial downregulation of expression of the E2f1-target gene *RnrS*, which occurs during cycles 15 and 16 prior to entry into $G1_{17}$, does not require Rbf1 or $p27^{Dap}$. This suggests a mechanism for Rbf1-independent control of E2f1 during early development. We show that E2f1 protein is destroyed in a cell cycle-dependent manner during S phase of cycles 15 and 16. E2f1 is destroyed during early S phase, and requires ongoing DNA replication. E2f1 protein reaccumulates in epidermal cells arrested in $G1_{17}$, and in these cells the induction of $p27^{Dap}$ activates Rbf1 to repress E2f1-target genes to maintain a stable G1 arrest.

KEY WORDS: *Drosophila*, E2F, pRb, Cell cycle, G1

INTRODUCTION

Proper control of cell cycle exit is an essential aspect of the development of all multicellular organisms. Cell cycle exit frequently occurs during G1 phase, and a stable G1 arrest is usually necessary for cell differentiation (Myster and Duronio, 2000). Multiple mechanisms contribute to stable G1 quiescence, and these mechanisms can be broadly defined as those that initiate the onset of G1 arrest and those that maintain G1 arrest. Disruption of either or both types of regulation can abrogate differentiation, block morphogenesis and contribute to the onset of cancer.

The initiation of G1 arrest involves the inhibition of G1 Cyclin–Cyclin-dependent kinase (Cyc-Cdk) complexes that promote entry into S phase. These complexes include CycD-Cdk4 and CycE-Cdk2 (Cdk2 is also known as Cdc2c – Flybase), which are negatively regulated by the cyclin-dependent kinase inhibitors (CKIs) $p16^{INK4a}$ and $p27^{Kip1}$, respectively (Sherr and Roberts, 1999). Whereas $p16^{INK4a}$ acts primarily as a tumor suppressor, the induction of p27 expression is required for proper cell cycle withdrawal and subsequent differentiation in a number of developing mammalian tissues, including the retina, the organ of Corti and skeletal muscle (Chen and Segil, 1999; Chu and Lim, 2000; Levine et al., 2000; Lowenheim et al., 1999; Zabludoff et al., 1998).

The maintenance of G1 arrest occurs through a distinct mechanism involving the repression of genes necessary for S phase, which are regulated by the E2F family of transcription factors. E2F activity is controlled mainly through interaction with members of the retinoblastoma (pRb) tumor suppressor or 'pocket protein' family (DeGregori, 2002; Dimova and Dyson, 2005; Trimarchi and Lees, 2002). During quiescence and early G1, hypophosphorylated pocket proteins form a complex with E2Fs that recruit co-repressors and result in the downregulation of E2F targets. In response to growth signals, G1 Cyc-Cdk complexes phosphorylate pocket proteins resulting in the dissociation of the repressive pocket protein-E2F complex and the induction of transcription of S phase genes. In lens cells, trophoblasts, keratinocytes and neural tissue, the maintenance of cell cycle arrest is compromised by the loss of pRb, presumably owing to an inappropriate increase in E2F activity and the consequent activation of replication genes (Jacks et al., 1992; MacPherson et al., 2003; Ruiz et al., 2004; Wu et al., 2003).

E2F activity can also be regulated independently of pocket proteins. E2F is a heterodimer composed of an E2f subunit and a Dp subunit that together are necessary for binding DNA (Trimarchi and Lees, 2002). During S phase, CycA-Cdk2 phosphorylates E2f-bound Dp resulting in dissociation of the E2f-Dp heterodimer from DNA (Dynlacht et al., 1994; Dynlacht et al., 1997; Krek et al., 1994; Krek et al., 1995). Other reports indicate that in mammalian cells, E2F proteins are destroyed in S-G2 via the ubiquitin-proteasome pathway (Campanero and Flemington, 1997; Hateboer et al., 1996; Hofmann et al., 1996; Marti et al., 1999). Similarly, E2f1 (E2f – Flybase) is destroyed at the G1-S transition in *Drosophila* imaginal disc cells (Asano et al., 1996; Heriche et al., 2003; Reis and Edgar, 2004), and this destruction involves the ubiquitin-proteasome pathway (Heriche et al., 2003). Whether these modes of E2F regulation contribute substantially to gene expression and cell cycle control during development is not known.

The cell cycles of early embryonic development display common features among a variety of animal species. In general, these cell cycles are very rapid and occur with the ubiquitous activity of key regulators such as E2F and CycE-Cdk2. In some instances (e.g. *Drosophila* and *Xenopus*), the earliest cell cycles lack measurable gap phases altogether. As development proceeds, different lineages first acquire additional cell cycle controls that result in the appearance of gap phases, and then undergo cell cycle exit and differentiation. The mechanisms contributing to specific changes in cell cycle regulation in particular tissue types during development remain incompletely understood.

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Accepted 14 November 2006

Drosophila embryos provide an excellent experimental system to address this issue because they execute a stereotyped, developmentally-controlled cell cycle program that is well-characterized (Lee and Orr-Weaver, 2003) (Fig. 1I). The first 13 cycles are rapid S-M cycles driven by ubiquitous maternal factors (Foe and Alberts, 1983). The first gap phase, G2, appears at the blastoderm stage during cell cycle 14 because of degradation of maternal *string* (*stg*) mRNA and protein (Edgar and Datar, 1996). *stg* encodes a Cdc25-type phosphatase that removes the inhibitory phosphates from Cdk1 (Cdc2 – Flybase) to allow entry into mitosis (Edgar and O'Farrell, 1989). After gastrulation begins, a pulse of zygotic transcription of *stg* in late G2 triggers the entry into mitosis during cycles 14, 15 and 16 (Edgar et al., 1994; Edgar and O'Farrell, 1990). In these so-called post-blastoderm division cycles there is no G1 phase, and S phase begins immediately after mitosis. G1 phase first appears during cell cycle 17, after which some cells (e.g. in the epidermis) remain arrested in $G1_{17}$ whereas others (e.g. in the midgut) re-enter S phase from $G1_{17}$ and begin endoreduplication cycles.

The regulation of *stg* establishes a paradigm for developmental control of the *Drosophila* embryonic cell cycle. The transition from ubiquitous, maternally-provided *stg* to regulated, zygotic expression of *stg* accounts for both the introduction of the first G2 phase and subsequent G2-M cell cycle regulation. This paradigm also applies to the introduction of G1-S regulation in cell cycle 17. Because Cyclin E is required for S phase in *Drosophila* (Knoblich et al., 1994), the change in activity of CycE-Cdk2 from ubiquitous (cycles 1-16) to cell cycle-regulated accounts for both the introduction of G1 phase in cycle 17 and subsequent regulation of the G1-S transition (Duronio and O'Farrell, 1995; Richardson et al., 1993; Sauer et al., 1995). This transition is achieved in part by zygotic transcription of *dacapo* (*dap*), which encodes the single *Drosophila* p27-like CKI (de Nooij et al., 1996; Lane et al., 1996). *dap* transcription is controlled by a complex cis-acting regulatory region that responds to developmental inputs that induce Dap production during cycle 16 (Liu et al., 2002; Meyer et al., 2002b). This results in the inhibition of CycE-Cdk2 and the appearance of G1 in cycle 17. Consequently, *dap*-mutant epidermal cells do not enter $G1_{17}$, but instead enter S_{17} immediately after the completion of M_{16} and undergo an ectopic cell division cycle (de Nooij et al., 1996; Lane et al., 1996).

The maintenance of a stable $G1_{17}$ arrest in the embryonic epidermis requires the function of Rbf1 (Rbf – Flybase), a *Drosophila* pRb homolog (Du et al., 1996a). Rbf1 negatively regulates the activity of E2f1. In *Drosophila,* E2f1 is necessary for the expression of replication factor genes including *Cyclin E*, although these genes are also regulated by additional factors such as Dref (Duronio et al., 1998; Duronio et al., 1995; Hirose et al., 1993; Royzman et al., 1997; Sawado et al., 1998; Yamaguchi et al., 1996). *Rbf1*-mutant embryos develop normally through cycle 17, and the epidermal cells are able to initiate $G1_{17}$ owing to the activity of Dap. However, some *Rbf1*-mutant epidermal cells fail to maintain G1 arrest and ultimately re-enter the cell cycle because of inappropriate expression of E2f1-target genes including *Cyclin E* (Du and Dyson, 1999). The developmental inputs and mechanisms that result in Rbf1 repressor function and the downregulation of replication genes are unknown. Here we show that, surprisingly, the initial downregulation of the E2f1-target gene *RnrS* prior to $G1_{17}$ does not require Rbf1 or Dap. Instead, loss of *RnrS* expression occurs coincident with the onset of S phase-coupled destruction of E2f1 protein, which may provide a mechanism for pRb-independent regulation of E2F activity.

MATERIALS AND METHODS

Drosophila strains

w^{1118}, *prd*-Gal4, *β tubulin* FLP, *w ovo*^{D} FRT 14A-B/C(1)DX, *y f*/Y; hsFLP, and *CycE*^{AR95}*/CyO* were obtained from the Bloomington Stock Center. UAS *dap*, *dap*^{4454}*/CyO*, *Df(1)biD3/FM7*, *dup*^{a1}*/CyO*, *dup*^{a3}*/CyO*, *arm*-Gal4 VP16/*TM3* and *E2f1*^{7172}*/TM3* have been described previously (de Nooij et al., 1996; Duronio et al., 1995; Lane et al., 1996; McEwen et al., 2000; Sigrist and Lehner, 1997; Spradling et al., 1995; Whittaker et al., 2000). *y w; stg*^{7B}*/TM3 e* as well as UAS *Rbf-280/TM3*, UAS *Rbf1*, and *Rbf1*^{14} FRT14A-B/*FM7* were kindly provided by Patrick O'Farrell and Wei Du, respectively (Du and Dyson, 1999; Edgar and O'Farrell, 1989; Xin et al., 2002). *dap*^{4454}*/CyO wg-lacZ, Df(1)biD3/FM7Actin*-GFP, *dup*^{a1}*/CyO wg-lacZ*, *dup*^{a3}*/CyO wg-lacZ*, *CycE*^{AR95}*/CyO wg-lacZ*, and *Rbf1*^{14} FRT 14A-B/*FM7 Actin*-GFP were constructed for this study. *Rbf1*^{14} germ line clones were generated as described (Du and Dyson, 1999). *stg*^{7B} *dap*^{4454} double-mutant embryos were unambiguously identified using Dap antibody staining and by the altered morphology caused by the *stg* $G2_{14}$ arrest phenotype.

RNA in situ hybridization and BrdU labeling

Embryos were dechorionated, fixed in 1:1 4% formaldehyde in PBS:heptane for 25 minutes, and devitellinized with methanol. For BrdU labeling, dechorionated embryos were permeabilized with octane, then pulse-labeled with 1 mg/ml BrdU for either 5 minutes or 15 minutes in Schneider's *Drosophila* medium prior to fixation. Embryos were stored in methanol at –20°C.

In situ hybridization with digoxigenin-labeled antisense RNA probes was performed as described (Kearney et al., 2004). Fluorescent detection of hybrids (FISH) was achieved with the TSA Fluorescence System (Perkin Elmer) using a 30-60 minute incubation in TSA-Cy3 or TSA-Fluorescein. For all triple fluorescent staining (i.e. FISH, anti-protein, anti-BrdU) except E2f1 or Dap plus FISH, embryos were first processed for FISH, then for immunodetection of proteins, and finally for BrdU detection by acid denaturation of chromosomes (Schubiger and Palka, 1987). For E2f1 or Dap detection plus FISH, the TSA Fluorescence System was first used for immunodetection of E2f1 or Dap, and then the embryos were fixed for 30 minutes in 4% formaldehyde to quench the peroxidase prior to FISH and BrdU detection.

Immunostaining

Embryos were rehydrated with PBS-0.1% Tween20 (PBS-T) and incubated with primary antibodies overnight at 4°C. Primary antibodies used were: mouse anti-BrdU monoclonal antibody (1:100, Becton Dickinson), rabbit anti-E2f1 (1:500 or 1:1000, gift of Maki Asano) (Asano et al., 1996), rabbit anti-phospho-tyrosine (1:100, Upstate), rat anti-phospho-tyrosine (1:50 or 1:100, R and D Systems), rabbit anti-β-galactosidase (1:200, Chemicon), mouse anti-phospho-Ser10-histone H3 (1:2000, Upstate), rabbit anti-GFP (1:2000, Abcam) and rabbit anti-Dap (1:600) (Lane et al., 1996). Secondary antibodies used were: goat anti-mouse Oregon Green (1:1000, Molecular Probes), goat anti-mouse-Cy5 (1:500, Jackson), goat anti-mouse-Cy3 (1:500, Jackson), goat anti-rabbit-Cy2 (1:500, Jackson), goat anti-rabbit rhodamine (1:1000, Molecular Probes), donkey anti-rat-Cy5 (1:500, Jackson), and goat anti-rabbit-Cy5 (1:500, Abcam). For detection of E2f1 and Dap, the TSA Fluorescence System (Perkin Elmer) was used with a biotin-conjugated anti-rabbit secondary antibody (1:1000, Chemicon). Stained embryos were mounted with Fluoromount-G (Southern Biotech) and visualized with either a Nikon Eclipse E800 microscope or a Zeiss LSM 510 scanning confocal microscope.

Co-immunoprecipitations and western blotting

Immunoprecipitations were performed with extracts from 0-4 hour and 5-8 hour w^{1118} embryos as described (Peifer et al., 1993), and analyzed by SDS-PAGE (7.5% precast gel, Biorad) and western blotting with mouse anti-Rbf1 (DX-3, 1:4) (Du et al., 1996a), rabbit anti-E2f1 (see above) and mouse anti-Dp (YUN1-3 1:4) (Du et al., 1996b). Secondary antibodies were ECL-sheep anti-mouse HRP (1:5000) and ECL-donkey anti-rabbit HRP (1:5000) from Amersham Biosciences.

RESULTS

Phosphorylation controls the activity of Rbf1 in the early embryo

The transcripts of E2f1-regulated replication factor genes are present during the first 16 embryonic cycles even though Rbf1 mRNA and protein are present continuously throughout all of early embryogenesis (Keller et al., 2005; Stevaux et al., 2002). This suggests that embryonic Rbf1 activity is regulated post-translationally. We therefore hypothesized that Rbf1 is hyperphosphorylated and thus inactivated until cycle 16 by constitutive G1 Cyclin-Cdk activity, resulting in ubiquitous expression of E2f1-target genes (Fig. 1I). To test this, we utilized a mutant version of Rbf1 (Rbf-280) containing mutations in four Cdk consensus sites that cannot be inhibited by the activity of G1 Cyclin-Cdk complexes such as CycE-Cdk2 (Xin et al., 2002). UAS *Rbf-280* was expressed with two strong drivers that are active during cycles 14-16, *prd*-Gal4 and *arm*-Gal4-VP16. E2f1 activity was monitored by in situ hybridization with a probe derived from the small subunit of ribonucleotide reductase (*RnrS*), a well-established E2f1-target gene (Duronio et al., 1995). UAS *Rbf-280* expression with *prd*-Gal4 resulted in the precocious termination of *RnrS* expression in alternating segments (Fig. 1A,C). Utilizing fluorescent detection of *RnrS* transcripts and BrdU labeling, we confirmed that the precocious termination occurs during cycle 15 (Fig. 1D). A similar but more widespread result was observed using the ubiquitous *arm*-Gal4-VP16 driver (Fig. 1E,G). Little change in *RnrS* expression was observed after expressing wild-type Rbf1 (Fig. 1B,F), indicating that the precocious termination of *RnrS* expression is specific to UAS *Rbf-280*. These results suggest that Rbf-280 can bypass the normal mechanism of Rbf1 control in the early embryo, and are consistent with the idea that Rbf1 is hyperphosphorylated and thus inactivated by constitutive Cyclin-Cdk activity in the early embryo to permit expression of replication factor genes such as *RnrS*.

Rbf1 phosphorylation prevents Rbf1 from binding to E2f1 (Du et al., 1996a; Xin et al., 2002). Therefore, our interpretation of the Rbf-280 results predicts that Rbf1-E2f1 complexes will not be present during early embryogenesis, and that these complexes will be detected only after the introduction of G1 control at ~7 hours of development. Consistent with this hypothesis, E2f1 and Rbf1 co-precipitate from 5-8 hour (cycles 16-17) but not from 0-4 hour (prior to cycle 16) embryo extracts (Fig. 1H). Dp co-precipitates with Rbf1 in both cases (Fig. 1H). The Rbf1-Dp interaction in 0- to 4-hour-old embryos is likely to represent the recently described Myb-MuvB-dREAM complex that contains E2f2-Dp-Rbf and which acts to repress many genes involved in developmental processes other than cell cycle progression (Korenjak et al., 2004; Lewis et al., 2004). We have been unable to detect hyperphosphorylated Rbf1 by reduced mobility on SDS-PAGE gels, as is commonly performed with mammalian pRb. Nevertheless, our results suggest that in early embryogenesis (cycles 1-16), Rbf1 is present in a hyperphosphorylated, inactive form that is not bound to E2f1.

The initial termination of E2f1-target gene expression does not require CycE-Cdk2 inhibition

In wild-type embryonic epidermis, the expression of E2f1 targets is terminated prior to $G1_{17}$, and Rbf1 is required to maintain repression of E2f1 targets during $G1_{17}$ (Du and Dyson, 1999; Duronio and O'Farrell, 1994; Richardson et al., 1993). Since our data imply that Rbf1 is hyperphosphorylated in the early embryo, we hypothesized that prior to the introduction of $G1_{17}$, Rbf1 is converted to a

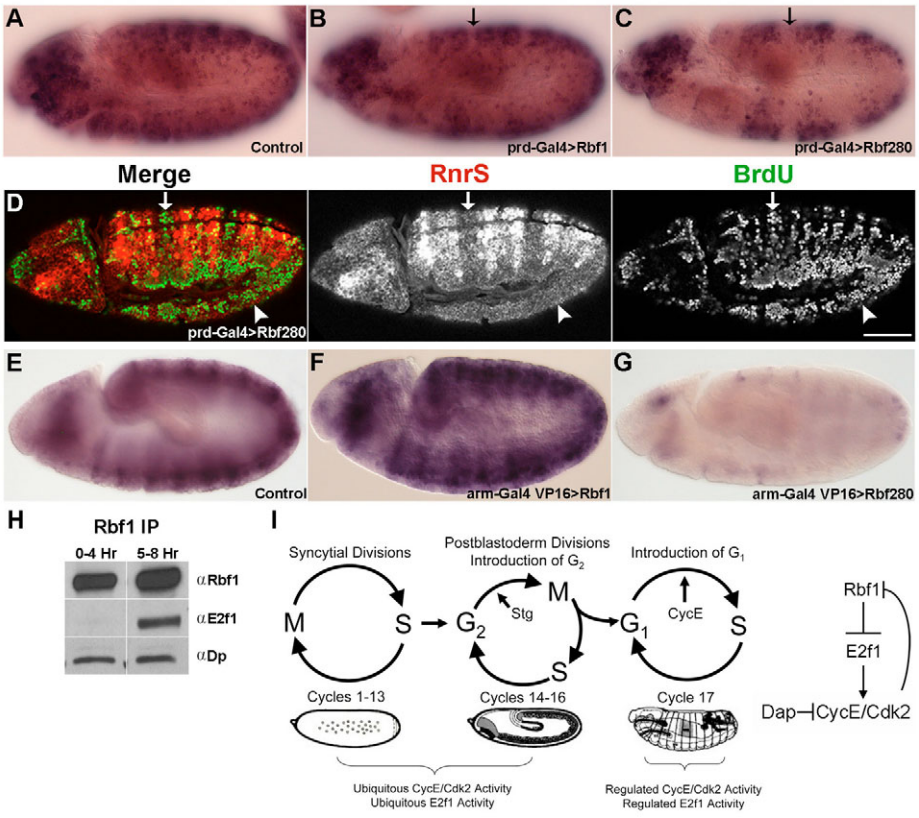

Fig. 1. Rbf1 activity is controlled by phosphorylation in the early embryo. (**A-G**) In situ hybridization of stage 10 embryos with an *RnrS* probe. (A) Sibling control embryo from a collection expressing UAS *Rbf1* with *prd*-Gal4. (B) UAS *Rbf1/prd*-Gal4. Arrow marks *paired*-expressing segment. (C) UAS *Rbf-280/prd*-Gal4. Arrow denotes the precocious termination of *RnrS* expression in a *paired*-expressing segment. (D) UAS *Rbf-280/prd*-Gal4 embryo pulse labeled for 15 minutes with BrdU (green). *RnrS* expression was detected by FISH (red). Arrow and arrowhead indicate cells in cycle 15 and 16, respectively. (E) Sibling embryo from a collection expressing UAS *Rbf*-280 with *arm*-Gal4 VP16. (F) UAS *Rbf1/arm*-Gal4 VP16. (G) UAS *Rbf-280/arm*-Gal4-VP16. (**H**) Rbf1 was immunoprecipitated from 0- to 4-hour-old and 5- to 8-hour-old w^{1118} embryo extracts, and the IPs were probed for the presence of E2f1 and Dp by western blotting. (**I**) Schematics of the embryonic cell cycle program and the regulation of E2f1 activity. Scale bar: 200 μm.

hypophosphorylated form that binds E2f1 and terminates E2f1-target gene expression. A possible mechanism for the conversion of Rbf1 to a hypophosphorylated form is the inhibition of G1 Cyclin-Cdk complexes, specifically CycD-Cdk4 and CycE-Cdk2, which in vertebrates are known to phosphorylate pRb (Dyson, 1998). Since the regulation of *RnrS* expression in the epidermis of both *CycD*- and *Cdk4*-mutant embryos is normal, the modulation of CycD-Cdk4 activity may not be part of the mechanism (Emmerich et al., 2004; Meyer et al., 2002a). By contrast, CycE-Cdk2, which can phosphorylate and inhibit Rbf1 (Du et al., 1996a), is inhibited just prior to the introduction of $G1_{17}$ by the developmentally-regulated induction of *dap* transcription during cycle 16 (de Nooij et al., 1996; Lane et al., 1996) (Fig. 1I). If the inhibition of CycE-Cdk2 activity by Dap is necessary for the accumulation of hypophosphorylated Rbf1 and the consequent suppression of E2f1 targets, then in *dap* mutants *RnrS* expression would not be terminated properly. However, we found that *RnrS* expression is downregulated in the epidermis of *dap* mutants prior to the completion of S_{16}, just as it is in wild-type embryos (Fig. 2A,B). Moreover, the termination of *RnrS* expression occured even though the epidermal cells of *dap*-mutant embryos enter an ectopic S_{17} (Fig. 2C,D).

A similar result is seen in the epidermis of *fizzy-related* (*fzr*; *rap* – Flybase) mutant embryos. *fzr* encodes *Drosophila* Cdh1, which during G1 phase targets mitotic cyclins for ubiquitination by the anaphase-promoting complex (APC/C) and subsequent destruction (Jacobs et al., 2002; Sigrist and Lehner, 1997). Similar to *dap* mutants, epidermal cells in *fzr* mutants fail to exit the cell cycle and inappropriately enter an ectopic S_{17}, which is likely to be driven by CycE-Cdk2 (Sigrist and Lehner, 1997). In spite of this, *RnrS* expression was seen to be properly downregulated in *fzr* mutants (Fig. 2E). Thus, although unrestricted CycE-Cdk2 activity can prevent the initiation of $G1_{17}$ in epidermal cells, E2f1-target gene expression is still terminated at the appropriate time. These data suggest that either the inhibition of CycE-Cdk2 does not result in the accumulation of hypophosphorylated Rbf1, or that a different mechanism is involved in the initial termination of E2f1-target gene expression.

Cell cycle-regulated destruction of E2f1 protein in the embryonic epidermis

One possible mechanism for the inhibition of E2f1 activity is the destruction of E2f1 protein. In both the eye and wing imaginal discs, E2f1 protein is destroyed at the G1-S transition and reaccumulates during G2 and M phase (Asano et al., 1996; Heriche et al., 2003; Reis and Edgar, 2004). We therefore postulated that E2f1 destruction during S phase of the post-blastoderm cell cycles contributes to the termination of E2f1-target gene expression in the epidermis. To examine this, we visualized E2f1 protein abundance by immunofluorescence in embryos that were pulse-labeled with BrdU (Fig. 3).

E2f1 protein is present throughout the embryo during early syncytial cycles 1-13 (data not shown). Notably, unlike imaginal disc cells, nuclear E2f1 was detected during S phase of cycles 13 and 14 (Fig. 3A,B). E2f1 protein accumulated to high levels in the nucleus during $G2_{14}$ (Fig. 3C), and then rapidly diminished as cells entered S_{15} (Fig. 3D). This effect is post-transcriptional, as E2f1 transcripts are ubiquitous during cycle 15 (Duronio et al., 1995), suggesting that E2f1 protein is destroyed upon entry into S phase. In addition, the

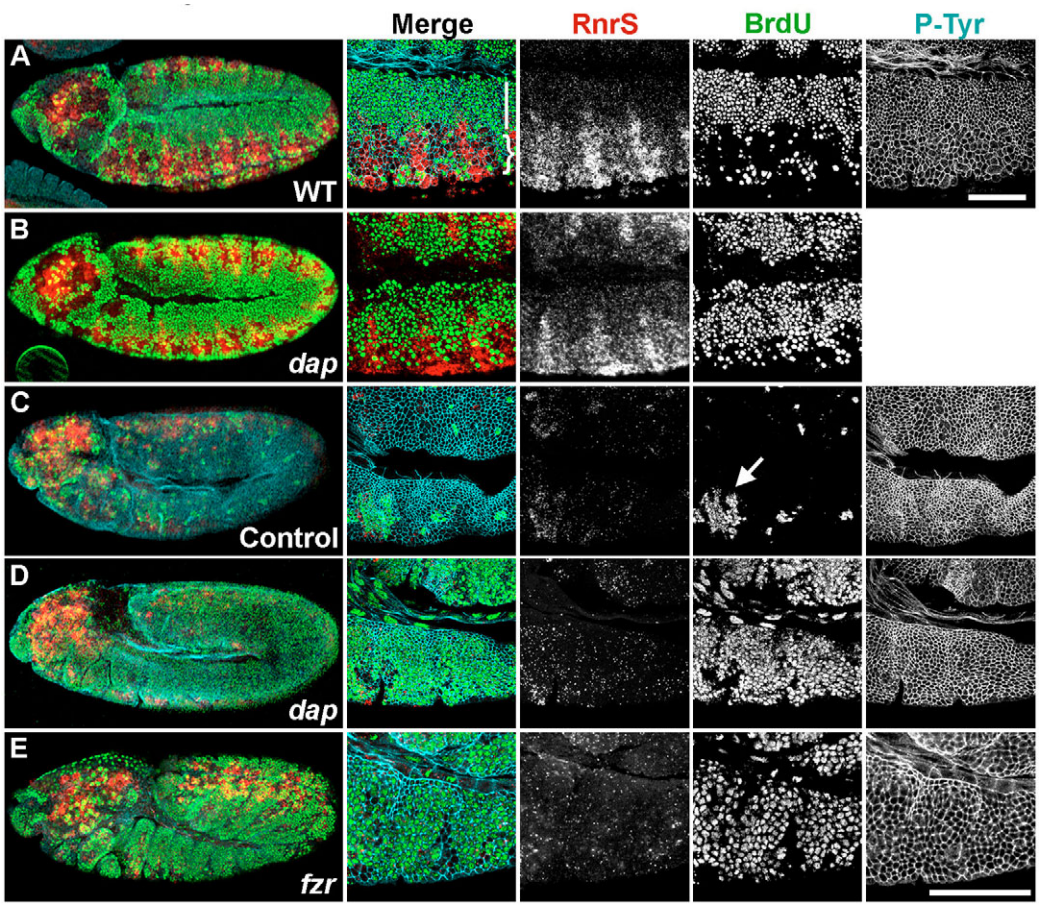

Fig. 2. E2f1-target gene expression is terminated in mutants containing ectopic CycE-Cdk2. Embryos were pulse-labeled with BrdU for 5 minutes (**A-D**) or 15 minutes (**E**) and were stained for BrdU incorporation (green) and phospho-tyrosine to highlight cell boundaries (cyan). *RnrS* expression was detected by FISH (red). (A) Stage 10 w^{1118} control embryo. The bar denotes S_{16} in the dorsal epidermis and the bracket marks cycle 15 in the ventral epidermis. (B) Stage 10 dap^{4454}/dap^{4454} embryo. Note that phospho-tyrosine staining was omitted because anti-β-galactosidase was used to distinguish CyO P[wg-lacZ]-containing embryos from the *dap* mutants. (C) Control embryo that is a sibling of the embryo in D. The arrow in the BrdU image denotes cells of the anterior spiracle primordium that normally enter S_{17}. (D) Stage 11 dap^{4454}/dap^{4454} embryo. (E) *Df(1)biD3/Df(1)biD3 fzr* mutant embryo. Scale bars: 50 μm.

DEVELOPMENT

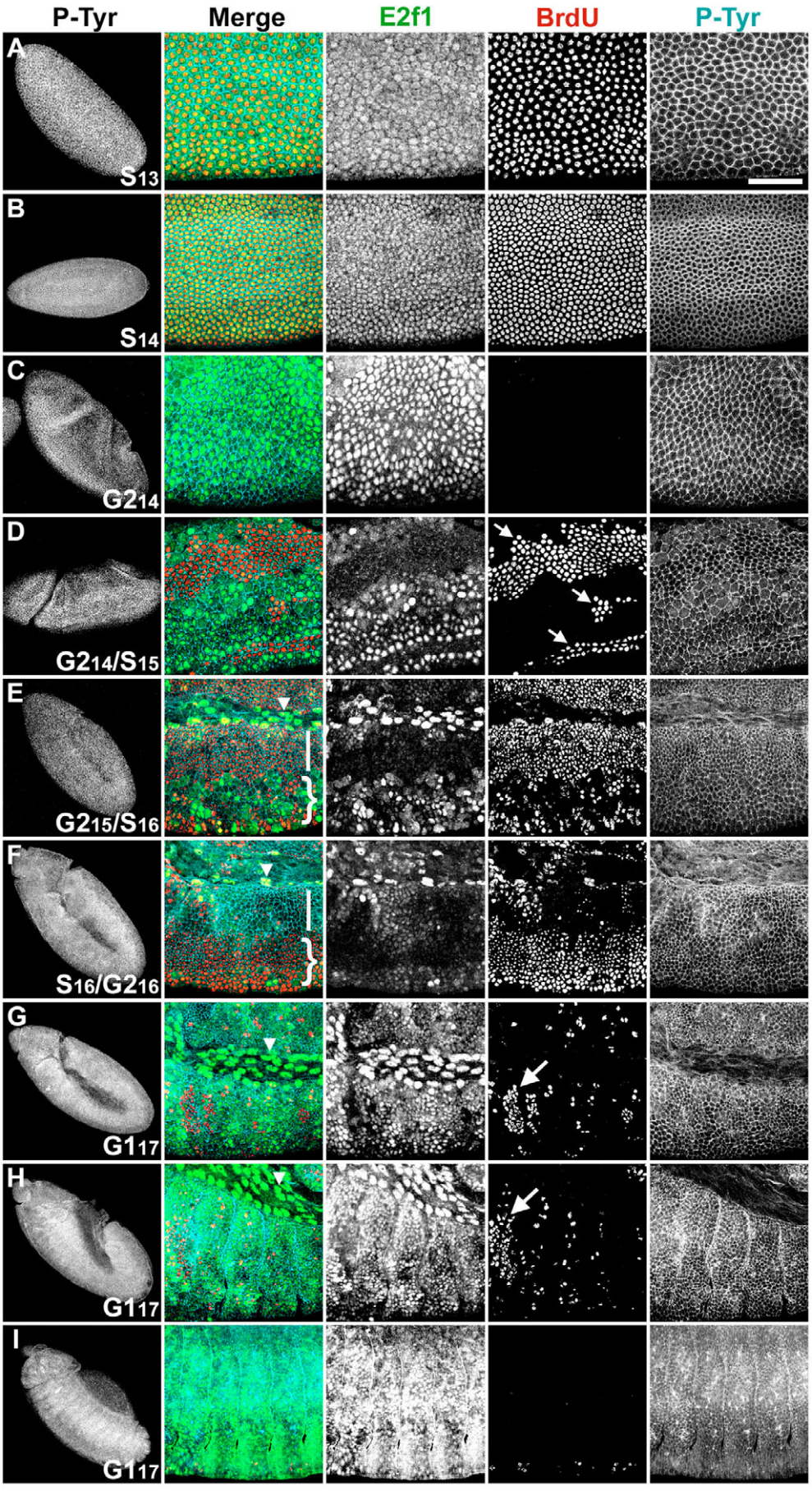

Fig. 3. E2f1 protein accumulation during embryogenesis. *w*^{1118} embryos were pulse-labeled with BrdU for 5 minutes and stained for E2f1 (green), BrdU incorporation (red) and phospho-tyrosine (cyan). Embryos undergoing (**A**) S_{13}; (**B**) S_{14}; and (**C**) $G2_{14}$. (**D**) S_{15} cells are indicated by arrows in the BrdU image; the remainder are still in $G2_{14}$. Note that entry into M_{14} is not synchronous throughout the embryo, resulting in groups of cells called mitotic domains that proceed through the cycle coordinately and that generate a reproducible and stereotypic pattern of BrdU incorporation [e.g. the top arrow indicates mitotic domain 11 (Foe, 1989)]. (**E**) Cycle 15 in the ventral epidermis (bracket) and S_{16} in the dorsal epidermis (bar). (**F**) S_{16} in the ventral epidermis (bracket) and $G2_{16}$-$G1_{17}$ in the dorsal epidermis (bar). Arrowheads in E-H indicate amnioserosa cells. (**G**,**H**) Although most cells of the epidermis have entered $G1_{17}$ (see Fig. 2C), some cells continue into cycle 17 (arrows in the BrdU image). (**I**) $G1_{17}$. Scale bar: 50 μm.

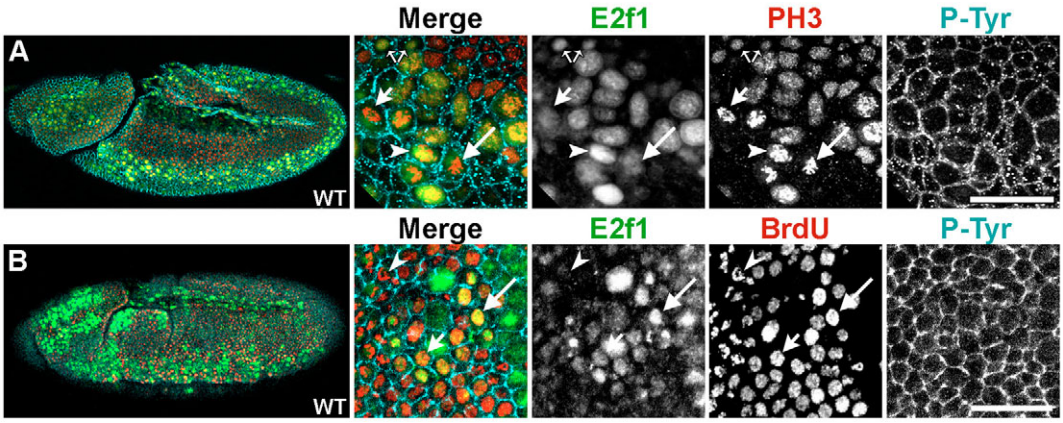

Fig. 4. E2f1 protein persists through mitosis into early S phase. (**A**) Stage 8 w^{1118} embryo labeled with E2f1 (green), phospho-histone H3 (red) and phospho-tyrosine (cyan). Prophase (arrowhead), metaphase (large arrow), anaphase (small arrow), and daughter cells in early interphase (double arrow) are indicated. (**B**) Stage 11 w^{1118} embryo labeled with E2f1 (green), BrdU (red; 5 minute pulse) and phospho-tyrosine (cyan). Early, mid, and late S phase are marked by a large arrow, a small arrow, and an arrowhead, respectively. Scale bars: 20 μm.

lack of S phase destruction of E2f1 in S_{13} and S_{14} suggests that zygotic gene expression, most of which begins during cycle 14, is necessary for the coupling of E2f1 destruction with S phase beginning in cycle 15.

E2f1 began to reaccumulate during G2 of cycle 15, but never attained the levels seen in G2 of cycle 14 (Fig. 3E), perhaps because of the short duration of $G2_{15}$. As in cycle 15, E2f1 protein abundance was low in S_{16}, but began to reaccumulate in $G2_{16}$ (Fig. 3E,F). By the time the epidermal cells entered $G1_{17}$, E2f1 protein had accumulated to a high level in the nucleus (Fig. 3G,H), and remained at this level at least until mid-embryogenesis (Fig. 3I). A group of cells in the first and second thoracic segments do not enter $G1_{17}$, but instead complete one more division cycle before arresting (Sauer et al., 1995). E2f1 protein was also downregulated during S phase in these cells (Fig. 3G,H, arrows). These data indicate that E2f1 protein abundance is inversely correlated with S phase during the post-blastoderm cell division cycles.

To determine the timing of E2f1 destruction more precisely, we compared E2f1 abundance with the pattern of BrdU incorporation as well as with phospho-histone H3 staining, which detects condensed mitotic chromosomes (Fig. 4). As reported for wing imaginal cells (Reis and Edgar, 2004), E2f1 protein was found to be abundant during mitosis. E2f1 was nuclear in early prophase prior to nuclear envelope breakdown (Fig. 4A, arrowhead). In metaphase and anaphase, E2f1 protein appeared more diffuse, most probably because of nuclear envelope breakdown (Fig. 4A, large and small arrows, respectively). E2f1 was present in newly formed daughter cells, suggesting that it is not destroyed by the APC/C during mitosis (Fig. 4A, double arrow). A high level of E2f1 protein was observed in cells in early S phase, characterized by uniform BrdU incorporation throughout the nucleus (Fig. 4B, large arrow). In mid-S phase, where BrdU incorporation was less uniform, there was a significant reduction in E2f1 protein (Fig. 4B, small arrow). By late S phase, where the more punctuate BrdU incorporation pattern marks late-replicating heterochromatin, there was very little E2f1 protein present (Fig. 4B, arrowhead). These data are consistent with the destruction of E2f1 protein after the initiation of S phase, and differ slightly from previous results in imaginal discs where no overlap between E2f1 staining and BrdU-labeling was detected (Heriche et al., 2003; Reis and Edgar, 2004). This difference may be due to the short embryonic cell cycle that lacks a G1 phase, as opposed to the canonical G1-S-G2-M disc cycles.

E2f1 staining in *E2f1*-mutant embryos was indistinguishable from wild type until S_{14} (data not shown), suggesting that maternal protein persists until S_{14}. *E2f1*-mutant embryos did contain a detectable amount of E2f1 protein in $G2_{14}$, but this was less than in sibling controls (see Fig. S1A,B in the supplementary material), indicating that zygotic E2f1 synthesis is responsible for a portion of the E2f1 protein found in $G2_{14}$. Zygotic *RnrS* mRNA rapidly accumulated in the epidermis during cycle 14, and then begin to decline during cycle 15 such that by the beginning of S_{16} these mRNAs were of very low abundance (Fig. 1D, arrowhead; Fig. 2A; see Fig. S2 in the supplementary material). This dynamic pattern of expression was not altered in *E2f1*-mutant embryos (see Fig. S1E,F in the supplementary material) (Duronio et al., 1995). These data suggest that maternal E2f1 is sufficient to induce early, zygotic transcription of E2f1 targets, and are consistent with the hypothesis that S phase-coupled destruction of E2f1 protein contributes to the decline of E2f1-regulated transcripts during cycle 15.

Destruction of E2f1 protein is S phase-dependent

The correlation between E2f1 disappearance and BrdU labeling suggests that either cell cycle progression into S phase or DNA synthesis per se triggers E2f1 destruction. To test if entry into S phase is required for the destruction of E2f1, we analyzed E2f1 protein levels in *stg* mutants, which arrest in $G2_{14}$ (Edgar and O'Farrell, 1990). E2f1 accumulated to a high level in the epidermis of *stg*-mutant embryos (Fig. 5A). Aminoserosa cells, which in wild-type embryos permanently exit the cell cycle in $G2_{14}$, also accumulated high levels of E2f1 (Fig. 3E-H, arrowheads). In *Cyclin E* mutants, E2f1 protein was not destroyed in the thoracic cells that normally enter a seventeenth division cycle (Fig. 3G,H), because these cells do not enter S phase (data not shown). These data suggest that the destruction of E2f1 in the epidermis requires entry into S phase.

To test if DNA synthesis is required for E2f1 destruction, we analyzed *double parked* (*dup*)-mutant embryos. *dup* encodes *Drosophila* Cdt1, a component of the prereplication complex (pre-RC) that is required for eukaryotic DNA synthesis. *dup*-mutant embryos develop normally through cycle 15, and then display

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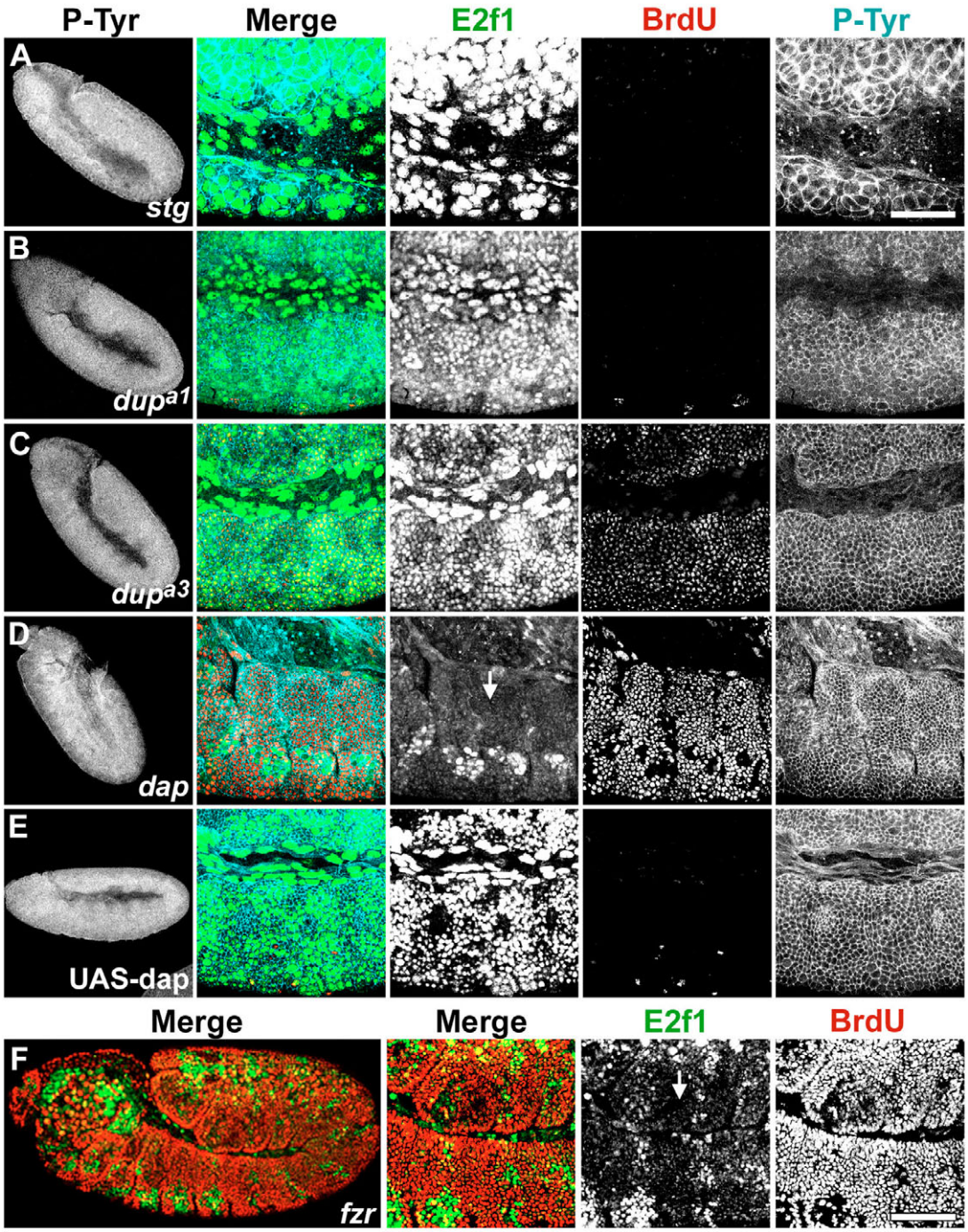

Fig. 5. E2f1 destruction is replication-dependent. (A-E) Stage 11 embryos were pulse-labeled with BrdU for 5 minutes, and stained for E2f1 (green), BrdU incorporation (red) and phospho-tyrosine (cyan). (**A**) stg^{7B}/stg^{7B}. (**B**) dup^{a1}/dup^{a1}. (**C**) dup^{a3}/dup^{a3}. (**D**) dap^{4454}/dap^{4454}; arrow indicates epithelial cells expressing low levels of E2f1. (**E**) UAS *dap/arm*-Gal4 VP16. (**F**) Stage 11 *Df(1)biD3/Df(1)biD3 fzr*-mutant embryos were pulse-labeled with BrdU for 15 minutes, and stained for E2f1 (green) and BrdU incorporation (red); arrow indicates epithelial cells expressing low levels of E2f1 similar to *dap* mutants. Scale bars: 50 μm.

impaired DNA replication in S_{16} causing cell cycle arrest and embryonic lethality (Garner et al., 2001; Whittaker et al., 2000). S_{16} is absent in dup^{a1}-null mutants, whereas dup^{a3} hypomorphic mutants display weak BrdU incorporation during a prolonged and partial S_{16} (Fig. 5B,C) (Garner et al., 2001). dup^{a1} mutants accumulated high levels of E2f1 in the epidermis, suggesting that DNA synthesis is necessary for E2f1 destruction (Fig. 5B). Interestingly, dup^{a3} mutants also accumulated high levels of E2f1 even though these epidermal cells were capable of incorporating some BrdU (Fig. 5C). This suggests that efficient progression through S phase is necessary to trigger E2f1 destruction and/or that Dup plays a more direct role in E2f1 destruction.

S phase-dependent destruction of E2f1 protein predicts that E2f1 levels will be low during the ectopic S_{17} that occurs in *dap* and *fzr* mutants. This would provide an explanation for the lack of E2f1-target gene expression even in the presence of the ectopic CycE-Cdk2 that

is predicted to prevent Rbf1 activation (Fig. 1I). Indeed, E2f1 protein abundance was low during ectopic S_{17} in the epidermis of both *dap* and *fzr* mutants (Fig. 5D,F, respectively). Conversely, Dap overexpression resulted in the accumulation of E2f1 protein throughout the epidermis, most likely because of the inhibition of S_{16} (Fig. 5E). These data are consistent with the hypothesis that the initial loss of E2f1-target gene expression results from the absence of E2f1, rather than from the appearance of hypophosphorylated Rbf1.

Rbf1 is not required for the initial termination of E2f1-target gene expression prior to $G1_{17}$ arrest

We have demonstrated that E2f1 protein is destroyed during S_{15} near the time when *RnrS* expression begins to decline. This is one cell cycle before Dap is induced to inhibit CycE-Cdk2 and trigger the onset of $G1_{17}$. If Dap expression and the inhibition of CycE-Cdk2 results in the accumulation of hypophosphorylated Rbf1 (Fig. 1I), then *RnrS* expression is normally terminated before Rbf1 becomes active. This model predicts that *RnrS* expression should terminate on schedule in *Rbf1*-mutant epidermal cells. Indeed, both E2f1 protein and *RnrS* transcripts were absent during S_{16} in *Rbf1*-mutant epidermal cells (Fig. 6A). Later, as E2f1 protein reaccumulated throughout the epidermis in $G2_{16}$ and $G1_{17}$, *RnrS* transcripts inappropriately reappeared in *Rbf1* mutants (Fig. 6B). This ectopic expression of E2f1-target genes ultimately results in cell cycle re-entry, as previously described (Fig. 6C, bracket) (Du and Dyson, 1999). Not all *Rbf1*-mutant epidermal cells re-enter S phase, suggesting that other inputs modulate the cell cycle response to Rbf1 loss. These may include cell-by-cell differences in the amount of E2f1, as we observed that cells with the most E2f1 were usually the same ones that entered S phase inappropriately. This is consistent with previous observations that transgene-mediated high level E2f1-Dp expression can drive most of the $G1_{17}$ epidermal cells into S phase (Duronio et al., 1996). These data indicate that *Rbf1* is not required for the initial termination of E2f1-target gene expression, but rather for sustained termination and stable G1 arrest.

Dap expression promotes conversion of Rbf1 to a repressor

Although our data suggest that E2f1-target genes are controlled independently of Rbf1 prior to cycle 17, they do not define the mechanism by which Rbf1 is converted to a repressor during $G1_{17}$. To address this issue, we re-evaluated the inhibition of CycE-Cdk2 activity by Dap. We hypothesized that developmentally-controlled Dap expression in cycle 16 does indeed convert Rbf1 into a repressor, but that Rbf1 is not required for the initial shut down of *RnrS* because other mechanisms, such as E2f1 destruction in cycles 15 and 16, are sufficient. Rather, Rbf1 is required to prevent the reactivation of E2f1-target genes as E2f1 protein reaccumulates during $G2_{16}$ and $G1_{17}$.

The phenotype of *stg* mutants allowed us to test this hypothesis. Previous experiments revealed that E2f1-target gene expression terminates on schedule in *stg* mutants even though *stg*-mutant epidermal cells arrest in $G2_{14}$ (Duronio and O'Farrell, 1994). This is an indication of a developmentally-timed event that occurs independently of cell cycle progression. The high level of E2f1 protein in *stg*-mutant epidermal cells (Fig. 5), which never enter S phase, would at first seem to be at odds with this result. However, developmentally controlled Dap expression in a *stg* mutant may inhibit CycE-Cdk2 and result in the accumulation of hypophosphorylated Rbf1 and the downregulation of E2f1 targets (Fig. 1I) (Meyer et al., 2002b). We therefore simultaneously examined Dap and *RnrS* expression in *stg* mutants. In the epidermis of *stg* mutants at the normal time of cycle 15 (i.e. after gastrulation and germ band extension), *RnrS* transcripts were abundant and Dap protein was not detected (Fig. 7A). Later, as Dap protein accumulated, *RnrS* expression decreased (Fig. 7B). To test whether loss of *RnrS* expression in *stg*-mutant embryos required Dap, we analyzed *stg dap* double-mutant embryos shortly after the time when Dap is first induced. *RnrS* was not suppressed in those cells of *stg dap* double-mutant embryos that corresponded to cells with high levels of Dap protein in *stg* single-

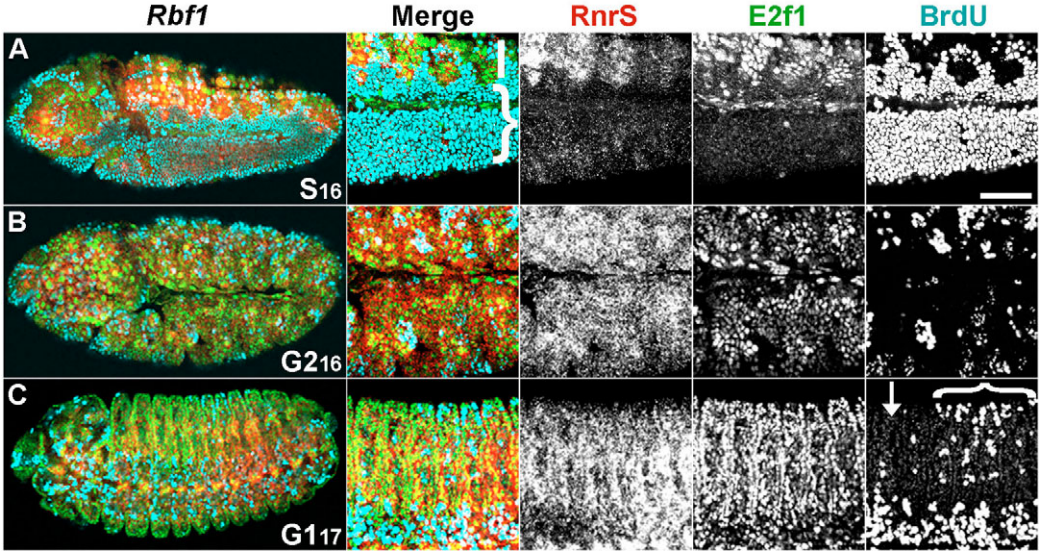

Fig. 6. The initial termination of E2f1-target gene expression does not require Rbf1. *$Rbf1^{14}$* maternal and zygotic null embryos were pulse-labeled with BrdU for 15 minutes and stained for E2f1 (green) and BrdU incorporation (cyan). *RnrS* expression was detected by FISH (red). (**A**) Stage 10 embryo; bracket marks the dorsal epidermis in S_{16} and the bar indicates cycle 15. (**B**) Stage 11 embryo; the epidermal cells are in $G2_{16}$. (**C**) Stage 13 embryo; arrow indicates epidermal cells arrested in $G1_{17}$ and the bracket denotes epidermal cells inappropriately incorporating BrdU. Scale bar: 50 μm.

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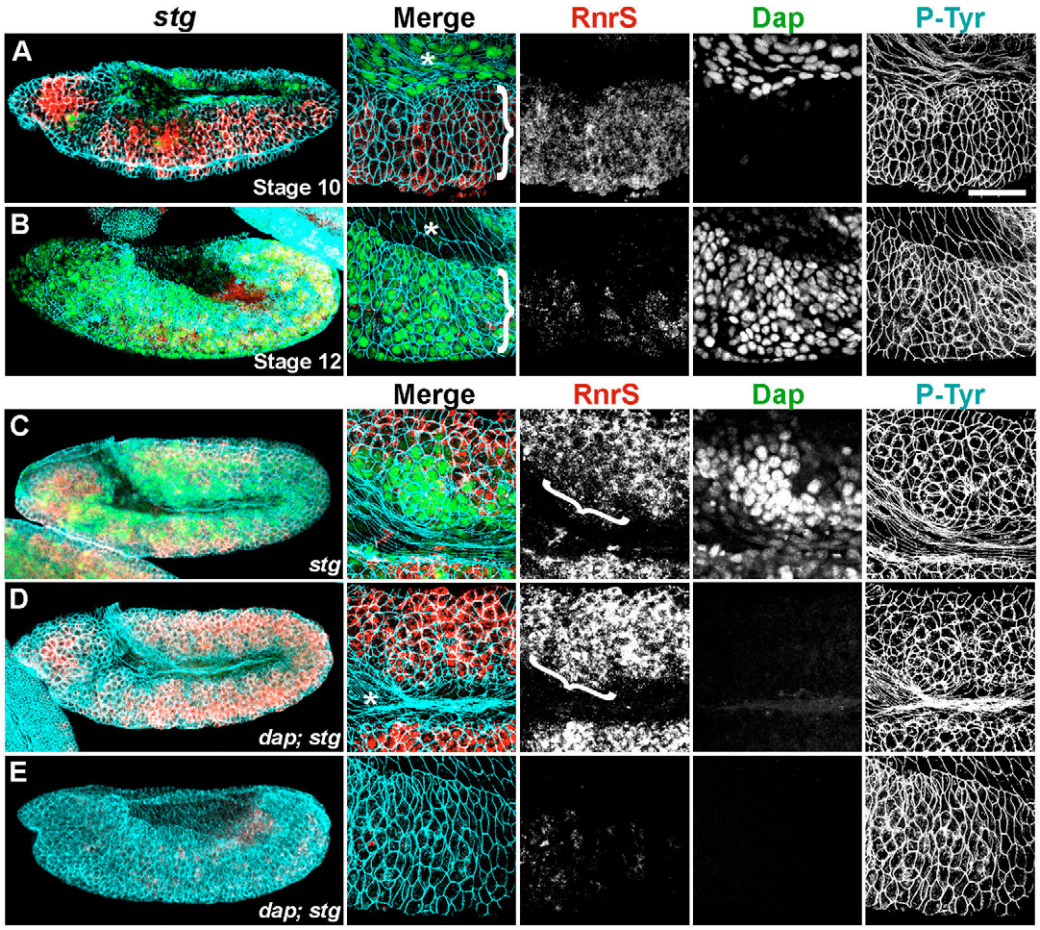

Fig. 7. Dap expression activates Rbf1. Embryos were stained for Dap (green) and phospho-tyrosine (cyan). *RnrS* expression was detected by FISH (red). (**A**) Stage 10 stg^{7B}/stg^{7B} embryo. (**B**) Stage 12 stg^{7B}/stg^{7B} embryo. Brackets mark the epidermal cells and asterisks denote $G2_{14}$-arrested aminoserosa cells. (**C-E**) Embryos from $dap^{4454}/+$; $stg^{7B}/+$ parents. (C) Stage 11 embryo with the stg^{7B}/stg^{7B} phenotype. (D) Stage 11 dap^{4454}/dap^{4454}; stg^{7B}/stg^{7B} embryo. C and D are siblings that are stage-matched based on age, morphology and phospho-tyrosine staining. Bracket in C indicates epidermal cells in which *RnrS* is starting to decline and Dap is expressed at high levels. Bracket in D shows the corresponding region in which *RnrS* levels remain high. Asterisk in D denotes aminoserosa cells. (E) Stage 12 dap^{4454}/dap^{4454}; stg^{7B}/stg^{7B} embryo. Scale bar: 50 μm.

mutant sibling embryos (Fig. 7C,D, bracket). These data are consistent with our model that the inhibition of CycE-Cdk2 by developmentally-controlled Dap expression results in the accumulation of Rbf1-E2f1 repressor complexes. However, as the *stg dap* mutant embryos aged, *RnrS* expression was eventually lost in many epidermal cells (Fig. 7E). This also occurred in the aminoserosa, which contains cells that have exited the cell cycle in $G2_{14}$ (Fig. 7D, asterisk). These data imply the existence of Rbf1-indepenent mechanisms to extinguish E2f1-target gene expression. Perhaps, when cells exit the cycle, Dap-mediated Rbf1 activation terminates E2f1-target gene transcription while additional mechanisms dramatically decrease mRNA stability.

Inefficient Rbf1 activation in *dup* mutants

For reasons that are unclear, *dup* mutants fail to terminate E2f1-dependent transcription in the epidermis (Whittaker et al., 2000). dup^{a1}-mutant epidermal cells failed to downregulate *RnrS* at the time of S_{16}, and dup^{a3} mutants continued to express *RnrS* during the prolonged and partial S_{16} (see Fig. S3A-C in the supplementary material). This may be explained by the high level of E2f1 protein that accumulates in *dup* mutants (Fig. 5). However, Dap protein accumulates during cycle 16 in *dup* mutants (data not shown), and this should result in the downregulation of E2f1 targets as in *stg* mutants. Rbf-280 expression using the *prd*-Gal4 driver suppressed the ectopic *RnrS* expression in dup^{a1} mutants, suggesting that E2f1 could still be repressed by hypophosphorylated Rbf1 in *dup* mutants (see Fig. S3D-G in the supplementary material). At later stages, *RnrS* transcripts eventually began to decline in *dup* mutants (data not shown). We suggest that in *dup* mutants, Rbf1 is still converted into an active, hypophosphorylated form in response to Dap expression, but that the termination of E2f1-dependent transcription occurs slowly because of the abnormally high level of E2f1 protein.

DISCUSSION

Our finding that $p27^{Dap}$ expression is not necessary for the downregulation of E2f1 targets was unexpected, based on the known regulatory circuitry of the pRb-E2F pathway (Fig. 1I). This result led us to hypothesize that mechanisms in addition to Rbf1 binding are used to control E2f1 activity in the early embryo. We found that E2f1 is destroyed during S phase of the post-blastoderm divisions in the embryonic epidermis, as was previously reported for cells in wing and eye imaginal discs (Asano et al., 1996; Heriche et al., 2003; Reis and Edgar, 2004). E2f1 destruction first occurs during S_{15}, at the same time that E2f1-regulated transcripts such as *RnrS* begin to decline. Because E2f1 functions as a transcriptional activator, and because we show that Rbf1 is not required for the initial decline in *RnrS* transcripts, we propose that the loss of E2f1 protein contributes to the initial termination of replication factor gene expression. Rbf1 is first required during development for the maintenance of $G1_{17}$ arrest and the continued repression of E2f1-target genes. Our data suggest that Rbf1 is converted to a repressor after the developmentally-induced expression of Dap, most likely because the consequent inhibition of

CycE-Cdk2 results in the accumulation of hypophosphorylated Rbf1. Dap expression accompanies the downregulation of *Cyclin E* transcription, and each of these mechanisms of inhibition of CycE-Cdk2 contributes to G1 arrest.

The high level of E2f1 protein in $G1_{17}$ epidermal cells may permit the formation of E2f1-Rbf1 complexes necessary to actively and stably repress replication factor genes during G1 arrest (Frolov and Dyson, 2004), and also provides a simple explanation for why the loss of Rbf1 function results in the ectopic expression of E2f1 targets (Du and Dyson, 1999). After hatching, and in response to the first instar larvae beginning to feed, the epidermal cells start to endoreduplicate. Thus, the accumulation of Rbf1-E2f1 complexes during G1 arrest may prepare cells for rapid production of replication factors and efficient re-entry into the cell cycle upon activation of G1 Cyclin-Cdk complexes after growth stimulation.

RnrS expression is lost in *E2f1* zygotic mutant embryos, but not until cell cycle 17 (Duronio et al., 1995). One interpretation of this result is that maternal stores of E2f1 are sufficient for the early induction of replication gene expression in the post-blastoderm divisions. Consistent with this, maternal E2f1 protein persists into cycle 14, coincident with the commencement of zygotic transcription of E2f1 targets such as *RnrS*. In addition, mutation of the E2f1-binding sites in the regulatory region of the *Pcna* gene (*mus209* – Flybase) is sufficient to abolish zygotic *Pcna* expression (Thacker et al., 2003). However, our data do not demonstrate a requirement for E2f1 for early zygotic *RnrS* expression, and E2f1 may be only one of several factors necessary for early zygotic expression of genes encoding replication factors (Hirose et al., 1993; Sawado et al., 1998; Yamaguchi et al., 1996). For instance, the transcription of *Cyclin E* requires E2f1 in embryonic endocycles, but also occurs independently of E2f1 via tissue-specific enhancer elements such as those operating in the CNS (Duronio and O'Farrell, 1995; Jones et al., 2000). Thus, any control of replication factor gene expression by E2f1 abundance may be modulated by other transcription factors, or bypassed entirely in certain cell types by E2f1-independent modes of expression.

Mechanisms of cell cycle-regulated E2f1 destruction

Our data suggest that E2f1 destruction is coupled to DNA synthesis. CycE-Cdk2 has been suggested as a possible cell cycle input for E2f1 destruction in imaginal cells because it is activated at the G1-S transition when E2f1 is destroyed (Heriche et al., 2003; Reis and Edgar, 2004). However, CycE-Cdk2 is continuously active during the embryonic post-blastoderm cell cycles, whereas E2f1 is destroyed only during S phase (Sauer et al., 1995). Thus, CycE-Cdk2 is unlikely to be the only signal, and actively replicating DNA may provide a necessary input into E2f1 destruction. This model is consistent with our observation that E2f1 destruction occurs after DNA synthesis begins, resulting in cells that are positive for both E2f1 and BrdU incorporation in early interphase.

Previous studies have suggested that mammalian E2f1 is degraded by the ubiquitin-proteasome pathway (Campanero and Flemington, 1997; Hateboer et al., 1996; Hofmann et al., 1996; Marti et al., 1999; Ohta and Xiong, 2001). In this pathway, E3 ubiquitin ligases bind to and mediate the ubiquitylation of specific proteins. The SCF class of cullin-dependent E3 ligases has been implicated in E2F1 destruction (Marti et al., 1999). In *Drosophila*, evidence from genetic and cell biology studies suggest that SCF^{SLMB} mediates E2f1 destruction at the G1-S transition in wing imaginal disc cells (Heriche et al., 2003). Although there is no evidence implicating a specific E3 ligase in the destruction of embryonic E2f1, there are interesting parallels with recent experiments describing the destruction of Cdt1/Dup. Like E2f1, Cdt1/Dup is degraded at the G1-S transition and cannot be detected during S phase (Thomer et al., 2004). In vertebrates, Cdt1 destruction is mediated by two independent and apparently redundant mechanisms: direct Cdk2 phosphorylation that targets Cdt1 to SCF^{SKP2}, and binding of PCNA to the Cdt1/Dup amino-terminus that targets Cdt1 to $Cul4^{DDB1}$ (Arias and Walter, 2006; Nishitani et al., 2006; Senga et al., 2006). This latter result is consistent with a recent study indicating that *Drosophila* Dup hyperaccumulates in cells where DNA synthesis is attenuated (May et al., 2005). Thus, more than one E3 ubiquitin ligase may participate in E2f1 destruction (Ohta and Xiong, 2001). Determining the molecular mechanism of E2f1 destruction should permit us to directly test whether prevention of E2f1 destruction would affect replication factor gene expression in the embryo.

pRb-independent E2F regulation and early animal development

E2F is necessary for the development of worms, flies and mice (DeGregori, 2002). Remarkably, however, pRb is not needed for the entirety of mouse embryonic development (Wu et al., 2003). This could in part be due to redundancy with other pRb family members, such as p107 and p130 (Dannenberg et al., 2004). Alternatively, a pRb-independent mechanism of regulating E2F activity may control S phase gene expression and cell cycle progression during early mammalian development. This idea is supported by experiments modeling the cell cycles of early vertebrate development in cell culture using murine embryonic stem cells (White et al., 2005). These pluripotent cells have a cell cycle composed mostly of S phase that is characterized by ubiquitous Cdk activity and the absence of CKIs (Faast et al., 2004; Savatier et al., 1996; Stead et al., 2002). As in the *Drosophila* embryo, E2F-regulated transcripts are also ubiquitous even though pRb family members are expressed (Savatier et al., 1994; Stead et al., 2002). Differentiation requires the lengthening of G1 and the negative regulation of Cdk2 activity, which is accomplished both by increases in the level of CKIs and by the downregulation of Cyclin E1 expression via inhibition of E2F (White et al., 2005). Thus, evolutionarily conserved regulatory mechanisms operating in early development may mediate the conversion from rapid cell cycles driven by intrinsic cues to slower, more highly regulated cycles that are influenced by extrinsic developmental and environmental cues.

We thank Maki Asano, Wei Du, Nick Dyson, Christian Lehner and Pat O'Farrell for reagents; Mark Peifer, Frank Conlon, Sima Zacharek and Sarah Radford for critical reading of the manuscript; members of the Duronio laboratory for helpful discussion; and Tony Perdue for assistance with confocal microscopy. This work was supported by grant GM57859 from the National Institutes of Health.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/3/467/DC1

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