Drosophila E2F1 is degraded during S phase in a PCNA-, Cul4-, and Cdt2-dependent manner

Shusaku Shibutani

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Approved by:

Robert J. Duronio

Victoria Bautch

Frank Conlon

Stephen Crews

Jeff Sekelsky

ABSTRACT

Shusaku Shibutani: *Drosophila* E2F1 is degraded during S phase in a PCNA-, Cul4-, and Cdt2-dependent manner
(Under the direction of Robert J. Duronio)

During the development of multicellular organisms, cell proliferation is tightly regulated by intrinsic and extrinsic cues, generating a spatiotemporal cell cycle pattern. For instance, cell cycles are very rapid during early embryogenesis, resulting in a sufficient number of cells for tissue formation. In contrast, cells that are going to differentiate usually arrest the cell cycle in G1 phase and subsequently enter the quiescent state (G0). Failure to maintain active cell cycles during early embryogenesis and to arrest the cell cycle before differentiation will cause destructive effects on tissue development and homeostasis. Since cells usually arrest in G1, an important decision step in the cell cycle is whether the cell stays in G1 phase or enters S phase. When G1 cells enter S phase, positive cell cycle regulators such as Cyclin E, RnrS, PCNA, and DNA polymerase are coordinately induced by the family of the E2F transcription factors.

In *Drosophila*, these genes are regulated by a single E2F (E2F1). During the early embryogenesis of *Drosophila*, E2F1-target genes are expressed ubiquitously, facilitating the rapid cell cycles. Later in embryogenesis, E2F1-target genes are downregulated before cells arrest in G1. This implies that during embryogenesis developmentally-regulated E2F1 activity causes this characteristic cell cycle pattern. In this thesis, we show that the initial downregulation of E2F1-target genes is preceded by the developmentally-regulated onset of

E2F1 destruction. Furthermore, we discovered that DNA replication induces E2F1 destruction in a PCNA-, Cul4-, and Cdt2-dependent manner. Expression of a stabilized form of E2F1 in the larval wing disc caused apoptosis and disrupted adult wing morphology, while expression in the larval salivary gland arrested the endocycle, a variant G1-S cell cycle that lacks mitosis and results in polyploidy. Taken together, our data suggests the existence of a robust negative feedback mechanism where E2F1 induces DNA replication, which in turn downregulates E2F1 by proteolysis, and this negative feedback loop is required for normal development of *Drosophila*.

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ago	archipelago
APC/C	Anaphase Promoting Complex/Cyclosome
arm	armadillo
β-gal	beta-galactosidase
BrdU	Bromodeoxyuridine
Cdc25	Cell division cycle 25
CDK	Cyclin-Dependent Kinase
cDNA	complementary DNA
Cdt1	Chromatin licensing and DNA replication factor 1
Cdt2	Denticleless homolog protein
CKI	Cyclin-Dependent Kinase Inhibitor
CNS	Central Nervous System
cpo	couch potato
Cul	Cullin
Cyc	Cyclin
Dap/$p27^{Dap}$	Dacapo
DAPI	4',6-diamidino-2-phenylindole
DDB1	DNA Damage Binding protein 1
DNA	Deoxyribonucleic acid
DP	DRTF1/E2F Dimerization partner 1
dDREAM	RBF, dE2F2, and dMyb-interacting proteins
DREF	DRE (DNA Replication-related Element) binding Factor

dsRNA	double-stranded ribonucleic acid
dup	double parked
E2F	E2 Factor
en	engrailed
FACS	Fluorescent-Activated Cell Sorting
FISH	Fluorescent In Situ Hybridization
fzr	fizzy-related
G	Gap
GFP	Green Fluorescent Protein
HA	Hemagglutinin
hid	head involution defective
Hsp70	Heat shock protein 70
htl	heartless
INK4	Inhibitor of Cdk4
Kip1	Kinase-inhibitory protein 1
M	Mitosis
MCM	Mini Chromosome Maintenance
mRNA	messenger ribonucleic acid
ORF	Open Reading Frame
PBS	Phosphate-Buffered Saline
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
PH3	Phospho-histone H3

PIP	PCNA-Interacting Protein
PMSF	phenylmethanesulphonylfluoride
PP1	Type 1 Protein Phosphatase
prd	paired
pre-RC	pre-Replication Complex
pRb	Retinoblastoma protein
ptc	patched
RBF	Retinoblastoma-family protein
RNAi	RNA interference
RnrS	Small subunit of Ribonucleotide reductase
RnrL	Large subunit of Ribonucleotide reductase
RT-PCR	Reverse Transcription Polymerase Chain Reaction
S	Synthesis
S2	Schneider 2
SAP130	Spliceosome-Associated Protein 130
SCF	Skp1-Cullin1-F box
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Skp1	S phase kinase-associated protein 1
Skp2	S phase kinase-associated protein 2
Slmb	Slimb (super-numary limbs)
sr	stripe
stg	string
UAS	Upstream Activator Sequence

WT	Wild Type

CHAPTER I

INTRODUCTION

Proper control of cell cycles is a fundamental feature of living organisms. For example, unicellular organisms respond to the surrounding environment; they actively proliferate in a nutrient-rich condition, and arrest the cell cycle when nutrients are limited. In the case of yeast, the cell cycle is regulated in response to mating pheromones (Humphrey and Pearce, 2005). On the other hand, multicellular organisms have evolved complex regulatory mechanisms for the cell cycle in order to form and maintain functional tissue morphology. The proliferation of cells in a tissue is orchestrated by various intrinsic and extrinsic cues (Lee and Orr-Weaver, 2003). In multicellular organisms, the cell cycle is typically rapid during early embryogenesis, resulting in a sufficient number of cells for tissue formation. Later in development, it becomes important to arrest the cell cycle before cells differentiate. The failure to arrest the cell cycle will lead to tissue deformation and can result in cancer development. Thus, understanding the mechanisms of cell cycle regulation in a multicellular organism is of particular importance for both the basic biology and medical research fields.

The regulation of the G1-to-S transition

The canonical cell cycle is composed of four phase: G1, S, G2, and M. During S phase, DNA synthesis takes place and the whole genome is replicated, resulting in two

identical sets of genomes in one nucleus. During M phase, the replicated genomes are divided into two nuclei, and the subsequent cytoplasmic division results in two daughter cells. G1 and G2 phases are defined as gap phases that separate S phase and M phase. In these gap phases, proteins required for the subsequent phase are actively synthesized. Previous studies have identified a large number of cell cycle regulators including Cyclin/Cyclin-dependent kinase complexes (Cyc/CDK) and E2 factor (E2F) transcription factors. Cyc/CDK complexes phosphorylate a plethora of proteins, and this phosphorylation coordinately drives the G1-to-S or G2-to-M transitions. Cyclins are destroyed each cell cycle, and because of this every cell cycle transition requires the new synthesis of Cyclins, which is achieved by the activity of a variety of transcription factors. One such transcription factor is E2F, which, together with its heterodimeric partner DP, induces transcription of genes involved in DNA synthesis, mitosis, apoptosis, DNA repair, and differentiation (Blais and Dynlacht, 2004; Iaquinta and Lees, 2007; Lee and Orr-Weaver, 2003; Stevaux and Dyson, 2002). E2F is negatively regulated by pocket protein family members including the retinoblastoma tumor suppressor protein (pRB), p107, and p130. To add further complexity, pocket proteins are negatively regulated by Cyc/CDK-mediated phosphorylation. Therefore, E2F, pocket proteins, and Cyc/CDK form a potential positive feedback loop (Figure 1.1).

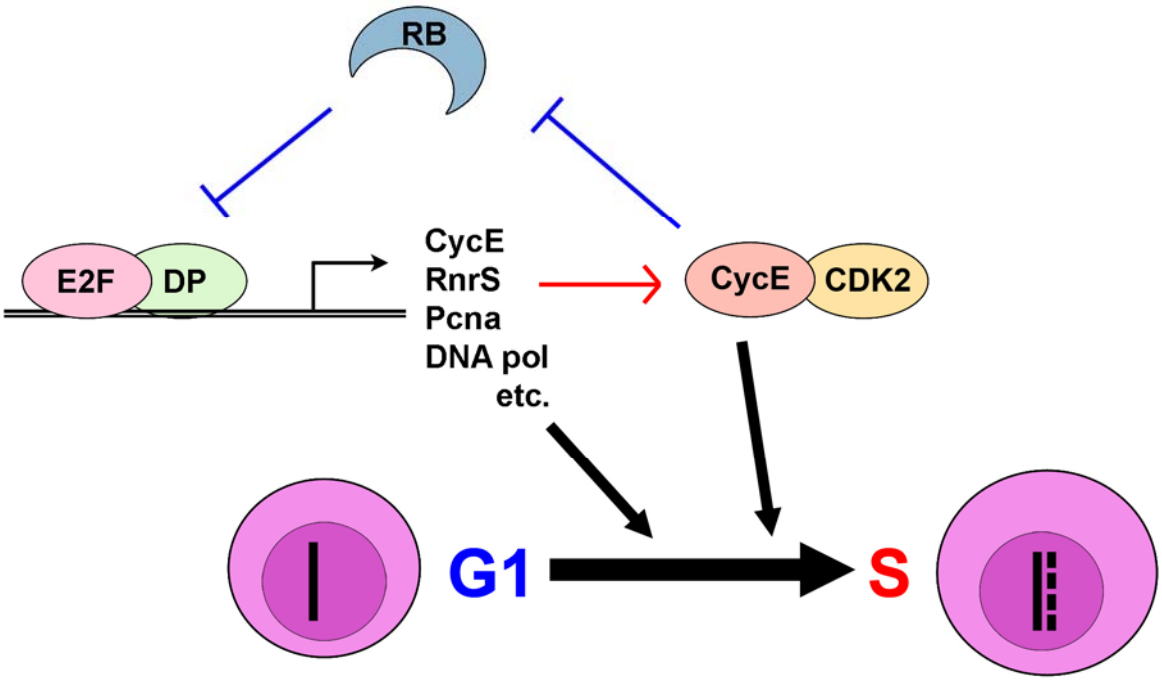

Figure 1.1: Regulators of the G1-to-S transition

The E2F transcription factor, together with its heterodimeric partner DP, induces DNA replication genes such as *CycE*, *ribonuclease reductase small subunit* (*RnrS*), *proliferating cell nuclear antigen* (*Pcna*), and *DNA polymerase*. Because of this, E2F acts as a strong positive regulator of the G1-to-S transition. E2F is negatively regulated by pocket protein family members (represented by RB in this figure). The phosphorylation of pocket proteins by Cyc/CDK complexes results in the dissociation of pocket proteins from E2F, allowing the induction of E2F-target gene transcription. Note that E2F, RB, and CycE/CDK2 form a potential positive feedback loop that promotes S phase entry.

It is thought that this potential positive feedback loop helps G1 cells to commit their entry into S phase (Malumbres and Barbacid, 2001; Yao et al., 2008). Once the activities of E2F and CycE/CDK2 reach a critical threshold, they synergistically activate one another, facilitating the robust entry into S phase. However, the activities of E2F and CycE/CDK2 must be suppressed both when the cell is in a quiescent state (G0 phase), and when DNA replication is initiated (S phase). Indeed, Cyc/CDK is negatively regulated by CDK inhibitors

such as $p16^{INK4a}$ and $p27^{Kip1}$ (Sherr and Roberts, 1999), and by the proteasome-mediated destruction of Cyclins (Nakayama and Nakayama, 2006). The negative regulation of Cyc/CDK occurs in a timely manner during the cell cycle, and the misregulation of these mechanisms often results in ectopic S phase (Chen and Segil, 1999; de Nooij et al., 1996; Lane et al., 1996; Lowenheim et al., 1999; Moberg et al., 2001). In addition to Cyc/CDK regulation, previous studies have identified mechanisms that negatively regulate E2F activity. The negative regulation involves pocket proteins, repressor E2Fs, proteasome-mediated destruction, CycA/CDK2-mediated phosphorylation, and transcriptional repression (see below for further details).

The regulatory mechanisms of mammalian and *Drosophila* E2Fs

In mammals, eight E2F genes have been identified, and can be generally categorized into two groups: activator E2Fs (1, 2 and 3a) and repressor E2Fs (3b and 4-8). When activator E2Fs are overexpressed in cultured cells, they often induce S phase entry, followed by apoptosis (Kowalik et al., 1995; Qin et al., 1994; Shan and Lee, 1994; Wu and Levine, 1994). Conversely, the triple knockout of *E2f1-3* in mouse embryonic fibroblasts completely arrests the cells cycle (Wu et al., 2001).

As mentioned above, the activity of these activator E2Fs must be suppressed when the cell is in the quiescent phase (G0 phase), and when DNA replication is initiated (S phase). Failure to suppress E2F activity in G0/G1 or S phase may cause ectopic S phase entry or re-replication, respectively. Indeed, a significant number of studies in mammals have revealed several mechanisms that suppress activator E2Fs.

First, activator E2Fs are repressed in G0/G1 by the binding of pRB to the C-termini of E2Fs. This binding interferes with the transcriptional activity of E2Fs. In addition, E2F-bound pRB recruits various chromatin regulatory complexes that contribute to the repression of transcription (Blais and Dynlacht, 2007; Frolov and Dyson, 2004). A widely-accepted mechanism to re-activate pRB-repressed E2Fs is that extracellular signals induce the transcription of CycD, which binds to and activates CDK4 and 6. CycD/CDK4 and CycD/CDK6 then hyperphosphorylate pRB, which leads to the dissociation of pRB from E2F (Frolov and Dyson, 2004). The importance of the pRB-mediated repression is inferred by the fact that many types of cancers have mutations in the *Rb* gene, and that the loss of *Rb* in model animals leads to failure to maintain cell cycle arrest (Jacks et al., 1992; MacPherson et al., 2003; Ruiz et al., 2004; Wu et al., 2003). Also, reduction of E2F activity can suppress tumor formation due to loss of pRB (Lee et al., 2002; Yamasaki et al., 1998; Ziebold et al., 2003). Second, activator E2Fs on gene promoters are replaced by repressor E2Fs during G0/G1, which results in the suppression of E2F-responsive promoters (Trimarchi and Lees, 2002). The third mechanism is the destruction of E2F1 protein via the ubiquitin-proteasome pathway in S/G2 phases. In mammals, the $Cul1^{SKP2}$ ubiquitin ligase complex has been linked to the degradation of E2F1. $Cul1^{SKP2}$ binds to the N-terminus of E2F1, leading to the ubiquitination and subsequent destruction of E2F1 (Marti et al., 1999). Other studies showed that the C-terminal truncation of E2F1, 2 and 4 stabilized these proteins, and E2F1 was protected from ubiquitination and destruction when bound by pRB (Campanero and Flemington, 1997; Hateboer et al., 1996; Hofmann et al., 1996). The fourth mechanism involves the phosphorylation of DP by CycA/CDK2 in S phase. DP forms heterodimers with E2Fs, and this heterodimerization is required for the binding of E2F/DP to DNA. In S phase,

CycA/CDK2 binds to the N-terminus of activator E2Fs and phosphorylates E2F-bound DP, leading to the dissociation of E2F/DP from the DNA (Dynlacht et al., 1994; Dynlacht et al., 1997; Krek et al., 1994; Krek et al., 1995). Lastly, a recent report showed that homo- and heterodimers formed by mouse E2F7/8 bind to the promoter of the *E2f1* gene and repress the transcription of *E2f1* in S/G2 (Li et al., 2008). The combinational loss of *E2f7* and *8* resulted in embryonic lethality with massive apoptosis induction, and the apoptosis was suppressed by additional deletion of *E2f1*, suggesting that the negative regulation of E2F1 by E2F7/8 is important for animal development.

Despite the large body of data that suggests suppression mechanisms of mammalian activator E2Fs, the redundancy that exists in mammals among the eight E2Fs, two DPs (DP1 and 2), and three pocket proteins (pRB, p107, and p130) makes it difficult to interpret the results. A more simplified E2F/DP/RB pathway has been characterized in *Drosophila*, providing an excellent model system to investigate the regulatory mechanisms of E2Fs. In *Drosophila*, two E2Fs (E2F1 and 2), one DP, and two pocket proteins (RBF1 and 2) have been identified (Lee and Orr-Weaver, 2003). E2F1, the only known activator E2F in *Drosophila*, shares major characteristics with mammalian activator E2Fs. It induces transcription of genes involved in DNA synthesis such as *Cyclin E*, *RnrS*, *Pcna*, and *DNA polymerase alpha* (Dimova et al., 2003; Duronio and O'Farrell, 1994), and regulates expression of pro-apoptotic genes such as *reaper* (Asano et al., 1996), *hid*, and *Dcp-1* (Moon et al., 2005). Mutation of *E2f1* is lethal and causes inhibition of S phase during embryogenesis (Duronio et al., 1995), and overexpression of *Drosophila* E2F1 can induce ectopic S phase entry and apoptosis (Asano et al., 1996; Du et al., 1996b; Duronio et al., 1996), supporting the idea that *Drosophila* E2F1 is a positive regulator of the G1-to-S

transition and apoptosis. Furthermore, the mechanisms to suppress activator E2Fs are conserved between mammals and *Drosophila* to some extent. Like mammalian activator E2Fs, *Drosophila* E2F1 is suppressed by RBF1-binding (Du et al., 1996a; Xin et al., 2002), and suppression of E2F1-target genes by E2F2 has been suggested (Frolov et al., 2001; Weng et al., 2003). The ubiquitin-mediated proteolysis of *Drosophila* E2F1 also exists and was previously linked to the $Cul1^{Slmb}$ ubiquitin ligase (Heriche et al., 2003). Although orthologs of E2F7/8 do not seem to exist in *Drosophila*, it has been reported that increased CDK activity represses the transcription of the *Drosophila E2f1* gene (Reis and Edgar, 2004). Unlike mammalian activator E2Fs, no known Cyclin-binding site has been found in *Drosophila* E2F1, and the dissociation of E2F1/DP from the DNA triggered by DP phosphorylation has not been reported in *Drosophila*.

Despite the simpler E2F/DP/RB pathway in *Drosophila*, the existence of the multiple layers of the E2F suppression mechanisms raises the question: What is the biological importance of each mechanism in regulating the suppression of E2F1 activity? A previous study has shown that an *E2f1* point mutant named $E2f1^{su89}$, which cannot bind to RBF1, is homozygously viable and fertile with no gross developmental defect (Weng et al., 2003), suggesting that the suppression of E2F1 by RBF1-binding is not required for normal development of *Drosophila*. Likewise, *E2f2* null mutant flies are viable with reduced fertility in females (Cayirlioglu et al., 2001; Frolov et al., 2001), suggesting that the suppression of E2F1 mediated by E2F2 is not essential during development. In addition, microarray data using RNAi-treated *Drosophila* S2 cells and *Rbf2* mutant embryos shows that E2F2 and its binding partner RBF2 normally regulates cell cycle-independent, differentiation-related

genes, which form a different gene cluster than E2F1-regulated, cell cycle-related genes (Dimova et al., 2003; Stevaux et al., 2005).

One possible mechanism that can compensate for the loss of RBF1- or E2F2-mediated E2F1 suppression is the ubiquitin-mediated destruction of E2F1. Previous studies showed that E2F1 is degraded during S phase in the eye disc (Asano et al., 1996) and wing disc (Heriche et al., 2003; Reis and Edgar, 2004). Previously, Heriche et al. linked the destruction of *Drosophila* E2F1 to the $Cul1^{Slmb}$ ubiquitin ligase (Heriche et al., 2003). They observed slight stabilization of E2F1 in wing discs overexpressing mouse Cul1, which may act as a dominant negative Cul1 in *Drosophila*, and in wing discs of a *slmb* mutant. However, the stabilization of E2F1 during S phase by mouse Cul1 overexpression and *slmb* mutation was incomplete (e.g. only 3% of S phase cells were E2F1 positive in mouse Cul1-overexpressing wing discs) (Heriche et al., 2003), suggesting the possibility that the S phase-dependent destruction of *Drosophila* E2F1 is also mediated by other mechanisms.

Drosophila embryos as an experimental model for studying E2F1 regulation

To investigate the regulatory mechanisms of *Drosophila* E2F1, we started with utilizing embryos as an experimental system. The *Drosophila* embryo provides an excellent model system to study the mechanism of E2F1 regulation in a developmental context. The cell cycle and the expression pattern of E2F1-target genes have been well-characterized in *Drosophila* embryogenesis (Lee and Orr-Weaver, 2003). In addition, we can take advantage of mutant fly resources and genetic tools, with which we can manipulate many aspects of the cell cycle in vivo.

The first 13 cycles of *Drosophila* embryogenesis are synchronous, rapid S-M cycles that lack obvious gap phases (Figure 1.2). These cycles are driven by maternal supplies of mRNAs and proteins (Foe and Alberts, 1983). After the S phase of cycle 14, the first gap phase $G2_{14}$ appears. It has been shown that $G2_{14}$ is caused by the degradation of maternal *string* (*stg*) mRNA and protein (Edgar and Datar, 1996). Stg is a *Drosophila* ortholog of Cdc25 phosphatase that activates CDK1 by removing the inhibitory phosphates. Stg is required for the G2-to-M transition, so that the degradation of maternal *stg* causes $G2_{14}$ introduction (Edgar and O'Farrell, 1989; Sigrist and Lehner, 1997). After gastrulation begins, the developmentally-regulated transcription of zygotic *stg* regulates the entry into mitosis during cycles 14, 15 and 16 (Edgar et al., 1994; Edgar and O'Farrell, 1990). After cycle 16, the first G1 phase appears in the epidermal cells of the embryo ($G1_{17}$), whereas the cells in the central nervous system (CNS) continue the S-G2-M cycle, and the cells in the midgut enter a G1-S cycle called the endocycle (also referred to as endoreduplication). The initiation of G1 phase is achieved by the developmentally-regulated transcription of *dacapo* (*dap*), which encodes the single *Drosophila* p27-like CDK inhibitor (de Nooij et al., 1996; Lane et al., 1996). Dap induces $G1_{17}$ because it specifically inhibits CycE/CDK2, which is required for entry into S phase. The $G1_{17}$-arrested epidermal cells remain in G1 for the rest of embryogenesis, and this maintenance of $G1_{17}$ requires the function of RBF1 (Du et al., 1996a). In *Rbf1* mutant embryos, the epidermal cells still initiate $G1_{17}$ due to the developmentally-regulated expression of Dap. However, some *Rbf1* mutant epidermal cells fail to maintain $G1_{17}$ and enter ectopic S phase due to the high activity of E2F1 (Du and Dyson, 1999).

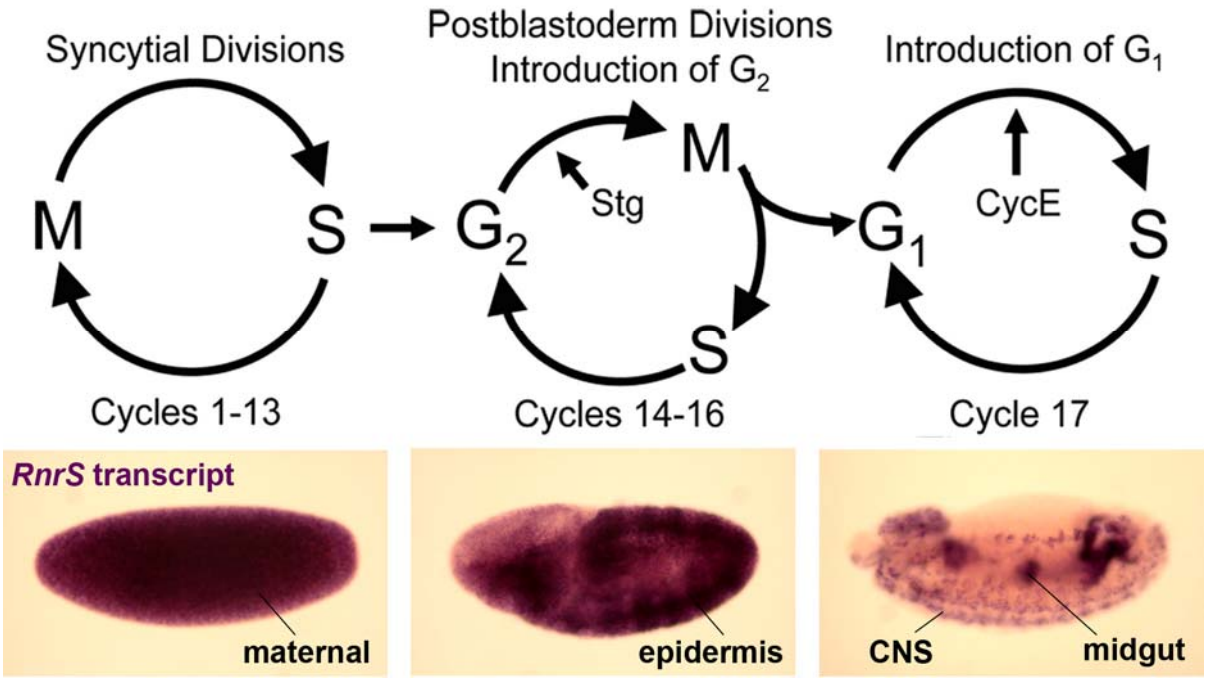

Figure 1.2: *Drosophila* embryonic cell cycles and E2F1-target gene expression

In the embryo, the first 13 cell cycles are rapid S-M cycles which occur in a common cytoplasm (syncytial divisions). These 13 cycles are regulated by maternal supplies of mRNA and protein. At cycle 14, degradation of maternal *stg* mRNA and Stg protein results in the introduction of G2. Cycles 14-16 (post-blastoderm divisions) are S-G2-M cell cycles controlled by the developmentally-regulated expression of Stg. At cycle 17, the first G1 appears in the epidermis ($G1_{17}$) due to the inhibition of CycE/CDK2 by the developmentally-regulated expression of Dap. The epidermal cells remain arrested in $G1_{17}$ for the rest of embryogenesis, whereas the cells in the CNS continue S-G2-M cycles and the cells in the midgut enter G-S cycles (endocycles).

Interestingly, it has been shown that these embryonic cell cycles correlate well with the expression pattern of E2F1-target genes such as *RnrS*, *Pcna*, *DNA polymerase*, and *CycE* (Figure 1.2). In the *Drosophila* embryo, these genes are specifically expressed in cells that are actively cycling. Conversely, the expression of these genes is suppressed in cells arrested in G1 (e.g. the epidermal cells in $G1_{17}$). However, the collection of mechanisms underlying this correlation remains incompletely understood.

Dissertation goals

In this thesis, I will describe the work that I performed with my collaborators addressing the regulation of E2F1 activity during *Drosophila* development. In Chapter II, we show that the initial downregulation of E2F1-target gene expression during embryogenesis does not require *Rbf1* or *dap*, both of which are inhibitory for the E2F1-CycE positive feedback loop. Instead, we propose that the developmentally-regulated onset of E2F1 destruction may be the mechanism regulating the initial downregulation of E2F1-target genes. In addition, we show that E2F1 destruction is strictly dependent on S phase. In Chapter III, we focus on the mechanism of S phase-dependent destruction of E2F1. We show that the destruction of E2F1 is mediated by PCNA, Cul4, and Cdt2 in a similar manner to the destruction of Cdt1, a protein of the pre-replication complex. Interestingly, the stabilization of E2F1 during S phase caused apoptosis in mitotically cycling cells in the wing disc, whereas it caused cell cycle arrest in endocycling cells in the larval salivary gland. In Chapter IV, we describe the finding of a genomic locus that is required for the early zygotic expression of *RnrS*. This implies that at this stage of development *RnrS* expression is controlled independently of E2F1, which may also explain the initial RBF1-independent downregulation of E2F1-target genes during embryogenesis. In Chapter V, these results will be discussed in a broad context.

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CHAPTER II

RBF1-INDEPENDENT TERMINATION OF E2F1-TARGET GENE EXPRESSION DURING EARLY *DROSOPHILA* EMBRYOGENESIS

Preface

This work was previously published and represents a co-first author effort between myself and my collaborator, Lisa Swanhart, a former graduate student in the lab.

I measured *RnrS* expression in embryos overexpressing UAS-*Rbf1-280* (Figure 2.1A-C), *dap* mutant embryos (Figure 2.2A-D), *dap;stg* double mutants (Figure 2.9C-E) and *dup* mutant embryos (Figure 2.10). I analyzed E2f1 protein dynamics throughout embryogenesis (Figure 2.3 and 2.4B). I also monitored E2f1 protein levels in a variety of mutant situations (Figure 2.5 and 2.7A-E). Lisa analyzed the physical interaction between Rbf1 and E2f1 (figure 2.1H) and the effects of UAS-*Rbf1-280* over-expression during early embryogenesis (Figure 2.1D-G). She measured the timing of *RnrS* decline in wild type embryos (Figure 2.6), *RnrS* expression and E2f1 protein levels in *fzr* (Figure 2.2E and 2.7F) and *Rbf1* mutants (Figure 2.8), and monitored Dap protein levels in *stg* mutants (Figure 2.9A and B) She also analyzed E2f1 protein dynamics during mitosis (Figure 2.4A). Both Lisa and I contributed to the writing of the manuscript while Robert Duronio conceived the project and finalized the manuscript.

Shibutani, S., Swanhart, L.M., and Duronio, R.J. (2007). Rbf1-independent termination of E2f1-target gene expression during early *Drosophila* embryogenesis. *Development* **134**, 467-78.

Abstract

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 17th 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 down regulation 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 re-accumulates in $G1_{17}$ arrested epidermal cells, and in these cells the induction of $p27^{Dap}$ activates Rbf1 to repress E2f1 target genes to maintain a stable G1 arrest.

Introduction

Proper control of cell cycle exit is an essential aspect of the development of all multi-cellular 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 (Cdk) complexes that promote entry into S phase. These kinases include Cyclin D/Cdk4 (CycD/Cdk4) and Cyclin E/Cdk2 (CycE/Cdk2), 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, hypo-phosphorylated pocket proteins form a complex with E2Fs that recruit co-repressors and results in the down-regulation of E2F targets. In response to growth signals, G1 Cyclin/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 due 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 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.

In a variety of animal species, the cell cycles of early embryonic development display several common features. 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.

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) (Figure 2.1I). 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 to allow entry into mitosis (Edgar and O'Farrell, 1989; Sigrist and Lehner, 1997). 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 $G1_{17}$ while 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, 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 $G1_{17}$ due 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 down-regulation of replication genes are unknown. Here we show that, surprisingly, the initial down regulation 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 the altered morphology caused by the *stg* $G2_{14}$ arrest phenotype.

RNA *in situ* hybridization and BrdU labeling

Embryos were dechorionated, fixed in a 1:1 mixture of 4% formaldehyde in PBS/heptane for 25 minutes, and devitellinized with methanol. For BrdU labeling, dechorionated embryos were permeabilized with octane, pulse-labeled with 1mg/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 minutes to 1 hour 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 immuno-detection 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 immuno-detection 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 re-hydrated with PBS/0.1% Tween20 (PBS-T) and incubated with primary antibodies overnight at 4˚C. Primary antibodies 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 (Lane et al., 1996) (1:600). Secondary antibodies 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% pre-cast 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 hyper-phosphorylated and thus inactivated until cycle 16 by constitutive G1 Cyclin/Cdk activity, resulting in ubiquitous expression of E2f1 target genes (Figure 2.1I). 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 (e.g. 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 (Figure 2.1A, C). Utilizing fluorescent detection of *RnrS* transcripts and BrdU labeling, we confirmed that the precocious termination occurs during cycle 15 (Figure 2.1D). A similar but more widespread result was observed using the ubiquitous *arm*-Gal4-VP16 driver (Figure 2.1E, G). Little change in *RnrS* expression was observed after expressing wild type Rbf1 (Figure 2.1B, 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 hyper-phosphorylated and thus inactivated by constitutive Cyclin/Cdk activity in the early embryo to permit expression of replication factor genes like *RnrS*.

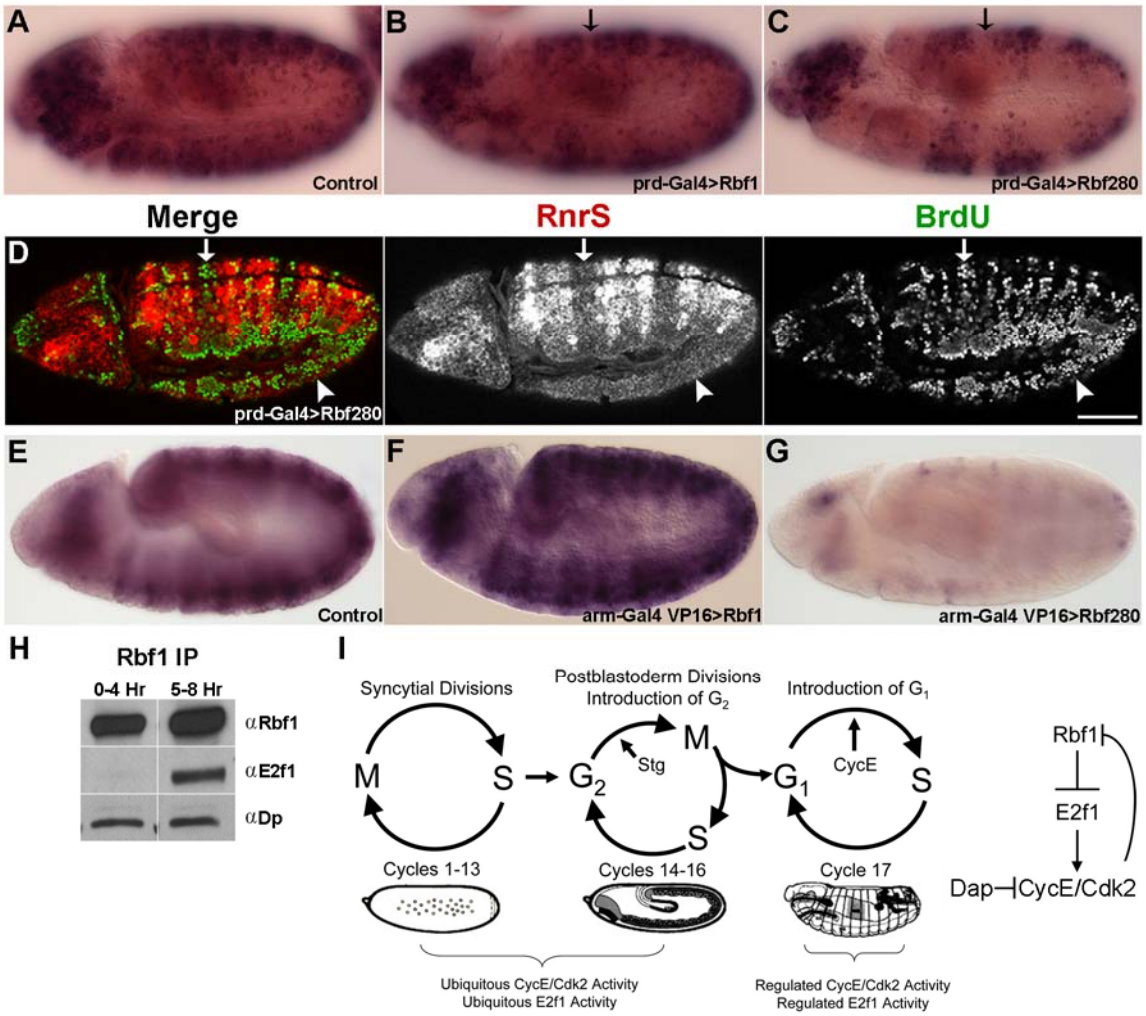


Figure 2.1: Rbf1 activity is controlled by phosphorylation in the early embryo. (A-G) in situ hybridization of stage 10 embryos with an *RnrS* probe. (A) Sibling control embryo from a collection expressing UAS-*Rbf1* with *prd*-Gal4. (B) UAS *Rbf1*/*prd*-Gal4. Arrow marks *paired*-expressing segment. (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 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 immuno-precipitated from 0-4 and 5-8 hour old w^{1118} embryo extracts, and the IPs were probed for the presence of E2f1 and Dp by western blotting. (I) Schematic diagrams of the embryonic cell cycle program and the regulation of E2f1 activity. Scale bar is 200 µm.

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 0-4 hour (prior to cycle 16) embryo extracts (Figure 2.1H). Dp co-precipitates with Rbf1 in both cases (Figure 2.1H). The Rbf1/Dp interaction in 0-4 hour old embryos likely represents 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 hyper-phosphorylated 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 hyper-phosphorylated, 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 hyper-phosphorylated in the early embryo, we hypothesized that prior to the introduction of $G1_{17}$ Rbf1 is converted to a hypo-phosphorylated form that binds E2f1 and terminates E2f1 target gene expression. A possible mechanism for the conversion of Rbf1 to a hypo-phosphorylated 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). In 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) (Figure 2.1I). If the inhibition of CycE/Cdk2 activity by Dap is necessary for the accumulation of hypo-phosphorylated Rbf1 and the consequent suppression of E2f1 targets, then in *dap* mutants *RnrS* expression would not be terminated properly. However, *RnrS* expression is down-regulated in the epidermis of *dap* mutants prior to the completion of S_{16} just as it is in wild type embryos (Figure 2.2A, B). Moreover, the termination of *RnrS* expression occurs even though the epidermal cells of *dap* mutant embryos enter an ectopic S_{17} (Figure 2.2C, D).

A similar result is seen in the epidermis of *fizzy-related (fzr)* mutant embryos. *fzr* encodes *Drosophila* Hct1/Cdh1, which during G1 phase targets mitotic cyclins for ubiquitination by the 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} that is likely driven by CycE/Cdk2 (Sigrist and Lehner, 1997). In spite of this, *RnrS* expression is properly down-regulated in *fzr* mutants (Figure 2.2E). Thus, while 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 hypo-phosphorylated Rbf1, or that a different mechanism is involved in the initial termination of E2f1 target gene expression.

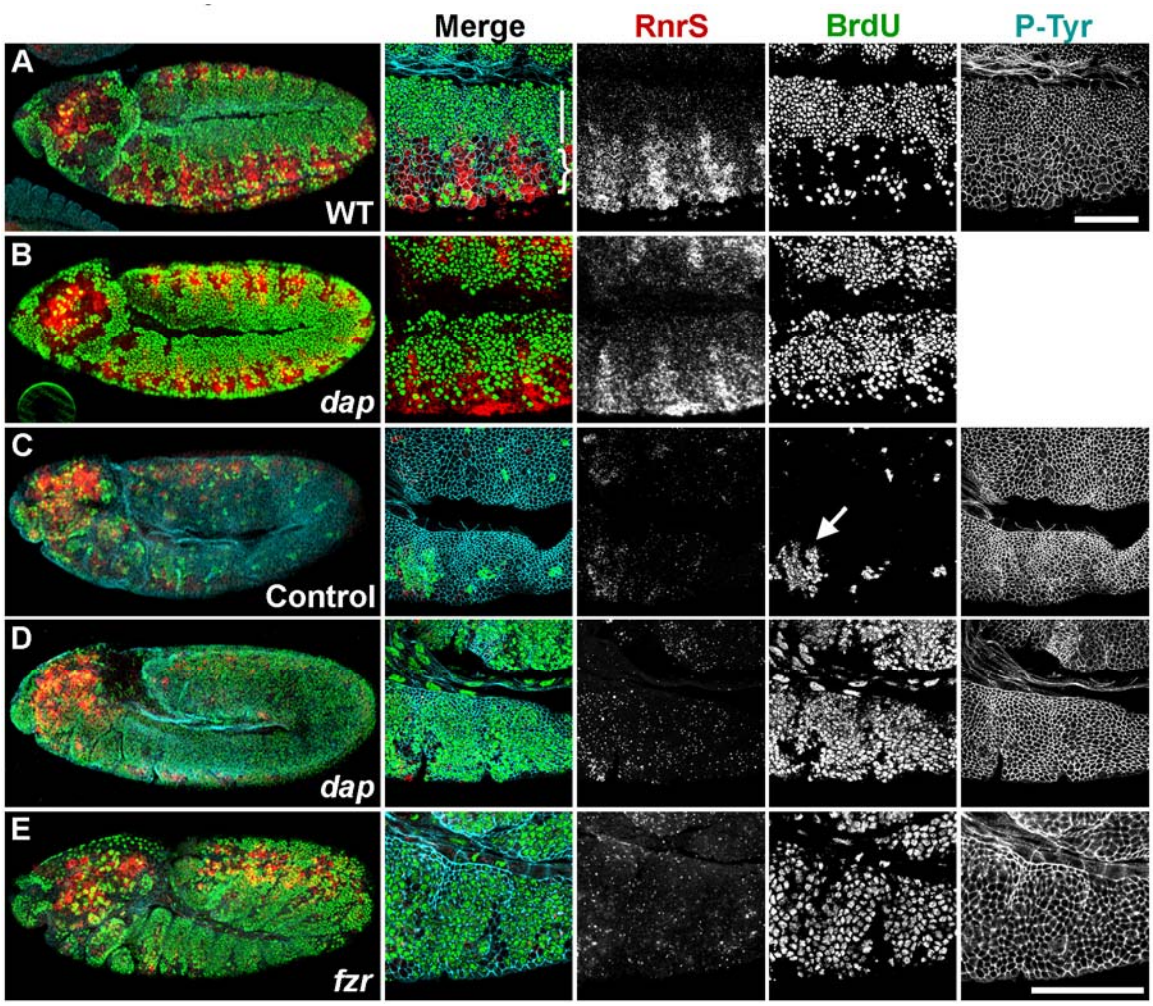


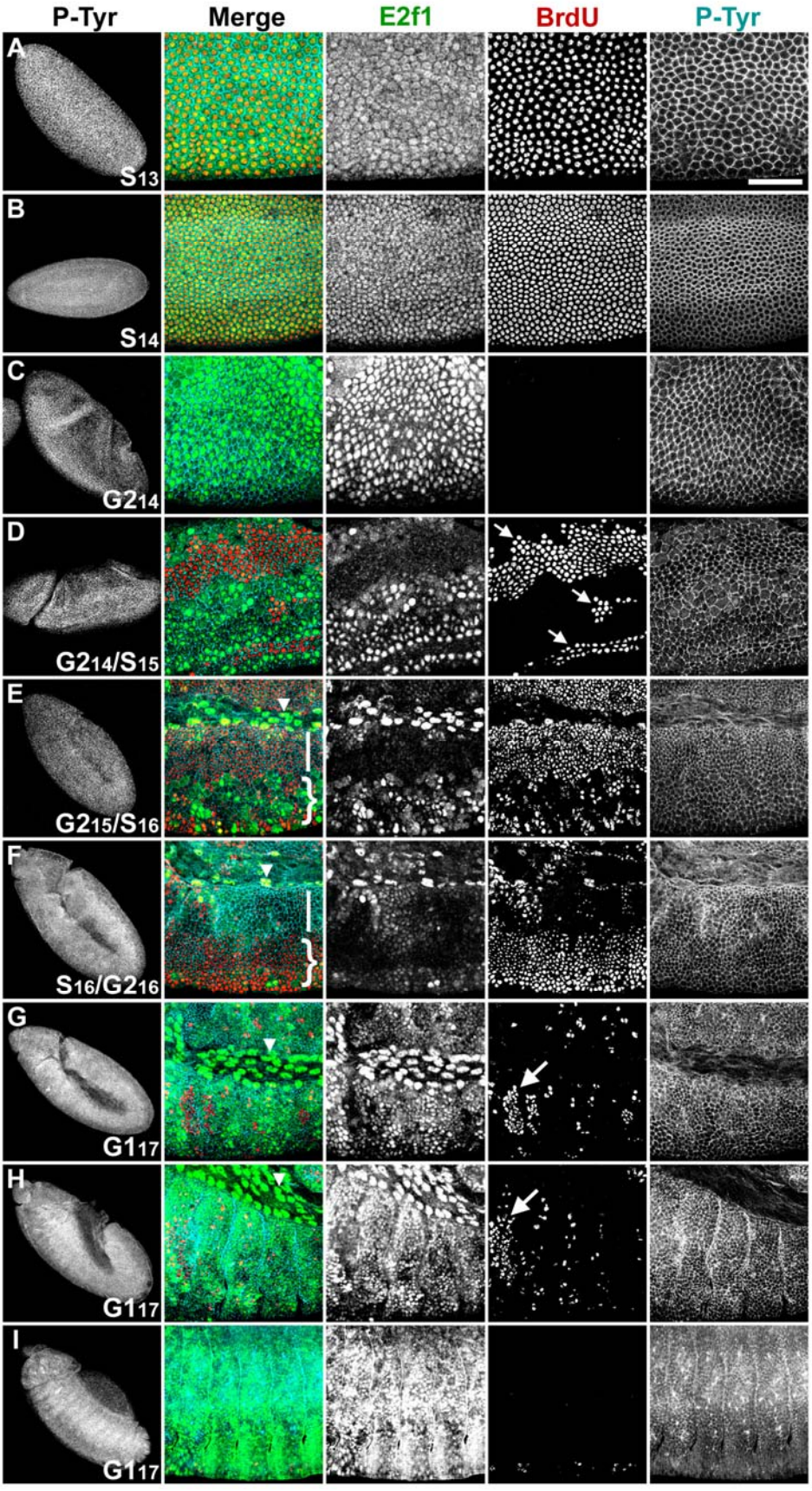
Figure 2.2: E2f1 target gene expression is terminated in mutants containing ectopic CycE/Cdk2. Embryos were pulse-labeled with BrdU for 5 minutes (A-D) or 15 minutes (E) and were stained for BrdU incorporation (green) and phospho-tyrosine to highlight cell boundaries (cyan). *RnrS* expression was detected 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 P-Tyr is absent because anti-β-Gal 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 panel D. The arrow 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 are 50µm.

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 re-accumulates 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 (Figure 2.3).

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 (Figure 2.3A, B). E2f1 protein accumulates to high levels in the nucleus during $G2_{14}$ (Figure 2.3C), and is then rapidly diminished when cells enter S_{15} (Figure 2.3D). This effect is post-transcriptional since 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 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 begins to re-accumulate during G2 of cycle 15, but never attains the levels seen in G2 of cycle 14 (Figure 2.3E), perhaps because of the short duration of $G2_{15}$. As in cycle 15, E2f1 protein abundance is low in S_{16}, but begins to re-accumulate in $G2_{16}$ (Figure 2.3E,F). By the time the epidermal cells enter $G1_{17}$, E2f1 protein has accumulated to a high level in the nucleus (Figure 2.3G, H), and remains at this level at least until mid-embryogenesis (Figure 2.3I). A group of cells in the first and second thoracic segments do


P-Tyr
Merge
E2f1
BrdU
P-Tyr
A
S13
B
S14
C
G214
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G214/S15
E
G215/S16
F
S16/G216
G
G117
H
G117
I
G117

Figure 2.3: 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). (A) Embryos undergoing S_{13}. (B) S_{14}. (C) $G2_{14}$. (D) S_{15} is indicated with arrows; the remaining cells 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) Most cells of the epidermis have entered $G1_{17}$, while, as described in Figure 2.2C, some cells continue into cycle 17 (arrows). (I) $G1_{17}$. Scale bar is 50 μm.

not enter $G1_{17}$, but instead complete one more division cycle before arresting (Sauer et al., 1995). E2f1 protein is also down-regulated during S phase in these cells (Figure 2.3G, 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 (Figure 2.4). As reported for wing imaginal cells (Reis and Edgar, 2004), E2f1 protein is abundant during mitosis. E2f1 is nuclear in early prophase prior to nuclear envelope breakdown (Figure 2.4A, arrowhead). In metaphase and anaphase, E2f1 protein appears more diffuse, likely due to nuclear envelope breakdown (Figure 2.4A, large and small arrows, respectively). E2f1 is present in newly formed daughter cells, suggesting that it is not destroyed by the APC/C during mitosis (Figure 2.4A, double arrow). A high level of E2f1 protein is present in cells in early S phase, which is characterized by uniform BrdU incorporation throughout the nucleus (Figure 2.4B, large arrow). In mid-S phase, where BrdU incorporation is less uniform, there is a significant reduction in E2f1 protein (Figure 2.4B, small arrow). By late S phase, where the more punctuate BrdU incorporation pattern marks late replicating heterochromatin, there is very little E2f1 protein present (Figure 2.4B, arrowhead). These data are consistent with the destruction of E2f1 protein after the initiation of S phase, and differs slightly from previous results in imaginal discs where no overlap between E2f1 staining and BrdU was detected (Heriche et al., 2003; Reis and Edgar, 2004). This difference may be due to the short embryonic cell cycle lacking a G1 phase as compared to the canonical G1-S-G2-M disc cycles.

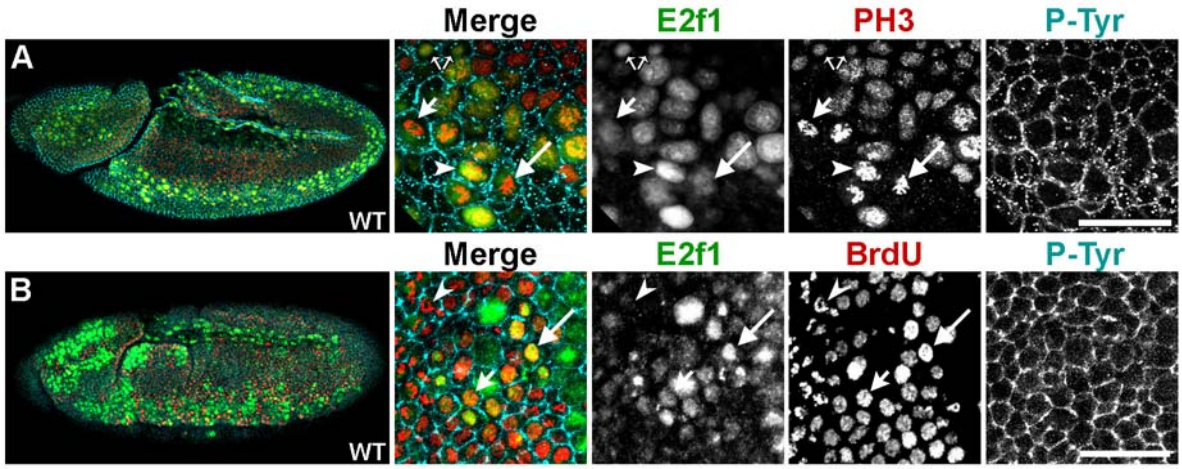

Figure 2.4: E2f1 protein persists through mitosis into early S phase. (A) Stage 8 w^{1118} embryo labeled with E2f1 (green), phospho-histone H3 (red), and phospho-tyrosine (cyan). Prophase (arrowhead), metaphase (large arrow), anaphase (small arrow), and daughter cells in early interphase (double arrow) 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 are 20µm.

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 contain a detectable amount of E2f1 protein in $G2_{14}$, but this amount is less than sibling controls (Figure 2.5A, B), indicating that zygotic E2f1 synthesis is responsible for a portion of the E2f1 protein found in $G2_{14}$. Zygotic *RnrS* mRNAs rapidly accumulate in the epidermis during cycle 14, and then begin to decline during cycle 15 such that by the beginning of S_{16} these mRNAs are very low (Figure 2.1D, arrowhead, Figure 2.2A, Figure 2.6). This dynamic pattern of expression is not altered in *E2f1* mutant embryos (Figure 2.5E, F) (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.

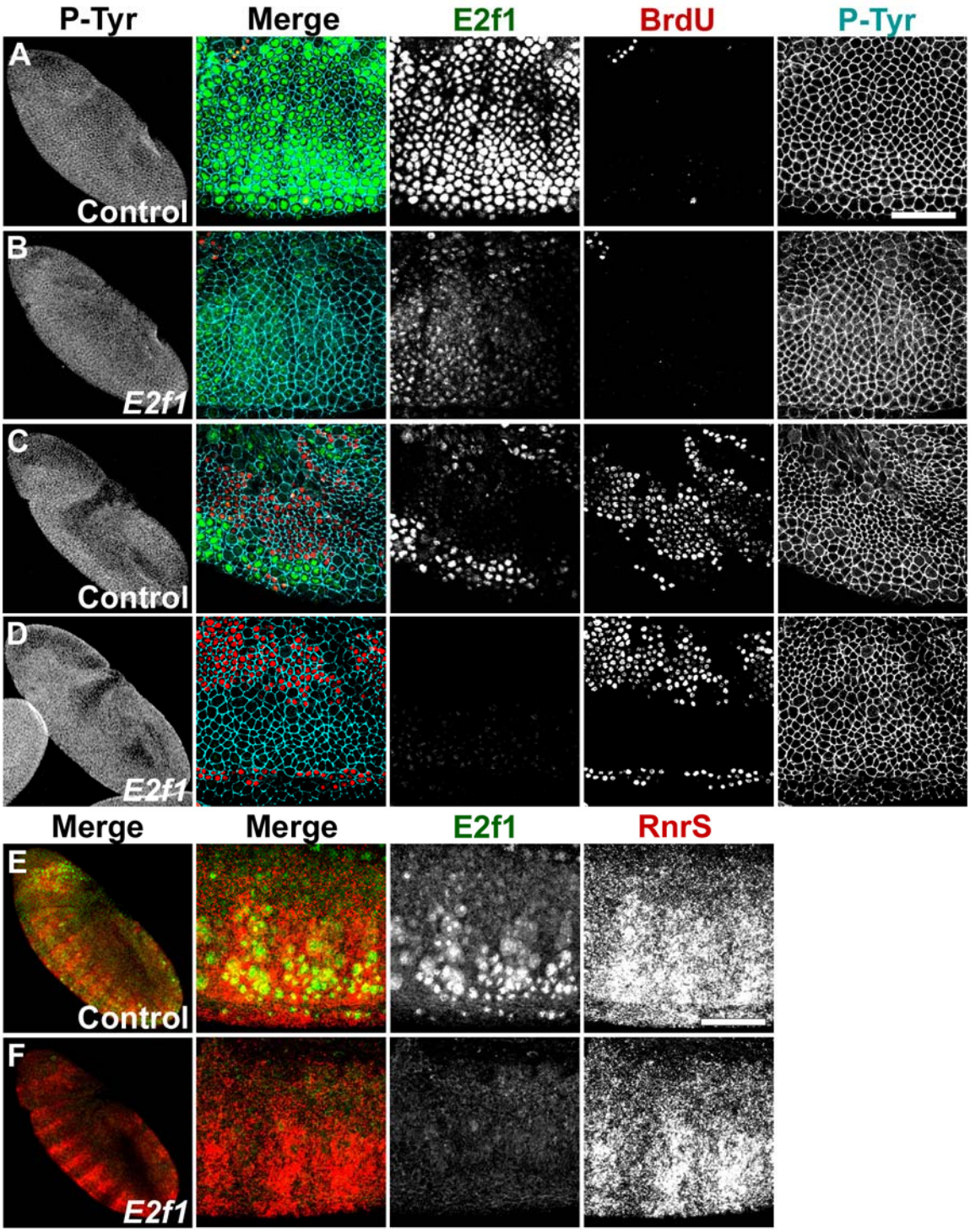

Figure 2.5: Maternal E2f1 protein is present at the onset of zygotic *RnrS* expression. (A-D) Embryos were pulse labeled for 5 minutes with BrdU and stained for E2f1 (green), phospho-tyrosine (cyan), and BrdU incorporation (red). (A) Sibling control at $G2_{14}$. (B) $E2f1^{7172}/E2f1^{7172}$ embryo at $G2_{14}$. (C) Sibling control at $G2_{14}/S_{15}$. (D) $E2f1^{7172}/E2f1^{7172}$ embryo at $G2_{14}/S_{15}$. *E2f1* mutants were identified by the reduction in E2f1 protein level. (E, F) Embryos were stained for E2f1 (green) and *RnrS* (red). (E) Sibling control. (F) $E2f1^{7172}/E2f1^{7172}$ embryo. Scale bars are 50 µm.

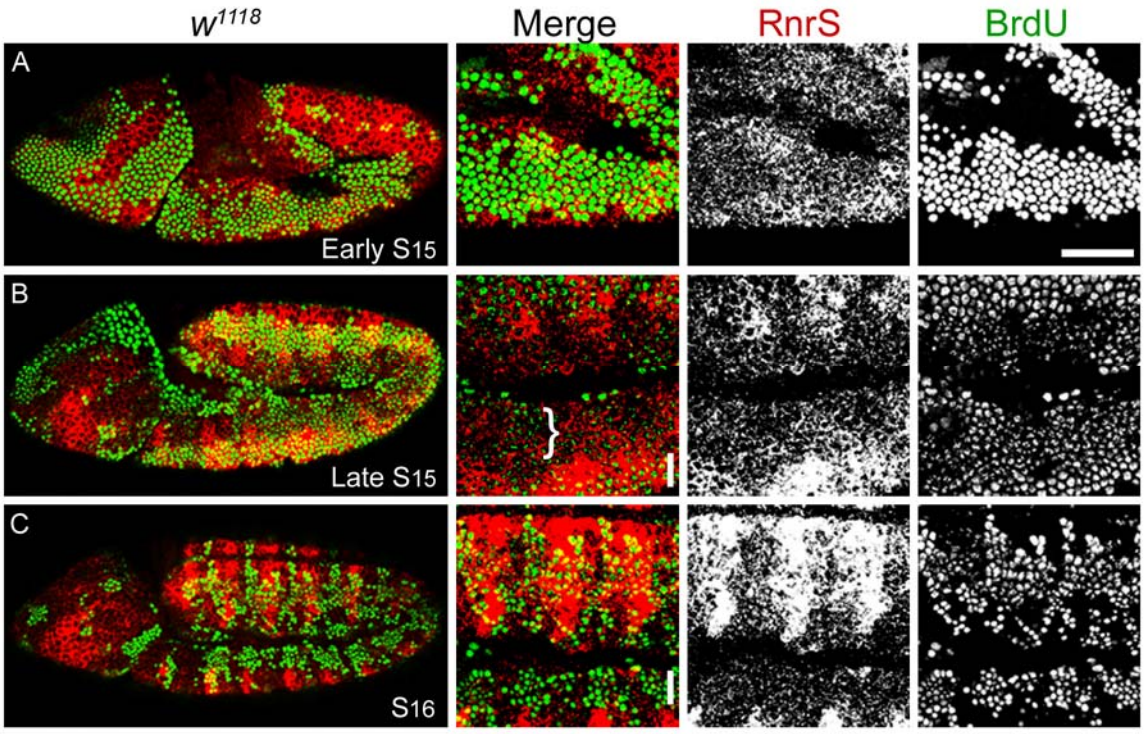

Figure 2.6: *RnrS* expression declines during cycles 15 and 16. (A-C) w^{1118} embryos were pulse-labeled with BrdU for 15 minutes and stained for BrdU incorporation (green). *RnrS* expression was detected by FISH (red). (A) Stage 9 embryo at early S_{15}. (B) Stage 9 embryo. The bracket denotes a region of late S_{15} and the line indicates cells in early S_{15}. (C) Stage 10 embryo. The line denotes a region of early S_{16}. Scale bar is 50 µm.

E2f1 protein destruction 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 accumulates to a high level in the epidermis of *stg* mutant embryos (Figure 2.7A). Aminoserosa cells, which in wild type embryos permanently exit the cell cycle in $G2_{14}$, also accumulate high levels of E2f1 (Figure 2.3E-H, arrowheads). In *Cyclin E* mutants, E2f1 protein is not destroyed in the thoracic cells that normally enter a 17^{th} division cycle (Figure 2.3G, 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 pre-replication complex (pre-RC) that is required for eukaryotic DNA synthesis. *dup* mutant embryos develop normally through cycle 15, and then display 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} (Figure 2.7B, C) (Garner et al., 2001). dup^{a1} mutants accumulate high levels of E2f1 in the epidermis, suggesting that DNA synthesis is necessary for E2f1 destruction (Figure 2.7B). Interestingly, dup^{a3} mutants also accumulate high levels of E2f1 even though these epidermal cells are capable of incorporating some BrdU (Figure 2.7C). 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 ectopic CycE/Cdk2 that is

predicted to prevent Rbf1 activation (Figure 2.1I). Indeed, E2f1 protein abundance is low during ectopic S_{17} in the epidermis of both *dap* and *fzr* mutants (Figure 2.7D, F, respectively). Conversely, Dap over-expression results in the accumulation of E2f1 protein throughout the epidermis, most likely because of the inhibition of S_{16} (Figure 2.7E). 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 hypo-phosphorylated Rbf1.

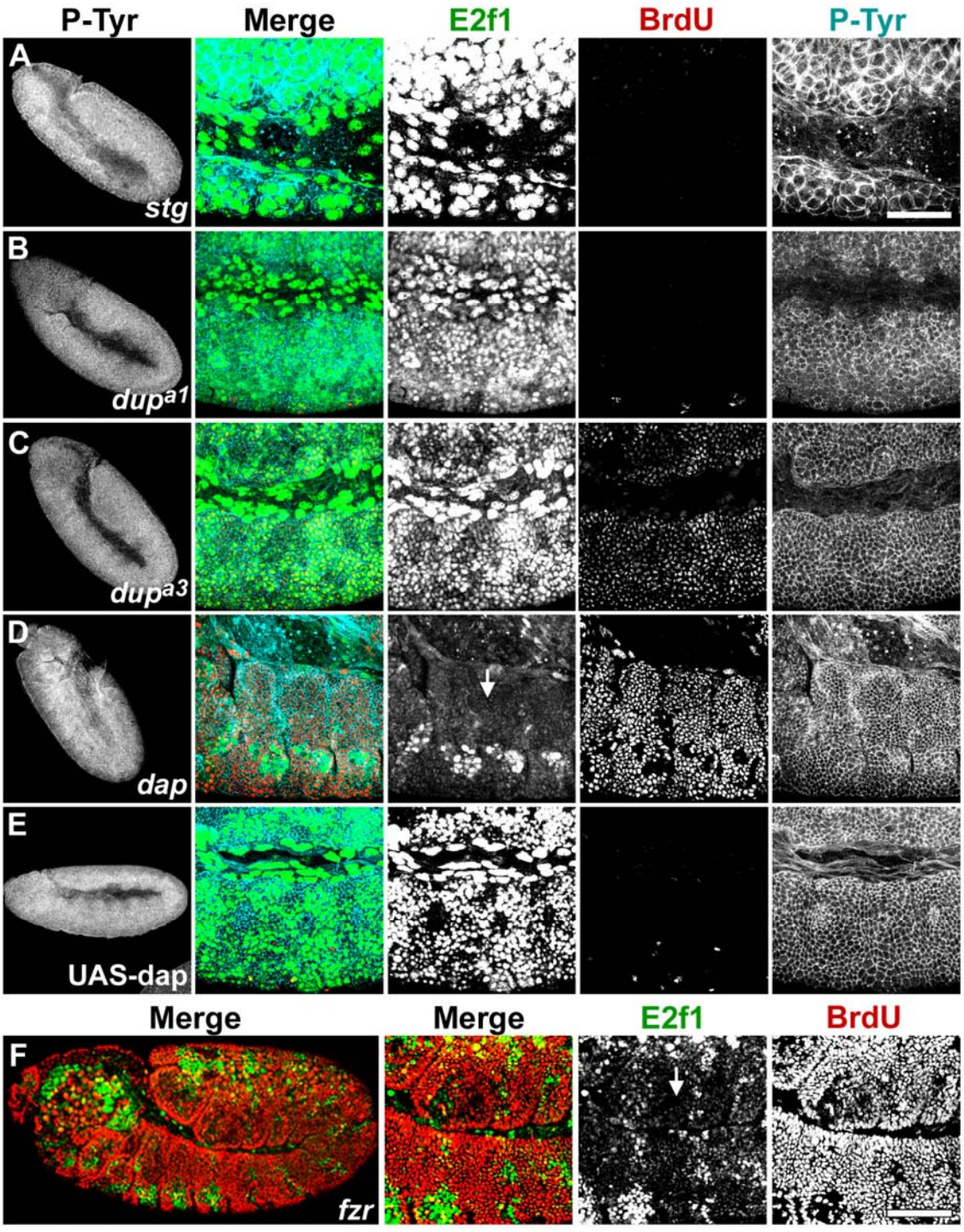

Figure 2.7: E2f1 destruction is replication-dependent. (A-E) Stage 11 embryos were pulse-labeled with BrdU for 5 minutes, and stained for E2f1 (green), BrdU incorporation (red), and phospho-tyrosine (cyan). (A) stg^{7B}/stg^{7B}. (B) dup^{a1}/dup^{a1}. (C) dup^{a3}/dup^{a3}. (D) dap^{4454}/dap^{4454}; 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* were pulse-labeled with

BrdU for 15 minutes, and stained with E2f1 (green) and BrdU (red). Arrow indicates epithelial cells expressing low levels of E2f1 similar to *dap* mutants. Scale bars are 50 μm.

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 hypo-phosphorylated Rbf1 (Figure 2.1I), 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 are absent during S_{16} in *Rbf1* mutant epidermal cells (Figure 2.8A). Later, as E2f1 protein re-accumulates throughout the epidermis in $G2_{16}$ and $G1_{17}$, *RnrS* transcripts inappropriately reappear in *Rbf1* mutants (Figure 2.8B). This ectopic expression of E2f1 target genes ultimately results in cell cycle re-entry, as previously described (Figure 2.8C, 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. This may include cell-by-cell differences in the amount of E2f1, since we observe that cells with the most E2f1 are usually the same ones that enter 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.

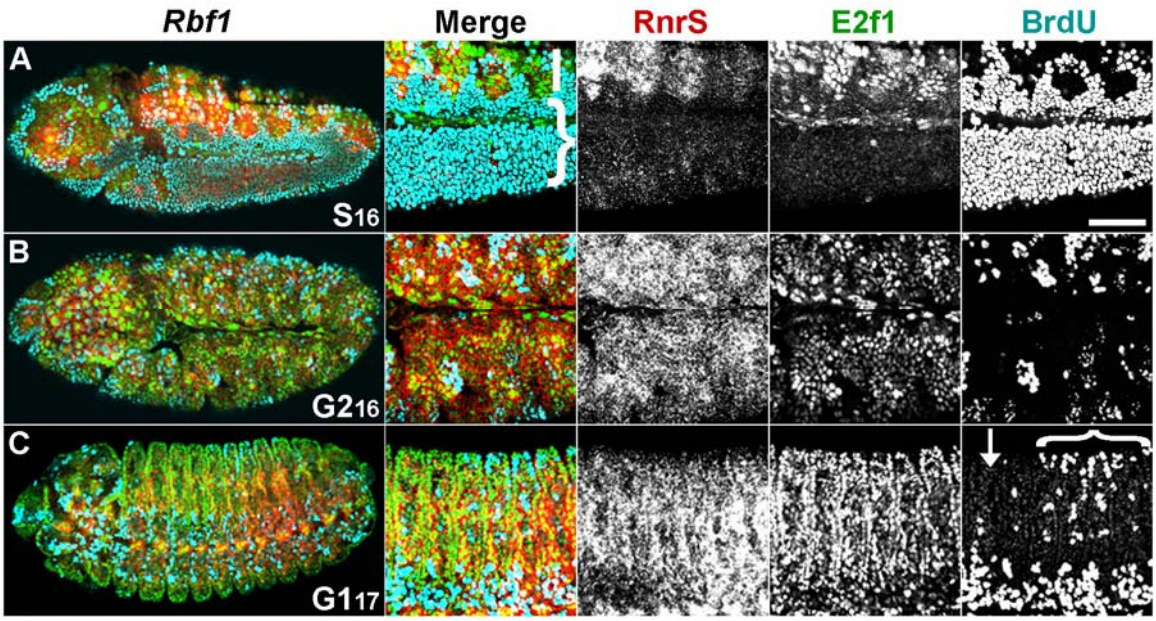

Figure 2.8: The initial termination of E2f1 target gene expression does not require Rbf1. *Rbf1*^{14} maternal and zygotic null embryos were pulse-labeled with BrdU for 15 minutes and stained for E2f1 (green) and BrdU incorporation (cyan). *RnrS* expression was detected by FISH (red). (A) Stage 10 embryo; 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 is 50μm.

Dap expression promotes conversion of Rbf1 to a repressor

While our data suggest that E2f1 target genes are controlled independently of Rbf1 prior to cycle 17, it does 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 to 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 re-accumulates 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 (Figure 2.7), 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 hypo-phosphorylated Rbf1 and the down regulation of E2f1 targets (Figure 2.1I) (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 are abundant and Dap protein is not detected (Figure 2.9A). Later, when Dap protein accumulates, *RnrS* expression decreases (Figure 2.9B). To test whether loss of *RnrS* expression in *stg* mutant embryos requires Dap, we analyzed *stg dap* double mutant embryos shortly after the time when Dap is first induced. *RnrS* is not suppressed in certain cells of *stg dap* double mutant embryos that correspond to cells with high levels of Dap protein in *stg* single mutant sibling embryos (Figure 2.9C, 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 *stg dap* mutant embryos age, *RnrS* expression is eventually lost in many epidermal cells (Figure 2.9E). This also occurs in the aminoserosa, which contains cells that have exited the cell cycle in $G2_{14}$ (Figure 2.9D, 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.

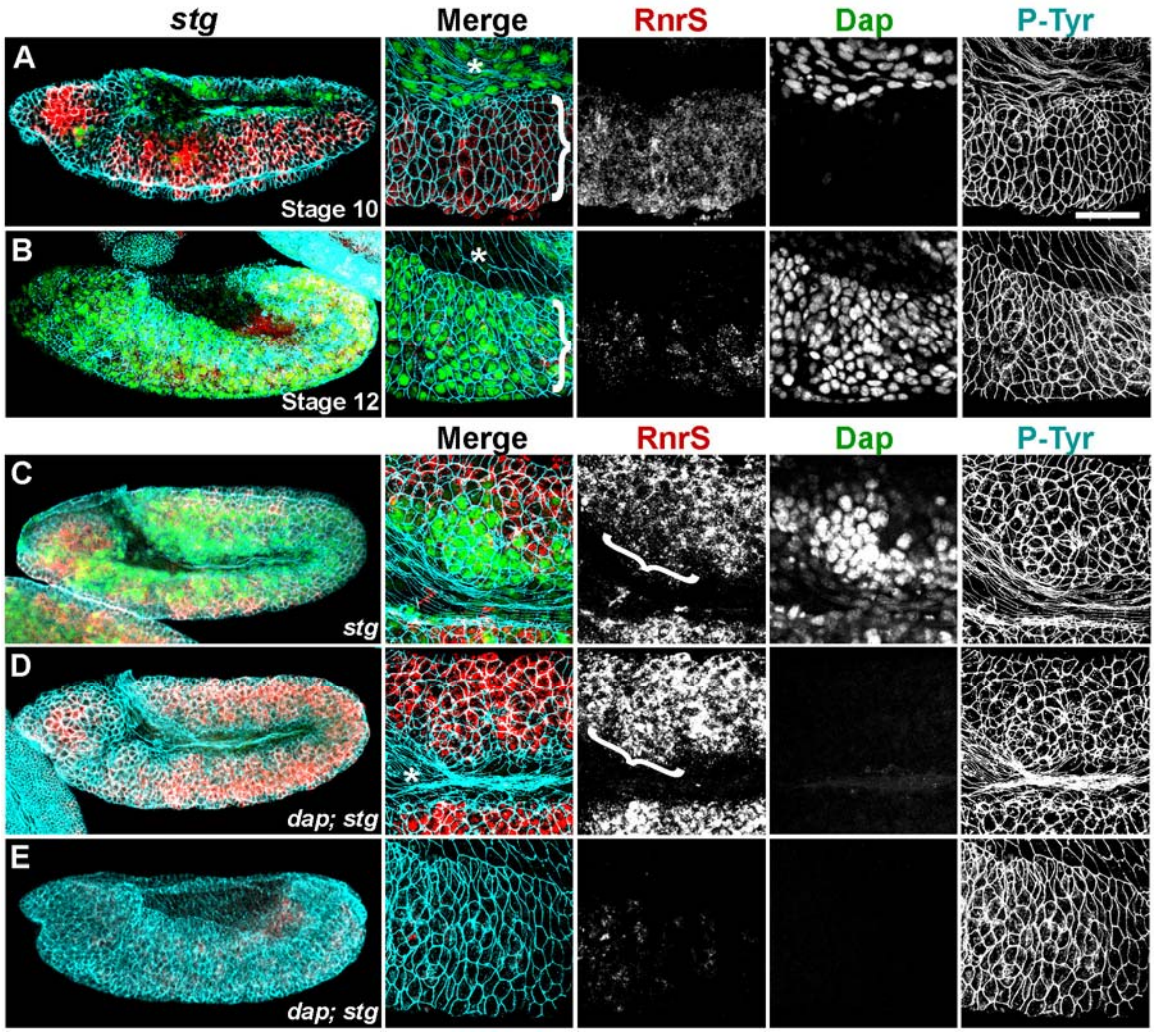

Figure 2.9: Dap expression activates Rbf1. Embryos were stained for Dap (green) and phospho-tyrosine (cyan). *RnrS* expression was detected by FISH (red). (A) Stage 10 stg^{7B}/stg^{7B} embryo. (B) Stage 12 stg^{7B}/stg^{7B} embryo. Brackets mark the epidermal cells and asterisks denote $G2_{14}$-arrested aminoserosa cells. (C-E) Embryos from $dap^{4454}/+$; $stg^{7B}/+$ parents. (C) Stage 11 embryo with 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 is 50μm.

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 fail to down-regulate *RnrS* at the time of S_{16}, and dup^{a3} mutants still express *RnrS* during the prolonged and partial S_{16} (Figure 2.10A-C). This may be explained by the high level of E2f1 protein that accumulates in *dup* mutants (Figure 2.7). However, Dap protein accumulates during cycle 16 in *dup* mutants (data not shown), and this should result in the down-regulation 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 can still be repressed by hypo-phosphorylated Rbf1 in *dup* mutants (Figure 2.10D-G). At later stages, *RnrS* transcripts eventually begin to decline in *dup* mutants (data not shown). We suggest that in *dup* mutants Rbf1 is still converted to an active, hypo-phosphorylated 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.

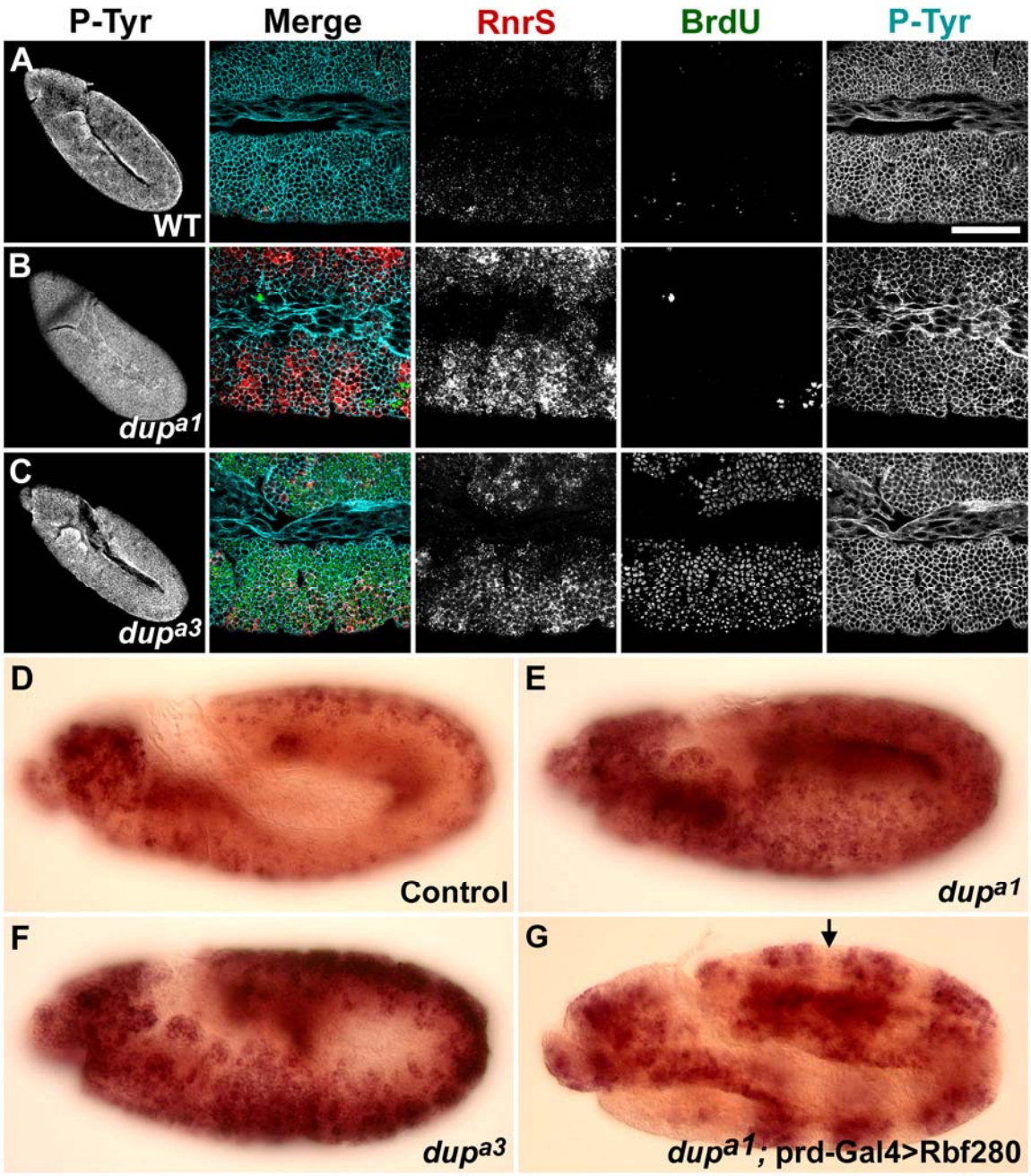

Figure 2.10: E2f1 target gene expression persists inappropriately in *dup* mutants. (A-C) Stage 11 embryos were pulse-labeled with BrdU for 5 minutes, and stained for BrdU incorporation (green), and phospho-tyrosine (cyan). *RnrS* expression was detected by FISH (red). (A) w^{1118}. (B) dup^{a1}/dup^{a1}. (C) dup^{a3}/dup^{a3}. (D-G) Histochemical detection of *RnrS* expression by in situ hybridization of dup^{a3} embryos (F) or embryos from dup^{a1}/+; UAS *Rbf-280*/+ females crossed to dup^{a1}/+; *prd*-Gal4/+ males (D, E and G). (D) Sibling control embryo. (E) Embryo with dup^{a1}/dup^{a1} phenotype. (F) dup^{a3}/dup^{a3} embryo. (G) dup^{a1}/dup^{a1}; UAS *Rbf-*

280*/prd*-Gal4 embryo. UAS *Rbf-280* expression suppresses ectopic *RnrS* caused by *dup* mutation (arrow). Scale bar in A-C is 50 μm

Discussion

Our finding that $p27^{Dap}$ expression was not necessary for the down regulation of E2f1 targets was unexpected based on the known regulatory circuitry of the pRb/E2F pathway (Figure 2.1I). This result led us to hypothesize that mechanisms in addition to Rbf1 binding were 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 like *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 hypo-phosphorylated Rbf1. Dap expression accompanies the down regulation of *Cyclin E* transcription, and each of these mechanisms of CycE/Cdk2 inhibition 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 like *RnrS*. In addition, mutation of the E2f1-binding sites in the regulatory region of the *PCNA* gene is sufficient to abolish zygotic *PCNA* expression (Thacker et al., 2003). However, our data do not demonstrate an E2f1 requirement 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*, genetic and cell biological evidence suggest that SCF^{SLMB} mediates E2f1 destruction at the G1/S transition in wing imaginal disc cells (Heriche et al., 2003). While 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 NH_2-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 hyper-accumulates 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 be due in part 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 (ES) 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 down-regulation 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.

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CHAPTER III

DROSOPHILA E2F1 IS DEGRADED DURING S PHASE IN A PCNA-, CUL4-, AND CDT2-DEPENDENT MANNER

Preface

This work represents a manuscript that is currently in preparation for publication. Along with my advisor Bob Duronio, I participated in the experimental design for this project. I performed the majority of the experiments described and wrote the manuscript. Jackson Turbyfill, an undergraduate student in the lab, analyzed the apoptosis and mitosis phenotype caused by the overexpression of PIP-3A mutant E2F1 in wing discs (Figure 3.8A). This work was carried out in collaboration with the Bruce Edgar lab in the Fred Hutchinson Cancer Research Center, who analyzed the larval salivary gland phenotype (Figure 3.9).

Abstract

Our previous finding suggests that the destruction of *Drosophila* E2F1 is dependent on DNA replication (Chapter III), leading us to investigate the mechanism that links DNA replication and E2F1 destruction. Here, using *Drosophila* S2 cells, we found that E2F1 is degraded during S phase in a PCNA-, Cul4-, and Cdt2-dependent manner. This suggests that a common destruction mechanism is used for the destruction of E2F1 and Cdt1, a member of the pre-replication complex. In addition, we found that the destruction of E2F1 is dependent on its partner DP, but not on CDK-mediated phosphorylation or RBF1-binding. The expression of a stabilized mutant of E2F1 in the wing imaginal disc induced massive apoptosis, resulting in abnormal morphology of the adult wing. This suggests that the S phase-dependent destruction of E2F1 is required for the normal development of *Drosophila*, and implicates the existence of a sensor that detects inappropriate E2F1 expression during S phase and subsequently activates apoptotic pathways.

Introduction

In the previous chapter, we showed that the initial downregulation of E2F1-target gene expression is independent of RBF1 and Dap. Instead, we showed the developmentally-regulated onset of E2F1 protein destruction as a possible mechanism regulating this initial downregulation of E2F1-target genes. Interestingly, the destruction of E2F1 was observed specifically during S phase, leading us to investigate the mechanism that links S phase and E2F1 destruction.

Mammalian E2F1 is degraded in S/G2 by the ubiquitin-mediated proteolysis. The $Cul1^{SKP2}$ ubiquitin ligase complex binds to the N-terminus of E2F1, and the N-terminal

truncation of E2F1 stabilized the protein (Marti et al., 1999). Other labs showed that the C-terminal truncation of mammalian E2F1, 2, and 4 stabilized these proteins, and that co-expression of pRB protected E2F1 from ubiquitination and subsequent destruction (Campanero and Flemington, 1997; Hateboer et al., 1996; Hofmann et al., 1996). In *Drosophila*, the $Cul1^{Slmb}$ ubiquitin ligase was previously linked to E2F1 destruction. Heriche et al. showed that E2F1 is stabilized by the overexpression of a putative dominant negative Cul1 (mouse Cul1) and by a mutation of *slmb*. However, the stabilization of E2F1 during S phase they observed was incomplete (e.g. only 3% of S phase cells were E2F1 positive in mouse Cul1-overexpressing wing discs) (Heriche et al., 2003), suggesting the existence of other ubiquitin ligases which target *Drosophila* E2F1 for destruction. In addition, *Drosophila* E2F1 is degraded in early S phase (Asano et al., 1996; Heriche et al., 2003; Reis and Edgar, 2004) (Figure 2.4), showing a difference in the timing of destruction between *Drosophila* and mammalian E2F1 (early S in *Drosophila* vs. S/G2 in mammals). Here we show that *Drosophila* E2F1 is degraded during S phase in a PCNA-, Cul4-, and Cdt2-dependent manner, which resembles the destruction of Cdt1, a pre-replication complex member (Arias and Walter, 2006; Jin et al., 2006; Senga et al., 2006). The destruction of E2F1 is also dependent on its partner DP, but not affected by mutations in the putative CDK phosphorylation sites or the RBF1-binding site. Furthermore, we examined the in vivo significance of E2F1 destruction and found that the stabilized version of E2F1 induces massive apoptosis when expressed in the wing imaginal disc, resulting in disturbed morphology of the adult wing. These results suggest that the S phase-dependent destruction of E2F1 is an essential feature for the normal development of *Drosophila*. We propose that

cells may possess an S phase-specific sensor which detects ectopic E2F1 activity and subsequently activates apoptotic pathways.

Materials and Methods

PCR primers

Primers for pENTR-D-TOPO cloning:

E2f1 (1-) pENTR forward

CACCATGTCCAAGTTCTTTGTGAATGTTGCC

E2f1 (47-) pENTR forward

CACCATGGTGGCCCGCAGACTCAACTA

E2f1 (93-) pENTR forward

CACCGGCGGCGTGGCAGCCCACC

E2f1 (139-) pENTR forward

CACCCAAAATCAGCAGCAACGCAAGG

E2f1 (231-) pENTR forward

CACCTCGCTGTCGACGCCCCAGCAAC

E2f1 (529-) pENTR forward

CACCCAGCAACAACAACAGTTGCTACAGC

E2f1 (-92) pENTR reverse

TCAGTTGCTGTTGCTGTCGCTGCTGC

E2f1 (-138) pENTR reverse

TCAGTGGTGGTGCTGCTGCTGCAG

E2f1 (-184) pENTR reverse

TCACGTCTGGTGGTGGGCGCTCTG

E2f1 (-230) pENTR reverse

TCAAAAGGGGTGATGCGATGCCGG

E2f1 (-528) pENTR reverse

TCACTGCTGCTGCTGGTTCAGATTATG

E2f1 (-805) pENTR reverse

TTAGGGTCCATAGGCATCCGAACCGAA

Cul4 pENTR forward

CACCATGAGTGCGGCCAAGAAGTACAAG

Cul4 pENTR reverse

TTATGCCACATAGTTGTATTGGTTTTG

Cdt2 (l(2)dtl) pENTR forward

CACCATGAACATTTACAACAAGTTGCGGGC

Cdt2 (l(2)dtl) pENTR reverse

TCAATCGCTGCCCACCGCCGTC

Primers for pDONR221 cloning:

Dp pDONR forward

GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGGCGCATTCGACGGGC

GGTAC

Dp pDONR reverse

GGGGACCACTTTGTACAAGAAAGCTGGGTTCAATCAATGTCGTCGTCCAG

CTC

Primers for mutagenesis:

E2f1 L786Q mutagenesis forward

CTATCCGTATGCGCAGAACGCGAACGAGG

E2f1 L786Q mutagenesis reverse

CCTCGTTCGCGTTCTGCGCATACGGATAG

E2f1 PIP-3A mutagenesis forward

GACCGGCAAATCCAACGATGCCACAAATGCCGCCAAGGTCAAACGTCGG
CCAC

E2f1 PIP-3A mutagenesis reverse

GTGGCCGACGTTTGACCTTGGCGGCATTTGTGGCATCGTTGGATTTGCCGG
TC

E2f1 PIP-7A mutagenesis forward

GGCGACCGGCAAATCCGCCGCAGCAGCGGCCGCGGCCAAGGTCAAACGT
CGG

E2f1 PIP-7A mutagenesis reverse

CCGACGTTTGACCTTGGCCGCGGCCGCTGCTGCGGCGGATTTGCCGGTCG
CC

E2f1 PIP-7del mutagenesis forward

GGCGACCGGCAAATCCAAGGTCAAACGTCGGC

E2f1 PIP-7del mutagenesis reverse

GCCGACGTTTGACCTTGGATTTGCCGGTCGCC

Primers for dsRNA synthesis:

Cul1 dsRNA forward

TAATACGACTCACTATAGGG CTGCTCAACGCAGACCG

Cul1 dsRNA reverse

TAATACGACTCACTATAGGG TGTCCTGCAGTTGCTGG

Cul4 dsRNA forward

TAATACGACTCACTATAGGG TTGGCCAAACGATTACTTGTGGG

Cul4 dsRNA reverse

TAATACGACTCACTATAGGG GAGAAGATTATGGCTCAGCG

Skp1 (SkpA) dsRNA forward

TAATACGACTCACTATAGGG TGCCCAGCATCAAGTTGCAATCTTC

Skp1 (SkpA) dsRNA reverse

TAATACGACTCACTATAGGG CTAGCTGTTTCAACTTAATGTTGGTC

Ddb1 dsRNA forward

TAATACGACTCACTATAGG CCCCGCTCCATTCTGATGACC

Ddb1 dsRNA reverse

TAATACGACTCACTATAGGG CTGCAGCAGCGTGATGGAGCGC

Cdt2 (l(2)dtl) dsRNA forward

TAATACGACTCACTATAGGG GCGGGCTCCGGCATACGCGGC

Cdt2 (l(2)dtl) dsRNA reverse

TAATACGACTCACTATAGG CGTGGCTGGAGCCCCAGGCCACG

Slmb dsRNA forward

TAATACGACTCACTATAGGG GGCCGCCACATGCTGCG

Slmb dsRNA reverse

TAATACGACTCACTATAGGG CGGTCTTGTTCTCATTGGG

Skp2 (CG9772) dsRNA forward

TAATACGACTCACTATAGGG TAGACCAGGTGCCCTCG

Skp2 (CG9772) dsRNA reverse

TAATACGACTCACTATAGGG GGTTGCTGGAATAAGATAGC

Ago dsRNA forward

TAATACGACTCACTATAGGG GATGCCCAGCTGCTAGC

Ago dsRNA reverse

TAATACGACTCACTATAGGG TGCTGGCGAGGGATTCG

Dp dsRNA forward

TAATACGACTCACTATAGGG GGCCCAGAACAAGTCCGAAATGG

Dp dsRNA reverse

TAATACGACTCACTATAGGG GGCAAGGTTTGGAGGCACCCAC

Rbf1 dsRNA forward

TAATACGACTCACTATAGGG AAGCTGGCGAAGAGGTAATAGCC

Rbf1 dsRNA reverse

TAATACGACTCACTATAGGG GCACACATAATATTTTGATCGAGGTG

Pcna dsRNA forward

TAATACGACTCACTATAGGG CAGGCCATGGACAACTCCCATG

Pcna dsRNA reverse

TAATACGACTCACTATAGGG TGTCTCGTTGTCCTCGATCTTGGG

lacZ dsRNA forward

TAATACGACTCACTATAGGG ACGCCGAACGATCGCCAGTTCTG

lacZ dsRNA reverse

TAATACGACTCACTATAGGG CGAGCCAGTTTACCCGCTCTGC

Primers for RT-PCR:

Cul1 RT-PCR forward

GCATCTGCAACTACCTGAATCGG

Cul1 RT-PCR reverse

CAGATTCTGAAACTCGGTGTGAAAG

Cul4 RT-PCR forward

CGTCACTATCTGGACTCGAGCAC

Cul4 RT-PCR reverse

CCAGATCGCAGTTTCATATCTACG

Ddb1 RT-PCR forward

GGTCTCTAAAGGTGGGAGTCG

Ddb1 RT-PCR reverse

GTAGATGGTCTCAAGATCAGGCAG

Cdt2 (l(2)dtl) RT-PCR forward

AATATTTTCGATGCCTCGAAGGTTG

Cdt2 (l(2)dtl) RT-PCR reverse

CACCTCCCACAGTCTGGCAGTG

Dp RT-PCR forward

GTACGACAACAACTGTGATCAAAAG

Dp RT-PCR reverse

TTCGCCAGTCTTGCCGGTGCC

Pcna RT-PCR forward

CTTCGATTGCAGCGACTCCGGC

Pcna RT-PCR reverse

AAATGTCAGCGTCACCGGCTCC

Slmb RT-PCR forward

CAACCACTATGCTGTACGACCCG

Slmb RT-PCR reverse

TCCGCCAGTTGTTCTCTATGCTG

Ago RT-PCR forward

GCTCGCGAGACGCAACCTTGAG

Ago RT-PCR reverse

GACCGCAGAATGATGCTTATTTGG

Skp2 (CG9772) RT-PCR forward

TCACTGTCGATGTCCCGTCAATC

Skp2 (CG9772) RT-PCR reverse

TTGTCCGCAAACCCAAATCTAGCC

Skp1 (SkpA) RT-PCR forward

TGCCCAGCATCAAGTTGCAATCTTC

Skp1 (SkpA) RT-PCR reverse

CAAATCCAATTCGTTCCCGAATCC

Rbf1 RT-PCR forward

GTGGTCAAGGGTAATTGTGTGTCC

Rbf1 RT-PCR reverse

CGAAGTTTTCGTTAGCCAATAGGCC

Plasmid vectors

The open reading frames (ORFs) of the wild type or ΔCDK mutant *E2f1* were amplified from pUAST-*E2f1* or pUAST-*E2f1*$^{\Delta CDK}$ (gifts from Dr. Bruce Edgar), respectively, and cloned into pENTR™/D-TOPO® (Invitrogen). The forward primers used to amplify *E2f1* were designed to contain CCAC at the 5' end for the directional cloning into pENTR. *E2f1* mutants containing L786Q, PIP-3A, PIP-7A and PIP-7del mutations were created from pENTR-*E2f1* using a QuikChange® II Site-Directed Mutagenesis Kit (Stratagene). For the deletion assay shown in Figure 3.1, the DNA sequences coding amino acids 1-230, 231-528, 529-805, 1-92, 47-138, 93-184 and 139-230 of *E2f1* were amplified from pENTR-*E2f1* and cloned into pENTR. The ORFs of *Cul4* and *Cdt2* (*l(2)dtl*) were amplified from cDNA clones (DGC clone LP02965 and LD21681, obtained from Open Biosystems) and cloned into pENTR. The ORF of *Dp* was amplified from a cDNA (gift from Dr. Nick Dyson) and cloned into pDONR™221 (Invitrogen) using the Gateway® BP Clonase™ II enzyme mix (Invitrogen). The ORFs inserted in pENTR or pDONR were confirmed by sequencing. To create expression vector plasmids, the pENTR or pDONR plasmids were recombined with pAGW (*Actin* promoter, N-term GFP), pHGW (*Hsp70* promoter, N-term GFP), pAMW (*Actin* promoter, N-term myc), pAHW (*Actin* promoter, N-term HA), or pPGW (UASp, N-term GFP) (provided by Dr. Terence Murphy using the Gateway® LR Clonase™ II enzyme mix (Invitrogen).

Cell culture and transfection

Schneider S2 cells were provided by Dr. Steve Rogers. Cells were routinely grown at room temperature in Schneider's *Drosophila* medium (Gibco) supplemented with 10% Fetal Bovine Serum (Sigma) and 1:200 Penicillin-Streptomycin (5 units/ml Penicillin, 5 μg/ml Streptomycin, Sigma). For transfection and RNAi assay, cells were grown at 28˚C.

For transfection, S2 cells diluted to 5×10^5 cells/ml were plated in 6 well plates (1.6 ml culture per well) and grown for a day until transfection. For transient transfection with pAGW, pAMW or pAHW plasmids, cells were transfected with 1.2 μg per well (pAHW-*Cul4* and pAHW-*Cdt2*, when transfected with pAMW-$E2f1^{PIP}$) or with 0.4 μg per well (other constructs) of plasmid using Effectene® (Qiagen), and subjected to analyses at 2 or 3 days after transfection. For stable transfection with pAGW or pHGW plasmids, cells were transfected with 0.4 μg per well of expression plasmid along with 0.02 μg per well of pCoHygro (Invitrogen). 2 or 3 days after transfection, the medium was replaced with fresh growth medium, and cells were allowed for 2 days to grow. Cells were then incubated in growth medium containing 500 μg/ml hygromycin B (Invitrogen) for more than 18 days for the selection of stably-transfected cells.

Flow cytometry

Transiently or stably transfected S2 cells were collected in microcentrifuge tubes and washed once with PBS at room temperature, fixed with ice-cold 1% paraformaldehyde in PBS for 30 min on ice, washed once with ice-cold PBS, and permeabilized with PBT (PBS containing 0.1% Tween20) for 15 min at room temperature. Then 3 μl per tube of 500 μg/ml DNase-free RNase (Roche) were added, followed by 30 min incubation at 37˚C. DNA was

stained with 400-500 µl per tube of ice-cold 15 µM propidium iodide in PBS overnight at 4˚C. Flow cytometry was performed using a CyAn (Dako), and the data was analyzed with the Summit 4.3 software (Dako). Percentages of G1, S, and G2 were calculated using the ModFit LT™ software (Verity Software House).

RNAi

Double stranded RNAs (dsRNAs) were transcribed from PCR products which are gene-specific sequences flanked by the T7 promoter sequences. PCR products were amplified from genomic DNA or cDNA clones. Transcription reaction was performed using the RiboMAX™ Large Scale RNA Production System-T7 (Promega) in 50 µl volume with 20 µl of PCR product. Synthesized RNAs were treated with 0.1 units/µl of RNase-free RQ1 DNase (Promega) for 15 min at 37˚C. Complementary RNA strands synthesized were denatured by incubating the RNAs for 2 min on a 95˚C heat block, and annealed to form dsRNAs by transferring the heat block to room temperature. dsRNAs were then cleaned with illustra™ Sephadex™ G-50 columns (GE Healthcare). Approximate concentrations of the dsRNAs were determined by comparing the intensity of RNA bands in an agarose gel using a dsRNA ladder (New England Biolabs) as a standard. The intensity of the RNA bands were analyzed using an AlphaImager™ 2200 (Alpha Innotech) and the AlphaEaseFC™ 3.2 software (Alpha Innotech).

For RNAi, S2 cells stably transfected with pHGW-*E2f1* were grown in Sf-900II serum free medium (Gibco) supplemented with 1:200 Penicillin-Streptomycin. Cells diluted to 1×10^6 cells/ml were plated in 6 well plates (1 ml per well), and grown for a day at 28˚C. dsRNAs of indicated amounts (see Figure 3.4) were then added to cells. After 2 days of

incubation at 28˚C, a subset of the cells was used for RT-PCR and Western blot, and the rest was heat-shocked for 30 min at 37˚C, fixed at 225 min after the end of heat-shock, and subjected to flow cytometry analysis.

Multiple alignment

Multiple alignment of E2F1 fragments shown in Figure 3.2 was created using ClustalW2 on the EMBL-EBI website (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

RT-PCR

Total RNA was extracted from S2 cells using TRIzol® (Invitrogen) according to manufacture's protocol. 1 μg of total RNA was used for reverse transcription with M-MLV reverse transcriptase (Invitrogen) in 20 μl volume. 0.5 μl of the 20 μl reaction was used for subsequent PCR with GoTaq® Flexi (Promega) for 27 or 30 cycles.

Co-immunoprecipitations and Western blotting

S2 cells transiently transfected with pAMW-*E2f1* constructs and/or pAHW constructs (*Cul4*, *Cdt2*, or *Dp*) were washed once with PBS and lysed with 500 μl of ice-cold lysis buffer (50mM Tris-HCl pH8.3, 150mM NaCl, 0.5% NP40) containing protease inhibitors (1 μg/ml leupeptin, 0.5 μg/ml pepstatin and 1 mM PMSF). After equalizing the concentrations of all samples, 300 μl of lysate was incubated overnight at 4˚C with 25 μl of Protein G Sepharose™ 4 Fast Flow (GE Healthcare) which was pre-incubated with a mouse anti-myc (1:500) antibody (gifts from Dr. Eric Wagner). Immunoprecipitates were then washed four times with lysis buffer, and analyzed by Western blot.

For Western blot using wing disc lysates, ~20 wing discs were dissected from 3rd instar larvae, and lysed with 200 µl of ice-cold lysis buffer (50mM Tris-HCl pH8.3, 150mM NaCl, 0.5% NP40) containing protease inhibitors. Protein concentrations were measured by the Bradford assay, and 5 µg of protein was loaded in each lane.

Cell/tissue lysates and immunoprecipitates were subjected to SDS-PAGE using 10% or 12% Tris-HCl Ready Gels (Bio-Rad). Proteins were transferred to Trans-Blot® 0.45 µm nitrocellulose membranes (Bio-Rad) and detected with the following primary antibodies: mouse anti-myc (1:2000, gift from Dr. Eric Wagner), mouse anti-HA (1:50000, gift from Dr. Eric Wagner), rabbit anti-Cul1 (1:250, Invitrogen-Zymed), rabbit anti-Cul4 (1:10000, gift from Dr. Sima Zacharek and Dr. Yue Xiong), mouse anti-Ddb1 (1:100**,** Invitrogen-Zymed), mouse anti-DP (YUN1-3, 1:4, gift from Dr. Nick Dyson (Du et al., 1996), rabbit anti-E2F1 (1:100, raised against full length *Drosophila* E2F1, and affinity purified against full length E2F1 polypeptide), and mouse anti-alpha tubulin (1:2000, Sigma). Secondary antibodies were ECL™ sheep anti-mouse HRP (1:2000) and ECL™ donkey anti-rabbit HRP (1:2000) from GE Healthcare.

***Drosophila* strains**

UASp-*E2f1*, UASp-*E2f1*$^{PIP\text{-}3A}$, UASp-*E2f1*$^{PIP\text{-}7A}$, and UASp-*E2f1*$^{PIP\text{-}7del}$ transgenic flies were created by injecting w^{1118} embryos with pPGW vectors containing the wild type, PIP-3A, PIP-7A or PIP-7del mutant *E2f1* ORFs, respectively. Injection was performed by Rainbow Transgenic Flies, Inc. (Newbury Park, CA). UAS-GFP, *prd*-GAL4/*TM3*, and *arm*-GAL4 were obtained from the Bloomington Stock Center. *en*-GAL4 was a gift from Dr. Steve Crews.

RNA in situ hybridization

In situ hybridization was performed as described (Shibutani et al., 2007). Embryos were dechorionated with 50% bleach, fixed in a 1:1 mixture of 4% formaldehyde in PBS/heptane for 25 min at room temperature, and devitellinized with methanol. Embryos were stored in methanol at -20˚C. A digoxigenin-labeled *RnrS* probe was synthesized by transcribing antisense RNA from a full length *RnrS* cDNA (DGC collection clone LD41588, obtained form Open Biosystems). Stained embryos were mounted with Fluoromount-G (Southern Biotech) and visualized with a Nikon Eclipse E800 microscope.

BrdU labeling and immunostaining

For staining embryos with anti-GFP, anti-phosphotyrosine and anti-BrdU, dechorionated embryos were permeabilized with octane, pulse-labeled with 1 mg/ml BrdU for 5 min in Schneider's medium prior to fixation. Embryos stored in methanol were re-hydrated with PBT and incubated with rabbit anti-GFP (1:10000, Upstate) overnight at 4˚C. To detect GFP, the TSA^{TM} Fluorescein System (Perkin Elmer) was used with a biotin-conjugated anti-rabbit secondary antibody (1:1000, Chemicon). Embryos were then incubated with rat anti-phosphotyrosine (1:100, R&D Systems), followed by donkey anti-rat-Cy5 (1:500, Jackson). After re-fixation with 4% formaldehyde in PBS for 25 min at room temperature, BrdU was detected by acid denaturation of chromosomes, mouse anti-BrdU (1:100, Becton Dickinson), and goat anti-mouse-Cy3 (1:500, Jackson).

For staining wing discs with anti-cleaved Caspase-3, flies were allowed to lay eggs for a day, and the eggs were incubated at 25˚C until they reach the 3rd instar larva stage. Wing discs were dissected from 3rd instar larvae in Schneider's medium, fixed with 6%

paraformaldehyde in PBS for 20 min at room temperature. After three washes with PBS, discs were incubated with 5% normal goat serum in PBT for 1h at room temperature, and incubated with rabbit anti-cleaved Caspase-3 (Asp175) (1:200, Cell Signaling Technology) overnight at 4˚C. Discs were then incubated with goat anti-rabbit-rhodamine (1:1000, Invitrogen-Molecular Probes), and 1 μg/ml DAPI.

For staining S2 cells with anti-BrdU, S2 cells stably transfected with pHGW-*E2f1* constructs were heat-shocked for 30 min at 37˚C, and pulse-labeled with 10 μg/ml BrdU in growth medium for the last 45 min prior to fixation. Cells were plated on a Concanavalin A-treated cover glass during incubation with BrdU, and fixed at 225 min after the end of the heat shock. Fixation was performed with 4% paraformaldehyde in PBS for 20 min at room temperature. Cells were then permeabilized with PBT for 15 min at room temperature, and the chromosomes were denatured by incubation with 50 units/ml RNase-free RQ1 DNase (Promega) in RQ1 DNase buffer (Promega) for 30 min at 37˚C. After blocking with 5% normal goat serum in PBT for 30 min at room temperature, cells were incubated with mouse anti-BrdU (1:100, Becton Dickinson) overnight at 4˚C. Cells were then incubated with goat anti-mouse-Cy3 (1:500, Jackson), and 5 μg/ml DAPI.

Stained embryos, wing discs and cells were mounted with Fluoromount-G. Embryos and wing discs were visualized with a Zeiss LSM 510 scanning confocal microscope. Cells were visualized with a Nikon Eclipse E800 microscope. Images were consistently modified with Photoshop® CS2 9.0 (Adobe)

Imaging of adult wings

Adult wings were dissected from CO_2 anaesthetized flies, mounted on a slide glass, and visualized with a Nikon Eclipse E800 microscope.

Results

Detection of the S phase-specific E2F1 destruction in S2 cells

In the previous chapter, we showed that *Drosophila* E2F1 protein destruction begins prior to differentiation during embryogenesis, and that this destruction is strictly dependent on S phase. We wished to understand the mechanism that underlies the S phase-specific destruction of E2F1. To this end, we first established a flow cytometry (FACS)-based experimental system that allows us to detect S phase-specific destruction of E2F1. S2 cells were transfected with plasmids to express GFP or GFP-E2F1 (GFP at the N-terminus of E2F1) under the control of the *Actin* promoter, and stable transfectants were selected with hygromycin. The transfected cells were fixed, stained for DNA, and analyzed by FACS. Figure 3.1 shows cell cycle profiles of all cells and GFP positive cells. GFP positive cells were defined by the cutoff value of GFP level above which very little non-transfected cells are detected (0.1% of total cells). These profiles show clear G1 and G2 peaks; G2 cells contain twice as much DNA as G1. S phase cells can be detected between these peaks on the histogram.

To visualize the S phase-specific destruction of GFP-E2F1, we overlapped the cell cycle profiles of all cells and GFP positive cells (note that the Y axis is shown in a relative scale between all cells and GFP positive cells, because the total number of the GFP positive cells is always less than that of all cells). This way, S phase-specific destruction of GFP-E2F1 was successfully visualized as a decrease in the S phase percentage of GFP-E2F1

positive cells (Figure 3.1A, bottom panel). Such a decrease was not observed in the GFP-only control (Figure 3.1A, top panel). In the following experiments, we used this FACS-based experimental system to investigate the mechanism of E2F1 destruction.

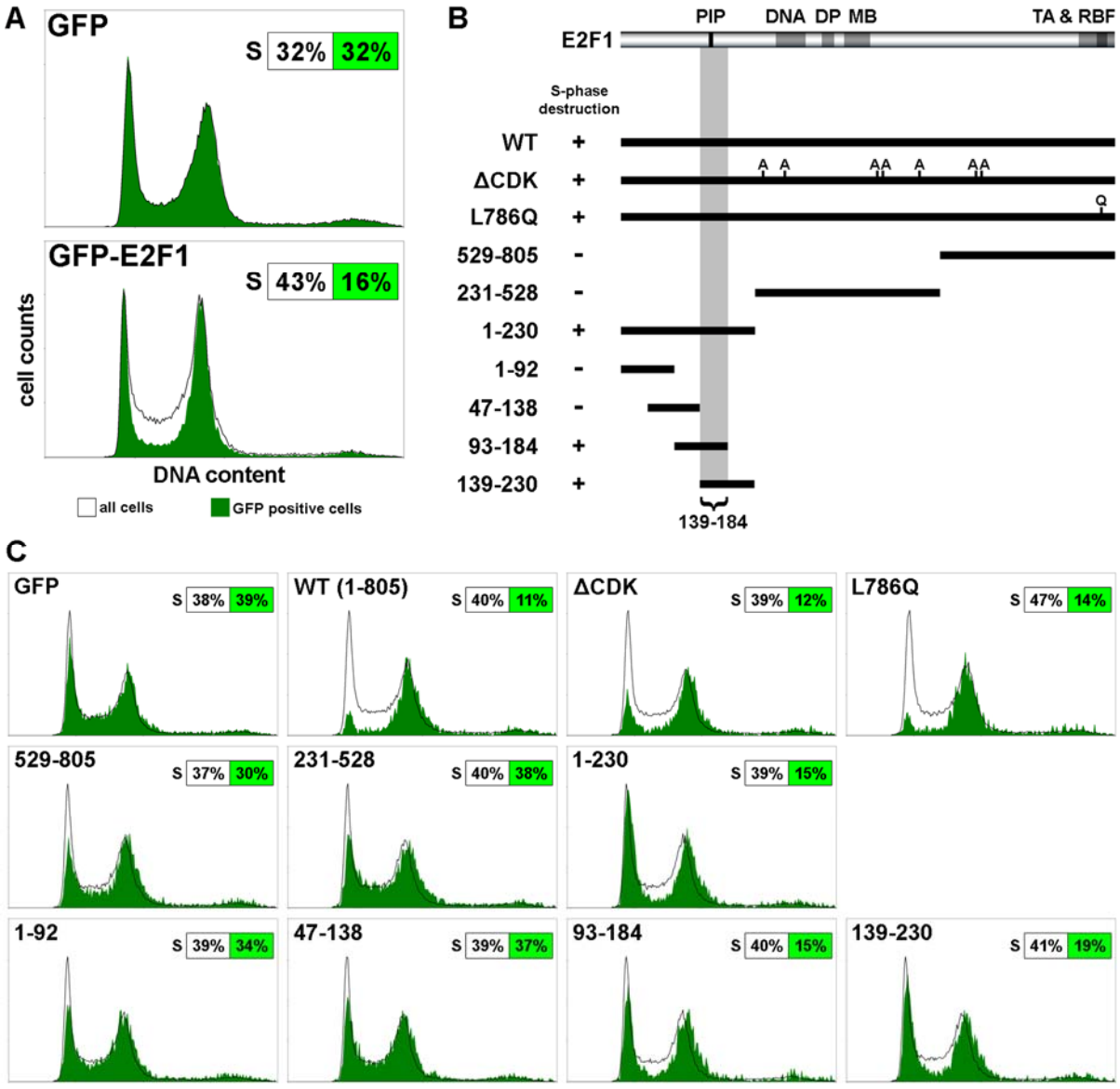

Figure 3.1: The S phase-specific destruction of E2F1 is regulated by its N-terminus in S2 cells

Cell cycle profiles of all cells (unfilled solid line), and GFP positive cells (filled in green). The GFP positive gate was determined as 99.9% of non-transfected cells are excluded from the gate. Note that the scales of Y axis are different between "all cells" and "GFP positive cells," since GFP positive cells are a subset of all cells.

Percentages of S phase cells for all cells and GFP positive cells are shown in insets. S phase-specific destruction is detected as a decrease in the S phase percentages in the GFP positive population.

(A) Cell cycle profiles of S2 cells stably transfected with pAGW (GFP under *Actin* promoter) or pAGW-*E2f1* (GFP-E2F1 under *Actin* promoter).

(B) A diagram of E2F1 constructs used in C. + and – indicate the existence of S phase-specific destruction of GFP-E2F1 variants according to the data shown in C. The shadowed region (amino acids 139-184) is indicates a necessary fragment for S phase-specific destruction. PIP, PCNA-interaction protein box. DNA, DNA-binding domain. DP, DP-dimerization domain. MB, marked box. TA, transactivation domain. RBF, RBF1-binding site.

(C) Cell cycle profiles of S2 cells transiently transfected to express GFP-E2F1 variants under the *Actin* promoter.

The N-terminus of E2F1 is sufficient to induce the S phase-specific destruction

Having established the experimental system, we sought to identify what aspect of the E2F1 protein is controlling its own destruction. We transfected S2 cells with the E2F1 variants shown in Figure 3.1B. These E2F1 variants were N-terminally tagged with GFP, and expressed under the *Actin* promoter. As a quick way to examine various constructs, we employed transient expression, instead of creating stable transfectants. Despite the low transfection efficiency (typically 5-10%), we were still able to observe S phase-specific destruction (compare the GFP-only control (GFP) and GFP-E2F1 wild type (WT) in Figure 3.1C).

The previous report by Reis et al. suggested that increased CDK activity downregulates E2F1 transcripts and proteins (Reis and Edgar, 2004). Also, it has been reported that Slmb, the substrate receptor for a Cul1-based ubiquitin ligase, binds to E2F1 in a phosphorylation-dependent manner (Heriche et al., 2003). If the phosphorylation of E2F1 by CDK promotes E2F1 destruction via Cul1-mediated ubiquitination, then mutations in the

CDK phosphorylation sites of E2F1 should stabilize the protein. To test this, all seven putative CDK phosphorylation sites (TP or SP) were mutated to alanines ($E2F1^{\Delta CDK}$). However, $E2F1^{\Delta CDK}$ was still degraded during S phase (Figure 3.1C). It should be noted that we observed a slight increase in the percentage of G1/early S phase cells in the GFP-$E2F1^{\Delta CDK}$ positive population (Figure 3.1C and 3.2D). However, we did not pursue to determine whether this increase is due to the moderate stabilization of E2F1 or it is within a range of experimental variability. Despite the possible stabilization of E2F1 in G1/early S phase, the data shows that CDK phosphorylation is not the main contributor of the E2F1 destruction during S phase.

We next tested $E2F1^{L786Q}$. This point mutation was found in the *E2f1*^{su89} mutant allele, and biochemical data indicate that this mutation abolishes the ability of E2F1 to bind RBF1 (Weng et al., 2003). It is suggested that human E2F1 is protected from ubiquitination and subsequent destruction when bound by pRB (Campanero and Flemington, 1997; Hateboer et al., 1996; Hofmann et al., 1996). If this is the mechanism that underlies the S phase-specific destruction (or G1/G2-specific protection) of *Drosophila* E2F1, then the L786Q mutation should destabilize E2F1 throughout the cell cycle. However, we observed a normal S phase destruction pattern of $E2F1^{L786Q}$ (Figure 3.1C), suggesting that the protection by RBF1 is not the mechanism that dictates the S phase-specific destruction of E2F1.

We then asked which region of the E2F1 protein is sufficient to induce destruction. To this end, we first examined amino acids 1-230, 231-528 and 529-805 (Figure 3.1C). Surprisingly, the 1-230 fragment, in which no conserved motifs have been reported, was sufficient to induce the S phase-specific destruction. In contrast, destruction was not observed for the 231-528 or 529-805 fragments. To further delineate the region, we created

four smaller, overlapping fragments within the 1-230 region (1-92, 47-138, 93-184 and 139-230), and found that the 93-184 and 139-230 fragments are degraded during S phase, whereas the 1-92 and 47-138 fragments are not (Figure 3.1C). These results suggest that there is a sequence that controls S phase-specific destruction within the region of amino acids 139-184.

The PIP box in E2F1 is required for the S phase-specific destruction

When the 139-184 region was aligned with the putative E2F1 orthologs from the 11 other *Drosophila* species, it was shown that the amino acid sequences within this region are highly conserved between these *Drosophila* species (Figure 3.2A). With help from Dr. Johannes Walter, we were able to find a putative "PIP box" motif (PCNA-interaction protein box) in this region. In this putative PIP box, the hydrophobic residue (I153 in *D. melanogaster*) and the following two consecutive aromatic residues (Y156 and Y157 in *D. melanogaster*) that characterize the PIP box are conserved in all *Drosophila* species. However, it does not contain the glutamine residue which is normally conserved in the PIP box (Moldovan et al., 2007). Previously, the PIP box was shown to be conserved in Cdt1, a member of the pre-replication complex. Cdt1 is degraded during S phase via the binding of Cdt1's PIP box to PCNA (Arias and Walter, 2006; Jin et al., 2006; Senga et al., 2006). These studies suggest that Cdt1 binds to S phase-specific, chromatin-bound PCNA, and this interaction triggers the ubiquitination of Cdt1 by the $Cul4^{Ddb1\text{-}Cdt2}$ ubiquitin ligase complex.

In order to test whether the putative PIP box is required for the S phase-specific destruction of E2F1, we created three different PIP mutant alleles of *E2f1* by mutagenesis, named PIP-3A, PIP-7A and PIP-7del (Figure 3.2A). PIP-3A contains three amino acid

mutations to alanines in the conserved I153, Y156 and Y157. The characteristics of these amino acids were also conserved in Cdt1's PIP box (Figure 3.2A). PIP-7A and PIP-7del were created because of the possibility that the PIP-3A mutation may not completely abolish the ability of E2F1 to bind factors involved in its destruction, such as PCNA.

We first attempted to obtain S2 transfectants which stably express GFP-E2F1^{PIP-3A} under the control of the *Actin* promoter. Cells were transfected with GFP, GFP-E2F1^{WT} or GFP-E2F1^{PIP-3A}. After selection with hygromycin, stable transfectants expressing GFP or GFP-E2F1^{WT} were obtained (Figure 3.1A). However, no selection-resistant cells of GFP-E2F1^{PIP-3A} were recovered, suggesting that the constitutive expression of E2F1^{PIP-3A} under the *Actin* promoter causes lethality, whereas cells can survive with the expression of wild type E2F1 under the same promoter.

To circumvent the lethality by E2F1^{PIP}, we employed inducible expression of transgenes under the control of the *Hsp70* promoter. Since the expression is induced only when cells are heat-shocked, the lethality caused by the expression of E2F^{PIP} should not be seen at maintenance temperatures (room temperature to 28˚C). Indeed, stable transfectants of the PIP mutants were obtained with the *Hsp70* promoter. To determine the optimal timing for detection of the S phase-specific destruction, a time course observation of GFP-E2F1^{WT} expression was performed (Figure 3.2B). Before heat shock treatment, cells expressed very low, if any, levels of GFP-E2F1^{WT} (Figure 3.2B and data not shown). Cells were treated with a 30 min heat shock at 37˚C to induce GFP-E2F1^{WT} expression. At 45 min post heat shock, GFP-E2F1^{WT} expression was seen in all cell cycle phases. At later time points (105, 165, 225 and 285 min), we observed a progressive decrease in the S phase percentage of GFP-E2F1^{WT} expressing cells, indicating that the destruction of GFP-E2F1^{WT} is a dynamic process. In

addition, we observed an increase in S phase population induced by GFP-E2F1^{WT} expression (29% S phase cells before heat shock vs. 42% at 285 min), suggesting that the GFP-tagged version of E2F1 retains the ability to promote the G1-to-S transition.

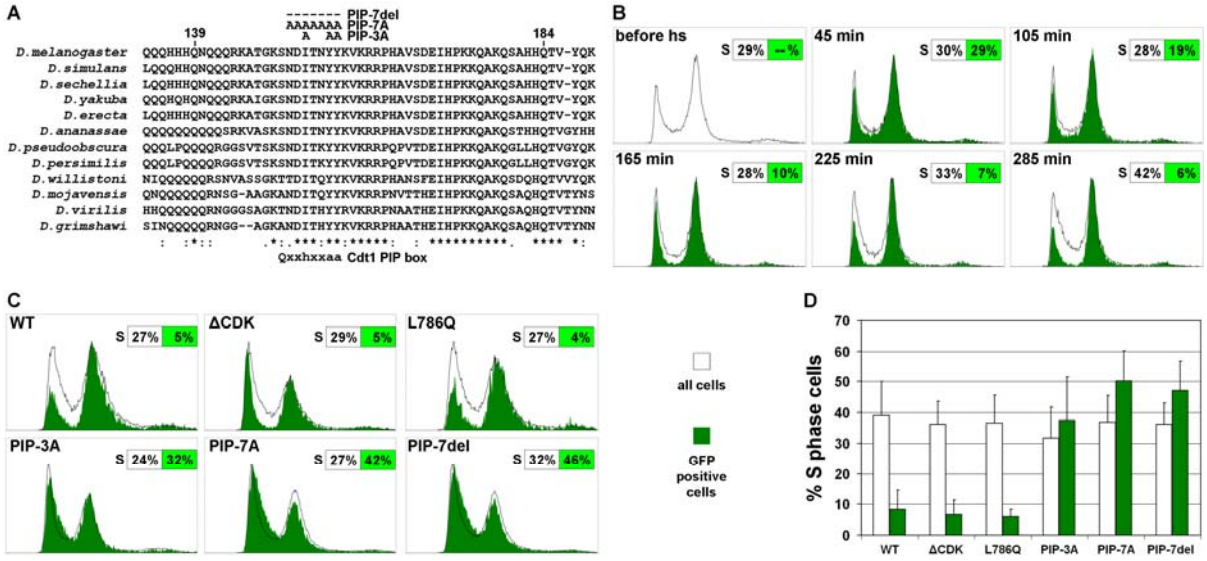

Figure 3.2: The PIP box is required for the S phase-specific destruction of *Drosophila* E2F1 in S2 cells

(A) A multiple alignment of E2F1 orthologs in 12 *Drosophila* species. Amino acids 139 and 184 of *D. melanogaster* E2F1 are indicated. A potential PIP box in E2F1 is aligned with the PIP box in Cdt1 (Arias and Walter, 2006; Senga et al., 2006). Q, glutamine. h, hydrophobic amino acid. a, aromatic amino acid. x, any amino acid. The PIP mutant E2F1 constructs created for this study are indicated above the alignment (PIP-3A, PIP-7A, and PIP-7del). "A" and "-" indicate mutation to alanines and deletion, respectively.

(B) Cell cycle profiles of S2 cells stably transfected to express GFP-E2F1^{WT} under the *Hsp70* promoter. Cells were heat-shocked for 30 min at 37˚C, and fixed at the indicated time points post heat shock.

(C) Cell cycle profiles of S2 cells stably transfected to express GFP-E2F1 variants under the *Hsp70* promoter. Cells were heat-shocked for 30 min at 37˚C, and fixed at 225 min post heat shock.

(D) Quantification of S phase percentages. The experiment described in C was repeated three times (one of which is shown in C), and the percentages of S phase cells were calculated for all cells (unfilled) and GFP positive cells (filled in green). Average percentages are shown, and error bars indicate standard deviations.

(B-D) The GFP positive gate was determined as 99.9% of non-heat-shocked transfected cells are excluded from the gate.

Based on the result of the time course experiment, we decided to use the time point of 225 min post heat shock for later analyses, because most of the S phase cells have degraded GFP-E2F1^{WT} at this time point (Figure 3.2B). S2 cells were stably transfected with *Hsp70*-controlled GFP-E2F1 variants (WT, ΔCDK, L786Q, PIP-3A, PIP-7A and PIP-7del). These stable transfectants were heat shocked and fixed at 225 min post heat shock. As shown in Figure 3.2C, S phase-specific destruction was observed in the GFP-E2F1 WT, ΔCDK, and L786Q stable transfectants. In contrast, the PIP mutant E2F1s were not degraded during S phase. The experiment was repeated three times to confirm reproducibility and quantified in Figure 3.2D. Furthermore, when cells were stained for BrdU incorporation (S phase marker), a significant number of GFP-E2F1/BrdU double positive nuclei were observed specifically for the PIP mutants (Figure 3.3). These results clearly show that the PIP box of E2F1 is required for the S phase-specific destruction of E2F1 in S2 cells.

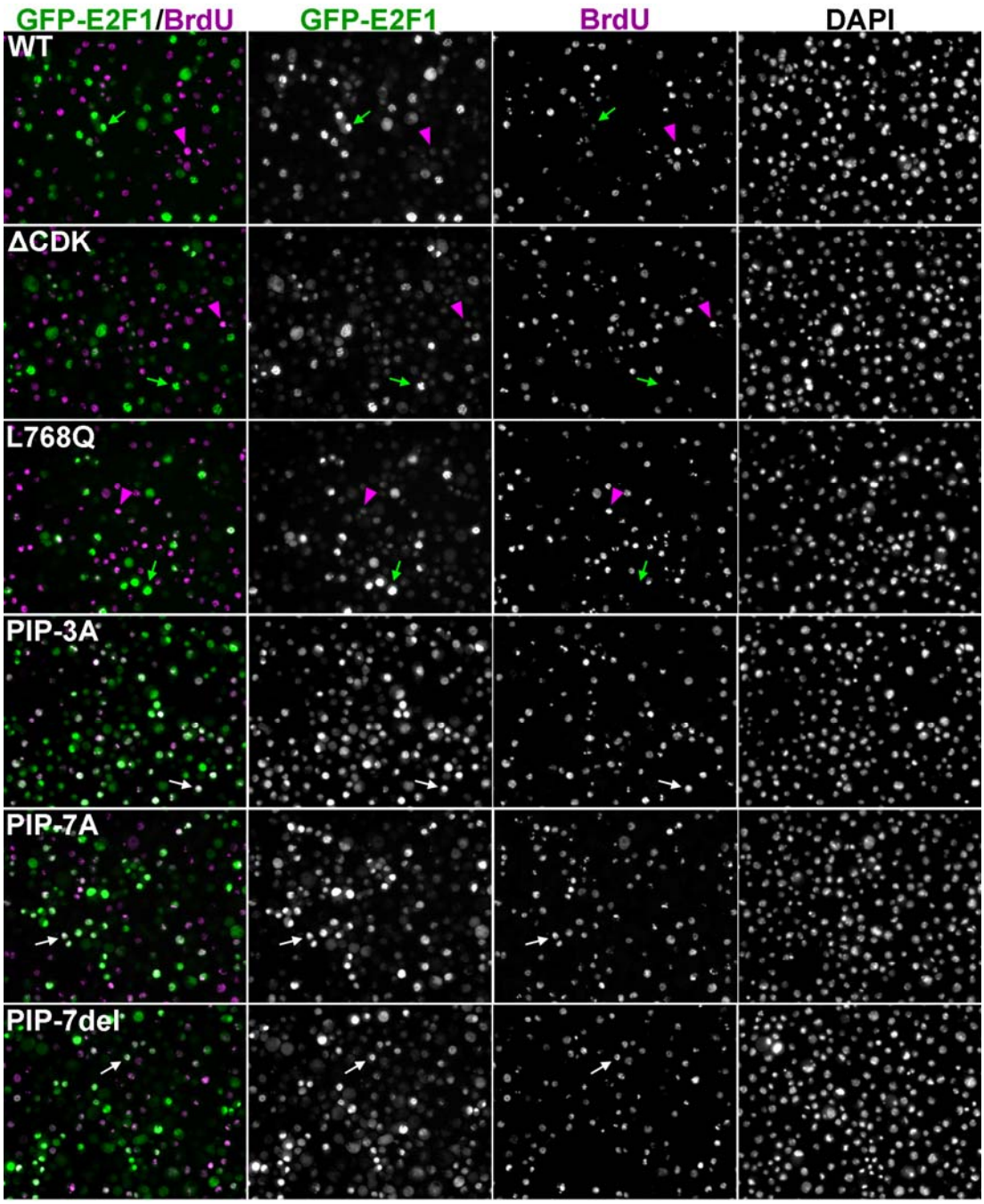

Figure 3.3: The PIP mutant E2F1 accumulates in the nucleus during S phase

S2 cells stably transfected to express GFP-E2F1 variants under the *Hsp70* promoter were heat shocked for 30 min at 37˚C, pulse-labeled with BrdU, and stained for BrdU incorporation (magenta). GFP fluorescence is shown in green. Wild type, ΔCDK mutant, and L786Q mutant E2F1 accumulate in the nucleus outside of S phase (green arrows) and degraded during S phase (magenta arrowheads). In contrast, PIP mutant E2F1s are stabilized and accumulated in the nucleus during S phase (white arrows).

The destruction of E2F1 requires Cul4, Cdt2, PCNA, and DP

The PIP box-dependent, S phase-specific destruction of *Drosophila* E2F1 led to the hypothesis that *Drosophila* E2F1 is degraded by the same pathway used for Cdt1 destruction, which involves the $Cul4^{DDB1\text{-}Cdt2}$ ubiquitin ligase complex and PCNA (Arias and Walter, 2006; Jin et al., 2006; Senga et al., 2006). To test this hypothesis, we knocked down *Cul4*, *Ddb1*, *Cdt2* and *Pcna* in S2 cells by RNAi. We also knocked down the genes encoding the Cul1 ubiquitin ligase complex members (*Cul1*, *Skp1*, *Slmb*, *Skp2*, and *Ago*), because $Cul1^{Slmb}$ and $Cul1^{Skp2}$ are suggested to be involved in the destruction of *Drosophila* E2F1 and human E2F1, respectively (Heriche et al., 2003; Marti et al., 1999). In addition, the knockdown of *Rbf1* and *Dp* was tested, because they play central roles in the E2F/DP/RB pathway, and because it has been suggested that human E2F1 is protected from destruction by pRB (Campanero and Flemington, 1997; Hateboer et al., 1996; Hofmann et al., 1996).

For the RNAi experiment, stable S2 transfectants of *Hsp70*-controled GFP-$E2F1^{WT}$ were incubated with dsRNAs for 2 days and tested for the S phase-specific destruction (Figure 3.4A). The successful knockdown of the RNAi-target genes was confirmed by RT-PCR (Figure 3.4B) and Western blot analysis (Figure 3.4C). As shown in Figure 3.4A, RNAi against *Cul4*, *Cdt2*, and *Pcna* stabilized GFP-$E2F1^{WT}$ during S phase. In agreement with our PIP mutant data (Figure 3.2) and previous work on Cdt1 destruction (Arias and Walter, 2006; Jin et al., 2006; Senga et al., 2006), our RNAi data suggest that *Drosophila* E2F1 is degraded during S phase via E2F1's binding to PCNA, which triggers the action of the Cul4-based ubiquitin ligase that contains Cdt2 as a substrate receptor.

Surprisingly, the knockdown of *Ddb1*, which encodes the only known Cul4-associated adapter protein, did not result in E2F1 stabilization (Figure 3.4A) even though the

RNAi successfully reduced *Ddb1* transcript and DDB1 protein to very low levels (Figure 3.4B and C). We reason that it is either because the *Ddb1* knockdown was not sufficient, so that residual *Ddb1* can still handle E2F1 destruction, or because there is another unidentified adaptor protein that can form a functional ubiquitin ligase complex with Cul4 and Cdt2.

Another unexpected finding was that the knockdown of *Dp* stabilized E2F1 during S phase (Figure 3.4A). Although the reason for this is currently unknown, the stabilization may be a direct consequence of the failure to form an E2F1/DP heterodimer, or an indirect effect of reduced E2F1-target gene transcription (discussed below).

Rbf1 RNAi did not stabilize E2F1, consistent with the normal destruction of the RBF1-binding defective mutant $E2F1^{L786Q}$ (Figure 3.1C). From these results, we conclude that the S phase-specific destruction of E2F1 is independent of RBF1 in S2 cells.

No obvious stabilization of E2F1 was observed in S2 cells with RNAi against the components of the Cul1 ubiquitin ligase complex (*Cul1*, *Skp1*, *Slmb*, *Skp2* and *Ago*). *Cul1* and *Slmb* RNAi may have slightly stabilized E2F1 during S phase, indicated by the slight increase in S phase percentages in the GFP positive population. This is consistent with a previous report that suggests the involvement of $Cul1^{Slmb}$ in E2F1 destruction (Heriche et al., 2003). However, the data presented here suggests that $Cul4^{Cdt2}$ and PCNA play more significant roles in E2F1 destruction than the Cul1-based ubiquitin ligases do.

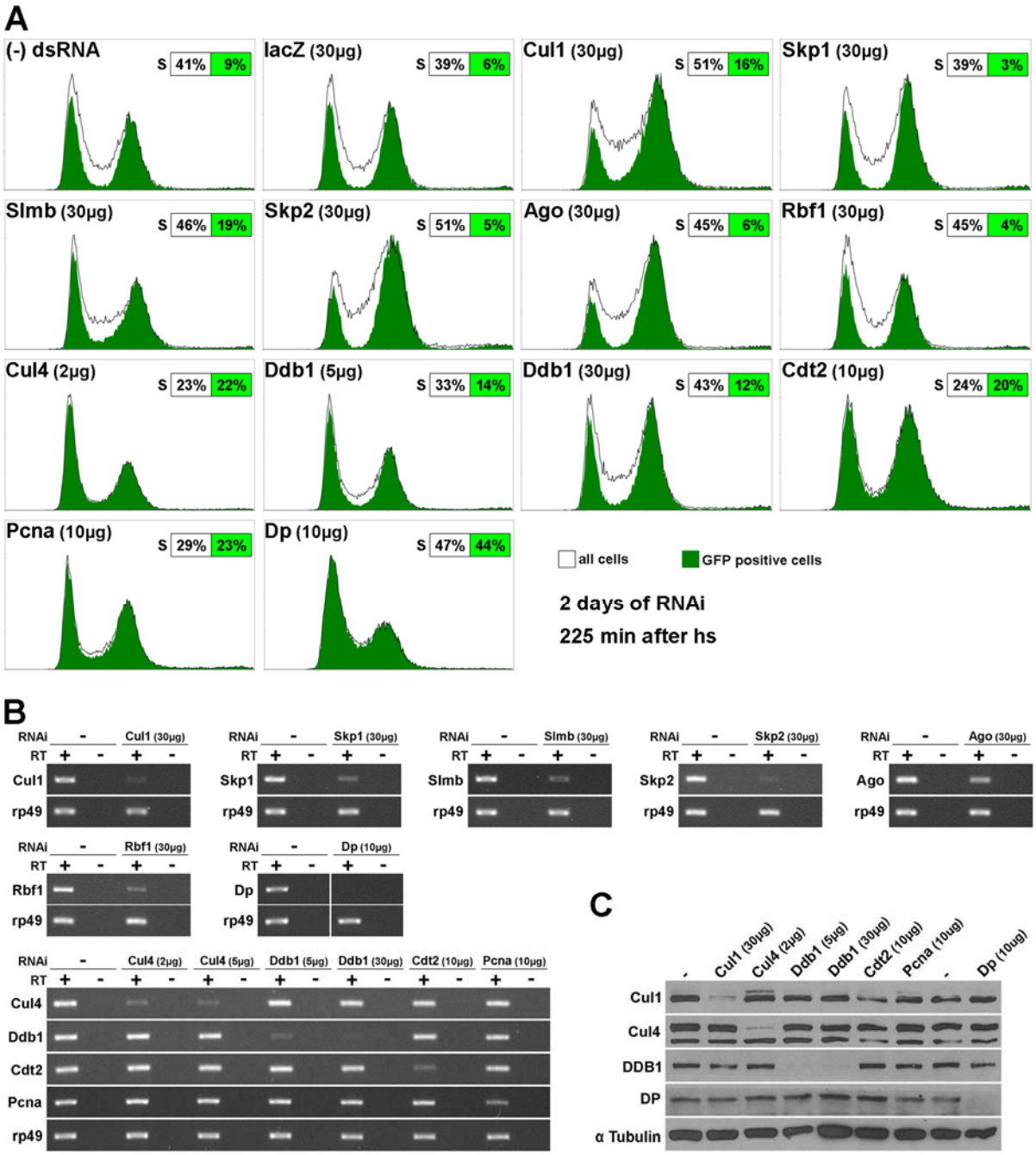

Figure 3.4: Knockdown of Cul4, Cdt2, PCNA, or DP stabilizes E2F1 during S phase in S2 cells

(A) Cell cycle profiles of S2 cells stably transfected to express GFP-E2F1 under the *Hsp70* promoter. Gene-specific dsRNAs of indicated amounts were added to cell culture. After 2 days of incubation with dsRNAs, GFP-E2F1^{WT} expression was induced by 30 min heat shock at 37˚C. Cells were fixed at 225 min post heat

shock. For the RNAi experiment, the GFP positive gate was determined as 99.97% (instead of 99.9%) of non-heat-shocked transfected cells are excluded from the gate.

(B) RT-PCR for the RNAi target genes. Mock templates without reverse transcriptase (RT-) were used to show the absence of genomic DNA contamination. *rp49* is shown as an internal control. Note that the RNAi for *Cul4*, *Ddb1*, *Cdt2* or *Pcna* does not affect the transcript levels of the other genes, suggesting that the effects of *Cul4*, *Cdt2* and *Pcna* knockdown on E2F1 destruction are independent with each other.

(C) Western blot of the RNAi target genes. α-tubulin was used as an internal control.

E2F1 interacts with Cul4 and Cdt2

To further examine the hypothesis that E2F1 is degraded through its binding to PCNA and to the Cul4 ubiquitin ligase complex, we employed a biochemical approach. myc-tagged E2F1 WT or PIP-3A were co-expressed with HA-tagged Cul4, Cdt2, or DP in S2 cells. Transfected cells were lysed and subjected to immunoprecipitation (IP) using an anti-myc antibody. The IPs and inputs were then analyzed by western blot using anti-myc and anti-HA antibodies (Figure 3.5). HA-Cul4 and HA-Cdt2 were co-immunoprecipitated with myc-E2F1$^{PIP\text{-}3A}$, as well as with myc-E2F1^{WT}, suggesting that the interaction between E2F1 and the Cul4^{Cdt2} complex is independent of E2F1's PIP box. These HA-constructs were not immunoprecipitated in the absence of myc-E2F1 co-expression, eliminating the possibility of non-specific interaction between the HA-constructs and anti-myc-conjugated beads.

Interestingly, the expression levels of myc-E2F1^{WT} and myc-E2F1$^{PIP\text{-}3A}$ were severely reduced when HA-DP was co-transfected. Also, the expression of HA-DP was reduced with the co-expression of myc-E2F1s. In spite of this, a significant amount of DP was co-immunoprecipitated with both myc-E2F1^{WT} and myc-E2F1$^{PIP\text{-}3A}$. Although the reason E2F1 expression levels are decreased by DP co-expression is currently unknown, this is consistent with the stabilization of E2F1 caused by DP knockdown (Figure 3.4A).

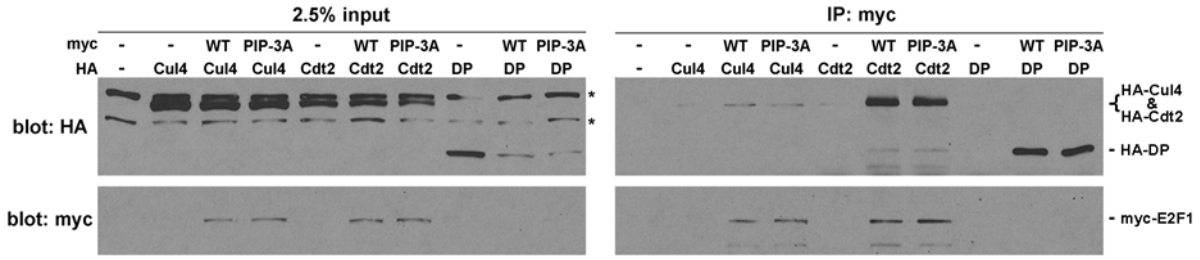

Figure 3.5: E2F1 interacts with Cul4 and Cdt2

Co-immunoprecipitation/Western blot showing interactions between myc-E2F1 (WT and PIP-3A) and HA-constructs (Cul4, Cdt2, and DP). S2 cells were transiently transfected to express myc-E2F1 and/or HA-constructs under the control of the *Actin* promoter. Transfected cells were lysed and immunoprecipitated for myc. Immunoprecipitates and 2.5% inputs were subjected to Western blot analysis using anti-HA or anti-myc antibodies. DP was used as a positive control as a known E2F1 binding protein. Asterisks indicate non-specific bands.

The PIP mutation stabilizes E2F1 during S phase in vivo

The S2 cell data suggests that E2F1 is degraded in a similar manner to Cdt1. To test if E2F1 is also degraded by the same mechanism in vivo, we created transgenic flies bearing UAS-GFP-*E2f1* WT, PIP-3A, PIP-7A or PIP-7del. These UAS flies were crossed to *engrailed* (*en*)-Gal4 driver flies to induce expression. In the embryo, GFP-E2F1^{WT} was degraded in the epidermal cells undergoing S phase of cycle 17 (Figure 3.6, arrows). In contrast, GFP-E2F1$^{PIP\text{-}3A}$ was stabilized in these S phase cells (Figure 3.6, arrowheads). Similar stabilization was also seen in GFP-E2F1$^{PIP\text{-}7A}$ and GFP-E2F1$^{PIP\text{-}7del}$ (data not shown). The result shows that E2F1 is degraded in the PIP box-dependent manner in vivo, as in S2 cells.

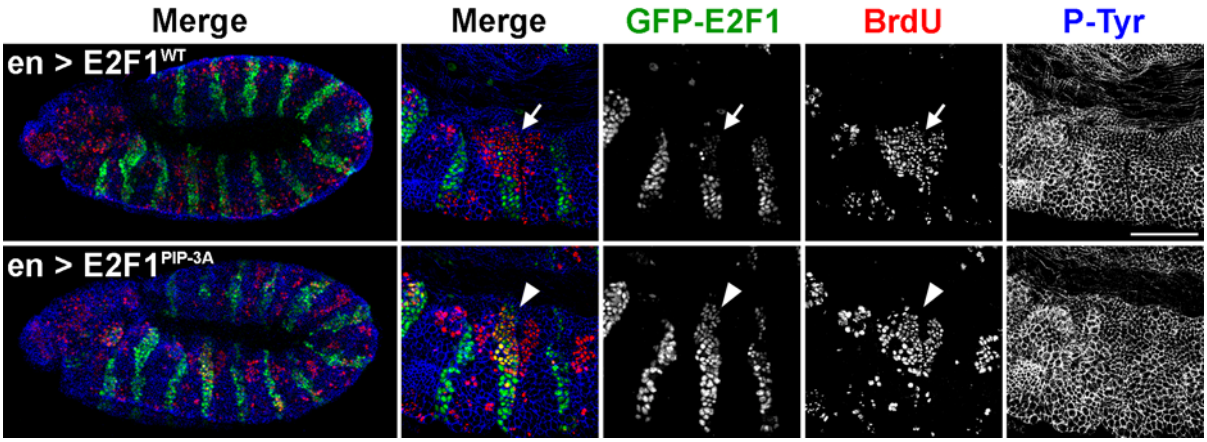

Figure 3.6. Mutations in the PIP box stabilize E2F1 during S phase in vivo

Embryos were pulse-labeled with BrdU for 5 min and stained for GFP (green), BrdU (red) together with phosphotyrosine (P-Tyr, blue) to visualize cell-cell boundaries. Embryos were collected from en>E2F1^{WT} (*en*-Gal4 mothers crossed to UAS-GFP-*E2f1*^{WT} males) and en > E2F1^{PIP-3A}. Arrows and arrowheads indicate a group of cells in the first and second thoracic segments undergoing S phase of cycle 17 (S_{17}). Note that GFP-E2F1 is degraded in S_{17} (arrows), whereas GFP-E2F1^{PIP-3A} is stabilized (arrowheads).

GFP-E2F1^{WT} and GFP-E2F1^{PIP} are functional as a transcription factor in vivo

Given that PIP mutant E2F1 is stabilized during S phase, we then examined the effect of overexpressing GFP-E2F1^{WT} or GFP-E2F1^{PIP} on the expression of E2F1-taget genes. To visualize E2F1's transcriptional activity, we stained embryos for transcripts of *RnrS*, a well-characterized E2F1-target gene (Dimova et al., 2003; Duronio and O'Farrell, 1994). Using *paired* (*prd*)-Gal4 driver, GFP-E2F1^{WT} or GFP-E2F1^{PIP} was overexpressed in seven stripes in the embryo. In these stripes, ectopic *RnrS* was induced by GFP-E2F1^{WT} or GFP-E2F1^{PIP-3A} (Figure 3.7A, top row). GFP-E2F1^{PIP-3A} induced slightly higher levels of *RnrS* than GFP-E2F1^{WT} does, when overexpressed with *prd*-Gal4. This tendency was more obvious when *armadillo* (*arm*)-Gal4 was used. *arm*-Gal4 induces ubiquitous expression of UAS-controlled genes throughout the embryo. The *arm*-Gal4-driven expression of GFP-E2F1^{WT} and GFP-

E2F1$^{PIP\text{-}3A}$ induced ectopic *RnrS* mainly in the midgut, a tissue that undergoes an endocycle (Figure 3.7A, middle and bottom rows). In the midgut of a wild type embryo at stage 14, *RnrS* expression is prominent in the central midgut, where cells are undergoing synchronous S phase (Figure 3.7A and 6B, wild type). The overexpression of GFP-E2F1^{WT} or GFP-E2F1$^{PIP\text{-}3A}$ induced ectopic *RnrS* in the anterior and posterior midgut. Notably, the induction levels of *RnrS* were significantly higher in GFP-E2F1$^{PIP\text{-}3A}$-expressing embryos than that of GFP-E2F1^{WT}-expressing ones. This tendency was consistently observed in four independent lines of UAS-GFP-*E2f1*^{WT} and UAS-GFP-*E2f1*$^{PIP\text{-}3A}$ each (data not shown). Furthermore, one line from UAS-GFP-*E2f1*$^{PIP\text{-}7A}$ and UAS-GFP-*E2f1*$^{PIP\text{-}7del}$ each was tested, and we observed similar levels of *RnrS* induction to UAS-GFP-E2f1$^{PIP\text{-}3A}$ (data not shown). These results suggest that GFP-E2F1^{WT} and GFP-E2F1^{PIP} are functional as a transcription factor in vivo, and that the stabilization of E2F1 by the PIP mutation enhances the ectopic induction of E2F1 target genes.

However, in the epidermis of late stage embryos (stage 12-14), *RnrS* transcripts were normally downregulated even with GFP-E2F1^{WT} or GFP-E2F1$^{PIP\text{-}3A}$ (Figure 3.7A for *arm*-Gal4 and data not shown for *prd*-Gal4). We attribute the absence of increased *RnrS* in late embryogenesis to the developmentally-regulated activation of RBF1 (Du and Dyson, 1999) and the limited amount of endogenous DP. Indeed, it has been shown that the overexpression of E2F1 by itself will not induce *RnrS* in late embryogenesis, unless DP is co-expressed (Duronio et al., 1996). In addition, there may be unidentified downregulation mechanisms which contribute to the absence of ectopic *RnrS* induction in the epidermis.

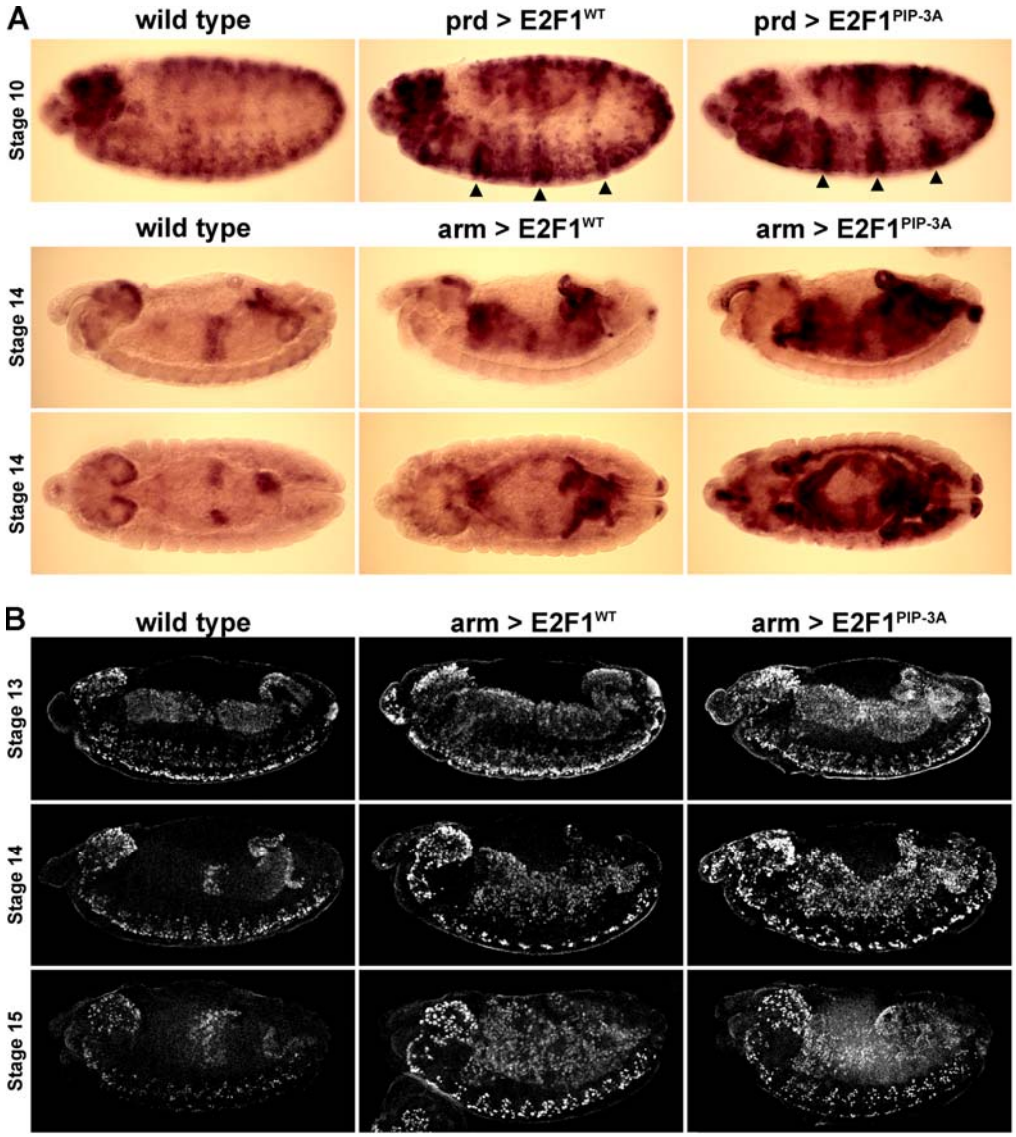

Figure 3.7: GFP-E2F1^{WT} and GFP-E2F1^{PIP} are functional as a transcription factor

(A) Embryos were stained for *RnrS* transcripts by in situ hybridization. Embryos were collected from wild type (w^{1118}), prd>E2F1^{WT} (*prd*-Gal4 mothers crossed to UAS-GFP-*E2f1* males), prd>E2F1^{PIP-3A}, arm>E2F1^{WT}, or arm>E2F1^{PIP-3A}.

Stage 10 embryos (top row) and stage 14 embryos (middle row, lateral views; bottom row; sagital views) are shown. Arrowheads show the induction of *RnrS* in cells where the *prd* promoter is activated.

(B) Embryos were pulse-labeled with BrdU and stained for BrdU incorporation. Embryos were collected from wild type, arm>E2F1^{WT}, or arm>E2F1^{PIP-3A}. Expression of GFP-E2F1^{WT} and GFP-E2F1^{PIP-3A} was confirmed by GFP staining (not shown).

The progression of endocycles in the gut is disturbed by the overexpression of GFP-E2F1^{WT} or GFP-E2F1^{PIP}

We next asked if the overexpression of GFP-E2F1^{WT} or GFP-E2F1^{PIP} leads to cell cycle defects. To address this question, we examined embryos stained for BrdU incorporation and for phospho-histone H3 (PH3), a mitosis marker. However, at least until stage 14, we did not detect ectopic S phase or mitosis in the epidermis overexpressing GFP-E2F1^{WT} or GFP-E2F1^{PIP} with *prd*-Gal4 or *arm*-Gal4 (data not shown). Although we did not detect defects in the mitotic cycle of the embryonic epidermal cells, the endocycle in the midgut was disturbed when GFP-E2F1^{WT} or GFP-E2F1^{PIP} was overexpressed with *arm*-Gal4 (Figure 3.7B and data not shown for PIP-7A and PIP-7del). This is consistent with the ectopic *RnrS* induction caused by GFP-E2F1^{WT} or GFP-E2F1^{PIP} (Figure 3.7A). In the embryo overexpressing GFP-E2F1^{WT} or GFP-E2F1^{PIP}, the S phase pattern was overall normal at stage 13 (Figure 3.7B). However, a synchronous gap phase that is seen in the anterior and posterior midgut of stage 14 and 15 wild type embryos was absent in the embryos overexpressing GFP-E2F1^{WT} or GFP-E2F1^{PIP-3A}. This result implies that proper endocycle progression requires the downregulation of E2F1 activity, as further examined in the larval salivary gland, another endocycling tissue (see discussion).

Stabilization of E2F1 during S phase activates an apoptotic pathway

Since we did not detect cell cycle defects in mitotic cycles of embryogenesis, we focused on the wing disc of 3rd instar larva. The cells in the wing disc undergo G1-S-G2-M mitotic cell cycles and will have been exposed to GFP-E2F1 overexpression for a longer time than embryos. We overexpressed GFP-E2F1^{WT} or GFP-E2F1^{PIP-3A} using *en*-Gal4, which

induces expression only in the posterior half of the wing disc. This allows us to compare the transgene-overexpressing posterior half and the non-expressing anterior half within one disc.

To ask whether GFP-E2F1^{WT} or GFP-E2F1^{PIP} induces ectopic mitosis index, we stained discs for PH3. We observed slight increase in PH3 positive nuclei in GFP-E2F1^{PIP-3A}-overexpressing cells (data not shown). This induction of mitosis may be underestimated since the overexpression of GFP-E2F1^{PIP-3A} induced massive apoptosis, as shown by the induction of cleaved Caspase-3 (apoptosis marker) (Figure 3.8A). Interestingly, the overexpression of GFP-E2F1^{WT} did not induce apoptosis, suggesting that the apoptosis is caused by the stabilization of E2F1 during S phase.

Furthermore, consistent with increased apoptosis, the morphology of the adult wing was disturbed in the posterior half of GFP-E2F1^{PIP-3A}-overexpressing adult flies, in contrast to normal morphology of GFP-E2F1^{WT}-overexpressing wings (Figure 3.8C). The severity of wing phenotype varies from mild (small size of the posterior wing, Figure 3.8C, third panel) to severe (shrunken wing morphology often with a blister in the posterior side, Figure 3.8C, bottom panel). This phenotype appears dependent on the environment; flies from crowded vials tend to have less severe phenotype. In this case, we often observed normal wing morphology even with the overexpression of GFP-E2F1^{PIP-3A} (data not shown). Nevertheless, these findings suggest that the S phase-specific degradation of E2F1 is required for the normal development of fly tissues, most likely because the ectopic expression of E2F1 during S phase activates apoptotic pathways.

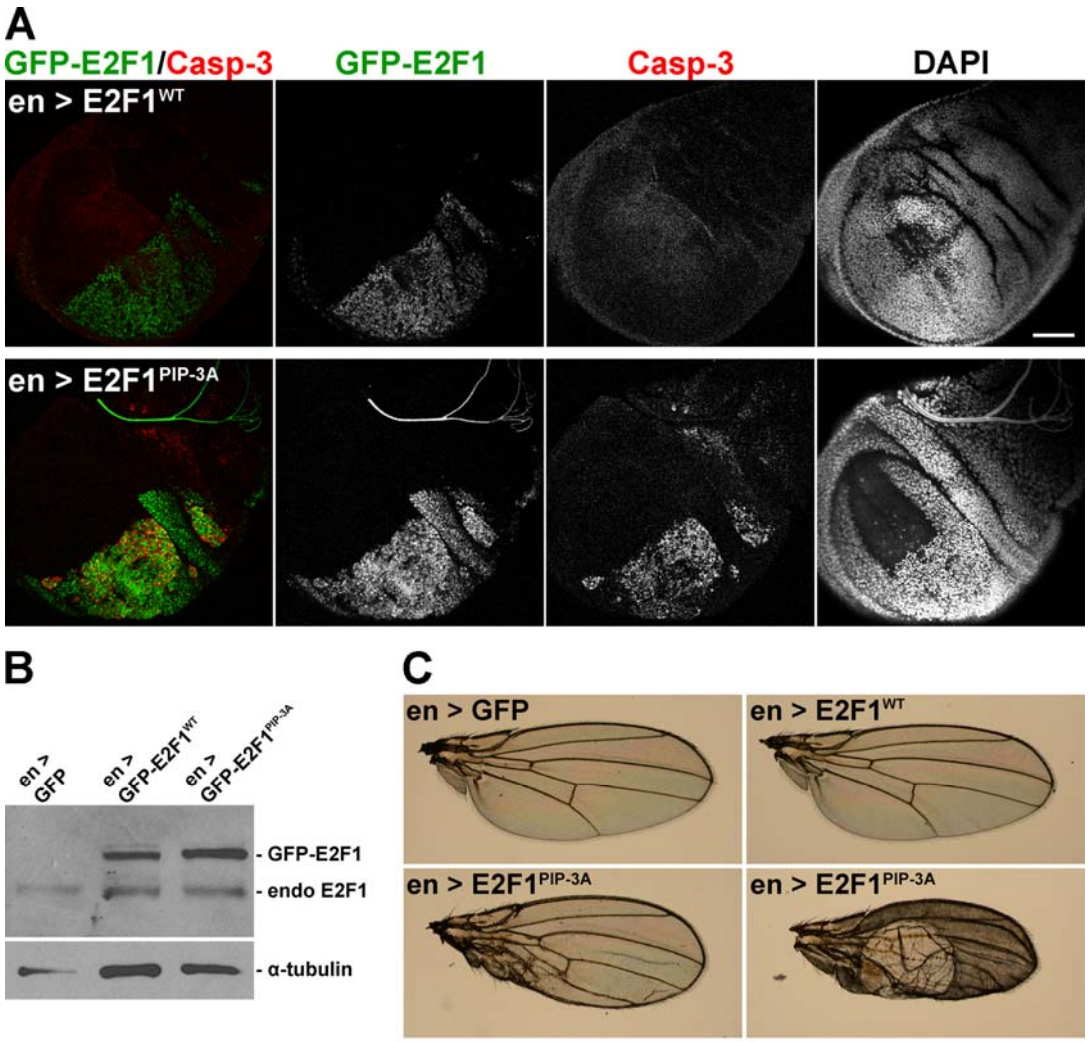

Figure 3.8: Stabilization of E2F1 during S phase induces apoptosis

(A) Wing discs from 3rd instar larvae were stained for cleaved Caspase-3 (red) and DNA (DAPI). Transgene expression is shown by GFP fluorescence (green). Anterior: top right. Posterior: bottom left. Note that Casp-3-positive cells are piled on the surface of the disc, as shown in the DAPI staining. These cells contain small and condensed nuclei, which is a characteristic of apoptotic cells.

(B) Wing discs dissected from 3rd instar larvae of en>GFP (*en*-Gal4 mothers crossed to UAS-GFP males), en>E2F1^{WT}, or en>E2F1^{PIP-3A} were lysed, and 5 µg of total protein was subjected to Western blot analysis. α-tubulin was blotted as a loading control.

(C) Wings of adult flies born from en>GFP, en>E2F1^{WT}, or en>E2F1^{PIP-3A}.

The morphology of the posterior side of the wing is disturbed in E2F1^{PIP-3A}-expressing flies. A wing with a moderate phenotype (left bottom panel) and severe phenotype (right bottom panel) are shown.

Discussion

In this chapter, we showed that *Drosophila* E2F1 is degraded during S phase in a PCNA-, Cul4-, and Cdt2-dependent manner, which resembles the destruction mechanism of Cdt1. (Arias and Walter, 2006; Jin et al., 2006; Senga et al., 2006). It is suggested that the interaction between Cdt1 and PCNA triggers the ubiquitination of Cdt1 by $Cul4^{DDB1\text{-}Cdt2}$. Since PCNA forms homotrimers around the DNA strand during S phase (Moldovan et al., 2007), this homotrimer may provide an S phase-specific binding site for Cdt1. Our data shown here suggests that a common mechanism is used for the destruction of *Drosophila* E2F1.

Normal E2F1 destruction in DDB1-depleted cells

Given that E2F1 is stabilized by RNAi against *Pcna*, *Cul4* or *Cdt2*, we were surprised that *Ddb1* RNAi did not stabilize E2F1 (Figure 3.4). DDB1 is the major adapter protein that mediates the assembly of Cul4 and Cul4-associated substrate receptors named DCAFs including Cdt2 into an active ubiquitin ligase (Jin et al., 2006). Indeed, DDB1 is required for the destruction of Cdt1 (Arias and Walter, 2006; Jin et al., 2006; Senga et al., 2006). The reason for the absence of E2F1 stabilization in DDB1-depleted cells is currently unknown. Although our RNAi depleted *Ddb1* mRNA and DDB1 protein almost to background levels (Figure 3.4B, C), it is possible that a very little amount of residual DDB1 is sufficient to handle E2F1 destruction. Alternatively, there may be other Cul4- and DCAF-associated adapter proteins that have not been identified.

DP-dependent, CDK- and RBF1-independent destruction of E2F1

Unexpectedly, the RNAi against *Dp* stabilized E2F1 during S phase (Figure 3.4A), and the co-expression of DP destabilized E2F1 (Figure 3.5). DP is a binding partner of E2F1, and E2F1/DP heterodimerization enhances their ability to bind DNA (Dynlacht et al., 1994). One possibility is that E2F1/DP induces transcription of genes involved in E2F1 destruction (e.g. Cdt2), so that DP indirectly enhances the destruction of E2F1. Another possibility is that the destruction of E2F1 directly requires DP-binding. In this case, E2F1's DNA-binding activity, which is facilitated by E2F1/DP dimerization, may be required for E2F1 destruction. However, this hypothesis is at a glance in conflict with the data that the 1-230, 93-184, and 139-230 fragments of E2F1 can be degraded during S phase, even though these N-terminal fragments do not contain either the DNA-binding domain or the DP-dimerization domain (Figure 3.1). This conflict can be formally reconciled by introducing a hypothetical signal sequence which inhibits E2F1 destruction. The data presented here can be explained if this hypothetical signal resides in the region C-terminal to 231 (231-805), and can be inactivated by DP-binding. In this model, the inhibitory signal in the full length E2F1 is suppressed by DP-binding, allowing the PIP-dependent destruction of E2F1. On the other hand, the 1-230 fragment can be degraded whether DP is present or not, since it does not contain the inhibitory signal.

In contrast, mutations in the consensus CDK phosphorylation sites did not result in significant stabilization of E2F1 (Figure 3.1, 3.2, and 3.3). Thus, the direct phosphorylation of E2F1 by CDK does not appear to be the major mechanism involved in E2F1 destruction. However, since CycE/CDK2 induces S phase entry and E2F1 is degraded in an S phase-dependent manner, CycE/CDK2 indirectly downregulates E2F1 via the induction of S phase. Previously, Reis et al. showed that increased CycE/CDK2 activity in the wing disc results in

the downregulation of *E2f1* transcripts (Reis and Edgar, 2004). Therefore, CycE/CDK2 can downregulate E2F1 levels at least in two ways: the transcriptional repression of the *E2f1* gene, and the induction of S phase entry, which in turn induces E2F1 destruction.

Previous studies have shown that human E2F1 can be protected by the co-expression of pRB (Campanero and Flemington, 1997; Hateboer et al., 1996; Hofmann et al., 1996). However, in our experiments a mutation in the RBF1-binding site of E2F1 ($E2F1^{L786Q}$) and the knockdown of *Rbf1* did not affect the destruction of E2F1 (Figure 3.1, 3.2, 3.3, and 3.4). Moreover, the overexpression of $RBF1^{280}$, an active form of RBF1 that has mutations in four CDK phosphorylation sites, did not stabilize E2F1 in the embryo (data not shown). This RBF1-independent destruction of *Drosophila* E2F1 may simply be explained by the fact that *Drosophila* E2F1 and human E2F1 are degraded by different machineries (Cul4 vs. Cul1). It is possible that the degradation of human E2F1 by the $Cul1^{SKP2}$ complex (and possibly by other ubiquitin ligase complexes) requires the access to E2F1's C-terminus, which can be blocked by the binding of pRB to E2F1's C-terminus.

E2F1-induced apoptosis

A large body of evidence suggests that activator E2Fs are positive regulators of apoptosis (Iaquinta and Lees, 2007). However, E2F-induced apoptosis is highly context-dependent, and little is known about the mechanism that underlies this context-dependency (Hallstrom et al., 2008; Iaquinta and Lees, 2007; Moon et al., 2006; Moon et al., 2005). Our data presented here shows that the expression of PIP mutant E2F1 induces massive apoptosis, whereas wild type E2F1 do not induce any apoptosis (Figure 3.8). This suggests that the stabilization of E2F1 during S phase activates apoptotic pathways. An interesting hypothesis

is that cells possess an S phase-specific sensor to detect ectopic E2F1 activity. When E2F activity remains high in S phase, cells may detect it and activate apoptotic pathways. Otherwise, elevated E2F1 activity may cause overproliferation. Moreover, failure to downregulate E2F1 activity during S phase may cause unwanted DNA re-replication since E2F1 can induce origin licensing factors such as $Cdt1^{Dup}$ and Cdc6.

Alternatively, E2F1-induced re-replication may be the cause of apoptosis. Although the overexpression of $E2F1^{WT}$ or $E2F1^{PIP}$ performed here appeared to have no detectable effect on mitotic cell cycles, it is possible that cells are so sensitive to re-replication that we could not detect obvious cell cycle defect before cells die by apoptosis. Future experiments will elucidate the mechanism underlying S phase-specific, E2F-induced apoptosis.

Significance of E2F1 destruction in endocycles

In the embryonic gut, E2F1 overexpression induced ectopic expression of *RnrS*, an E2F1-target gene. As a probable consequence, the synchronous cell cycle progression of the endocycle was disrupted (Figure 3.7). We did not observe obvious differences between the cell cycle phenotypes caused by GFP-$E2F1^{WT}$ and GFP-$E2F1^{PIP}$, at least until stage 15. This may be due to the high sensitivity of embryonic gut cells to increased E2F1 levels. However, a critical difference has been observed between GFP-$E2F1^{WT}$ and GFP-$E2F1^{PIP}$ when overexpressed in the larval salivary gland (Figure 3.9). The cells in the salivary gland undergo asynchronous endocycles to achieve very high levels of polyploidy (~2048C) (Edgar and Orr-Weaver, 2001), making the larval salivary gland an excellent experimental model to study endocycle regulation. Previous studies have shown that the oscillation of CycE/CDK2 activity is required for endocycle progression in this tissue (Follette et al., 1998; Weiss et al.,

1998). Consistently, low CDK activity during early G1 is thought to allow the assembly of the pre-replication complex, which is required for the origin firing in the following S phase (Blow and Dutta, 2005). However, the mechanism that generates this oscillation of CycE/CDK2 has not been fully understood. The data presented here provides a model for the regulation of an endocycle; E2F1 induces CycE expression, which induces S phase entry. During S phase E2F1 is downregulated by proteolysis, resulting in CycE downregulation. This simple regulatory system composed of E2F1, CycE/CDK2, and DNA replication may be the central mechanism that regulates endocycles in this tissue and possibly in other tissues (Kim et al. manuscript in preparation).

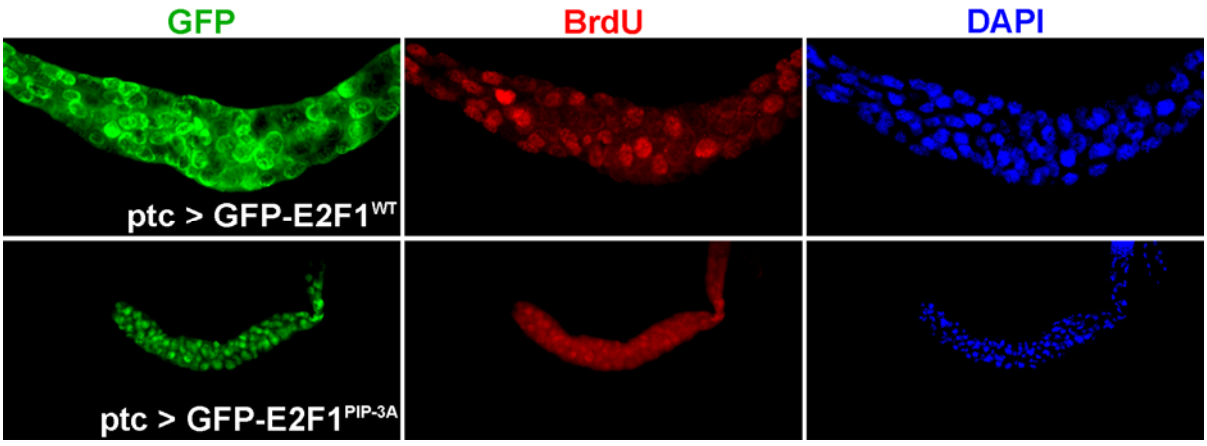

Figure 3.9: Stabilization of E2F1 in the larval salivary gland arrests the cell cycle

GFP-E2F1^{WT} or GFP-E2F1$^{PIP\text{-}3A}$ (green) was expressed in the salivary gland with *ptc*-Gal4 driver. Salivary glands were dissected from 3rd instar larvae, pulse-labeled with BrdU (red), and stained with DAPI (blue). Note that GFP-E2F^{WT} is degraded in BrdU positive nuclei (top panels). In contrast, GFP-E2F1$^{PIP\text{-}3A}$-expressing salivary glands show reduced BrdU incorporation, resulting in decreased polyploidy and small size of the tissue. (Courtesy of Dr. Bruce A. Edgar)

Taken together, our data indicate that E2F1 degradation is dependent on DNA replication and requires a Cul4-based ubiquitin ligase. This provides a model of negative

feedback mechanism where E2F1 induces DNA replication, which in turn downregulates E2F1. This negative feedback is required for preventing apoptosis in a mitotic cycle (larval wing disc) and for the cell cycle progression of an endocycle (larval salivary gland). In addition, the $E2F1^{PIP}$ mutants created in this study will provide a useful tool for future experiments. It is also interesting to investigate whether the destruction of activator E2Fs is required for normal cell cycle progression in other species.

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CHAPTER IV

E2F1-INDEPENDENT REGULATION OF *RNRS* TRANSCRIPTION DURING EARLY EMBRYOGENESIS

Preface

This chapter describes a project on early expression of the E2F1-target gene *RnrS* that I initiated and that will be continued by others in the lab. I performed the majority of experiments shown in this chapter. Pam Gasdaska, a research technician in the lab, molecularly mapped the deleted regions of *Df(3R)sr16* and sr^{461} mutants. (Figure 4.4) She also examined the expression of *CG14317* transcripts (data not shown).

Abstract

During *Drosophila* embryogenesis, DNA replication-related, E2F1-target genes are expressed specifically in cells that are actively replicating DNA. It has been assumed that the expression of these genes is regulated mainly by E2F1. However, here I describe a genomic locus that is required for the early zygotic expression of *RnrS*, a gene well-characterized as an E2F1-target gene. A deletion of the locus does not affect E2F1 expression, suggesting an E2F1-independent mechanism of early zygotic *RnrS* expression. Using an overlapping set of deletions in the region we identified 19 candidate genes for the *RnrS* phenotype.

Introduction

During the early embryogenesis of *Drosophila*, E2F1-target genes such as *RnrS*, *Pcna*, *CycE*, and *DNA polymerase alpha* are expressed continuously during the G2-regulated, post-blastoderm cell cycles, which lack G1 phase. This expression is thought to facilitate the rapid cell cycle during early embryogenesis. Although this expression pattern of these E2F1-target genes has been described in detail (Duronio and O'Farrell, 1994; Lee and Orr-Weaver, 2003), the responsible mechanisms remain to be understood.

As shown in Figure 4.1, embryos contain maternal supplies of these transcripts at the beginning of embryogenesis (Figure 4.1, stage 1). These maternal supplies are cleared from the embryo by cycle 14 (Figure 4.1, stage 5). For the rest of embryogenesis, the transcripts of E2F1-target genes are transcribed from zygotic loci (Figure 4.1, stage 6-14).

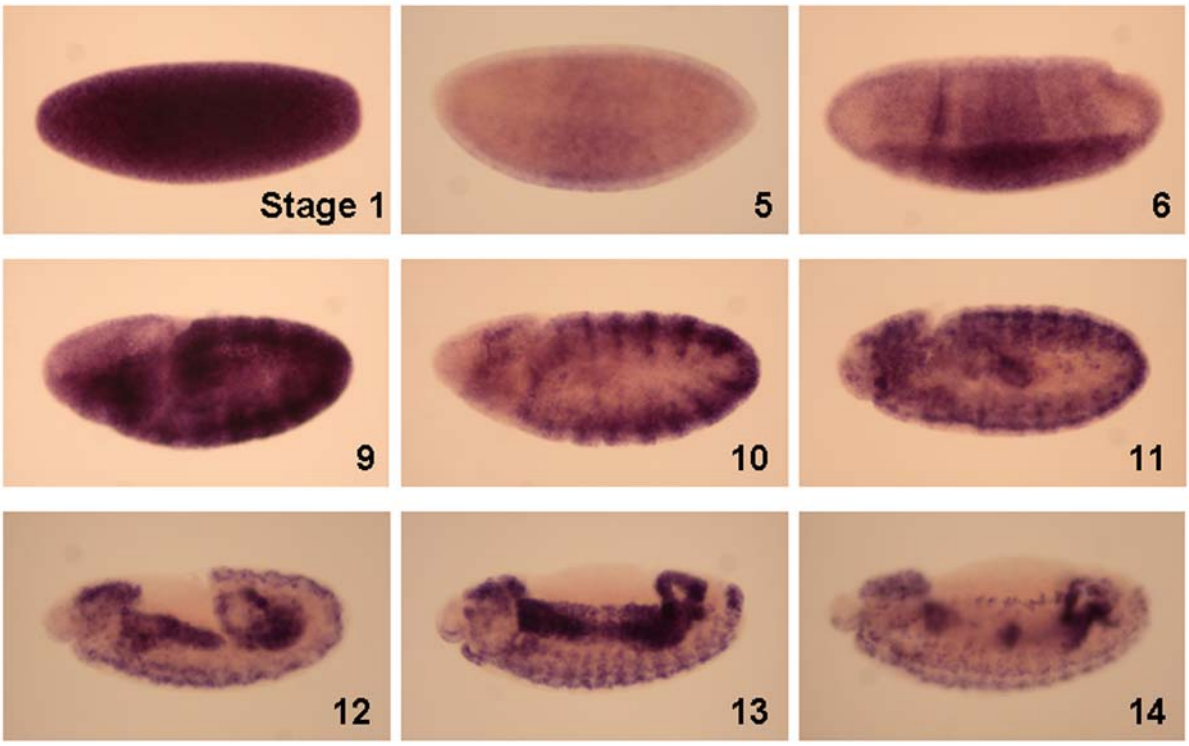

Figure 4.1: The expression pattern of *RnrS* transcripts during *Drosophila* embryogenesis

Wild type embryos were stained for *RnrS* transcripts by in situ hybridization. Stages are determined by the morphology of the embryo and the staining pattern of *RnrS*.

In *E2f1* zygotic mutant embryos, the zygotic transcription of E2F1-target genes is severely reduced in late embryogenesis (late stage 11~) (Duronio et al., 1995). However, these transcripts are expressed at normal levels during the early embryogenesis (stage 6-11) of *E2f1* mutants (Figure 2.5) (Duronio et al., 1995). One possible explanation is that maternal E2F1 protein is sufficient to induce early expression of E2F1-target genes even in the absence of zygotic E2F1. Indeed, residual maternal E2F1 protein can be observed in *E2f1* zygotic mutants until it is degraded during S phase of cycle 15 (stage 8-9) (Figure 2.5). Although the residual E2F1 protein is very little, it may be sufficient to induce the initial transcription of E2F1-target genes. Alternatively, the early transcription of E2F1-target genes

(i.e. stages 6-10) may be independent of E2F1 and regulated by other mechanisms. E2F1-independent regulation of E2F1-target genes may appear counterintuitive because these E2F1-target genes are all very sensitive to E2F1 levels, which provides a model whereby cell cycle genes are coordinately expressed in a timely manner by one key factor (E2F1). However, in a screening experiment, I found a genomic locus that is required for the early zygotic transcription of *RnrS*. This finding raises the possibility that the early expression of *RnrS* is independent of E2F1.

Materials and Methods

Drosophila strains

Df(3R)ED5780, *Df(3R)Exel6177*, *Df(3R)Exel6178*, *Df(3R)DG2*, *Df(3R)DG4*, *Df(3R)P14*, *Df(3R)sr16*, *Df(3R)LK19-1*, *Df(3R)Cha7*, $cpo^{BG02810}$, cpo^{01432}, $DNaseII^{lo}$, $DNaseII^{B571}$, $CG7785^{f06154}$, htl^{d07110}, sr^{461}, sr^{155}, and sr^{03999} were obtained from the Bloomington Stock Center.

RNA in situ hybridization and immunostaining

In situ hybridization and immunostaining was performed as described (Shibutani et al., 2007). Embryos were dechorionated with 50% bleach, fixed in a 1:1 mixture of 4% formaldehyde in PBS/heptane for 25 min at room temperature, and devitellinized with methanol. Embryos were stored in methanol at -20˚C. A digoxigenin-labeled *RnrS* probe was synthesized by transcribing antisense RNA from a full length *RnrS* cDNA (DGC collection clone LD41588, obtained from Open Biosystems). For the E2F1/RnrS/phosphotyrosine triple staining, the TSA Fluorescence System (Perkin Elmer) was used for detection of E2F1 and

RnrS. Antibodies used are: rabbit anti-E2F1 (1:500, gift from Dr. Maki Asano), goat anti-rabbit-biotin (1:1000, Chemicon), mouse anti-phosphotyrosine (1:1000, Upstate), and donkey anti-mouse-Cy5 (1:500, Jackson). Stained embryos were mounted with Fluoromount-G (Southern Biotech) and visualized with a Nikon Eclipse E800 microscope or a Zeiss LSM 510 scanning confocal microscope. Images were consistently modified with Photoshop® CS2 9.0 (Adobe)

Results

I performed a screening experiment using *Drosophila* deficiency mutants with the intention of identifying a protein phosphatase 1 (PP1) subunit required for normal regulation of E2F1-target genes during embryogenesis. We performed the screening based on the hypothesis that RBF1 is activated by a PP1 complex during late embryogenesis, resulting in the suppression of E2F1-target genes. This initial hypothesis was disproved by Lisa Swanhart, a former graduate student in the lab who showed that PP1 is not required for the suppression of E2F1-target genes during embryogenesis (Swanhart et al 2007). Nevertheless, in the screening I found a deficiency mutant that shows decreased *RnrS* transcription during early embryogenesis (Figure 4.2, Df(3R)DG2). In this mutant, *RnrS* transcripts were severely reduced during early embryogenesis (stage 6-11). Interestingly, *RnrS* expression in the mutant was normal in the gut and central nervous system in late embryogenesis (Figure 4.2). In addition, when the mutant embryos were stained for transcripts of other E2F1-target genes (*RnrL*, *CycE*, *Pcna*, *Mcm2*, and *dup*), I observed normal levels of these transcripts (data not shown). In the mutant, neither the *RnrS* gene nor the *E2f1* gene is deleted. In addition, E2F1 protein levels were normal (Figure 4.3), suggesting that E2F1 is not sufficient to induce early

zygotic *RnrS*. Taken together, the early zygotic expression of *RnrS* was severely reduced in the mutant in an E2F1-independent manner, and this phenotype is specific for *RnrS* expression among E2F1-target genes tested.

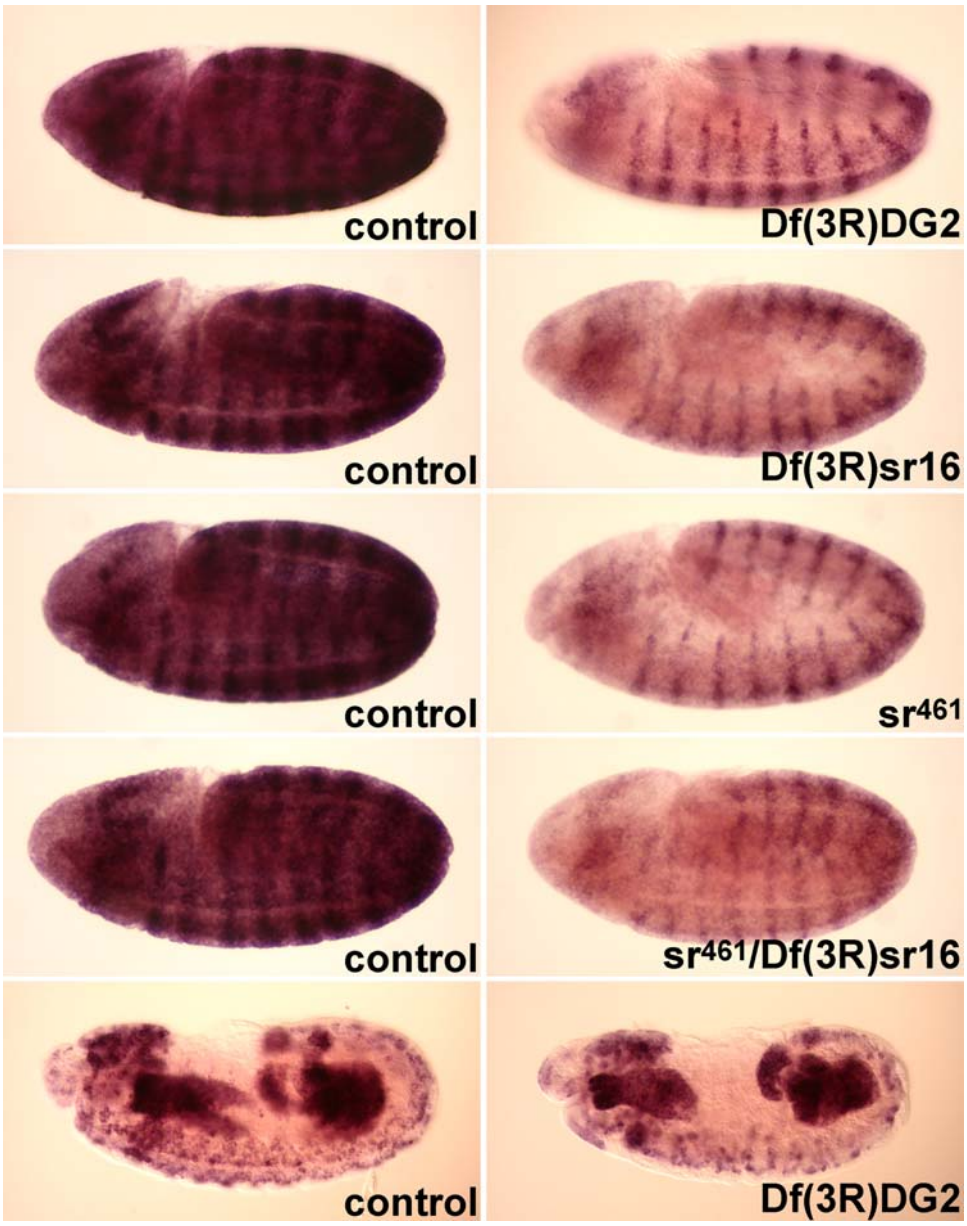

Figure 4.2: *RnrS* phenotypes in deficiency mutants

Embryos were stained for *RnrS*. In each row, the left panel is a sibling control of the homozygous mutant in the right panel. Top four rows show stage 10 embryos, and the bottom row shows stage 12 embryos.

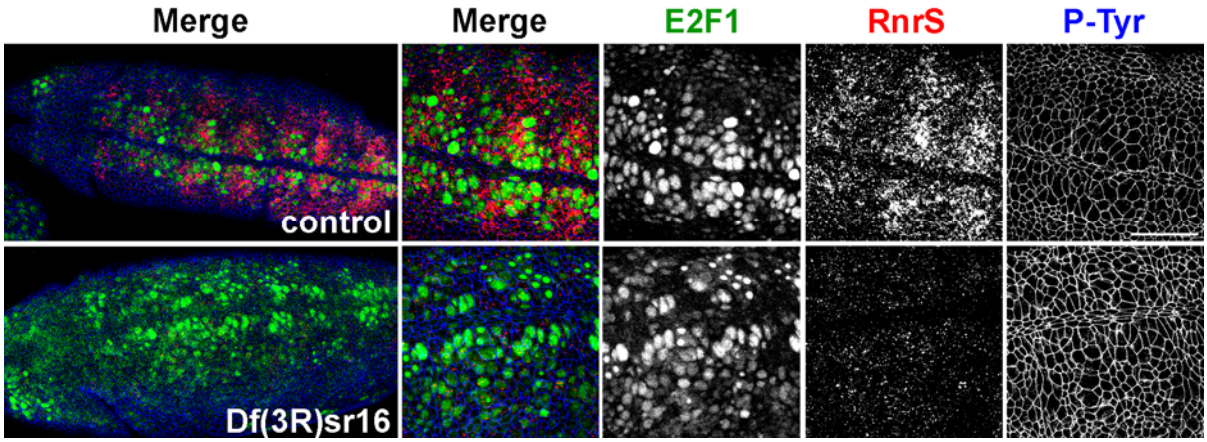

Figure 4.3: E2F1 expression in the mutant embryo

Embryos were stained for E2F1 (green), *RnrS* (red), and phosphotyrosine (P-Tyr, blue). A embryo homozygous for *Df(3R)sr16* (bottom panels) and a sibling control embryo (top panels) are shown. Note that the expression of E2F1 is not affected in the mutant. Scare bar: 50μm.

To narrow down the region responsible for the phenotype, we examined embryos of various other deficiency mutants. The result is summarized in Figure 4.4. Of the deficiency mutants tested, the DG2, P14, sr16, and DG4 deficiency mutants showed decreased *RnrS* expression, whereas the others (ED5780, Exel6117, LK19-1, Exel6178, and Cha7) did not show the phenotype. The Exel6177 and Exel6178 deficiency mutants, both of which did not show the *RnrS* phenotype, are annotated with the exact break points. The Exel6177 deficiency eliminates *CG14318*, but not the next gene *CG7713*. The Exel6178 deficiency eliminates the whole open reading frame of *CG7215* and the translation start site of *CG7217*, but does not eliminate the next gene, *Cbp20*. Thus, we can accurately narrow down the region of interest to the 23 genes from *CG7713* (locus = 90C5) to *Cbp20* (90E7). From these 23 genes, we could further eliminate 4 genes (*CG14316* to *Cbp20*) by PCR-based mapping of the sr16 deficiency (data not shown), leaving 19 genes (*CG7713* to *stripe* (*sr*)) as possible candidate genes. Furthermore, the DG4 deficiency, which shows the *RnrS* phenotype, has

been cytologically mapped to 90D2-4/90F3-6. From this, we inferred that 7 genes from *couch potato* (*cpo*) (90D1-6) to *sr* (90E2-4) are the most likely candidate genes for the *RnrS* phenotype (Figure 4.4).

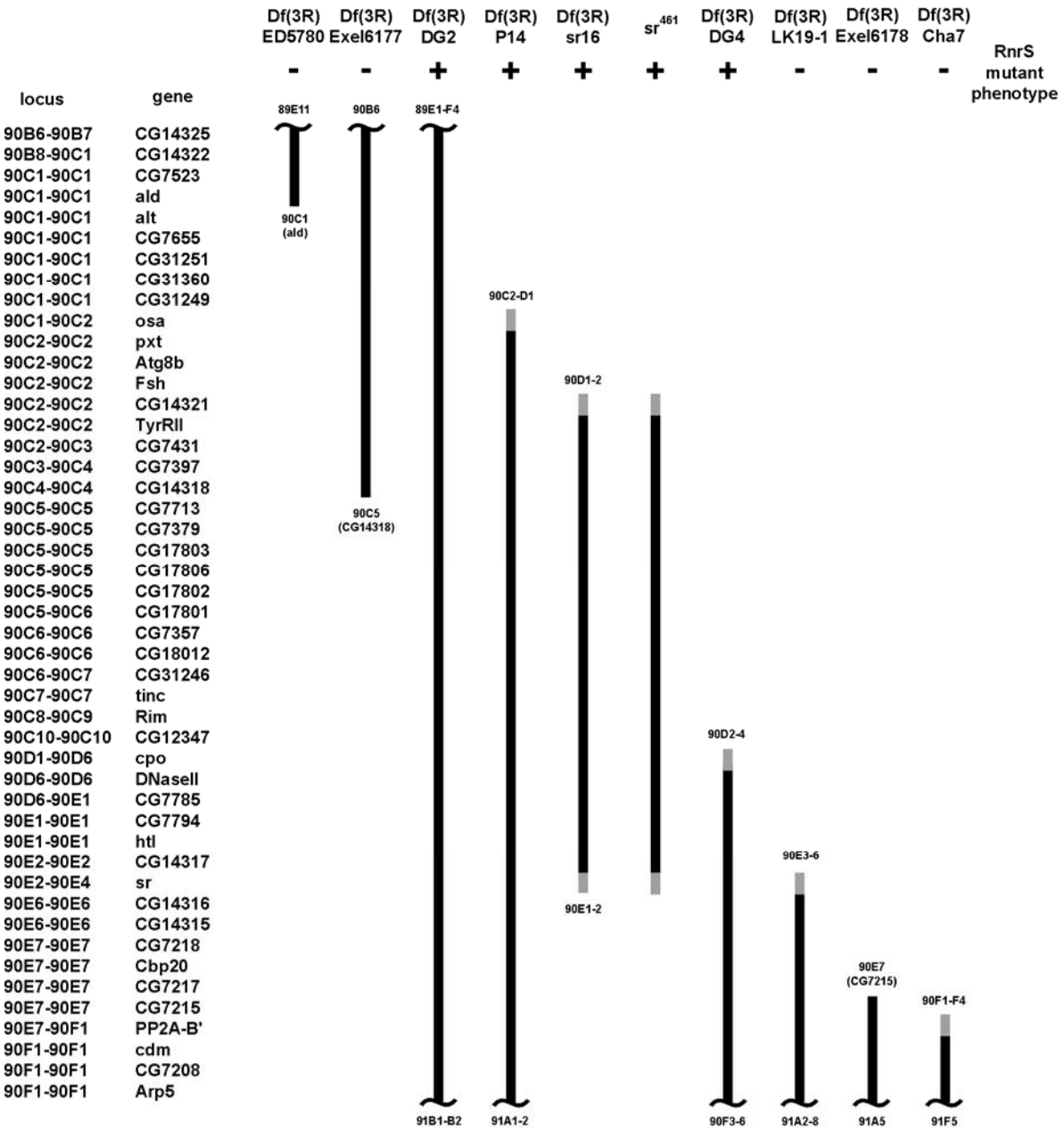

Figure 4.4: The mapping of the locus showing the *RnrS* phenotype

Chromosome deficiencies are shown in black solid bars with the breakpoint locus at each end of the bar. The gene name in the parenthesis below the breakpoint locus indicates the gene deleted by the deficiency. Deficiencies with molecularly-mapped breakpoints are shown with black ends, whereas ones with

cytologically-mapped breakpoints (mapped by chromatin banding pattern of polytene chromosomes in the salivary gland) are shown with gray ends. Note that *Df(3R)sr16* (previously mapped to 90D1-2/90E1-2) and sr^{461} are re-mapped by PCR to 90C2-4/90E2-4.

Among these 7 most likely candidate genes, mutant flies were available for *cpo*, *DNase II*, *CC7785*, *htl*, and *sr* (homozygous lethal: $cpo^{BG02810}$, cpo^{01432}, htl^{d07110}, sr^{461}, sr^{155}, and sr^{03999}, homozygous viable: $DNaseII^{lo}$, $DNaseII^{B571}$, and $CG7785^{f06154}$). No mutants were available for *CC7794* and *CG14317*. We examined *RnrS* transcription of these mutant embryos, and found that one *sr* mutant allele, sr^{461}, shows the *RnrS* phenotype (Figure 4.2), whereas the other *sr* alleles do not. However, later we found that the sr^{461} allele is a deletion mutant which deletes at least 19 genes (from CG14318 to CG14317, data not shown). This is consistent with our complementation tests, where sr^{461} partially complemented two other *sr* alleles: sr^{03999} (P-element insertion) and sr^{155} (point mutation), and failed to complement with *cpo* and *htl* (Table 4.1). Moreover, sr^{03999} and sr^{155} did not show the *RnrS* phenotype, suggesting that *sr* is not responsible for the *RnrS* phenotype.

	Df(3R) ED5780	Df(3R) DG2	Df(3R) P14	Df(3R) sr16	cpo	htl	sr[461]	sr[03999]	sr[155]	Df(3R) DG4	Df(3R) Exel6178	Df(3R) Cha7
Df(3R)ED5780												
Df(3R)DG2	N/D											
Df(3R)P14	N/D	no										
Df(3R)sr16	yes	no	no									
cpo	N/D	no	no	no								
htl	N/D	no	no	no	yes							
sr[461]	yes	no	no	no	no	no						
sr[03999]	yes	no	no	yes *	yes	yes	yes **					
sr[155]	yes	no	no	no	yes	yes	yes ***	no				
Df(3R)DG4	N/D	no	no	no	no	no	no	no	no			
Df(3R)Exel6178	yes	N/D	N/D	yes	N/D	N/D	yes	yes	yes	N/D		
Df(3R)Cha7	N/D	no	no	yes	yes	yes	yes	yes	yes	no	N/D	

Table 4.1 The complementation test of the mutants in the suspected locus for the *RnrS* phenotype

yes: complemented, no: failed to complement, N/D: not determined. Asterisks indicate partial complementation. *: 70/419 (non-balanced/balanced), **: 89/439, ***: 27/587.

Discussion

Our search for a PP1 subunit that regulates RBF1 activity during embryogenesis revealed an unexpected phenotype: decreased zygotic *RnrS* transcription during early embryogenesis. This was surprising for us, because *RnrS* is a well-characterized E2F1-target gene (Dimova et al., 2003; Duronio and O'Farrell, 1994), and it is widely accepted that *RnrS* and other E2F1-target genes are mainly regulated by E2F1. The mutants that show the *RnrS* phenotype do not bear mutations in the *E2f1* gene, and E2F1 protein levels were normal in the mutants, leading us to hypothesize an E2F1-independent regulation of "E2F1-target genes" during early embryogenesis. Interestingly, in the mutants, other E2F1-target genes are expressed in a normal pattern throughout embryogenesis. Moreover, the transcription of *RnrS* is decreased in the mutants only during early embryogenesis. These findings suggest a gene-specific, stage-specific regulation of *RnrS* expression.

What then is the mechanism underlying this *RnrS* phenotype? Formally, 19 genes from *CG7731* to *sr* are candidate genes based on molecular mapping (see results). Of these 19 genes, 7 genes including *cpo*, *DNase II*, *CG7785*, *CG7794*, *htl*, *CG14317*, and *sr* were the most likely candidates based on a cytologically-mapped deficiency (DG4). Among these most suspected genes, mutant flies of *cpo*, *DNase II*, *CC7785*, *htl*, and *sr* were available and none of these mutants showed the *RnrS* phenotype. Therefore, 2 genes (*CG7794* and *CG14317*) remain as the most likely candidates so far. The protein BLAST search using *CG7794* and *CG14317* showed homology to α-tubulin, and no conserved mammalian orthologs, respectively. Interestingly, the BioGRID yeast two hybrid database (http://www.thebiogrid.org) shows the interaction of CG14317 with cell cycle regulators (CDK2, Cul2, SkpA (Skp1), SkpB, and SkpC) and with transcription factors (TATA-binding

protein associated factor 11 and Tramtrack69) (Giot et al., 2003; Stanyon et al., 2004). Furthermore, an in situ hybridization experiment showed that the expression timing of CG14317 correlates well with the onset of the *RnrS* phenotype; it is ubiquitously expressed only during early embryogenesis (data not shown).

Although the *RnrS* phenotype discovered here suggests that E2F1 is not sufficient for the early zygotic transcription of *RnrS*, it does not exclude the possibility that E2F1 is required for the early zygotic transcription of E2F1-target genes. We cannot test this idea simply by using zygotic *E2f1* mutant embryos because maternal E2F1 protein remains until stages when the early zygotic transcription starts (Figure 2.5). Embryos of maternal and zygotic *E2f1* null mutant die during very early embryogenesis, making it impossible to determine the *RnrS* phenotype of these mutant embryos.

Although we are currently investigating the possible E2F1-independent regulation during early embryogenesis, it should be noted that it is also formally possible that the *RnrS* phenotype is caused by a slight decrease in E2F1 activity. In this scenario, the mutations eliminate a gene that supports E2F1 activity during early embryogenesis. *RnrS* may be the only E2F1-target gene that is sensitive to the slight reduction of E2F1 activity, and therefore the only gene whose expression is affected. These possibilities will be further examined in future experiments in our laboratory.

Acknowledgements

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CHAPTER V

DISCUSSION AND FUTURE DIRECTIONS

The E2F/DP/RB pathway plays a central role in cell cycle regulation. However, the regulation of the pathway in vivo has yet to be fully understood. In this thesis, I started with the question of how E2F1 activity is downregulated during *Drosophila* embryogenesis, and discovered a developmentally-regulated onset of E2F1 destruction (Chapter II). In the course of the study, we found that the destruction of E2F1 occurs specifically during early S phase. Our attempt to understand this S phase-specific E2F1 destruction led to the discovery of the mechanism; E2F1 is degraded during S phase through its interaction with PCNA, Cul4, Cdt2, and DP. We further showed that the stabilization of E2F1 during S phase induces apoptosis (Chapter III). In addition, we identified a genomic locus that is required for the early zygotic transcription of *RnrS*, an E2F1-target gene (Chapter IV). This finding raised the possibility of an E2F1-independent mechanism that is necessary for the early zygotic transcription of "E2F1-target genes".

E2F1 destruction as a mechanism of E2F1-target gene downregulation during *Drosophila* embryogenesis

During *Drosophila* embryogenesis, E2F1-target genes are expressed specifically in cells that are actively cycling (Duronio and O'Farrell, 1994). One way to explain the correlation between E2F1-target gene expression and active cell cycle is that E2F1 activity is

developmentally regulated and, as a consequence, the cell cycle follows. This cause-consequence relationship is supported by the fact that *E2f1* mutant embryos show impaired DNA replication when the maternal supply of E2F1 is depleted (Duronio et al., 1995).

Then, what is the mechanism that regulates the spatiotemporal pattern of E2F1-target gene expression during embryogenesis? In Chapter II, we showed RBF1-independent, Dap-independent downregulation of E2F1-target genes during early embryogenesis. This finding was unexpected for us, since RBF1 and Dap are major inhibitors of the potential E2F1-CycE/CDK2 positive feedback loop. In the search for an alternative mechanism of E2F1 downregulation, we found developmentally-regulated E2F1 destruction (Figure 2.3), leading to the hypothesis that the initial downregulation of E2F1-target genes is caused by this onset of E2F1 destruction. Although we successfully identified the mechanism of E2F1 destruction (Chapter III), the developmental signal regulating the onset of E2F1 destruction is currently unknown. The signal may be the downregulation of a maternal factor(s) that protects E2F1 from destruction and/or the induction of a zygotic gene(s) that is required for the destruction of E2F1.

The best possible experiment to test the requirement of E2F1 destruction will be to stabilize E2F1 protein during embryogenesis. If E2F1 destruction is required for the initial downregulation of E2F1-target genes, then the stabilization of E2F1 protein should result in the failure to downregulate E2F1-target genes. This experiment became technically possible when we discovered the mechanism of E2F1 destruction and successfully created a stabilized form of E2F1 (PIP mutant E2F1). However, the experiments employed in this thesis did not provide sufficient data to conclude about this hypothesis. We saw a normal initial downregulation of *RnrS* transcripts even with GFP-E2F1^{PIP} overexpression although de-

repression of *RnrS* at later stages was observed with the *prd*-Gal4-driven overexpression of GFP-E2F1^{WT} or GFP-E2F1^{PIP} (Figure 6A). This may imply that the initial downregulation of E2F1-target genes is not caused by the destruction of E2F1. Instead, other mechanisms may control this initial downregulation of E2F1-target genes. Although this is contradictory to our initial hypothesis stated in Chapter II, the later finding of E2F1-independent expression of *RnrS* (Chapter IV) indicates that E2F1-target genes can be regulated independently of E2F1 during early embryogenesis. Alternatively, the normal initial downregulation of *RnrS* may be due to the low expression of GFP-E2F1s under the control of a weak UAS sequence (UASp), which we employed for the studies described here. Higher levels of transgene expression will be achieved by the use of UASt, which may provide sufficient levels of expression in the embryo to test whether E2F1 destruction is required for the initial downregulation of E2F1 target genes. To confirm this, we are currently in the process of creating transgenic flies bearing UASt-GFP-*E2f1s*. However, we will not be able to test the hypothesis if overexpression with UASt induces too high expression, so that even the overexpression of E2F1^{WT} prevents the initial downregulation of E2F1-target genes.

E2F1-independent regulation of E2F1-target genes during *Drosophila* embryogenesis

In addition to E2F1 destruction as an inhibitory mechanism of E2F1 activity, we discovered a possible E2F1-independent mechanism that positively regulates "E2F1-target genes". As described in Chapter IV, we identified a genomic locus that is required for the early zygotic expression of *RnrS*. This locus does not contain the *E2f1* gene, and E2F1 protein expression was normal in the deletion mutant of the locus (Figure 4.4). Thus, the data suggests that E2F1 is not sufficient for the early zygotic expression of *RnrS*. However, it

should be noted that this does not exclude the possibility that E2F1 is "required" for the early zygotic expression of *RnrS*. Therefore, the discovery of the locus does not reject our hypothesis that the initial downregulation of zygotic E2F1-target genes is caused by the onset of E2F1 destruction. We hypothesize that the locus contains a gene(s) that positively regulates the early zygotic expression of *RnrS*. As candidate genes, we have identified 19 genes in the locus. The discovery of the responsible gene will provide insight into the regulatory mechanism of E2F1-target genes during early embryogenesis.

The mechanism of E2F1 destruction

In the work described in Chapter III, we established a FACS-based experimental system by which S phase-specific destruction of E2F1 can be robustly detected. Using this system, we discovered that the destruction of E2F1 is dependent on PIP box, PCNA, Cul4, and Cdt2. Since the destruction of Cdt1 is also dependent on PIP box, PCNA, Cul4, and Cdt2 (Arias and Walter, 2006; Jin et al., 2006; Senga et al., 2006), we think that it is reasonable to conclude that *Drosophila* E2F1 and Cdt1 are degraded by a common mechanism.

Interestingly, E2F1 degradation appears dependent on its partner DP. E2F1 was stabilized when *Dp* was knocked down (Figure 3.4), and was destabilized when DP was co-expressed (Figure 3.5). This led to the hypothesis that E2F1 degradation requires its binding to DP (and possibly to DNA). However, this hypothesis by itself cannot explain the fact that the N-terminal fragments of E2F1 (1-230, 93-184 and 139-230), which lacks both the DNA-binding domain and DP-dimerization domain, can still be degraded during S phase (Figure 3.1). As discussed in Chapter III, this may imply the existence of a signal sequence which resides in the 231-805 region and inhibits E2F1 destruction. The signal may be inactivated by

DP-binding, allowing the PIP box-dependent destruction of E2F1. This hypothesis can explain the fact that the N-terminal fragments are degraded during S phase in the absence of the DP-binding domain. In this hypothesis, the N-terminal fragments can be degraded since they do not contain the inhibitory signal, but full length E2F1 needs to be bound by DP in order to be degraded. It is interesting to test whether these N-terminal fragments are stabilized when a mutation is inserted to the PIP box. The hypothesis predicts that the N-terminal fragments will be stabilized by a mutation in the PIP box. In addition, it will be informative to examine the stability of mutant E2F1s which lack the DNA-binding domain or the DP-dimerization domain. The DNA-binding domain will not be required for E2F1 destruction if E2F1/DP dimerization by itself is sufficient to inactivate the putative inhibitory signal sequence. Alternatively, the DNA-binding domain will be required if DNA-binding, which is facilitated by E2F1/DP dimerization, is required for bypassing the inhibitory signal. Another hypothesis is that E2F1/DP induces transcription of genes involved in E2F1 destruction such as Cdt2. In this case, *Dp* knockdown will stabilize all the E2F1 constructs including the N-terminal fragments.

Since DDB1 is so far the only known adaptor protein that mediates the association of Cul4 and Cdt2 (Jin et al., 2006), it was surprising for us to find that *Ddb1* knockdown did not result in the stabilization of E2F1 (Figure 3.4). One possibility is that the knockdown did not sufficiently reduce DDB1, even though successful DDB1 knockdown was confirmed by Western blot analysis (Figure 3.4). Alternatively, it is also possible that other DDB1-like proteins can compensate for the loss of DDB1. Interestingly, a recent report showed that a DDB1-like protein named SAP130 (Spliceosome-associated protein 130) interacts with Cul4a as well as Cul1 and 2 (Menon et al., 2008). SAP130 appears to be highly conserved in

Drosophila (CG13900, 75% homology to human SAP130 amino acid sequence). It is interesting to test whether *CG13900* knockdown or *Ddb1*/*CG13900* double knockdown stabilizes E2F1 during S phase in our S2 cell system.

The *Drosophila*-specific evolution of E2F1 destruction mechanism

Drosophila E2F1 and mammalian E2F1 appear to be degraded via different ubiquitination pathways. Although PIP box is conserved in Cdt1 in mammals, flies, zebrafish, frog and worm (Arias and Walter, 2006; Senga et al., 2006), so far we did not find a putative PIP box in E2Fs of other species. This may indicate that the mechanism for E2F destruction has evolved in *Drosophila* differently from other organisms. Indeed, mammalian E2F1 is degraded in S/G2 (Marti et al., 1999) unlike *Drosophila* E2F1, which is degraded in early S phase (Asano et al., 1996; Reis and Edgar, 2004) (Figure 2.3). Consistent with this, the ubiquitination of mammalian E2F1 is linked to $Cul1^{SKP2}$ (Marti et al., 1999), whereas our RNAi data indicated little contribution of Cul1 or SKP2 to the destruction of *Drosophila* E2F1 destruction (Figure 3.4). In addition, Ohta and Xiong showed that the in vitro ubiquitination of human E2F1 was enhanced by the addition of Cul1, 2, 3 and 5, but not by Cul4a (Ohta and Xiong, 2001), as opposed to our data that suggests the Cul4-dependent destruction of *Drosophila* E2F1.

Apoptosis induced by E2F1 in mitotic cycles

Activator E2Fs can act as positive regulators of apoptosis by inducing pro-apoptotic genes (Asano et al., 1996; Iaquinta and Lees, 2007; Moon et al., 2005; Muller et al., 2001). However, E2F-induced apoptosis is highly context-dependent, and little is known about the

mechanism that underlies this context-dependency (Hallstrom et al., 2008; Iaquinta and Lees, 2007; Moon et al., 2006; Moon et al., 2005). The data presented here suggests that E2F1 induces apoptosis when it is stabilized during S phase (Figure 3.8). Importantly, overexpression of GFP-E2F1^{WT} did not induce apoptosis even though it is expressed at comparable levels to GFP-E2F1^{PIP} outside S phase (Figure 3.8), reinforcing the idea that E2F1 overexpression outside S phase is not apoptosis-inducing.

In general, E2F's transcriptional activity reaches its peak in late G1, when activator E2Fs replace repressor E2Fs, and pRB dissociates from these activator E2Fs. This high E2F1 activity in late G1 is thought to facilitate S phase entry and progress (Trimarchi and Lees, 2002). We hypothesize that the activity of activator E2Fs is suppressed during S phase (and G2 in mammals) by the destruction of E2Fs via the ubiquitin-proteasome pathway in conjunction with the transcriptional repression of the *E2f1* gene by E2F7/8 (mammals) or by CDK activity (*Drosophila*). Our results suggest that, when E2F activity remains high in S phase, cells may detect it and activate apoptotic pathways, otherwise the high E2F activity may cause overproliferation of cells, and possibly DNA re-replication through its induction of replication initiation factors such as Cdt1/dup. Alternatively, E2F1-induced re-replication may be the cause of apoptosis. If this is the case, wing disc cells expressing GFP-E2F1^{PIP} may have died by apoptosis before any re-replication is detected (Figure 3.8).

The existence of an S phase-specific, apoptosis-inducing sensor mechanism for E2F1 activity is supported by the study by Asano et al. They showed that the heat shock-induced expression of *Drosophila* E2F1 in the eye disc induces apoptosis. However, the cells within or anterior to the morphogenetic furrow (a zone composed of cells in developmentally-

regulated G1) did not undergo E2F1-induced S phase or subsequent apoptosis (Asano et al., 1996).

The question of how cells detect ectopic E2F1 activity during S phase will require future investigation to be answered. At least, a cell cycle-independent, constant threshold of E2F1 activity cannot be a sufficient explanation, because cells do not activate apoptotic pathways in late G1, when E2F1 activity peaks.

Unlike in wing discs, no apoptosis induction was observed even when GFP-E2F1^{PIP} was overexpressed in embryos (data not shown). As discussed above, this may reflect the fact that the UASp/Gal4 system we employed induces relatively low levels of transgene expression. However, our finding described in Chapter II indicates that E2F1 destruction does not occur even in S phase prior to cycle 15 (Figure 2.3). In spite of this, apoptosis is not seen in early embryogenesis. One possibility is that during early embryogenesis, cells possess a mechanism to escape from E2F1-induced apoptosis, so that they do not suffer from apoptosis even with high levels of E2F1 throughout the cell cycle. This escaping mechanism may be required to achieve very rapid cell cycles during early embryogenesis. After cells differentiate and obtain tight regulation by external signals, cells with inappropriately high levels of E2F1 during S phase may have to be eliminated in order to prevent overproliferation and/or DNA re-replication caused by elevated E2F1 activity.

The significance of E2F1 destruction in endocycles

Our data shown here suggest that endocycles are more susceptible to an increase in E2F1 level. In the embryonic gut, the synchronicity of endocycles was perturbed even with GFP-E2F1^{WT} overexpression (Figure 3.7), making it impossible to compare the effects of

GFP-E2F1^{WT} and GFP-E2F1^{PIP} in this tissue. However, our collaborative work with Dr. Bruce Edgar has shown that the expression of GFP-E2F1^{PIP} arrests the endocycles in the larval salivary gland, whereas the expression of GFP-E2F1^{WT} does not (Figure 3.9). The onset of the cell cycle arrest caused by GFP-E2F1^{PIP} is slow; scattered DNA replication was still observed at early larval stages (~72 hours after egg deposition), and the complete arrest was seen at later larval stages (~96 hours) (Kim et al. manuscript in preparation). This slow onset of the cell cycle arrest may explain the absence of cell cycle arrest in the embryonic gut overexpressing GFP-E2F1^{PIP}.

Concluding Remarks

The work presented in this thesis has advanced our understanding about E2F1 regulation in the context of development. We showed evidence that the initial downregulation of E2F1-target genes during embryogenesis may be caused by the developmentally-regulated destruction of E2F1. Furthermore, we have identified the mechanism for E2F1 destruction using a FACS-based experimental system which was established for this study. It should be noted that this experimental system can be readily applied to investigation of other cell cycle-regulated proteins. Our data indicates that *Drosophila* E2F1 is degraded in a DNA replication-dependent manner. This finding revealed the existence of a robust negative feedback mechanism embedded in the central cell cycle regulatory machineries; E2F1 induces DNA replication, which in turn downregulates E2F1. This negative feedback is required for preventing apoptosis in a mitotic cycle (larval wing disc) and for the cell cycle progression of an endocycle (larval salivary gland). Additionally, E2F1^{PIP} mutants created in this study will provide a useful tool for future experiment.

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