S PHASE-COUPLED E2F1 DESTRUCTION ENSURES HOMEOSTASIS IN PROLIFERATING TISSUES

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ABSTRACT

Jean Marie Davidson: S PHASE-COUPLED E2F1 DESTRUCTION ENSURES HOMEOSTASIS IN DEVELOPING TISSUES
(Under the direction of Robert J. Duronio)

A fundamental question in biology seeks to understand the balance acheieved by rapid proliferation and careful regulation of genomic integrity that is essential for generating and maintaining complex multi-cellular organisms. Cells require the ability to divide incredibly quickly, especially during early development, to achieve the appropriate size in a short amount of time. However, rapid proliferation is dangerous. Defects may arise when cells duplicate rapidly and if these defective cells are not recognized there may be serious consequences for the health of the organism. Therefore the cell has in place many checkpoints and strategies to balance proliferation with careful regulation. Here we present a novel mechanism to address this issue. The transcription factor E2f1 is a potent activator of cell cycle progression and critical for normal G_1-S transitions. It is carefully regulated prior to the transition by Rbf1 and many upstream effectors. Recently, the mechanism of its destruction during S phase has been described. We describe the consequences of blocking this normal destruction. Surprisingly, the programmed cell death that arises from stabilization of E2f1 is not solely due to it's transcriptional activity, but instead relies on an elegant mechanism to "sense" inappropriate E2f1 levels to activate apoptosis before any potential defects of hyper-proliferation may occur. Therefore E2f1 acts both to drive proliferation and tissue growth, but also as a reporter molecule for defects in normal cell

cycle progression which can result in the clearing of that damaged cell to maintain tissue homeostasis. We propose that this mechanism is in place in order to balance the rapid proliferation required for normal development with the fundamental requirement of genomic integrity.

ACKNOWLEDGEMENTS

A million thanks to everyone who helped me get here today. It is a long journey and takes the support of people who think you can do it, even when you don't. Thanks to all the members of Duronio lab, past and present, for being a lovely community of scientists who taught me so much. Special thanks to Deirdre Tatomer, my collaborator, friend, cheerleader, and favorite person to talk science with. My time here was greatly enriched because of her friendship and I feel extremely fortunate to have shared this experience with her. A huge thank you to my mentor Bob Duronio. He taught me so much, challenged me to be my best, and gave me incredible opportunities to learn, grow, and become a member of the scientific community. It is a tricky relationship to navigate, but I feel very fortunate to call him my friend.

My family has always supported me with unfaltering dedication and I thank them for teaching me I could do anything I set my mind to and to follow my dreams. From my 7th grade quantum mechanics presentations, to my very own microscope in my room, to getting me cow brains to dissect, to long hikes hearing me babble on about ferns, they have always encourgaged me. It is hugely empowering to be so supported.

And of course, a universe of graditude to my partner, Trent. There aren't the words to express how much his love and companionship have meant to me throughout this journey. I am thrilled to see what our future holds for us.

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LIST OF ABBREVIATIONS

APC/C	Anaphase Promoting Complex/Cyclosome
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
CRL	Cullin-RING E3 ubiquitin Ligases
Cul	Cullin
Cyc	Cyclin
Dap/p27^{Dap}	Dacapo
DAPI	4',6-diamidino-2-phenylindole
DCAF	Ddb1 and Cul4 Associated Factors
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
EdU	5-Ethyl-2'-deoxyuridine
en	engrailed
FACS	Fluorescent-Activated Cell Sorting
fzr	fizzy-related
G	Gap
GFP	Green Fluorescent Protein
GMR	Glass Multimer
HA	Hemagglutinin
hid	head involution defective
Hsp70	Heat shock protein 70
INK4	Inhibitor of Cdk4
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
pre-RC	pre-Replication Complex
pRb	Retinoblastoma protein
RBF	Retinoblastoma-family protein
RNAi	RNA interference
RnrS	Small subunit of Ribonucleotide reductase
RT-PCR	Reverse Transcription Polymerase Chain Reaction
S	Synthesis
S2	Schneider 2
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
SLBP	Stem loop binding protein
Slmb	Slimb (super-numary limbs)
stg	string
UAS	Upstream Activator Sequence
WT	Wild Type

CHAPTER I

INTRODUCTION

Establishing and maintaining proper patterning, timing, and tissue organization are critical for normal development. This process requires precise control of cell proliferation and cell death. These seemingly opposing influences must be balanced in order to achieve homeostasis. Inappropriate activation, or repression, of either can have severely detrimental consequences. Loss of proliferation is associated with senescence and some early aging disorders such as progeria, whereas hyper-proliferation is a classical hallmark of tumorgenesis and cancer (Burtner and Kennedy, 2010; Hanahan and Weinberg, 2011). A host of complicated decisions must occur to transition from a single cell after fertilization into a complex, multi-cellular, organism. The proliferation of cells in a tissue is regulated by various intrinsic and extrinsic cues. In multicellular organisms, the cell cycle is usually 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. Specific adult cell populations must maintain the ability to re-enter the cell cycle upon mitogenic signaling, especially rapidly proliferating epithelial tissues such as skin, lung, and intestine. In contrast, other cells must exit the cell cycle definitively and their proliferation could have severe consequences (Hanahan and Weinberg, 2011). The failure to arrest the cell cycle when required may cause neoplastic growth and eventual cancer development. Another mechanism of ensuring tissue homeostasis is programmed cell death, or apoptosis (Fuchs and Steller, 2011). Cells that can no longer contribute to the health of the organism

are specifically culled before they can harm the surrounding tissue, or ignore regulatory cues. There is also a requirement for apoptosis during normal development, as rapid morphological changes occur that require the loss of specific cell populations. Again, mis-regulation of this powerful molecular mechanism has potentially serious consequences during development and the adult life of the organism. Therefore, it is critical to understand the mechanisms at work to balance the proliferation, arrest, and the critical role of programmed cell death during development to understand how homeostasis is maintained, and how disruptions of these mechanisms may act as initiating events in disease.

The regulation of the G1-to-S transition

The canonical cell cycle is made 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. There are many factors that regulate this canonical cell cycle, most of which tend to function at the major transitions including the G_1 to S transition and the G_2 to M transition. Some of these factors include transcription factors and Cyclin/Cyclin dependent kinase complexes (Cyc/Cdk). Cyc/CDK complexes phosphorylate a plethora of proteins, and this phosphorylation coordinately drives these transitions into a new cell cycle phase. 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 at the G1-S transition (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.

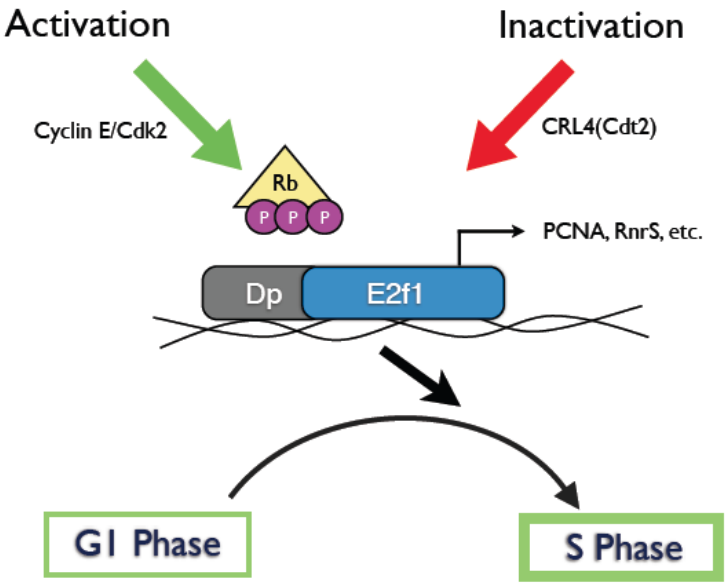

Figure 1.1: Roles and Regulations of *Drosophila* E2f1. The E2F transcription factor, together with its heterodimeric partner DP, induces DNA replication genes such as, *ribonuclease reductase small subunit* (*RnrS*), *proliferating cell nuclear antigen* (*Pcna*), and *DNA polymerase*. Because of this, E2f1 acts as a strong positive regulator of the G1-to-S transition. E2f1 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 E2f1-target gene transcription

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.

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 has initiated DNA replication (S phase). Failure to suppress E2F activity in S phase may cause ectopic S phase re-entry or re-replication. 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).

Upon entry in to S phase, there are specific mechanisms to disrupt E2F activity. The first 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). It is predicted that this free dimer is then targeted for destruction via the ubiquitin-proteosome pathway during S phase. 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). As a final means of protecting against inappropriate E2F activity, 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). Recent

reports have 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.

It becomes evident, based on the multiple mechanisms in place to ensure E2F activity is down-regulated upon S phase entry, that there must be critical consequences of inappropriate activator E2F function. However, 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 directly address this question. A more simplified E2F/DP/RB pathway has been characterized in *Drosophila melanogaster*, providing an excellent model system to investigate the regulatory mechanisms of E2Fs. In *Drosophila*, two E2Fs (E2f1 and E2f2), one DP, and two pocket proteins (RBf1 and Rbf2) 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). 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. Furthermore, the mechanisms to suppress activator E2Fs are conserved between mammals and *Drosophila* to some extent. Like mammalian activator E2Fs, *Drosophila* E2f1 is repressed via RBF1-binding (Du et al., 1996a; Xin et al., 2002), and E2f2 has been shown

to suppress the transcription of E2f1-target genes (Frolov et al., 2001; Weng et al., 2003). However, other aspects of E2f1 biology were not conserved, namely the down-regulation upon S phase. Unlike mammalian activator E2Fs, no known Cyclin-binding site has been found in *Drosophila* E2f1. Previous work has suggested a ubiquitin-mediated proteolysis of *Drosophila* E2f1 dependent on the $Cul1^{Slmb}$ ubiquitin ligase (Heriche et al., 2003), though more recently a new mechanism has emerged, where $CRL4^{Cdt2}$ targets E2f1 for rapid ubiquitination and destruction during S phase (Shibutani et al., 2008). Orthologs of E2F7/8 do not seem to exist 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 are the biological consequences of disrupting E2f1 S phase-coupled destruction?

Ubiquitin Mediated Proteolysis

It is well established that ubiquitin-mediated degradation of proteins plays an critical role in various processes including cell cycle progression, transcription and DNA replication and repair (Nakayama and Nakayama, 2006). Recent evidence supports a growing role for the E3 ubiquitin ligase, Cullin-4, in conjunction with the substrate recognition factor Cdt2 ($CRL4^{Cdt2}$), for the degradation of multiple cell cycle-regulated proteins to in order to prevent hyper-proliferation, DNA damage, and eventual genomic instability (Abbas and Dutta, 2011). This ubiquitin-mediated proteolysis is required for normal cell cycle progression by destroying specific cell cycle proteins in order to maintain the coordinated oscillations required. Here, we briefly highlight the current knowledge about the various roles of the

$CRL4^{Cdt2}$ E3 ubiquitin ligase, and how its activity contributes both to normal cell cycle progression and to the preservation of genome integrity.

The ubiquitylation reaction involves the covalent attachment of the small 76 amino-acid ubiquitin moiety on a specific lysine residue in the target substrate protein. For substrates destined for degradation via the 26S proteasome, polyubiquitin chains are assembled through the successive attachment of the ubiquitin molecules through the formation of isopeptide bonds between the C-terminus of the incoming ubiquitin to lysine 48 of the attached ubiquitin (Havens and Walter, 2011). The formation of this polyubiquitin chain marks the substrate protein for proteolysis. Polyubiquitylation is achieved through three distinct and consecutive enzymatic steps where ubiquitin is first activated by an E1 ubiquitin-activating enzyme followed by the transfer of the activated (AMP-charged) ubiquitin from the E1 enzyme to an E2 ubiquitin-conjugating enzyme. Finally, the ubiquitin is transferred from the E2 enzyme to the substrate through the activity of an E3 ubiquitin ligase (Abbas and Dutta, 2011). The ability of E3 ubiquitin ligases to recognize, with high specificity, a relatively large number of substrates accounts for the diverse structural complexity of this group of proteins.

Cullin-RING E3 ubiquitin Ligases (CRLs) represent the largest and most complex family of E3 ubiquitin ligases and play significant roles in multiple physiological processes including transcription, differentiation, cell cycle control, proliferation, apoptosis and tumorigenesis. This class of E3 ubiquitin ligases include cullin 1, 2, 3, 4A, 4B, 5 and cullin 7 as well as the cullin-like proteins PARC and APC2 (Abbas and Dutta, 2011; Baker, 2007). Several CRLs and many of their target protein substrates are conserved throughout evolution. The core CRL4 ubiquitin ligase complex is composed of one of two scaffold proteins (Cul4A

or Cul4B), Ddb1 (damage-specific DNA binding protein-1), an adaptor protein which functions to bridge one of many substrate recruiting factors (DCAFs; Ddb1 and Cul4 Associated Factors) to the Cul4 E3 subunit, and a small RING finger protein (Rbx1/2) required for the recruitment of a corresponding E2 ubiquitin-conjugating enzyme (UBC) (Jackson and Xiong, 2009). There are at least 49 known DCAFs or WDR (WD repeat-containing proteins) proteins that function as substrate recognition factors to recruit substrates to the CRL4 ubiquitin ligase complex (Higa et al., 2006b).

The CRL4 E3 ligase orchestrates a variety of physiological processes including DNA replication, transcriptional regulation, apoptosis and a number of DNA repair processes. Recent work demonstrated that CRL4 is critical for preventing genomic instability through its ability to promote the ubiquitin-dependent proteolysis of Cdt1, a replication initiation protein that is essential for pre-RC (pre-replication complex) assembly and the recruitment of the replicative helicase MCM2-7 at replication origins (Abbas and Dutta, 2011; Havens and Walter, 2009; Higa et al., 2006a). Cells that are deficient in Cul4 exhibit re-replication and genomic instability reminiscent to that seen in cells overexpressing the replication initiation factor Cdt1 (Arias and Walter, 2005; Jin et al., 2006).

Following Cdt1, a small but growing list of proteins have been shown to be targeted for destruction by $CRL4^{Cdt2}$ ubiquitination. $CRL4^{Cdt2}$ promotes the ubiquitin-dependent degradation of several of its substrates not only in response to genotoxic stress, but also in unperturbed proliferating cells, specifically during the S phase of the cell cycle. The CDK inhibitor p21 (Jorgensen et al., 2011; Nishitani et al., 2008), the histone monomethyl transferase Set8/Pr-Set7, the *C. elegans* the bypass polymerase, (Acharya et al., 2008), and *Drosophila* E2f1 (Shibutani et al., 2008) have been shown to be targets. While these proteins

seem to have a variety of roles, they all coalesce on regulation of cell cycle progression, arrest following genotoxic stress, and overall maintenance of genome integrity (Havens and Walter, 2011). The coupling of destruction of these proteins via $CRL4^{Cdt2}$ to a specific phase of the cell cycle or after DNA damage relies on an elegant coupling of this mechanism to chromatin loaded PCNA (Arias and Walter, 2006). Recent crystal structure studies have shown that only once PCNA (Proliferating Nuclear Antigen) is associated with chromatin, there is the formation of the appropriate hydrophobic pocket that can recognize a specific amino-acid sequence referred to as the PIP degron (Havens and Walter, 2009). This degron contains both amino acids that interact with chromatin loaded PCNA (PIP box), as well as amino acids that extend into the nucleoplasm to recruit the substrate receptor, Cdt2, initiating the recruitment of the entire $CRL4^{Cdt2}$ complex. The PIP degron is highly conserved between all $CRL4^{Cdt2}$ targets and temporally connects the destruction of these substrates to S phase, as well as after DNA damage, when DNA replication relies on PCNA loading (Figure 1.2).

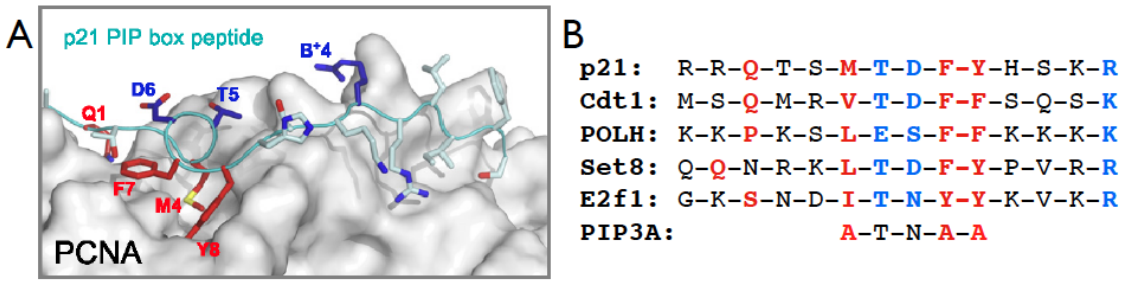

Figure 1.2: PIP Degron Interacts with Chromatin Associated PCNA A) Structure of chromatin associated PCNA demonstrating hydrophobic pocket in which PIP degrons interact. Amino acids in red associate directly with PCNA pocket, while amino acids shown in blue extend into the nucleoplasm and interact directly with substrate receptor protein, Cdt2. Figure from (Havens and Walter, 2009) B) Conservation of PIP degron among five target proteins. Colors of amino acids are the same as in A. PIP3A mutation in *Drosophila* E2f1, the substitution of three amino acids for alanine, completely blocks E2f1 S phase coupled destruction.

Drosophila embryos as an experimental model for studying E2F1 regulation

The various stages of *Drosophila* development provide an excellent model system to study the mechanism of E2f1 regulation in different contexts. The various cell cycle

pathways and regulations 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. 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 (discussed in Chapter IV).

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). Interestingly, it is only after the onset of zygotic transcription that E2f1 is cleared from S phase cells. Prior to this onset, E2f1 protein may co-localize with BrdU

positive cells (Shibutani et al., 2007), however following the first gap phase ($G2_{14}$), E2f1 is targeted for destruction upon subsequent S phase entry (Figure 1.2B). The mechanism that activates this destruction upon zygotic expression is as yet unknown, but will be discussed further in the future directions.

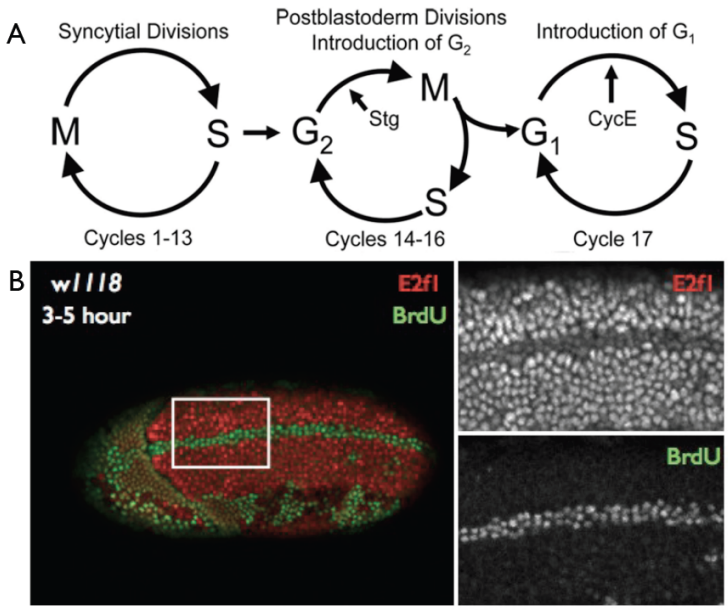

Figure 1.3: *Drosophila* embryonic cell cycles and E2f1 Destruction A) Schematic of various cell cycle s utilized during *Drosophila* embryonic development. B) A 3-5 hour wild type (w1118) embryo. BrdU (green) is a nucleotide analogue that incorporates into replicating DNA and labels cells undergoing S phase. E2f1 protein (red) as measured by a polyclonal antibody generating by the Duronio lab. Those cells in the midline marked with BrdU have entered S_{15}, the first S where E2f1 is targeted for destruction. The onset of this mechanism is still unclear and will be discussed later.

Drosophila Imaginal Discs as an Experimental Model for Studying Tissue Homeostasis

During the development of homometabolous insects such as *Drosophila melanogaster*, specific cells are fated during embryogenesis to contribute to adult structures (Baker, 2007). These are called imaginal cells and make up imaginal tissues that are contained within the body of larvae. During pupation, these tissues are often broken down but will provide the signals and tissues to rapidly grow the correct adult structure. Polarity, symmetry, and size cues are established during larval and embryonic development (Figure

1.3). Imaginal tissues have long served as an important tool for understanding the mechanisms controlling cell cycle regulation, size determination, and development. We will focus on wing imaginal discs and eye imaginal discs as they provide distinct advantages for the analysis of stabilization of E2f1 protein on normal tissue homeostasis.

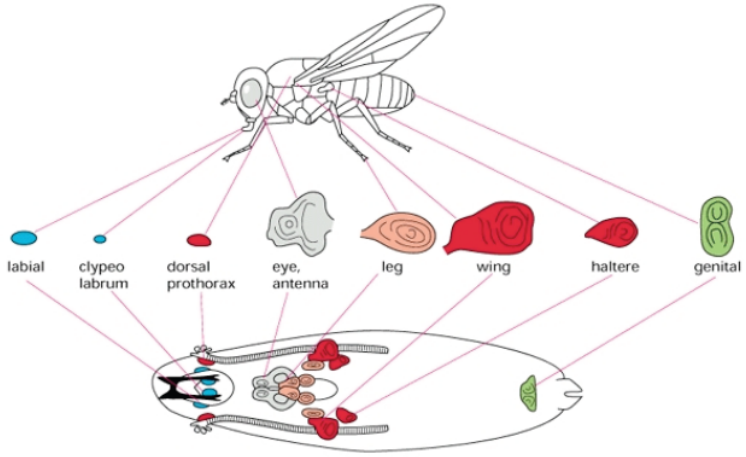

Figure 1.4: *Drosophila* Larval Imaginal Structures Schematic of the imaginal tissues found in Drosophila larvae during development and the adult structures they give rise to. Discussed further in this chapter are wing and eye discs.

Imaginal Wing Discs

Initiating as an approximately 50 cell-primordium, wing imaginal discs will undergo rapid mitotic cell cycles to proliferate to a nearly 50,000 cell epithelial monolayer at the end of larval development (Neufeld et al., 1998). Upon pupation, this disc will give rise to the adult wing. During larval growth, cell cycles are rapid and asynchronous, with an estimated total of 9.1 cell divisions (Martin et al., 2009). Early divisions occur 5.5 hours per complete cell cycle, while cycles may extend to nearly 30 hours towards late larval development. There are specific polarity patterns established during imaginal disc origination that will persist throughout larval growth and dictate the orientation of the adult wing. These patterns are carried out by spatial expression of several secreted proteins, such as *hedgehog*, *wingless*,

and *decapentaplegic* (Neufeld et al., 1998). The most prominent is the formation of the anterior and posterior compartments, which is established within the wing primordial and persists until the adult structure. Due to their well-characterized cell cycle profiles and developmental patterning, imaginal wing discs have long served as an excellent model for understanding rapidly proliferating developing tissues and which molecular mechanisms are at work to ensure their homeostasis. Interestingly, there is relatively little apoptosis seen normally in developing disc tissues. It has been shown, however, that these cells are poised to enter programmed cell death pathways in response to cells which have atypical cell division rates or acquired genomic damage. Despite robust apoptosis, these discs are able to form the correct adult structures. Recent work has shown that UV damage which results in up to 50% cell death within the imaginal disc can still result in a normal adult wing (Rusconi et al., 2000). The mechanism at work is known as compensatory proliferation, which is discussed further later. Due to all these characteristics, imaginal wing discs have served as an excellent model to study the balance of proliferation, arrest, and apoptosis required for complex tissues to develop rapidly.

Imaginal Eye Discs

Unlike their relatively unorganized cell cycle program of the wing, the eye imaginal disc is exquisitely useful due to its' precise spatial and temporal cell cycle patterns (Roignant and Treisman, 2009). This disc arises from an approximately 20 cell epithelial primordium in the embryonic blastoderm. By late larval development, the disc contains nearly 2000 cells and has a distinct pattern of differentiation. A dorsal ventral furrow forms and progresses from posterior to anterior, driven by *hedgehog* function (Baker, 2007). This furrow is the

area where specific cells differentiate to a photoreceptor fate. Those cells anterior to the furrow are synchronously dividing, but without any pattern. Cells within the furrow are arrested in G1, and as the furrow progresses, some cells re-enter the cell cycle in what is called the second mitotic wave (SMW) while others differentiate into various photoreceptor cell populations (Roignant and Treisman, 2009). The power of analyzing the effects of E2f1 stabilization in this tissues falls with the genetic tool, GMR-gal4. This activator promotes UAS dependent gene expression only after the morphogenic furrow has progressed posteriorly. Therefore, it allows precise, acute, measurements of the consequences of stabilized E2f1 protein. Previous studies have investigated the role of E2f1 in the eye disc. Asano and colleagues 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 did not undergo E2f1-induced S phase or subsequent apoptosis (Asano et al., 1996).

Cell Competition, Apoptosis, and Compensatory Proliferation in Development and Cancer

Developmental biologists began studying the role of cell competition on normal development in multicellular systems when Morata and Ripoli (Morata and Ripoll, 1975) utilized the well-characterized development of Drosophila wing discs to uncover dominant mutations called "*Minute*." These mutations reduce the rate of cell division in a cell-autonomous manner. Therefore the heterozygous mutants develop more slowly, yet are viable and eventually reach normal body size. By studying clones of heterozygous cells surrounded by wild-type neighbors, they discovered that *minute* mutant cells were eliminated from the clones, and replaced with wild-type cells from the surrounding tissue. They noticed

that surrounding cells were dividing with greater frequency to replace the slow growing minute mutant cells. This observation was called "cell competition." Interestingly, this process has been implicated in cells with tumor-suppressor mutations being replaced by surrounding wild-type cells as an intrinsic tumor-suppression mechanism to eliminate precancerous cells (Tamori and Deng, 2011). However, this mechanism requires that cells are able to sense mutant cells and clear them from the population, while relying on compensatory proliferation to fill the resulting gaps to ensure normal tissue homeostasis.

A recently well-studied example of this process is termed "compensatory proliferation." In this situation, instead of slow growth, certain cells are cleared from the tissue via apoptosis (Martin et al., 2009). However, as they undergo the well-controlled cascade responsible for the activation of apoptosis, specific extra-cellular mitogens are released from the dying cells and initiate proliferation of neighboring cells (Rusconi et al., 2000). This cell-cell communication is critical to ensure those dying cells are replaced and there are a sufficient number of cells for tissue formation, but also allows that developing tissue to cull cells which may have acquired mutations that could have potentially deleterious consequences on the overall health of the organism. Cells that have induced apoptosis, but are prevented from carrying out the complete program via expression of the baculovirus p35 pan-caspase inhibitor result in what has been called "undead" or "zombie" cells (Martin et al., 2009). These cells initiate compensatory proliferation, but as they are not cleared from the tissue, continue to signal and lead to eventual hyperplasia.

Tumors initiate when proliferation is uncontrolled, often by transformed cells with activated oncogenes or inactivated tumor-suppressor proteins, and malignant neoplasias arise from these mutant cells that have lost the ability to assemble and form normal functional

tissues (Weinberg, 2007). Cancer, therefore, is a disease that arises when mutations affect intrinsic mechanisms of tissue-integrity and organ-size control. Regulated cell competition, apoptosis, and compensatory proliferation act in concert drive sufficient proliferation to generate adult structures, and maintain those structures through the life of the organism, but also to prevent accumulated mutations from allowing proliferation to proceed unchecked.

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 present the specific details of two assays critical to our investigations, flow cytometry measurement of S phase coupled destruction of GFP tagged proteins in both Drosophila S2 cells as well as dissociated Drosophila imaginal wing discs. In Chapter III, we focus on the biological consequences of disrupting of S phase-dependent destruction of E2F1. We show that the destruction of E2F1 is required for the homeostasis of rapidly developing tissues, such as imaginal wing discs. Interestingly, the stabilization of E2F1 during S phase caused apoptosis, even when E2f1 was lacking activity as a transcription factor. Instead, we propose a mechanism where E2f1/Rbf1 interactions act to induce apoptosis by relieving repression of a pro-apoptotic gene, *hid*. In Chapter IV, we describe a comprehensive review of the mechanisms behind and biological requirements for atypical cell cycles during normal development and in disease states. We cover a wide spectrum of mechanisms for inducing endoreplication, possible functions in development, and scenarios where these atypical cycles could contribute to disease. In Chapter V, these results will be discussed in a broad context and ongoing research questions will be described.

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CHAPTER II

IN-VIVO ASSAY OF S PHASE-COUPLED DESTRUCTION

Preface

This work was previously published as an in depth technical article. It is based on cell culture flow cytometry assays established by a former graduate student Shu Shibutani, and expanded by myself into imaginal discs dissociation FACS.

The S2 flow cytometry assay was initally carried out by Shu Shibutani, and previously published (Shibutani et al., 2008). I contributed to the writing of the manuscript while Robert Duronio conceived the project and finalized the manuscript. I expanded this technique into dissociated imaginal discs to utilize this technique to measure cell cycle perturbances in-vivo. This was fundamental for experiments described in Chapter III as well as a significant contribution to a manuscript developed by a former graduate student, Harmony Salzler.

Davidson, JM., and Duronio, R.J. (2011). Using Drosophila S2 Cells to Measure S phase-Coupled Protein Destruction via Flow Cytometry. Methods in Molecular Biology (Clifton, NJ). 782: 205.

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Abstract

Cell proliferation depends on the timely synthesis and destruction of proteins at specific phases of the cell cycle. Recently it was discovered that the destruction of several key cell cycle regulatory proteins during S phase is coupled directly to DNA replication. These proteins harbor a motif called a PIP degron that mediates binding to chromatin bound PCNA at replication forks and recruits the $CRL4^{Cdt2}$ E3 ubiquitin ligase. These interactions comprise an elegant mechanism for coupling DNA replication with ubiquitylation and subsequent proteolysis by the 26S proteasome. Here we describe a flow cytometry-based method using *Drosophila* S2 and wing imaginal discs cells that recapitulates S phase-specific protein proteolysis. Because of the high degree of evolutionary conservation of the PIP degron and $CRL4^{Cdt2}$ and the ease of culturing and inhibiting gene function by RNAi in S2 cells, as well as the genetic tools available *in-vivo*, our flow cytometric method should serve as a general tool for determining whether any eukaryotic protein is subject to replication-coupled protein destruction.

Introduction

Accurate progression through the cell cycle depends on the timely synthesis and destruction of a myriad of proteins. A classic example is the B-type cyclins, which are targeted for destruction via the APC/C E3 ubiquitin ligase complex during the metaphase to anaphase transition to ensure completion of mitosis. Cell cycle regulators are also targeted for destruction in other phases of the cell cycle. Recently it was discovered that the proteolysis of a small set of proteins is coupled directly to DNA synthesis occurring during S phase or DNA repair. These proteins include the pre-replicative complex component Cdt1

(Arias and Walter, 2006; Hall et al., 2008; Higa et al., 2006; Hu and Xiong, 2006; Jin et al., 2006; Nishitani et al., 2006; Senga et al., 2006), the cyclin-dependent kinase inhibitor p21 (Abbas et al., 2008; Kim et al., 2008; Nishitani et al., 2008), the *C. elegans* translesion DNA polymerase Pol-H (Kim and Michael, 2008), and the *Drosophila melanogaster* transcription factor E2f1 (Shibutani et al., 2008). There is increasing evidence that these proteins must be destroyed during S phase to ensure normal cell cycle progression.

The mechanism of destruction used by this group of proteins couples DNA replication with ubiquitin-mediated proteolysis. Each of these four proteins contains a short (approximately 14 amino acid) motif called a PIP degron that is required for replication-coupled destruction. The PIP degron has two critical features: a PIP box that confers binding to a hydrophobic pocket on PCNA (proliferating cell nuclear antigen) and key basic residues flanking the PIP box that recruit the multi-subunit $CRL4^{Cdt2}$ E3 ubiquitin ligase, presumably through direct interaction with the substrate receptor, Cdt2 (Havens and Walter, 2009). PCNA loads onto DNA as a homotrimer and serves both as a processivity clamp for DNA polymerase during DNA synthesis and as a platform to recruit various PIP box-containing proteins to replicating DNA (e.g. topoisomerase and histone deacetyltransferase) (Moldovan et al., 2007). Current data support a model whereby proteins with a PIP degron interact with PCNA and become ubiquitylated by $CRL4^{Cdt2}$ only when PCNA is assembled on chromatin at replication forks. This results in an elegant mechanism for coupling ongoing DNA replication with ubiquitylation and subsequent proteolysis by the 26S proteasome (Figure 2.1).

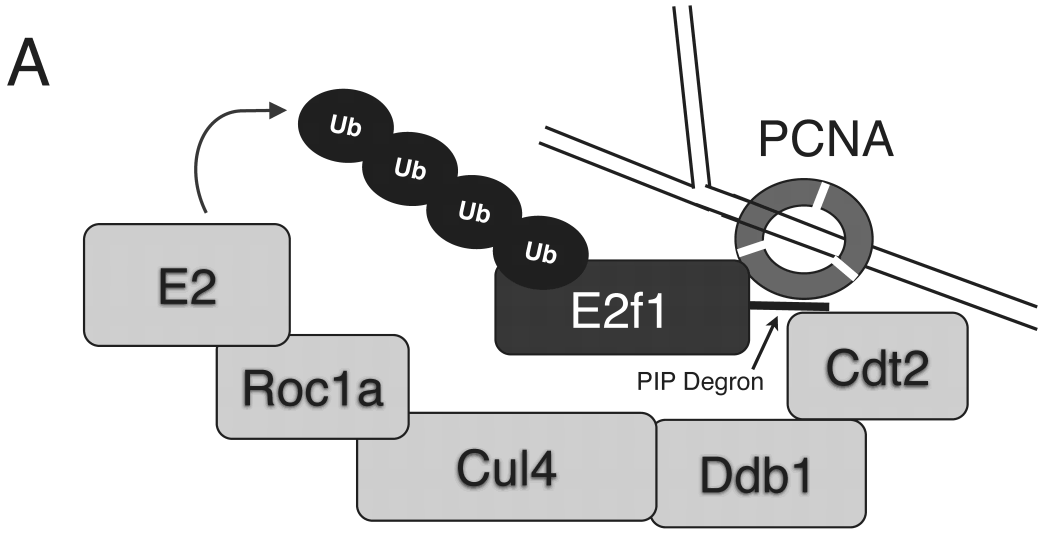

Figure 2.1: A model of CLR^{Cdt2} mediated destruction of E2f1.

Drosophila E2f1 is a member of the E2F family of transcriptional activators, which play a pivotal role in the progression of the G1-S transition (van den Heuvel and Dyson, 2008). Like mammalian E2Fs, E2f1 is inhibited prior to S phase via interaction with the retinoblastoma protein (pRb) homolog, Rbf1. Activation of cyclin-dependent kinases during G1 results in hyper-phosphorylation of Rbf1, thereby relieving E2f1 repression and activating a transcription program that promotes entry into S phase. E2f1 is then rapidly destroyed during S phase (Asano et al., 1996; Heriche et al., 2003; Reis and Edgar, 2004; Shibutani et al., 2007). We hypothesized that S phase-specific destruction provides an important Rbf1-independent negative regulation of E2f1. In order to test this, we developed a facile method for using exogenous GFP-E2f1 fusion proteins and flow cytometry of cultured S2 cells that recapitulates the S phase-specific destruction of E2f1 observed *in vivo*. This method has the capacity to rapidly test whether specific domains within E2f1 or trans-

acting factors (e.g. components of $CRL4^{Cdt2}$) are required for destruction of E2f1 during S phase. Our assay allowed us to discover a PIP degron within E2f1 and to demonstrate that PCNA and $CRL4^{Cdt2}$ are necessary for S phase-specific destruction of E2f1 (Shibutani et al., 2008).

In this chapter we describe our method for determining whether a protein is destroyed specifically during S phase and whether this destruction requires components of the $CRL4^{Cdt2}$ E3 ligase. We discuss the method of generating stably transfected S2 cell lines, generating and treating those cell lines with dsRNA that will knockdown components of the $CRL4^{Cdt2}$ E3 ligase, inducing expression of GFP-fusion proteins, and submitting fixed and stained cells to flow cytometric analysis to ascertain their cell cycle profile and quantitatively measure S phase-specific protein destruction. Because of a high degree of evolutionary conservation and the ease of culturing and inhibiting gene function by bathing S2 cells in dsRNA, our flow cytometric method should serve as a general tool for determining whether any eukaryotic protein is subject to the replication-coupled, PIP degron/$CRL4^{Cdt2}$-mediated destruction mechanism.

MATERIALS

***Drosophila* S2 Cell Culture:** *Drosophila* S2 Cells (Invitrogen). SF-900 III SFM culture medium (Gibco, Invitrogen). Penicillin-Streptomycin: Solution stabilized, sterile-filtered, with 10,000 units penicillin and 10mg streptomycin/mL (Sigma, St. Louis, MO)., 25 cm^2 Corning Cell Culture Flasks (Sigma). Incubators with proper humidity (28°C/100% air incubator for normal growth, 37°C/100% for heat shock induction).

Creating Double Strand RNA for RNAi: T7 RNA Polymerase (New England Biolabs), RNAsein® Ribonuclease Inhibitor (Promega), 10x Transcription Buffer: 400mM Tris pH

8.0, 150 mM $MgCl_2$, 50mM DTT, 0.5 mg/mL BSA. DNase 1U/ul (Promega), NTPs set 100mM Solutions (Fermentas), Phenol:Chloroform:Isoamyl Alcohol 25:24:1 saturated with 10 mM Tris, pH 8.0, 1 mM EDTA. (Sigma Aldrich), dsRNA ladder (New England Biolabs), AlphaImager™ 2200 (Alpha Innotech) and ImageQuant 5.2 software (GE Life Science)

Generating Stable Lines: pHGW: Gateway-compatible destination vector developed by Dr. Terence Murphy (http://www.ciwemb.edu/labs/murphy/Gateway%20vectors.html). Available upon request. This vector allows NH_2-terminal GFP fusion driven by the *Hsp70* promoter, with Ampicillin resistance. Other vectors allow COOH-terminal fusion, which may be necessary for certain proteins depending on the stability of the fusion protein and the location of the PIP degron (e.g. NH_2-terminal GFP fusion may mask an NH_2-terminal PIP degron). Gateway® LR Clonase™ II enzyme (Invitrogen). pCoHygro plasmid (Invitrogen) 4526bp with Ampicillin resistance. Hygromyosin B (Invitrogen). Amaxa Nucleofectin® V for *Drosophila* S2 Cell transfection (Lonza). Nucleofector® apparatus (Lonza). 6 well culture plate (Corning). 2ml round bottom tubes (Eppendorf). $25cm^2$ cell culture flasks (Corning).

Fixing S2 Cells: 32% Paraformaldehyde (Electon Microscope Sciences), 1X PBS-T: 1X Phosphate Buffered Saline with 1%/vol Tween20 (Promega), DNase-free RNase (Roche), Propidium Iodide: 1.0 mg/mL solution in water (Invitrogen), store at 4° C protected from light.

Flow Cytometric Analysis: Dako CyAn Flow Cytometry System (Dako, Beckman-Coulter), Summit 4.3 Flow Cytometer Data Collection software (Dako, Beckman-Coulter), Falcon 35-2002 round bottom 6-mL polystyrene tube $12x75mm^2$. If using a different flow cytometer system, use appropriate tube required for that cytometer. ModFit LT™ (Verity Software

House) Required parameters: height and area of fluorescence emission signal for GFP or propidium iodide signal. Height of forward and side scatter.

6. Nomenclature: Forward Scatter (FS); parameter to measure light scattered less than 10˚ FS measures relative cell size. Side Scatter (SS); light scattered at a 90˚ angle. Measures complexity of cellular membranes combined to measuring clumping of cells. Height (H) is the maximum amplitude of the emission signal. Area (A) is the area of the signal. Event Count measures the number of cells within to a certain parameter.

METHODS

Maintaining Drosophila S2 Cells in Culture

Cells were routinely grown at room temperature in SF-900 *Drosophila* medium (Gibco) without serum and 1:200 Penicillin-Streptomycin (5 units/ml Penicillin, 5 μg/ml Streptomycin, Sigma). Schneider's *Drosophila* medium (Gibco) supplemented with 10% Fetal Bovine Serum (Sigma) can also be used, but may interfere with RNAi and transfection efficiency. There are many multiparameter flow cytometer systems available that are functional to collect cell cycle data. The main requirement is that the system be able to excite and detect GFP signal versus Propidium Iodide (PE) staining. Similarly, many operating systems are publically available for cytometric analysis, including FlowJo (TreeStar) and TestDNA (Verity Software).

Passage cells when density is between 6 to 20 x 10^6 cells/mL and split at 1:2 to 1:5 dilution. S2 cells do not grow well when seeded at a density below 5 x 10^5 cells/ml. Cells will being to disassociate from the flask when overcrowded, however these cells are still alive. It is a good idea to avoid allowing the cultures to become overcrowded.

Simple laminar flow hoods are sufficient when working with *Drosophila* S2 cells, as they are not thought to carry vectors of human disease. However, good sterile technique should be utilized to prevent contamination of the cells. Once contaminate, there is little to no possibility of recovery. Therefore, it is a good idea to freeze an aliquot of all cell lines generated. We recommend freezing 2 x 10^7 cells from a single plate in 2.5 mL of freezing medium in 5 x 0.5 mL aliquots and storing in liquid nitrogen. When removing cells from the flask, tap the flask several times to dislodge adherent cells. Use a pipette to wash the surface where cells adhere before transferring cells to a new flask.

Creating double-strand RNA and RNAi on S2 Cells

Generate primers to amplify target genes using primers that contain a T7 promoter sequence (labeled in Italics). Below are the sequences used to generate dsRNA against components of the $CLR4^{Cdt2}$-mediated destruction machinery. Note that an extended knockdown of PCNA is predicted to eventually result in an S phase arrest, thereby precluding detection of S phase-coupled destruction because of a depletion of S phase cells from the population. However, we were able to find conditions of PCNA knockdown that stabilized E2f1 during S phase prior to extensive cell cycle arrest (Shibutani et al., 2008). A similar situation occurs with Cul4 knockdown, which eventually will result in G1 arrest.

a. Cul4 dsRNA Forward:

*TAATACGACTCACTATAGGG*TTGGCCAAACGATTACTTGTGGG

b. Cul4 dsRNA Reverse:

*TAATACGACTCACTATAGGG*GAGAAGATTATGGCTCAGCG

c. Cdt2 dsRNA Forward:

*TAATACGACTCACTATAGGG*GCGGGCTCCGGCATACGCGGC

d. Cdt2 dsRNA Reverse:

*TAATACGACTCACTATAGG*CGTGGCTGGAGCCCCAGGCCACG

e. PCNA dsRNA Forward:

*TAATACGACTCACTATAGGG*CAGGCCATGGACAACTCCCATG

f. PCNA dsRNA Reverse:

*TAATACGACTCACTATAGGG*TGTCTCGTTGTCCTCGATCTTGGG

Double stranded RNAs (dsRNAs) are transcribed from PCR products generated by gene-specific primers containing T7 promoter sequences. PCR products are amplified from genomic DNA or cDNA clones. cDNA clones are available from the Drosophila Genomics Resource Center (https://dgrc.cgb.indiana.edu/). RNAi causes a reduction in gene expression and is not equivalent to a complete loss of function. Western blot analysis after dsRNA treatment is recommended to determine the efficiency of knockdown. Various responses to RNA interference occur depending on the threshold of activity necessary for each particular gene product.

Transcription reaction is performed by combining the following: 2mM (final concentration) each NTP, 10μL 10x Transcription Buffer, 0.5 μl RNasein, 1 μg purified DNA template (e.g. via phenol:chloroform extraction), 1 μl T7 RNA polymerase with sterile water to make 100μl total reaction volume. Incubate at 37˚ C for 6 hours (reaction may become cloudy). Add 1μl DNase and incubate at 37˚C for 30 minutes. Annealed RNA strands are generated by boiling samples in a beaker of water to remove secondary structure and slowly returning to room temperature. Purify dsRNA reaction using phenol:chloroform:isoamyl alcohol 25:24:1.

Approximate concentrations of the dsRNAs are determined by comparing the intensity of band of 1μl of RNA sample in an agarose gel using a dsRNA ladder as a standard. The intensity of the RNA bands is analyzed using by imaging the AlphaImager™ and quantified with the ImageQuant 5.2 software. Since RNase is highly ubiquitous and ssRNA is unstable, we recommend wearing gloves and maintaining a clean environment when working with RNA. DEPC-treated water and RNAse-free plastics should be used when generating dsRNA. dsRNA itself is fairly stable, however multiple freeze-thaw cycles should be avoided.

Generating Stable Lines

The open reading frame of wild type Drosophila *E2f1* was amplified from pUAST-*E2f1* and cloned into pENTR™/D-TOPO©. The forward primers used to amplify E2f1 are designed with a CACC at the 5' end for directional cloning into pENTR. To create expression vector plasmids, pENTR *E2f1* was recombined with the Gateway expression vector pHGW, which contains an NH_2-terminal GFP tag and a *Hsp70* promoter, using the Gateway® LR Clonase™ II enzyme mix. Split cells 2-3 days prior to transfection to ensure they are growing well and not overcrowded. Ensure that the entire Nucleofector® Supplement is added to the Nucleofector® Transfection solution and allowed to reach room temperature prior to beginning transfection protocol. The transfection solution should be kept at 4°C otherwise and will expire within 3 months of the supplement being added.

Prepare 6-well plates by filling appropriate number of wells with 1ml of SF-900 SFM culture media and allow it to come to room temperature. Count an aliquot of cells and determine density. Use $1x10^6$ cells per sample, and spin down at 500xg for 5 minutes at room temperature. Completely remove supernatant and flick tube to break up pellet.

Resuspend cells in 100 μl room-temperature Nucleofector® Solution per sample. Avoid leaving cells in this solution for longer than 15 minutes. Combine cell solution with 2 μg pENTR *E2f1* and 0.5 μg pCoHygro and transfer solution to cuvette supplied in Nucleofection® Kit V, ensuring cell/DNA solution covers bottom of the cuvette with no air bubbles. Close cuvette with cap.

Select Nucleofector® Program G-030. Insert cuvette into apparatus and apply the program by pressing the X-button. Take cuvette out once transfection is successfully completed. Immediately add 500 μl of room temperature SF-900 SFM media to cuvette and gently transfer to prepared 6 well plate, using supplied pipettes. Final volume is 1.6 mL/well. Incubate cells in a humidified 25°C/100% air incubator without $C0_2$ for 2-3 days. Collect cells and centrifuge for 5 minutes at 500xg. Replace with 2mL of fresh SF-900 SFM media and transfer to a new 6 well plate. Let grow 2-3 days in a humidified 25°C/100% air incubator.

Collect cells into two 2mL round bottom Eppendorf tubes and centrifuge for 5 minutes at 500xg. To one sample, add 2mL of SF-900 SFM with 50μg/mL Hygromyosin B. To the other, add only SF-900 SFM, in case selection kills the culture. Grow in 6 well plate for 2-3 days in humidified 25°C/100% air incubator. Other selection methods are available. Methotrexate resistance plasmids: pHGCO, pHCO, p8hCHO, actDHFR, (available from Drosophila Genomics Resource Center (DGRC)). Methotrexate (Sigma) (L-amethopterin) used at a final concentration of 2×10^{-7} M (store stock solution protected from light at -20°). α-Amantinin resistance plasmids: pPC4 is available at DGRC. α-Amantinin is used at a final concentration of 5-10 μg/mL. Blastocidin S resistance: pCoBlast (available from Invitrogen). Blastocidin S used at approximatedly 5 μg/mL (per Invitrogen).

Once cells reach confluency within several days, transfer to 25 cm^2 cell culture flask and split cells 1:5 every week. Maintain 50mg/mL Hygromyosin B in SF-900 SFM media. Initial transfection efficiency should be around 50-60%, this can be checked by removing an aliquot, subjecting the cells to a 37°C heat shock for 30 minutes, waiting an hour and visualizing GFP signal under a microscope. Once population is nearly homogeneous for containing the plasmid, experiments may begin.

RNAi and Heat Shock Induction

3-4 days prior to the experiment, count and plate stably transfected pHGW-*E2f1* and pHGW-only cells at $1x10^6$ cells/mL. Add 1mL to each well of a 6 well plate. Let cells grow overnight in a humidified 28°C/100% air incubator. The following day, add the appropriate amount of dsRNA. The normal range of dsRNA is 2-30 μg dsRNA per treatment (e.g. Cul4 knockdown required 2μg whereas Cdt2 and PCNA required 10 μg). Treat each sample with dsRNA two consecutive days while incubating at 28°C/100% air. A non-specific control (e.g. LacZ) should also be used to measure effect of dsRNA treatment. A subset of the cells may be collected and lysed for western blot analysis to measure efficiency of dsRNA knockdown. Optimization of the amount of dsRNA, and length of treatment, required to sufficiently knockdown a protein of interest may be required. Prior to heat shock induction, a control sample of cells may be taken to measure baseline cell cycle profile.

Transfer the rest of the cells to a 37°C/100% air incubator for 30 minutes to induce *Hsp70* expression. After 30 minutes, return cells to 28°C/100% air incubator and remove cells for appropriate time course.

Fixing S2 Cells for Flow Cytometric Analysis

Collect all the cells from each well by pipetting gently up and down and transfer each to a labeled 2ml round bottom Eppendorf tube. Centrifuge tubes for 5 min at 500xg. Pour out supernatant, flick tube gently to break up pellet. Wash cells with 500µl 1X phosphate buffered saline solution (PBS), centrifuge 5 minutes at 2300 rpm and gently tap tube to break up pellet. Add 500 µl 1% paraformaldehyde in ice cold 1X PBS and keep on ice for 30 min. Make the paraformaldehyde solution immediately prior to use by combining 700µl 10x PBS, 218µl 32% paraformaldehyde stock solution and bringing total volume to 7 mL. Centrifuge tubes at 4° for 5 minutes at 500xg. Remove paraformaldehyde solution into proper waste container. Gently tap tube to break up pellet. Wash cells with ice cold 1X PBS. Centrifuge tubes at 4° for 5 minutes at 500xg, pour out supernatant and flick to break up pellet. Add 500µl 1X PBT (PBS with 0.1% Tween20), let sit 15 minutes at room temperature. This increases the permeability of the cell membranes. Centrifuge for 5 minutes at 500xg at room temperature. Remove supernatant and tap tube to break up pellet. Add 3ul DNase-free RNase. Incubate for 30 minutes at 37° C. It is critical to degrade RNA as propidium iodide binds equally to DNA and RNA. Add 5µl 15 mM Propidium Iodide and 500µl ice cold 1X PBS. Leave at 4° C overnight. Fixed and stained cells can be kept up to a week until flow cytometric analysis, kept at 4° C and protected from light. Propidium iodide is a DNA intercalating agent and must therefore be treated appropriately and discarded in a suitable manner.

FLOW CYTOMETRIC ANALYSIS OF S2 CELLS

We recommend proper training from be received prior to beginning a flow cytometry experiment. The following method outlines a protocol for measuring cell cycle profiles in S2 cells on a CyAn (Dako, Beckman Coulter), but does not address all the complexities of flow cytometric analyis or the various other operating systems that can be used.

Transfer all cells to Falcon tubes appropriate for the flow cytometer being used. Break up any clumps of cells prior to analysis by gently vortexing tubes. Establish the correct protocol using Summit 4.3 software. GFP detection uses a 488-nm laser and detection through a 530/30 bandpass filter. Propidium iodide uses a 488-nm laser and detection through a 630/30 bandpass filter. Create an acquisition template with plots by selecting "Create Histograms" from the menu options and create the following plots measuring the following parameters (x and y axis respectively): SS Area vs. FS Area, PI Lin vs. PI Area, PI Area vs. GFP Log, Event Count vs. GFP Log, Event Counts vs. PI Area, Even Count vs. PI Area (of only GFP positive gate). Save this template as a new protocol (Figure 2.2).

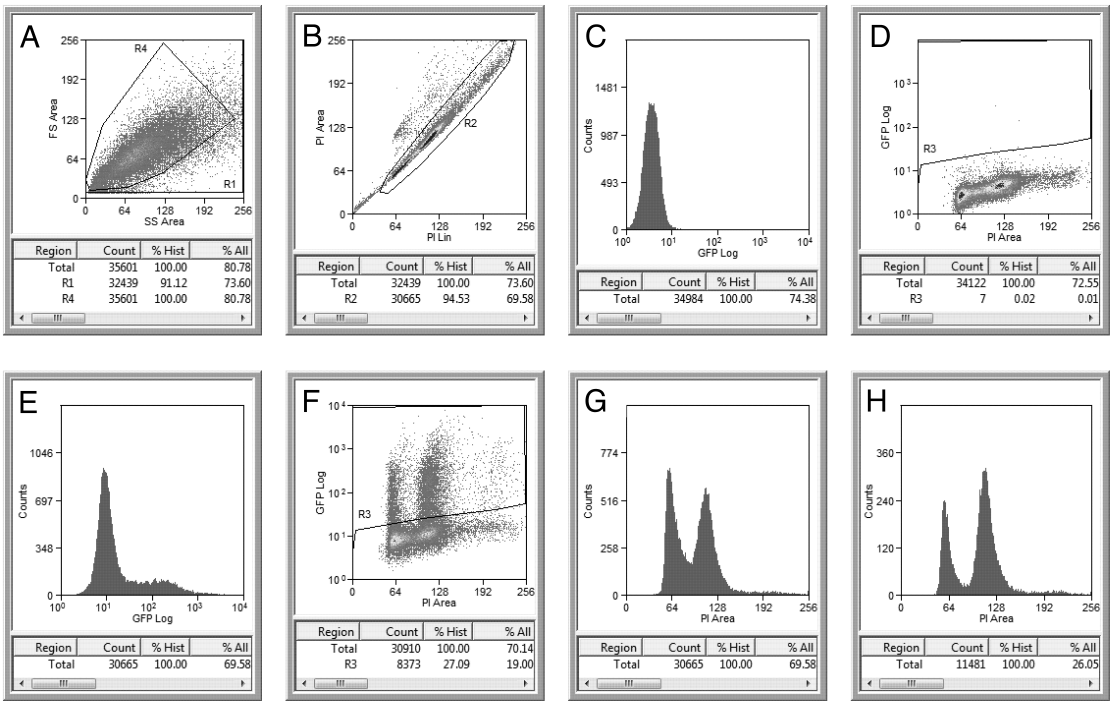

Figure 2.2: Gating protocol for generating a cell cycle profile of *Drosophila* S2 cells, and measuring GFP fluorescence intensity using Summit software (X and Y-axis respectively). A: SS Area vs. FS Area. Gate R1 and R4 to collect only cells, not debris. B: PI Linear vs. PI area. Gate R2 to only collect single cells, not doublets or triplets, which may skew laser intensity readings. C: GFP log vs. Counts on untransfected control cells. GFP laser intensity should be set so the peak falls between 10^0-10^1. D: PI Area vs. GFP log on untransfected control cells. Set R3 gate so that no more than 0.05% of untransfected cells falls in the GFP positive range. E: GFP log vs. Counts for GFP-E2f1 transfected cells. Note shift of GFP intensity to 10^2 range. F: PI area vs. GFP log measurement of GFP-E2f1 transfected cells. Note shift of cell population now within the GFP positive gate (27% of all cells). G: PI area vs. Counts of all cells measured. First peak (64) represents with G1, second peak (128) represents G2/M. H: PI area vs Counts of GFP positive only cells. Note that fewer cells appear in the area between the G1 and G2/M peaks, reflecting the targeted destruction in S phase of GFP-E2f1.

Using the Gate Logic Builder and the Gate Scheme applications, create the following gates on your histograms, as follows;

a. SS Area vs. FS Area: Avoid bottom left corner where debris will be measured (R1).

b. PI Lin vs. PI Area: Avoid doublets and clumped cells (R2).

c. PI Area vs. GFP Log: Create a gate that will select only GFP positive cells (the threshold will be determined by running the untransfected control).

Under the Sample menu, assign a root file name, a starting file number and a data storage location. Limit total events collected (<1,000,000). Under the Preferences menu, set

parameters to “Auto-Start” and “Auto-Boost.” However, occasionally manual Boost will be required to activate the collection. Run the untransfected control cells to set the GFP negative parameter. Aim for less than 0.05% of cells falling above the GFP cut off in the untransfected samples. Adjust laser voltage to generate single peak in the GFP Log vs. Counts histogram that falls between 10^0-10^1. Adjust gates to ensure that no debris and only single cells are being collected. Once parameters are set for negative control, do not adjust for the remainder of the experiment.

When acquiring cells, ensure that cells following “Boost” are not collected and cells run through the analysis between 200-400 cells per second. Running the cells too quickly will decrease the accuracy of the data due to decreasing the time for excitation and detection. Run pHGW-only (GFP only) control to measure efficiency of transfection and heat shock induction. Aim to collect at least 10,000 experimental events (GFP positive cells after all the gating). The minimum number needed for statistical analysis is 4,000. 20,000 cells is optimal. Ensure that data is properly stored after each collection before starting the next. Take care that the gates ensure that only the experimental data is saved, not debris, which could quickly overfill your saving capacity. In between samples, activate “Backflush” under the Acquisition menu to clear out any residual cells. Rinse the CyAn uptake nozzle with distilled, deionized, water.

DATA ANALYSIS

There are two options for analyzing flow cytometry data to visualize cell cycle coupled destruction, such as is seen with *Drosophila* E2f1. Visually, two histograms can be overlaid, using the Summit 4.3 software, and color labeled to distinguish the total cell

population as compared to the GFP-positive population alone. This provides an easy way to quickly visualize S phase specific destruction.

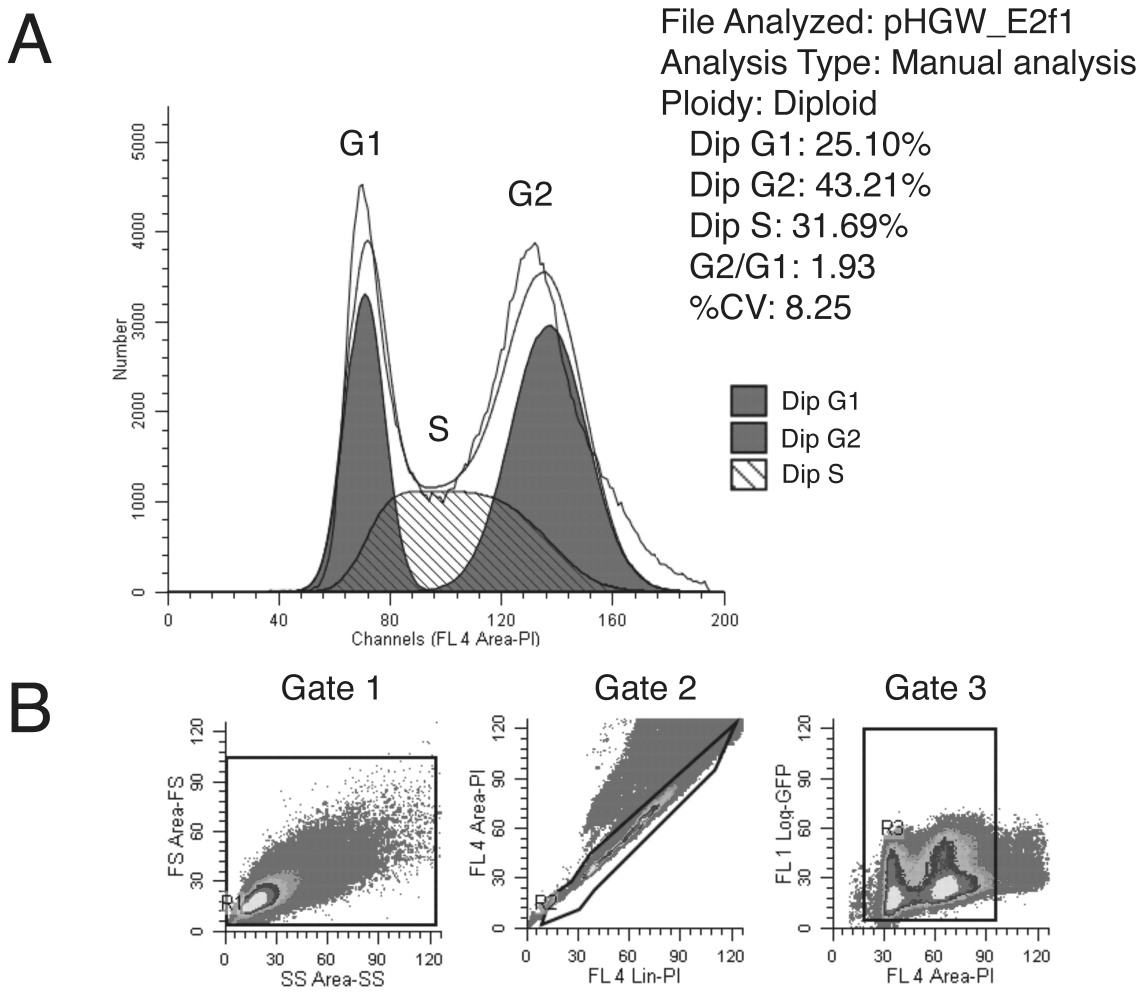

Figure 2.3: ModFit LT analysis. A: Histogram representing the calculated percentage of cells in each phase of the cell cycle and the confidence in these values. Shaded peaks represent the calculated profile of G1 and G2 peaks, dashed lines represent the calculated percentage of cells in S phase. B: The gating required to analysis flow cytometry samples (X and Y-axis, respectively). Gate 1: SS area vs. FS area. Gate 2: PI Linear vs. PI Area. Gate 3: Area PI vs. GFP Log. Include all cells in this gate to get a total cell population profile, and gate only the GFP positive cells to measure the cell cycle profile of GFP-E2f1 transfected cells.

To observe more subtle changes in the cell cycle, or for a more quantifiable approach, statistical software can calculate the percentages of cells in each phase of the cycle, in addition to apoptotic (i.e. sub-G_1) cells. This will be discussed further. Open ModFit LT software and open the file to analyze. Select Area PI as the Parameter for Analysis. Define three gates as follows (X and Y-axis respectively): Gate 1: SS Area vs. FS Area. Gate 2:

Linear PI vs. Area PI. Gate 3: Area PI vs. Log GFP. Adjust the gates to closely reflect how the data was collected (Figure 2.3).

Initially, include all cells in Area PI vs. Log GFP gate. This will give you the whole cell cycle profile. Open Mod window to set the parameters. For S2 cells, use the following constraints: Linearity: 1.93, Standards: 0, Number of cycles: 1, Model Template: Diploid, Range Positions: Compute Range Positions. This will allow the computer to appropriately predict G1 and G2 peaks. Utilizing Auto-Aggregation, Auto-Debris and Apoptosis modeling functions is depended on the samples and the data required. Select Range, and adjust the automatically applied G1 and G2 ranges to most accurately fit the data. Then select Fit, and the software will calculate the percentage of cells in G_1, S, and G_2/M. The strength of confidence is an %CV value, which should be under 10 for high confidence in the data. Low experimental cell numbers can reduce this confidence value.

Repeat this with the GFP positive only cell population to compare the values. A S phase-coupled destruction will result in a significant decrease in GFP positive cells in S phase. RNAi that depletes a component of the destruction mechanism will result in a flow cytometry profile that resembles a GFP-only control. Basic spreadsheet software (eg. Excel) can create graphical representations of the percentages of cells in each phase.

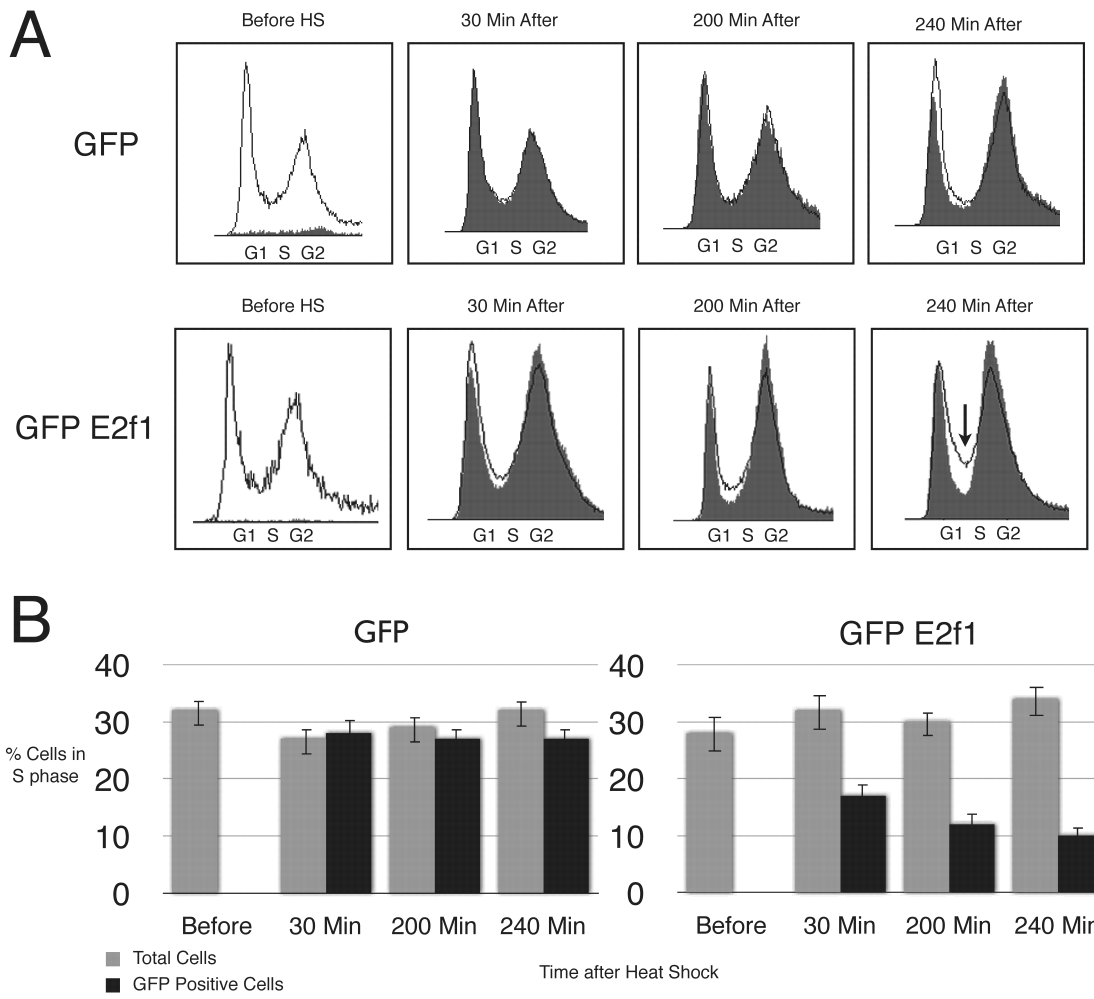

Figure 2.4: Representations of flow cytometric data. A) Time course of induction of GFP and GFP-E2f1 expression in stable S2 cell lines. Overlay of total cell population (unfilled histogram) with GFP positive cells (filled histogram). Time points taken before heat shock induction, and for representative time points. Note that by 240 minutes after expression, an S phase coupled destruction of GFP-E2f1 is noticeable (arrow). B) Graphical representation of data collected in ModFit analysis. Percentages of cells in S phase of the total cell population compared to the GFP positive cell population, over the time course. Note the sharp decline in GFP-E2f1 cells. Error bars represent one standard error.

Flow Cytometric Analysis of Dissociated Imaginal Discs

A powerful application of this method utilizes dissociated imaginal discs instead of S2 cell culture to observe cell cycle conditions in a developing organism. While many of the steps are similar as presented above, we will highlight the specific technique of obtaining and staining disociated cells. This technique contributed significantly to the following chapter, as well as the work of a fellow lab mate, Harmony Salzler.

Culture *Drosophila* according to normal protocol. This method allows for either dissociation of the entire disc and measuring the effects of a specific mutant genotype (Salzler et al., 2009) or driving expression of a GFP-tagged transgenic protein in the posterior compartment of the imaginal disc using UAS/En-Gal4. In either situation, it is best to grow robust cultures at room temperature, clear all wandering larvae at the beginning of the day, and then collect larvae which crawl up within a few hours to ensure they are all of the same stage. Pick as many as you can feasibly dissect in 30 minutes. In PBS, quickly dissect out the imaginal discs with out bringing any connective tissue or fat bodies. Leaving the haltare disc attached is fine. Transfer to an eppendorf tube after coating pipet tip with carcasses to ensure discs will not stick to pipet.

Aspirate PBS and replace with the following solution: PBS containing 0.05% Trypsin- EDTA (Gibco), and 1X Hoechst 33342 DNA binding dye (Sigma) and rock for 3 hours on rotating platform. Do not flick or vortex sample. To verify dissociation, small aliquots can be removed and visualized with microscopy. Once cells are nearly homogeneous, transfer to appropriate flow cytometry sample tube. If there are visible clumps still present, pass sample gently through a 22 micron mesh filter. Proceed with flow cytometry analysis. However, instead of fixed cells and propidium iodine marking DNA content, these cells are unfixed and use Hoeschts which must be measured with a 335nm wavelength. Therefore, it is imperative to quickly sort cells, as extended trypsinization results in cell death and to use a flow cytometer equipt with a UV laser Otherwise, parameters described above should be followed. Again, it is possible to measure the effects of specific genotypes on the entire disc cell cycle profile, as shown below with SLBP mutants causing cell cycle defects (Salzler et al., 2009) relative to wild type.

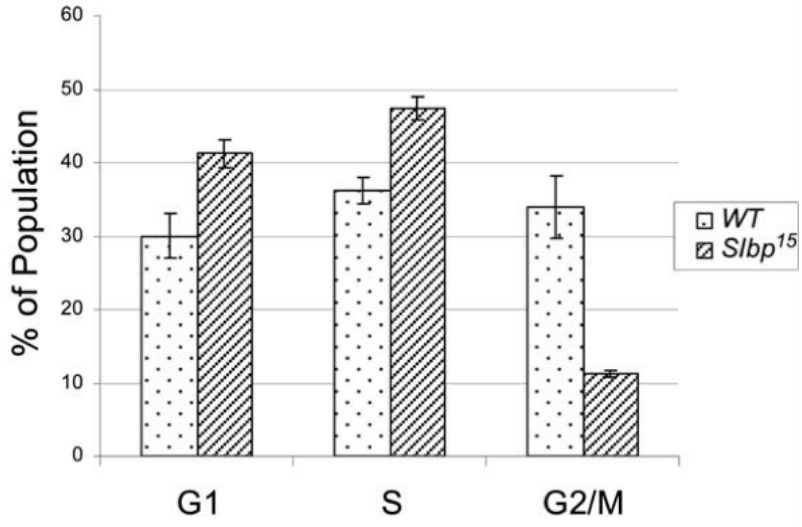

Figure 2. 5 FACS analysis of *wt* and *Slbp*^{15} mutant wing discs. Each bar represents the mean of three independent experiments where over 4,000 cells were analyzed per genotype.

Conversely, one can measure the effects of transgenes only expressed in the posterior compartment, utilizing the anterior portion of the disc as an internal control. This is acheieved by expressing GFP-tagged ectopic proteins with the En-Gal4 promoter. This will be discussed much further in the following chapter.

DISCUSSION

Here we present a detail methods protocol for utilizing S2 cells or imaginal discs and flow cytometry tools to measure the specific cell cycle regulated destruction of certain proteins. These tools have made significant contributions to several publications and will continue to be a critical technique in future investigations. Future directions can include the dissociation with other imaginal tissues (such as eye discs) and measuring the proteolysis of other target proteins.

ACKNOWLEDGMENTS

We are grateful to Aida Flor A. de la Cruz and Bruce A. Edgar for their contributions to establishing dissociated discs for use in flow cytometry. We also thank Shu Shibutani for initially utilizing S2 cells to measure tagged protein destruction. Thanks to Barry Udis, Nancy Fisher, Joan Kalnitsky, and Lisa Bixby at UNC Flow Cytometry Core Facility for their technical assistance.

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CHAPTER III

BIOLOGICAL CONSEQUENCES OF E2f1 STABILIZATION

PREFACE

This work is currently in press in the journal PLoS Genetics. All experiments were conducted by myself, but this follows on work established by a former graduate student, Shu Shibutani (Shibutani et al., 2008). The project was conceived and written by myself and Robert J. Duronio.

Davidson, J.M., and Duronio, R.J. (2012). "S Phase-Coupled E2f1 Destruction Ensures Homeostasis in Proliferating Tissues." PLoS Genetics (in press).

ABSTRACT

Precise control of cell cycle regulators is critical for normal development and tissue homeostasis. E2F transcription factors are activated during G1 to drive the G1-S transition, and then inhibited during S phase by a variety of mechanisms. Here, we genetically manipulate the single *Drosophila* activator E2F (E2f1) to explore the developmental requirement for S phase-coupled E2F down-regulation. Expression of an E2f1 mutant that is not destroyed during S phase drives cell cycle progression and causes apoptosis. Interestingly, this apoptosis is not exclusively the result of inappropriate cell cycle progression, because a stable E2f1 mutant that cannot function as a transcription factor or drive cell cycle progression also triggers apoptosis. This observation suggests that the inappropriate presence of E2f1 protein during S phase can trigger apoptosis by mechanisms that are independent of E2F acting directly at target genes. The ability of S phase-stabilized

E2f1 to trigger apoptosis requires an interaction between E2f1 and the *Drosophila* pRb homolog, Rbf1, and involves induction of the pro-apoptotic gene, *hid*. Simultaneously blocking E2f1 destruction during S phase and inhibiting the induction of apoptosis results in tissue overgrowth and lethality. We propose that inappropriate accumulation of E2f1 protein during S phase triggers the elimination of potentially hyperplastic cells via apoptosis in order to ensure normal development of rapidly proliferating tissues.

Introduction

During development, cells continually integrate extrinsic and intrinsic signals that control cell growth, proliferation and apoptosis. Mitogenic signals that drive growth and cell proliferation are balanced with apoptotic signals that eliminate damaged or unneeded cells. Genetic changes that inappropriately stimulate cell proliferation, reduce apoptosis, or both disrupt this homeostasis and result in aberrant development or neoplastic diseases like cancer [1]. Understanding the mechanisms that exist to maintain such homeostasis is thus an important area of investigation.

The balance between cell proliferation and cell death in growing tissues must ultimately function through key regulators of the cell cycle. These regulators include the E2F family of transcription factors, which control the expression of many genes responsible for cell proliferation, differentiation and apoptosis [2]. E2Fs are highly conserved proteins that act as either activators or repressors of transcription based on protein partners and structural features. As key mediators of cell proliferation and apoptosis, tight regulation of E2F activity is essential for normal development in mammals, flies, worms, and plants [2,3]. The best-characterized mode of regulation involves members of the retinoblastoma (pRb) tumor

suppressor protein family, which bind to and inhibit those members of the E2F family that dimerize with DP proteins [2]. In addition, pRb family/E2F complexes function as transcriptional repressors [4]. Loss of pRb function causes ectopic proliferation and apoptosis that is partially repressed by reducing E2F activity [5].

pRb family regulation of E2F occurs in quiescent cells and during G1 phase. Several pRb-independent mechanisms have been described that regulate activator E2Fs outside of G1, including Cyclin A/Cdk2-dependent phosphorylation of the DP subunit [6,7,8], SCF^{Skp2}-directed proteolysis [9,10], antagonism by the atypical E2F7 and E2F8 proteins [4,11,12], and binding to DP-4 [13]. These mechanisms are thought to down-regulate transcriptional activation by E2Fs during S phase or after DNA damage. In particular, disruption of Cyclin A/Cdk2 phosphorylation of E2F1 causes S phase defects and apoptosis in mouse cells, as does simultaneous loss of E2F7 and E2F8 [7,8,11]. In addition, E2F7/8 mutation in mice results in lethality, indicating that E2F7/8 play an essential role in the E2F regulatory network during development [11]. Mouse mutant genotypes that would specifically determine the contribution to development of Cyclin A/Cdk2 phosphorylation or the other modes of pRb-independent E2F inhibition have not been developed.

Here we examine the function of pRb-independent E2F regulation in developing *Drosophila* tissues, where E2F regulatory pathways are simpler than in mammals. While eight mammalian E2F genes encode nine distinct proteins (5 activators and 4 repressors), *Drosophila* encodes a single E2F activator (E2f1) and a single E2F repressor (E2f2), both of which bind the single Dp protein [2]. The primary cell cycle regulator is E2f1/Dp, which activates the transcription of replication factor genes and is negatively regulated by Rbf1, one of the two *Drosophila* pRb family members [14]. *E2f1* mutant cells proliferate poorly

[15,16,17], in part because of E2f2-mediated repression [18,19]. Conversely, over-expression of E2f1 can drive cells into S phase [20,21,22]. E2f1 over-expression also induces apoptosis [17,20,21], and this may reflect the positive role E2f1 plays in developmentally controlled and DNA damage induced apoptosis [23,24,25,26]. While many S phase and apoptotic transcriptional targets of E2f1 have been described [27,28], the aspects of E2f1 regulation that coordinate the expression of these targets in rapidly growing tissues to achieve the proper balance of cell proliferation and apoptosis are not well understood.

In addition to the evolutionarily conserved pRb mode of activator E2F regulation, *Drosophila* E2f1 is inhibited by rapid destruction during early S phase [20,29,30,31]. We recently determined that this S phase destruction is mediated by a "PIP degron" in E2f1 [32]. PIP degrons promote direct binding to DNA-loaded PCNA and the subsequent recruitment of the $CRL4^{Cdt2}$ ubiquitin E3 ligase, thereby coupling proteolysis with DNA synthesis that occurs during S phase or after DNA damage [33,34]. *Drosophila* E2f1 thus joined a small but growing number of proteins involved in genome duplication and maintenance that are regulated by $CRL4^{Cdt2}$ [33,34].

We previously demonstrated that expression of an S phase-stabilized E2f1 causes cell cycle acceleration, apoptosis, and developmental defects [32]. Because similar levels of wild type E2f1 expression, which is degraded during S phase, do not induce these phenotypes, we concluded that accumulation of E2f1 during S phase is poorly tolerated during development. However, we did not determine whether apoptosis and the developmental defects were a consequence of changes to the cell cycle in response to hyperactive E2f1 transcriptional activity, or to some other consequence of E2f1 accumulation during S phase. To explore this issue, we used assays in larval imaginal discs to understand the *in vivo* consequences of

stabilizing E2f1 during S phase in developing tissues, focusing specifically on which activities of the E2f1 protein (e.g. DNA binding or Rbf1 binding) were responsible for the deleterious phenotypes resulting from stabilization during S phase.

We demonstrate here that the apoptosis and developmental defects caused by accumulation of E2f1 protein during S phase do not require E2f1's ability to induce transcription and cell cycle progression. Instead, apoptosis may occur via alleviation of Rbf1-dependent repression of the pro-apoptotic gene *hid*. We also show that simultaneously stabilizing E2f1 in S phase and blocking apoptosis results in extensive tissue overgrowth. We propose that inappropriate S phase accumulation of E2f1 protein in proliferating *Drosophila* cells triggers a form of proliferative stress, and that the cells experiencing this stress are consequently eliminated via apoptosis in order to prevent hyper-proliferation and maintain homeostasis during rapid tissue growth.

MATERIALS AND METHODS

Molecular Biology

E2f1 constructs were generated and expressed using pENTR TOPO (Invitrogen) and Gateway-compatible P element vectors (http://www.ciwemb.edu/labs/murphy/Gateway%20vectors.html).

Cell Culture and Transfection

For S phase-coupled protein destruction analysis, S2 cells stably transfected with *hsp70* constructs were heat shocked for 30 minutes at 37°C, which results in GFP or GFP-E2f1 expression in all cells of the population, and allowed to recover at room temperature for

200 minutes prior to analysis by flow cytometry. During the 200 min chase period GFP-E2f1 is destroyed in S phase cells while GFP is not, as measured by the percentage of GFP(+) cells in each phase of the cell cycle. For cell cycle analysis, S2 cells were transfected with plasmid DNA expressing GFP or GFP-E2f1 encoding mRNA from the *Actin5C* promoter and analyzed by flow cytometry 48 hours later.

Flow Cytometry

For flow analysis of wing imaginal discs, at least 15 third instar larvae of the appropriate genotype were dissected in PBS. 30 imaginal discs were collected and immediately dissociated in PBS containing 0.05% Trypsin- EDTA (Gibco), and 1X Hoechst 33342 DNA binding dye (Sigma) and rocked for 3 hours at room temperature. The dissociated tissue was then immediately analyzed using a LSRII Flow Cytometer and Diva software (Becton Dickinson). Cell cycle profiles were calculated using FlowJo™ Software. Percentages of G1, S, and G2 cells were calculated using Modfit LT software (Verity Software House). P values for all experiments were calculated by student's T test.
S2 cells stained with propidium iodide were analyzed by flow analysis as previously described [32] using the Cyan flow cytometer with Summit 4.3 software (Deko).

qRT-PCR

Total RNA was extracted from 30 third instar wing imaginal discs using Trizol reagent (Invitrogen) and tissue was sheared with eight passes through a 25-gauge needle. 0.75 ug of total RNA was used for reverse transcription with RevertAid Reverse transcription kit (Fermentas). The resulting cDNA was used for qRT-PCR performed using an ABI prism 7700 Sequence Detection system. Relative levels of specific mRNAs were determined by detection of Maxima Sybr Green (Fermentas). Primers are listed in Table 1. Comparative CT

methods were used to quantify levels versus control Rp49 mRNA using the manufacturer's protocol.

Drosophila genetics and cytology

Transgenic flies were generated by injecting UASp-E2f1 plasmids into w^{1118} (Best Gene *Drosophila* Injection Services, Chino Hills, CA). UAS-GFP, Engrailed-Gal4, UAS-RFP and UAS-p35 stocks were obtained from the Bloomington Stock Center. For antibody staining, imaginal discs were dissected from third instar larvae in PBS, fixed in 6% paraformaldehyde for 20 minutes at room temperature, then permeabilized for 20 minutes in PBS-1.0% Triton-X. Wing discs were incubated overnight with mouse anti-GFP (1:1000, Abcam) and rabbit anti-cleaved Caspase-3 (Asp175) (1:200, Cell Signaling Technology) at 4°C. Secondary antibodies were goat anti-mouse Oregon Green 488 (1:2000 Invitrogen) and goat anti-rabbit Rhodamine (1:2000 Invitrogen) for 1 hour at room temperature. Eye discs were dissected, incubated in 10ug/mL EdU (Click-iT™ EdU Alexa Fluor® 594, Invitrogen) for 30 minutes, fixed and permeabilized as described above. EdU was detected according to manufacture protocol. To detect mitosis, eye discs were incubated overnight at 4°C with rabbit anti-PH3 (1:1000, Abcam) and then with goat anti-rabbit Rhodamine (1:1000 Invitrogen) for 1 hour at room temperature. For DNA damage detection, rabbit anti-p-H2Av antibody from Kim McKim's lab was incubated over night at 4°C at 1:1000 and then goat anti-rabbit Rhodamine (1:1000 Invitrogen). DAPI was added for DNA detection (1:1000 Invitrogen) for 2 minutes. Tissue samples were analyzed with a Zeiss LSM 510 scanning confocal microscope. Quantification of CC3 and p-H2Av foci was collected by projecting confocal images that were one micron apart through the eye disc of 6 discs per genotype and

using ImageJ software to count all foci above threshold detection posterior to morphogenetic furrow. 7 images per disc projected for p-H2Av, 6 images per disc for CC3. Graph shown represents the average number of foci of those 6 discs.

Western Blot and Co-immunoprecipitation

30 third instar larvae wing imaginal discs were dissected in PBS then dissociated by eight passes through a 25 gauge needle after addition of ice-cold NP40 buffer with protease inhibitors aprotinin (1:1000), leupeptin (1:1000) and PMSF (1:100). E2f1 protein levels were measured with affinity-purified rabbit anti-E2f1 raised against full-length *Drosophila* E2f1 (1:1000)[32] overnight at 4°C and anti-rabbit HRP secondary (1:10,000 GE Healthcare) for 1 hour at room temperature. B-tubulin was used as loading control (1:1000, Abcam) with anti-rabbit HRP secondary (1:10,000 GE Healthcare). Co-immunoprecipitation was performed by co-transfecting S2 cells with 2μg Myc-E2f1 and 1μg HA-Dp or HA-Rbf1 using the Amaxa transfection system (Lonza) and incubating the cells for 24 hours at 28°C. S2 cells were lysed on ice using NP40 buffer with the protease inhibitor cocktail described above. 10% of each total extract was subjected to western blot analysis with mouse anti-Myc (1:2000 UNC Hybridoma) or mouse anti-HA (1:2000, UNC Hybridoma). Secondary antibodies were ECL donkey anti-mouse HRP (1:10,000, GE Healthcare) and ECL donkey anti-rabbit HRP (1:10,000, GE Healthcare). The remainder of the extract was incubated overnight at 4°C with 0.5 μg mouse anti-Myc antibody (UNC Hybridoma) and 1/10 volume Protein G Sepharose 4 Fast-Flow beads (GE Healthcare).

Results

An in vivo assay for S phase-coupled E2f1 destruction

In order to examine the biological functions of $CRL4^{Cdt2}$-mediated destruction of E2f1 during tissue growth and development, we examined larval wing imaginal discs, which grow from a ~50 cell primordium to a ~50,000 cell epithelial monolayer via canonical G1-S-G2-M cell division cycles and then differentiate into the adult wing during pupal development [17,35]. Imaginal disc growth is highly tuned to modulate the balance between proliferation and apoptosis in response to particular stimuli. A dramatic example is the ability of wing discs to utilize "compensatory proliferation" in order to achieve normal wing development when as many as 50% of the disc cells have been killed via apoptosis following ionizing radiation [36]. This is possible because *Drosophila* apoptotic cells release mitogens such as Dpp and Wg that signal neighboring cells to begin proliferating and replace the dying cells [37,38,39]. We utilized this well characterized, rapidly proliferating tissue to examine the consequences of disrupting the normal S phase-coupled destruction of E2f1 (Figure 1A). We sought to determine the extent to which this destruction contributes to the balance between proliferation and apoptosis.

We previously established an assay for E2f1 destruction during S phase using flow cytometry of cultured *Drosophila* S2 cells expressing GFP-E2f1 fusion proteins [32]. In this assay, a mutation of E2f1 predicted to disrupt interaction with PCNA ($GFP\text{-}E2f1^{PIP\text{-}3A}$) or a mutation predicted to abrogate $CRL4^{Cdt2}$ binding ($GFP\text{-}E2f1^{R161A}$) blocks S phase destruction (Figure S1A, B) [40]. We adapted this assay to wing imaginal discs in order to establish a quantifiable assay for measuring E2f1 destruction *in vivo*. We used *engrailed*-Gal4 (en-Gal4) to induce GFP or GFP-E2f1 fusion protein expression (e.g. "en-Gal4 > GFP") in all cells of

the posterior compartment of the disc (Figure S1C). Wing discs were dissected from third instar larvae, dissociated into individual cells by trypsin digestion, and subjected to flow cytometry after staining cells with a DNA binding dye [41]. We were able to directly compare the cell cycle profile of GFP-expressing posterior compartment cells to GFP-negative, anterior compartment control cells from the same tissue (Figure S1D-F). Because GFP is stable throughout the cell cycle, all posterior compartment S phase cells from en-Gal4 > GFP discs were also GFP-positive (Figure S1D, G). In contrast, en-Gal4 > GFP-E2f1 posterior compartment cells with an S phase DNA content were unlikely to be GFP-positive, because GFP-E2f1 is destroyed during S phase (Figure S1E, G). Only ~12% of all GFP-E2f1 expressing cells in the posterior compartment were also in S phase, whereas ~27% of GFP-expressing cells were in S phase (Figure S1G). This S phase destruction requires an intact PIP degron, as expression of GFP-E2f1$^{PIP\text{-}3A}$ resulted in an amount of GFP-positive posterior compartment S phase cells similar to GFP controls (Figure S1F, G). (For the rest of this manuscript we will refer to stabilized E2f1$^{PIP\text{-}3A}$ as E2f1^{Stable}). These data extend our previously published wing disc experiments, in which we measured the effects of E2f1^{Stable} expression on cell cycle progression by flow cytometry, but not directly on E2f1 destruction [32].

We previously showed that E2f1^{Stable} expression accelerates cell cycle progression by using en-Gal4 to drive expression of GFP or GFP + GFP-E2f1 fusion proteins together in the posterior compartment of wing imaginal discs [32]. To measure such cell cycle effects for this study, we switched to co-expressing RFP with GFP or GFP-E2f1 fusion proteins (Figure S1C). By determining the number of RFP-positive cells in each phase of the cell cycle via DNA content, we can obtain a cell cycle profile of all posterior compartment cells. E2f1

stimulates cell cycle progression in wing imaginal disc cells by reducing the duration of G1 phase [17]. Therefore, by comparing the number of RFP-positive cells with G1 DNA content after expression of GFP or GFP-E2f1, we are able to quantify the extent to which E2f1 expression affects the cell cycle. For example, expression of either GFP-E2f1 or GFP-E2f1^{Stable} caused a decrease in the percentage of cells in the population with a G1 DNA content compared to GFP expression alone (~11% versus ~28%, respectively; Figure S1H), indicating that both wild type and S phase-stabilized E2f1 proteins are equally able to increase the rate of wing disc cell cycle progression by reducing G1 length, as we previously described [32].

E2f1 domain mutations disrupt critical E2f1 functions

We previously demonstrated that in addition to an increase in the rate of cell proliferation, ectopic expression of E2f1^{Stable} in wing imaginal discs caused an increase in apoptosis [32]. Interestingly, under the conditions of these experiments, expression of wild type E2f1 did not induce apoptosis although it did increase the rate of proliferation. We therefore hypothesize that E2f1^{Stable} -induced apoptosis is not merely a consequence of increased cell proliferation resulting from excess E2f1 activity, but that the stabilization of E2f1 specifically in S phase triggers cell death.

To explore this phenomenon further, we constructed variant forms of E2f1^{Stable} in which key E2f1 activities--DNA binding, Rbf1 binding, and transactivation--were disrupted in order to determine those aspects of E2f1 function that are necessary for E2f1^{Stable} -induced phenotypes (Figure 1B). To disrupt DNA binding, we mutated to alanines four amino acids in the highly conserved RRXYD motif (R292, R293, Y295 and D296) that make direct contact

with bases in the E2F recognition sequence (E2f1$^{DBD\ Mut}$) [42]. Mutation of the E2F RRXYD motif was previously demonstrated to block DNA binding [43]. To disrupt interaction with Rbf1, we engineered into our constructs a previously characterized missense mutation (L786Q) within the COOH-terminal Rbf1-binding domain of E2f1 that disrupts normal Rbf1-E2f1 interaction but leaves E2f1 transactivation intact (E2f1$^{Rb\ Mut}$) [44]. Because this single amino acid change does not completely eliminate Rbf1-E2f1 interaction (see Figure 2E), we also engineered into our constructs a previously described mutation (*E2f1^{i2}*) that inserts a stop codon at amino acid Q527 [45]. This allele produces a truncated protein lacking the COOH terminal 1/3 of E2f1, thereby eliminating both transactivation function and Rbf1 binding. We will refer to this allele as E2f1^{Trunc}.

We first determined whether the mutations we engineered affected GFP-E2f1 and GFP-E2f1^{Stable} activity as predicted. We generated UAS-transgenic lines and selected for analysis those that expressed equivalent amounts of GFP-E2f1 mRNA when driven with en-Gal4 (Figure 1C). Each GFP-E2f1^{Stable} mutant protein accumulated to a similar level that was 30-40% higher than either GFP-E2f1 or endogenous E2f1 (Figure 1D). This increase in protein level is consistent with stabilization only during S phase, which represents about one third of the total cell cycle length (Figure 1F, GFP only).

We next assessed the ability of the E2f1 mutant proteins to drive cell cycle progression and to activate E2f1 target gene expression. The GFP-E2f1$^{Rb\ Mut}$ and GFP-E2f1$^{Stable/Rb\ Mut}$ Rbf1 binding mutants with intact transactivation domains were able to promote cell cycle progression (Figure 1E). In contrast, expression of either GFP-E2f1 or GFP-E2f1^{Stable} proteins with mutations that disrupt the transcriptional activity of E2f1, either by blocking

DNA binding (GFP-E2f1$^{DBD\ Mut}$) or removing the transactivation domain (GFP-E2f1^{Trunc}), failed to shorten G1 (Figure 1E). Identical results were obtained using S2 cells (Figure S1I).

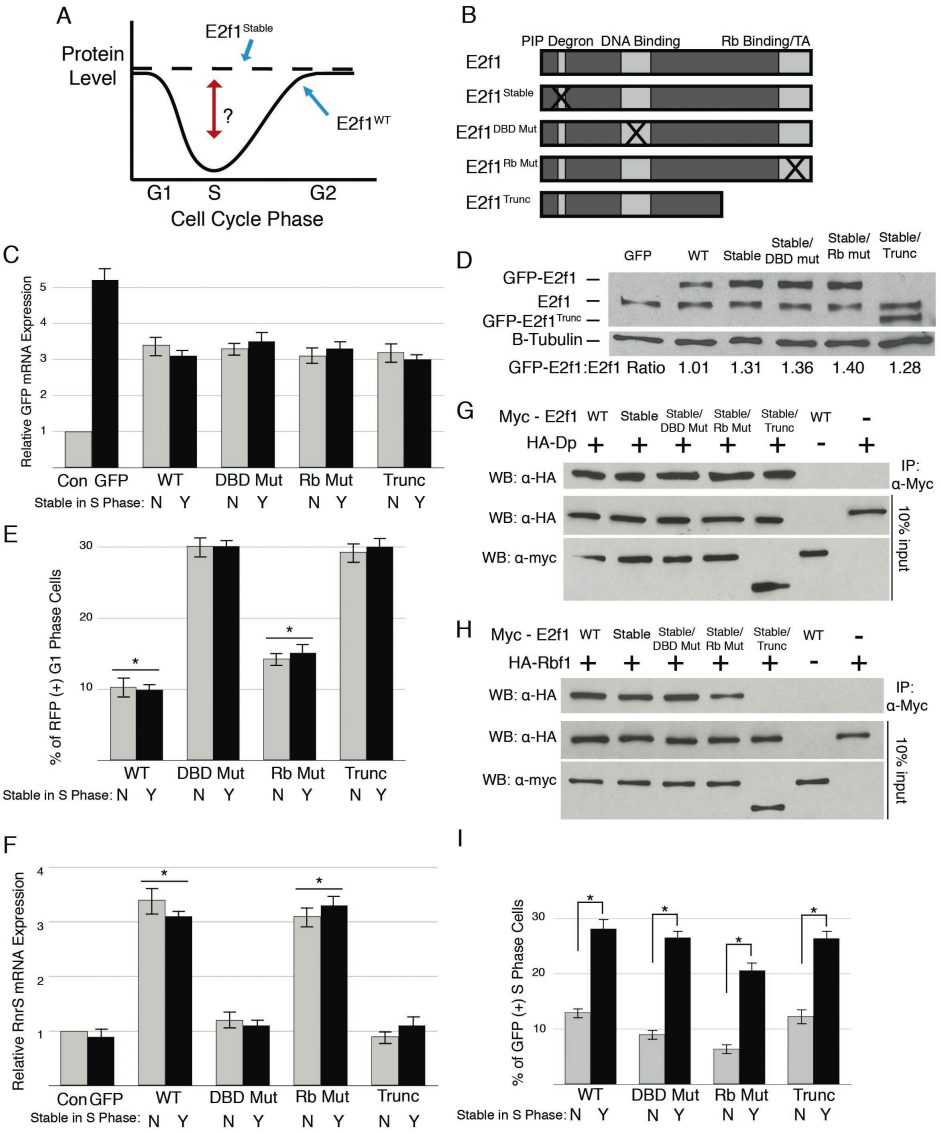

Figure 3.1: Domain mutations disrupt critical E2f1 functionsA) Schematic of the experimental paradigm. B) Schematic representation of E2f1 alleles used in this study. C) qRT-PCR quantification of GFP-containing mRNA in en-Gal4 wing discs expressing GFP or the indicated GFP-E2f1 fusion proteins that lack (grey; "N") or contain (black; "Y") the PIP-3A mutation (Figure S1A) relative to a non-transgenic w^{1118} control (Con). Error bars represent the standard error of three independent experiments. These designations will be used throughout the remaining figures. UAS-GFP expression was greater than any E2f1 construct because the UASt promoter was used rather than UASp. D) Anti-E2f1 western blot measuring GFP-E2f1 and endogenous E2f1 expression in third instar imaginal wing discs. The ratio of transgene expression to endogenous E2f1 expression is shown below. E) Quantification by flow cytometry of RFP-positive G1 cells from trypsin-dissociated en-Gal4, UAS-RFP wing discs expressing GFP or the indicated GFP-E2f1 fusion proteins. * $p < 0.001$ as compared to GFP-E2f1 expression. F) qRT-PCR quantification of *RnrS* mRNA in en-Gal4 wing discs expressing GFP or the

indicated GFP-E2f1 fusion proteins. G, H) Co-immunoprecipitation analysis of Myc-E2f1 and HA-Dp (G) or HA-Rbf1 (H) from transiently transfected S2 cells. I) Quantification by flow cytometry of GFP-positive S phase cells from trypsin-dissociated en-Gal4 wing discs expressing GFP or the indicated GFP-E2f1 fusion proteins. * $p < 0.001$ compared between stabilized and normally degraded proteins.

Mutations that disrupt DNA binding or transactivation should prevent E2f1 from activating expression of replication factor genes. To test this prediction, we measured the level of *RnrS* mRNA, a well-known E2f1-regulated transcript [15]. While expression of GFP did not change the level of *RnrS* mRNA, both GFP-E2f1 and GFP-E2f1^{Stable} expression resulted in a ~3 fold increase in *RnrS* mRNA in wing imaginal discs (Figure 1F). Similar to the cell cycle progression results, those GFP-E2f1 or GFP-E2f1^{Stable} mutant derivatives that are predicted to be deficient for E2f1 transcriptional activity (GFP-E2f1$^{DBD\ Mut}$ and GFP-E2f1^{Trunc}) were unable to induce *RnrS* expression, while the Rbf1 binding point mutant (GFP-E2f1$^{Rb\ Mut}$) induced *RnrS* expression similarly to GFP-E2f1 (Figure 1F). Thus, the introduction of the S phase stabilizing mutation did not alter the transcriptional activity of E2f1.

E2f1 requires dimerization with Dp for transcriptional activity and Rbf1 binding for normal regulation in G1 phase [46]. To determine whether our mutations affected Dp or Rbf1 binding, we transiently transfected Myc-E2f1 with either HA-Dp or HA-Rbf1 into S2 cells and performed co-immunoprecipitation assays. All of the E2f1^{Stable} mutant proteins bound Dp equivalently to wild type E2f1 (Figure 1G). Likewise, we found that E2f1, E2f1^{Stable}, and E2f1$^{Stable/DBD\ Mut}$ precipitated similar amounts of Rbf1 (Figure 1H). In contrast, E2f1$^{Stable/Rb\ Mut}$ precipitated a reduced amount of Rbf1 relative to E2f1, and the truncated E2f1$^{Stable/Trunc}$ showed no ability to precipitate Rbf1 (Figure 1H). These data indicate that we have successfully created PIP degron mutant derivatives of E2f1 that have the predicted effects on

the ability to activate transcription and drive cell cycle progression (GFP-E2f1$^{Stable/DBD\ Mut}$), bind Rbf1 (E2f1$^{Stable/Rb\ Mut}$), or both (E2f1$^{Stable/Trunc}$).

E2f1 destruction does not require DNA binding or interaction with Rbf1

We next asked whether any of these mutations affected S phase-coupled E2f1 destruction. Using either the wing disc or S2 cell flow cytometric assays, we found that E2f1$^{DBD\ Mut}$, E2f1$^{Rb\ Mut}$, and E2f1^{Trunc} are each destroyed during S phase in a PIP degron-dependent manner (Figure 1I, S1J) [32]. These data indicate that neither the DNA binding, Rbf1 interaction, or transactivation domains of E2f1 are required for S phase-coupled destruction. We previously demonstrated that E2f1 destruction during S phase requires Dp [32], a result that could be interpreted as a requirement for E2f1/Dp DNA binding [34]. However, an alternative interpretation from our observations that the E2f1$^{DBD\ Mut}$ protein binds Dp and is destroyed normally during S phase is that E2f1/Dp heterodimers are the preferred substrate of CRL4^{Cdt2}. In addition, these data suggest that a nuclear pool of E2f1/Dp that is not bound to DNA can interact with PCNA at replication forks and recruit the ubiquitylation machinery.

Rbf1 binding but not DNA binding is required for S phase-stabilized E2f1 to induce apoptosis

As we showed previously [32], GFP-E2f1^{Stable} induces apoptosis in wing imaginal discs although expression of GFP-E2f1 or GFP does not (Figure 2A-C). We hypothesized that some activity of E2f1 is necessary to cause cell death only when the protein is inappropriately stabilized in S phase. To determine which functional domains of GFP-

E2f1^{Stable} were required to induce apoptosis, we expressed GFP-E2f1^{Stable} variants containing each of the three mutations described above and stained wing imaginal discs with anti-cleaved caspase 3 antibodies (CC3). We first examined the E2f1 DNA binding domain mutant. As expected, GFP-E2f1$^{DBD\ Mut}$, which does not function as a transcription factor or cell cycle regulator, did not induce apoptosis (Figure 2D). Very surprisingly, however, we detected robust CC3 staining when this protein was stabilized during S phase with the PIP3A mutation (GFP-E2f1$^{Stable/DBD\ Mut}$) (Figure 2E). This result indicates that apoptosis in response to stabilizing E2f1 in S phase is neither a consequence of aberrant cell cycle progression or E2f1 target gene expression, nor is it solely due to gross over-expression as the normally degradable E2f1$^{DBD\ Mut}$ did not cause this phenotype.

We next addressed whether GFP-E2f1^{Stable}-induced apoptosis requires an interaction with Rbf1. Expression of GFP-E2f1$^{Rb\ Mut}$ did not induce apoptosis (Figure 2F). The S phase-stabilized Rbf1 binding mutant GFP-E2f1$^{Stable/Rb\ Mut}$ caused an increase in CC3 staining compared to controls, but less than we observed with either GFP-E2f1^{Stable} or GFP-E2f1$^{Stable/DBD\ Mut}$ expression (Figure 2G). Intriguingly, this effect suggested that the ability of S phase-stabilized E2f1 to induce apoptosis requires an interaction with Rbf1 but *not* the ability of E2f to bind to E2F response elements at target genes or to shorten G1 phase. To test the role of the E2f1-Rbf1 interaction further, we examined the C-terminally truncated GFP-E2f1$^{Stable/Trunc}$ protein, which is devoid of Rbf1 binding. Neither expression of the GFP-E2f1^{Trunc} nor the GFP-E2f1$^{Stable/Trunc}$ protein resulted in an increase in CC3 staining (Figure 2H, I). Importantly, both the GFP-E2f1$^{Stable/Rb\ Mut}$ and the GFP-E2f1$^{Stable/Trunc}$ proteins were expressed at levels equivalent to the GFP-E2f1^{Stable} and GFP-E2f1$^{Stable/DBD\ Mut}$ proteins that induce apoptosis (Figure 1D).

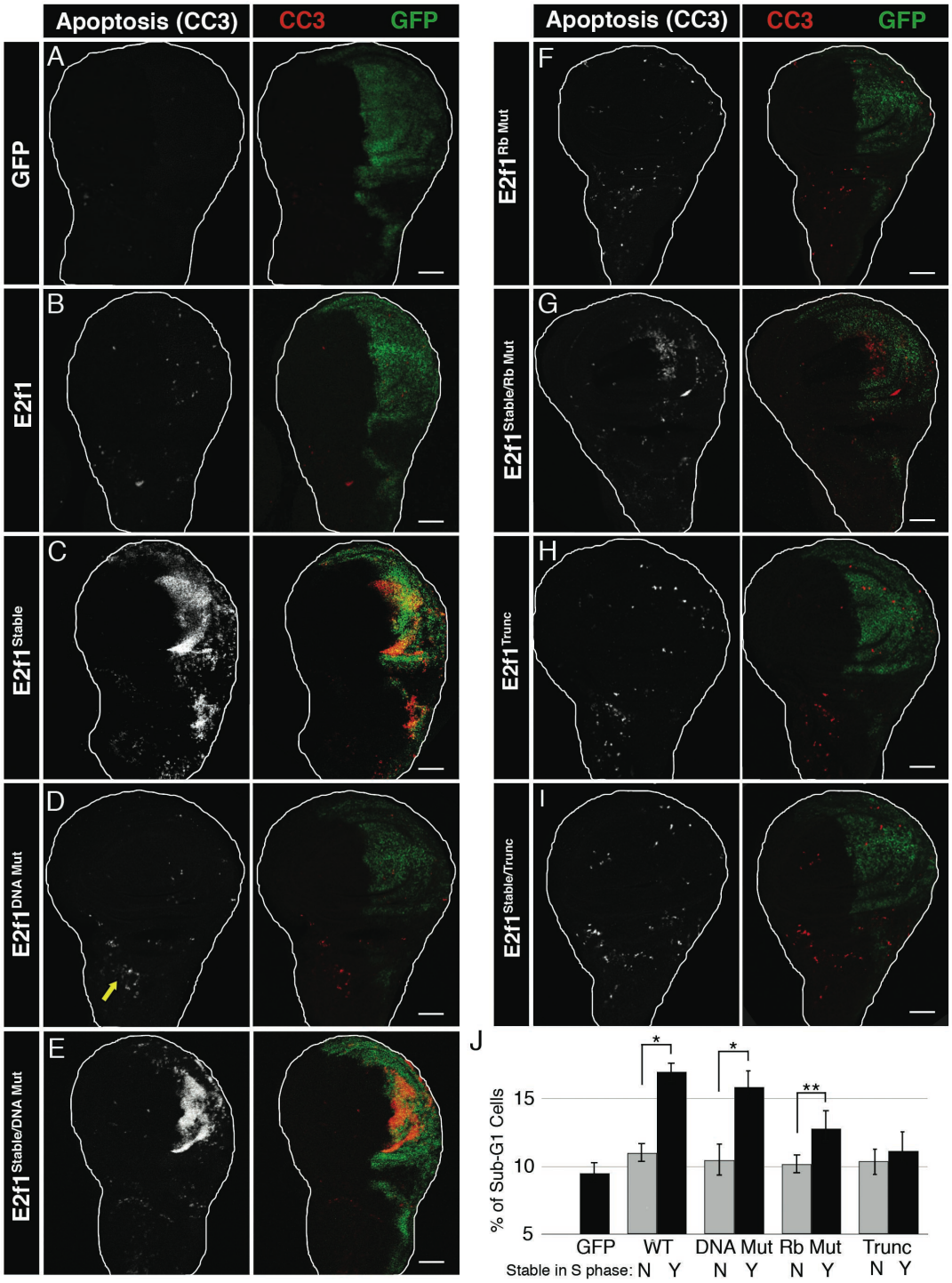

Figure 3.2: E2f1^{Stable}-induced apoptosis requires Rbf1 binding but not DNA binding A-I) Detection of apoptosis via Cleaved Caspase-3 (CC3, red) staining of third instar larval wing imaginal discs expressing the indicated GFP-E2f1 (GFP, green) proteins with en-Gal4. Arrow in D indicates an example of apoptosis observed in wild type wing discs. Bars = 50mM. J) Quantification by flow cytometry of GFP-positive apoptotic cells from trypsin-dissociated en-Gal4 wing discs expressing GFP or the indicated GFP-E2f1 fusion proteins. Error bars represent the standard error of three independent experiments. ** $p < 0.01$, * $p < 0.001$.

To quantify the apoptosis induced by different GFP-E2f1 proteins, we measured the number of cells within a specific range of sub-G1 DNA content via flow cytometry of dissociated wing discs. By this assay, we detected ~5% apoptotic cells in GFP-expressing control discs, which likely reflects both the normal low levels of apoptosis present in unperturbed discs (e.g. arrow Figure 2D) and the consequences of the extensive trypsinization required for dissociation (Figure 2J). GFP-E2f1 caused only a slight increase in sub-G1 cells relative to GFP controls, as did the transcriptionally inactive GFP-E2f1$^{DBD\ Mut}$ (Figure 2J). In contrast, and in correspondence with the CC3 staining of intact discs, expression of GFP-E2f1^{Stable} or GFP-E2f1$^{Stable/DBD\ Mut}$, which lacks a functional DNA binding domain, caused a significant increase in the apoptotic population of cells relative to controls (Figure 2J). The E2f1$^{Stable/Rb\ Mut}$ Rbf1-binding mutant triggered apoptosis, but less so than GFP-E2f1 proteins with a wild type Rbf1 binding domain, and the GFP-E2f1$^{Stable/Trunc}$ Rbf1-binding deficient mutant did not significantly increase apoptosis above controls (Figure 2J). These data indicate that interaction with Rbf1 is required for S phase-stabilized E2f1 to induce apoptosis. They also suggest that cells have a mechanism to detect aberrant E2f1 protein levels during S phase that is independent of E2f1's role as a transcription factor.

E2f1^{Stable} causes defects in the first cell cycle after induction of its expression

Our experiments thus far utilize en-Gal4 to drive GFP-E2f1 expression continuously in the posterior compartment during growth of the wing imaginal disc. Because this expression initiates very early during development, we cannot determine whether phenotypes arise in the very first cell cycle after stabilizing E2f1 during S phase, or result from E2f1^{Stable} expression over many cell cycles. To address this issue, we took advantage of the distinct cell cycle

program of eye imaginal discs. During third instar larval development, a wave of differentiation associated with a coordinated cell shape change called the morphogenetic furrow (MF) sweeps across the eye disc from posterior to anterior over a period of two days [47]. Cells anterior to the MF are undifferentiated and undergo asynchronous cell proliferation, while cells posterior to the MF differentiate into the neurons and other specialized cell types of the compound eye. Cells within the MF arrest in G1 phase, and as they exit the MF some cells remain in G1 and differentiate while others synchronously reenter a final cell division cycle prior to terminal differentiation called the "second mitotic wave" (SMW) (Figure 3A) [48].

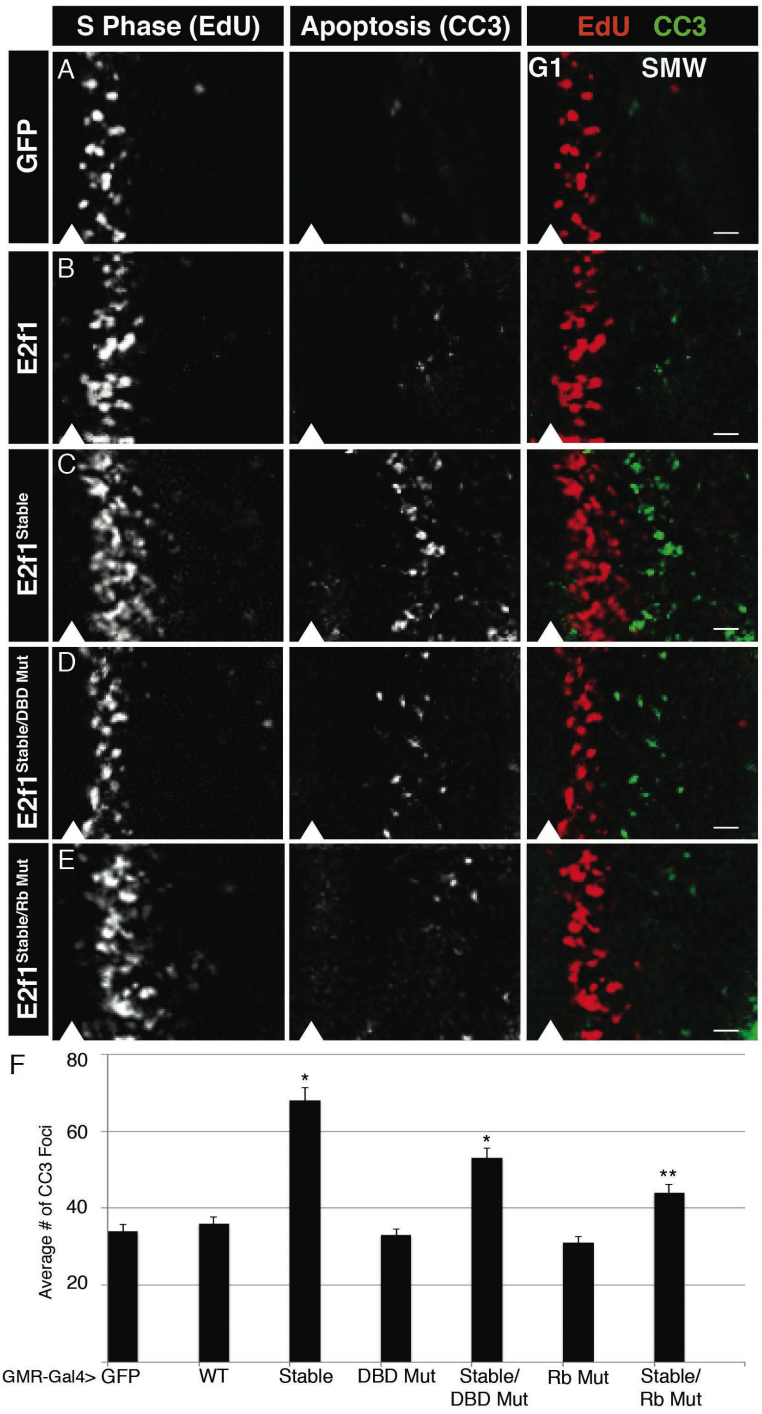

Figure 3.3: E2f1^{Stable} acts acutely to trigger apoptosis A-E) Detection of S phase by EdU labeling (red) and apoptosis by CC3 staining (green) in GMR-Gal4 third instar larval eye imaginal discs expressing GFP or the indicated GFP-E2f1 fusion proteins. Arrowheads indicate the position of the MF, with anterior to the left and posterior to the right. Bars = 5mM. F) Quantification of the number of CC3 positive cells posterior to the MF. * $p < 0.001$ relative to UAS-E2f1 expression.

The GMR-Gal4 driver is activated in late G1 cells of the MF and remains on in all cells posterior to the MF (Figure S2A). By using GMR-Gal4 we could examine the very first cell cycle after expression of the E2f1 transgenes. Normal eye discs have a very organized and stereotypical pattern of S phase in the SMW, and very few cells enter apoptosis immediately posterior to the MF (Figure 3A). Expression of GFP-E2f1 resulted in minimal changes to S phase of the SMW and no significant increase in apoptosis posterior to the MF (Figure 3B, F). (Note that others have demonstrated previously that co-expression of E2f1 and Dp with GMR results in ectopic S phase in the MF and apoptosis [21].) In contrast, expression of $E2f1^{Stable}$ disrupted the normal S phase pattern: we observed an increase in the number of cells entering S phase as well as an expansion of the zone of EdU labeling posterior to the MF, suggesting an increase in the length of S phase (Figure 3C). The changes in the S phase pattern caused by GFP-$E2f1^{Stable}$ were accompanied by an increase in DNA damage, as measured by anti-phospho-H2Av staining (Figure 4A-C, F), and apoptosis posterior to the MF (Figure 3C, F). There was no change in the number of cells entering mitosis posterior to the MF, as measured by anti-phospho-histone H3 staining (Figure S2B), suggesting that cells die before entering mitosis. In addition, $E2f1^{Stable}$ did not induce apoptosis when expressed in G1-arrested epidermal cells in the embryo (Figure S3), suggesting that apoptosis may be S phase specific. These data suggest that the presence of stabilized E2f1 in even a single S phase can disrupt cell cycle progression, induce DNA damage, and result in apoptosis. Importantly, however, DNA damage and apoptosis does not occur in <u>all</u> of the cells expressing $E2f1^{Stable}$, much like we observed by flow cytometry in the wing discs (Figure 2J).

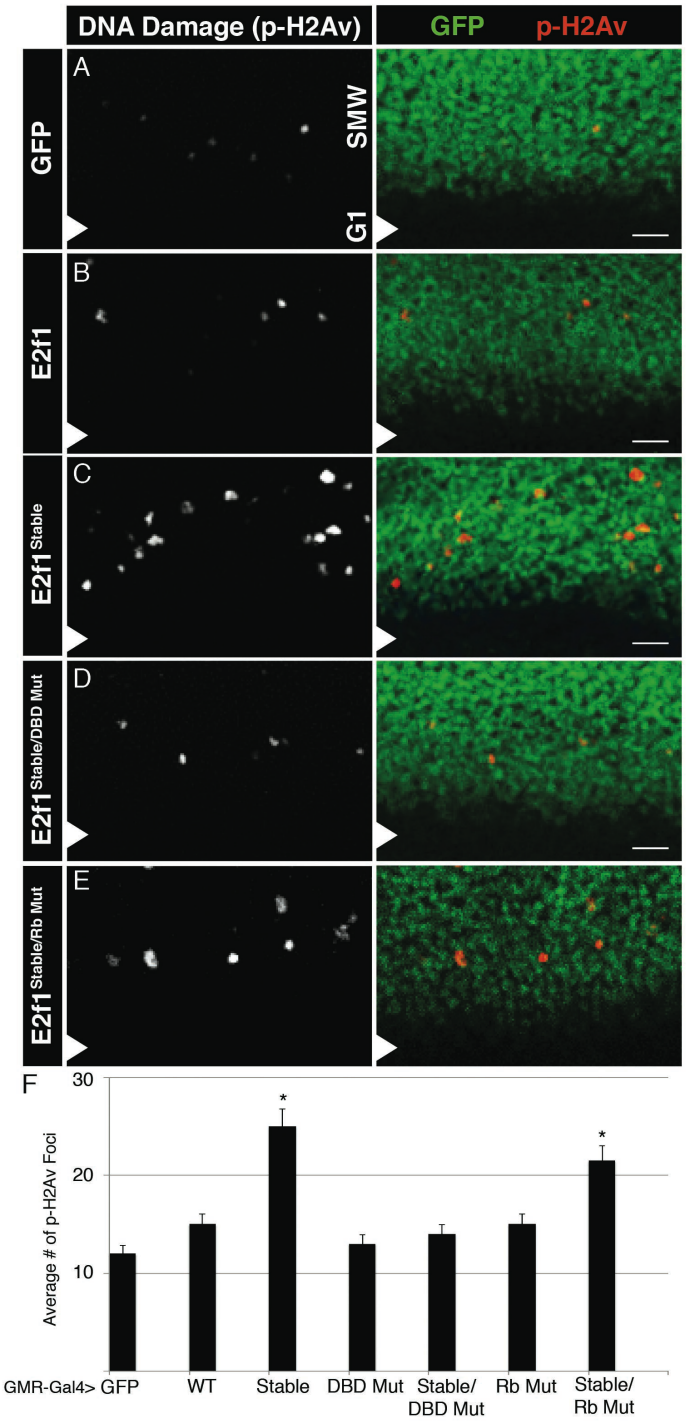

Figure 3.4: E2f1^{Stable} causes DNA damage A-E) Detection of DNA damage by anti-phospho-H2Av staining (red) in GMR-Gal4 third instar larval eye imaginal discs expressing GFP or the indicated GFP-E2f1 fusion proteins (green). Arrowheads indicate the position of the MF, with anterior to the bottom and posterior to the top. Bars = 10mM. F) Quantification of the number of phospho-H2Av positive cells posterior to the MF. * $p < 0.001$ relative to UAS-E2f1 expression.

E2f1^{Stable} induces apoptosis in two ways in eye discs

We next asked whether the DNA damage and apoptosis observed after S phase stabilization of E2f1 resulted from aberrant cell cycle progression. Expression of GFP-E2f1$^{Stable/DBD\ Mut}$ did not perturb the organization of S phase in the SMW (Figure 3D) or result in an increased number of phospho-H2Av foci (Figure 4D, F), likely because this protein does not alter cell cycle progression. Thus, the DNA damage observed with E2f1^{Stable} was most likely due to proliferation defects, because mutants that failed to shorten G1 did not induce phospho-H2Av. On the other hand, when compared to controls, GFP-E2f1$^{Stable/DBD\ Mut}$ expression did not cause an increase in phospho-H2Av foci (Figure 4D, F), although it still resulted in an increase in apoptosis posterior to the MF Figure 3D, F). These data suggest that stabilizing E2f1 in S phase can trigger apoptosis independently of cell cycle effects. The level of apoptosis in GMR > GFP-E2f1$^{Stable/DBD\ Mut}$ discs was somewhat less than in GMR > GFP-E2f1^{Stable} discs, suggesting a contribution from proliferative stress that is dependent on E2f1 DNA binding (Figure 3F). As in wing discs, apoptosis required an interaction with Rbf1 because GFP-E2f1$^{Stable/Rb\ Mut}$ expression resulted in reduced apoptosis compared to GFP-E2f1^{Stable} (Figure 3E, F). Taken together, these data suggest that two factors contribute to apoptosis when E2f1 is stabilized in S phase in the SMW: proliferative stress caused by aberrant E2f1 activity that leads to DNA damage, and a mechanism independent of E2f1 DNA binding activity that relies on interaction with Rbf1.

We previously reported that E2f1^{Stable} expression in the posterior compartment of the wing discs did not increase the amount of detectable DNA damage [32]. Our eye discs results prompted us to reexamine this issue. Using a different source of anti-phospho-H2Av antibody, we detected an increase in phospho-H2Av foci in wing imaginal discs following

expression of GFP-E2f1^{Stable}, and as in the eye discs this amount was more than with GFP-E2f1 expression (Figure S4).

Apoptosis requires full length E2f1^{Stable}

Our data are consistent with the hypothesis that an interaction between S phase-stabilized E2f1 and Rbf1 triggers apoptosis, even when E2f1^{Stable} cannot bind DNA and is functionally inactive as a transcription factor. This result suggests that cells can specifically detect and respond to E2f1/Rbf1 complexes that inappropriately assemble in S phase. However, another possibility is that over-expression of any Rbf1 binding protein would trigger apoptosis. To distinguish between these possibilities, we utilized a NH_2-terminally truncated allele of E2f1 (E2f1$^{336-805}$) that we previously characterized [49]. E2f1$^{336-805}$ contains only the C-terminal half of the E2f1 protein, and thus lacks the PIP degron and DNA binding domain but retains the Rbf1 binding and transactivation domains (Figure 5A). We hypothesized that this protein would interact with Rbf1 during S phase, but not trigger apoptosis because of the absence of a domain necessary for cells to detect the E2f1^{Stable}/Rbf1 complex. Indeed, en-Gal4 expression of E2f1$^{336-805}$ failed to induce apoptosis (Figure 5B), even though this protein accumulated to levels similar to GFP-E2f1^{Stable} (Figure 5C) and efficiently interacted with Rbf1 in co-immunoprecipitation assays (Figure 5D). These data indicate that interaction with Rbf1 is not by itself sufficient to induce apoptosis, and suggest that full-length E2f1^{Stable} is specifically recognized by cells to induce apoptosis.

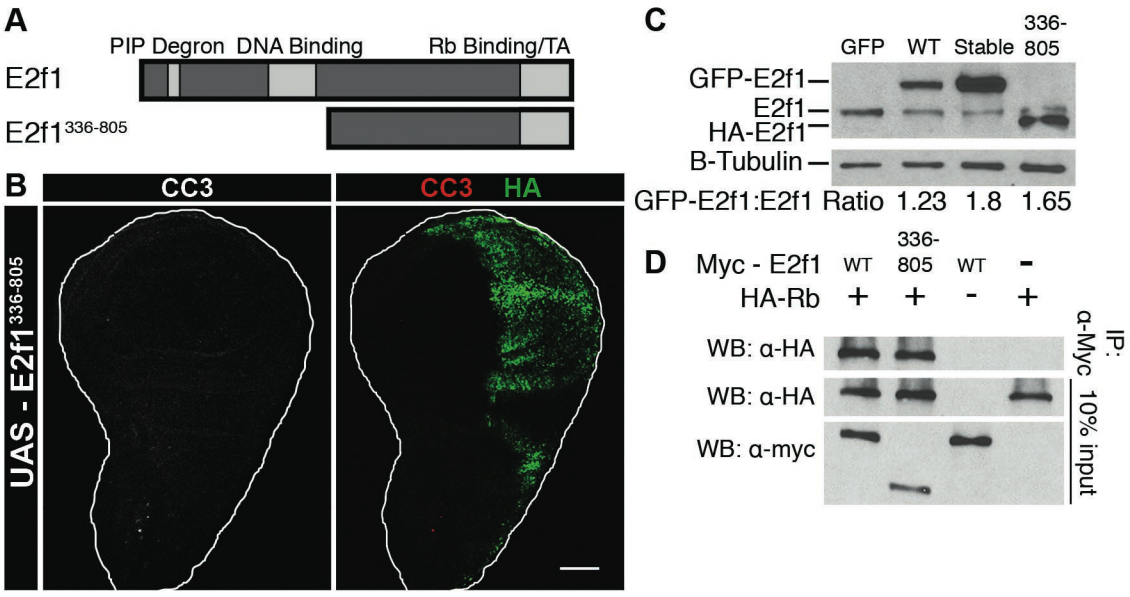

Figure 3.5: Induction of Apoptosis requires full length E2f1^{Stable} A) Schematic of the E2f1$^{336-805}$ mutant protein, which contains an NH_2-terminal HA tag. B) Detection of apoptosis via Cleaved Caspase-3 (CC3, red) staining of third instar larval wing imaginal discs expressing HA-E2f1$^{336-805}$ (anti-HA, green) with en-Gal4. Bar = 50 μm. C) Anti-E2f1 western blot of third instar imaginal wing discs expressing GFP-E2f1, GFP-E2f1^{Stable}, or HA-E2f1$^{336-805}$. D) Co-immunoprecipitation analysis of Myc-E2f1 and HA-Rbf1 from transiently transfected S2 cells.

Stabilizing E2f1 during S phase causes apoptosis by inducing *hid* expression

What mechanism could explain the induction of apoptosis upon stabilization of a transcriptionally inert, but Rbf1 binding-proficient, E2f1 protein during S phase? Recent work from several laboratories showed that loss of Rbf1 function causes apoptosis in several developmental contexts by triggering expression of the pro-apoptotic gene, *hid* [18,24,25,50,51]. Hid is homologous to SMAC/Diablo family proteins that function to antagonize IAPs, which act to block activator caspases. *hid* expression triggers an apoptotic cascade by antagonizing DIAP1, thus releasing inhibition of the initiator caspase Dronc and activating the effector caspase Drice [36,52].

We hypothesized that GFP-E2f1^{Stable} or GFP-E2f1$^{Stable/DBD\ Mut}$ binds to Rbf1 and disrupts its function, resulting in activation of *hid* expression. This hypothesis predicts that

GFP-E2f1^{Stable} or GFP-E2f1$^{Stable/DBD\ Mut}$ expression will increase *hid* expression, while E2f1 mutants that cannot bind Rbf1 will fail to increase expression. To test this prediction, we used qRT-PCR to measure the levels of *hid* mRNA in wing imaginal discs expressing the various GFP-E2f1 transgenes with en-Gal4. Consistent with our hypothesis, there was a two-fold increase in *hid* mRNA in GFP-E2f1^{Stable}- or GFP-E2f1$^{Stable/DBD\ Mut}$ -expressing discs relative to those expressing GFP-E2f1 or GFP-E2f1$^{DBD\ Mut}$ (Figure 6A). Similar levels of *hid* induction were previously observed following ionizing radiation treatments that trigger apoptosis [24]. *hid* expression was not significantly increased by the GFP-E2f1 mutants lacking normal Rbf1 binding activity (Figure 6A). To test whether the *hid* de-repression was a specific response to stabilizing E2f1 in S phase, we measured expression of another pro-apoptotic gene, *reaper*, which is not de-repressed by *Rbf1* mutation [24]. We detected no increase in *reaper* mRNA in discs expressing any GFP-E2f1 transgene (Figure 6B).

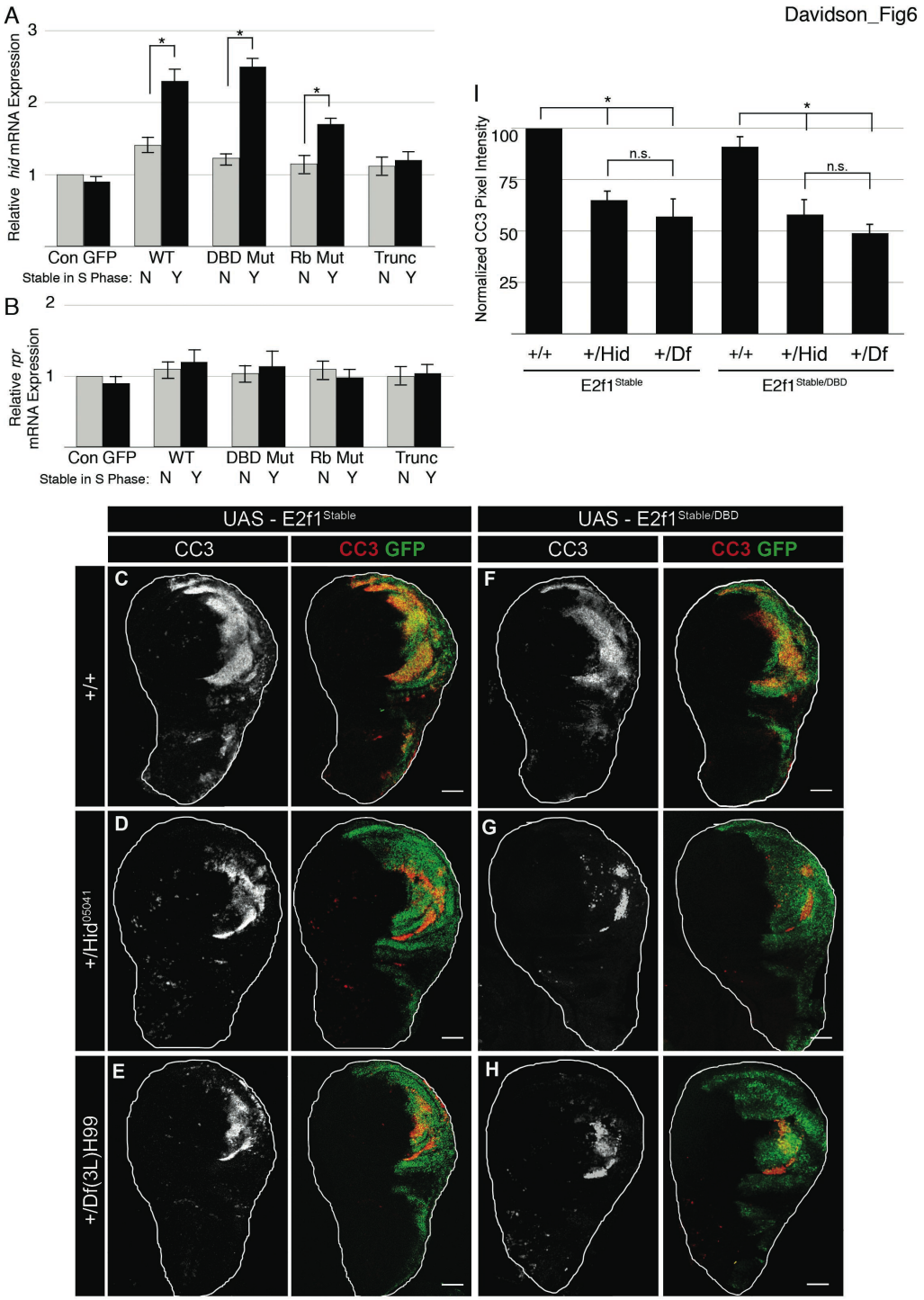

Figure 3.6: Stabilizing E2f1 during S phase induces *hid* expression A, B) qRT-PCR quantification of *hid* mRNA (A) or *rpr* mRNA (B) in en-Gal4 wing discs expressing GFP or the indicated GFP-E2f1 fusion proteins that lack (grey) or contain (black) the PIP3A mutation relative to a non-transgenic w^{1118} control (Con). * $p < 0.001$. C-I) Detection of apoptosis via Cleaved Caspase-3 (CC3, red) staining of third instar larval wing imaginal discs expressing the indicated GFP-E2f1 (GFP, green) proteins with en-Gal4. En-Gal4 > $E2f1^{Stable}$ (C-E) or $E2f1^{Stable/DBD}$ (F-H) in either a wildtype *hid* background (+/+), or heterozygous Hid^{05141}/+ or *Df(3L)H99*/+ backgrounds. I) Quantification of CC3 pixel intensity as measured using ImageJ. All genotypes were normalized against $E2f1^{Stable}$; +/+ cleaved caspase-3 levels. * $p < 0.001$. n.s. not significant. n=12 discs for each genotype.

To test if *hid* expression contributed to E2f1^{Stable}-induced apoptosis, we determined whether reducing *hid* gene dose would result in a decrease in apoptosis. We utilized two different *hid* alleles: *hid*^{05014}, containing a transposable element insertion between amino acids 105 and 106 in the open reading frame that effectively reduces *hid* expression [53], and *Df(3L)H99*, which deletes the entire *hid* gene as well as the neighboring pro-apoptotic genes, *reaper* and *grim* [53,54]. Wing discs heterozygous for either *hid* allele contained a significantly reduced amount of apoptosis after GFP-E2f1^{Stable} or GFP-E2f1$^{Stable/DBD}$ expression compared to controls (Figure 6C-H, I). Quantification of CC3 staining revealed no significant difference between the results obtained with *hid*^{05014} (Figure 6D, G, I) and *Df(3L)H99* (Figure 6E, H, I). This result suggests that *grim* and *reaper* do not contribute as substantially as *hid* to E2f1^{Stable}-induced apoptosis, consistent with our failure to detect an increase in *reaper* expression by E2f1^{Stable} and its derivatives (Figure 6B). These data support the idea that stabilizing E2f1 during S phase results in disruption of Rbf1 function leading to de-repression of *hid* expression and apoptosis.

E2f1^{Stable} induces hypertrophy when cells are prevented from executing apoptosis

Why would *Drosophila* cells induce a potent activator of apoptosis in response to elevated E2f1 protein levels during S phase? We considered the possibility that a small number of individual cells in a growing population of adult precursor cells, like those in wing imaginal discs, might stochastically experience hyper-expression of E2F that would manifest as the presence of excess E2f1 protein in S phase. Such cells would be eliminated by apoptosis, thereby helping to maintain growth homeostasis by suppressing the appearance of

potentially hyperplastic cells that could lead to aberrant overgrowth. If this was a developmentally important event, then blocking the ability of tissues to eliminate such cells by apoptosis should disrupt normal development.

To test this idea, we used en-Gal4 to co-express GFP-E2f1 transgenes in wing imaginal discs together with baculovirus p35, which efficiently blocks apoptosis in *Drosophila* cells [55]. Expressing p35 together with GFP had no deleterious effects on wing disc development (Figure 7A). In contrast, GFP-E2f1/p35 co-expression resulted in a range of morphological defects caused by hyperplastic growth. While some GFP-E2f1/p35-expressing discs appeared normal, most displayed various degrees of overgrowth in the posterior portion of the disc (Figure 7B). We quantified this overgrowth by microscopically measuring posterior compartment "thickness", which we defined as the sum of the number of confocal sections one micron apart required to image through the entire posterior compartment. Using this measurement we binned the discs into four phenotypic categories: normal (<9 μm), mild (9-11 μm), moderate (12-15 μm), and severe (>15 μm) (Figure 7D). GFP-E2f1^{Stable}/p35 expression caused a more severe phenotype than did GFP-E2f1/p35 expression. None of the discs were normal, and a larger percentage of the discs fell into the severe overgrowth category (Figure 7C, D). In addition, GFP-E2f1^{Stable}/p35 expression caused the appearance of a unique fifth phenotype in ~1/3 of the discs, which we called "arrest" (Figure 7C, D). In these discs the posterior compartment was almost absent, as confirmed by co-expression of RFP. We speculate on the origin of this class of discs in the Discussion. Expression of p35 together with either GFP-E2f1 or GFP-E2f1^{Stable} caused 100% lethality. Importantly, the hyperplastic growth induced by GFP-E2f1 or GFP-E2f1^{Stable} required the normal transcriptional and cell cycle-promoting activity of E2f1, as co-expression

of p35 with GFP-E2f1$^{DBD\ Mut}$ or GFP-E2f1$^{Stable/DBD\ Mut}$ resulted primarily in normal wing discs and did not cause lethality (Figure 7D). These data indicate that the developmental effects of E2f1 hyper-activity during tissue growth are exacerbated by simultaneously blocking apoptosis and E2f1 destruction in S phase.

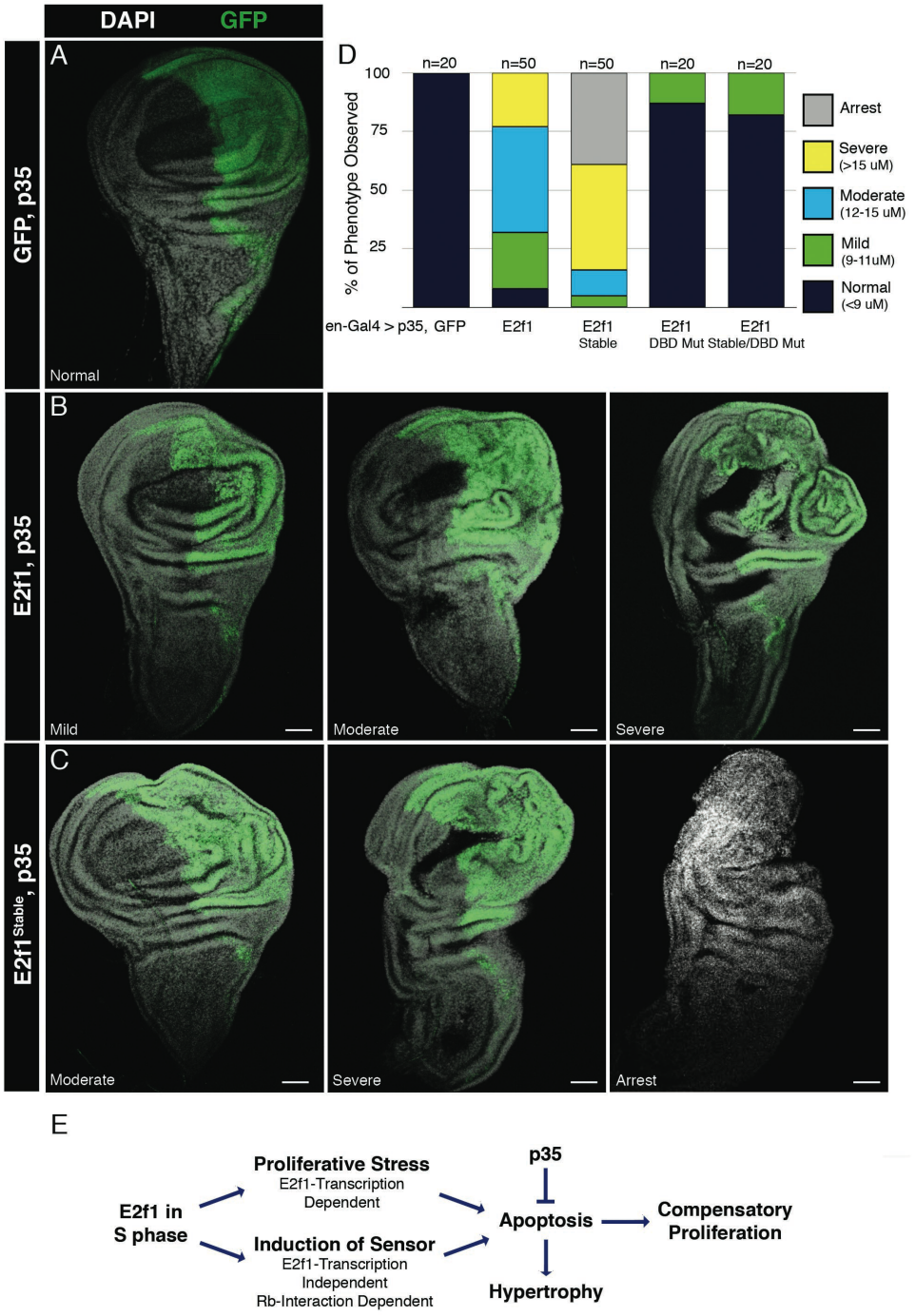

Figure 3.7: E2f1^{Stable} causes homeostasis defects and tissue hyperplasia A-C) Detection of GFP or the indicated GFP-E2f1 proteins (green) in en-Gal4, UAS-p35 wing discs stained with DAPI (white). Scale bars indicate 50 μm. D) Quantification of morphological defects by microscopically measuring the thickness of the posterior compartment of the indicated en-Gal4 > GFP-E2f1 wing discs. Measurements were obtained by counting the number of 1 micron sections required to visualize all the way through the posterior compartment of the tissue. Bars = 50mM. E) E2f1^{Stable} induces apoptosis in two ways.

DISCUSSION

We show here that stabilizing the single *Drosophila* activator E2f1 in S phase results in apoptosis that is necessary to prevent hypertrophy of wing imaginal discs. We conclude from these data that hyper-accumulation of E2f1 during S phase represents a form of proliferative stress during development that is sensed by the apoptotic machinery and results in the elimination of cells with excess E2f1 activity to maintain homeostasis during tissue growth.

S phase-coupled E2f1 destruction helps balance cell proliferation with apoptosis

What might be the function of a *Drosophila* cell's ability to detect abnormal accumulation of E2f1 protein during S phase and subsequently trigger apoptosis? One possibility is that accumulation of E2f1 during S phase resembles instances of abnormally high E2f1 activity that might occur sporadically during rapid growth of a population of precursor cells such as those in the wing imaginal disc. These events could be caused by stochastic or even genetic changes that affect either *E2f1* gene transcription or the ability of the CRL4^{Cdt2}/PCNA pathway to destroy E2f1 after replication factor genes are activated in late G1. The cell's ability to detect E2f1 accumulation in S phase clears these potentially hyperplastic cells from developing tissues via apoptosis, consequently contributing to the balance between cell proliferation and cell death that is necessary for normal tissue growth.

Growing *Drosophila* imaginal discs possess another mechanism of homeostasis in which a process of compensatory proliferation is activated in order to achieve normal tissue development when as many as 50% of cells are killed by external stimuli like radiation-induced DNA damage [56]. Indeed, in spite of high levels of apoptosis (15% of the cells), 50% of en-Gal4 > E2f1^{Stable} progeny survive until adulthood with about 2/3 of these surviving flies containing wings with somewhat mild morphological defects [32]. Blocking apoptosis with baculovirus p35 when E2f1^{Stable} is expressed shifts the cell proliferation/apoptosis balance too strongly in favor of cell proliferation, resulting in massive hypertrophy and 100% lethality.

p35 is a broad caspase inhibitor that blocks effector caspase activity at a step downstream of their proteolytic activation [55]. Therefore, cells expressing p35 can initiate apoptosis, but lack the capacity to complete it and are referred to as "undead cells." These undead cells produce signals that stimulate unaffected neighboring cells to proliferate [36]. Thus, the dramatic hypertrophy we see in E2f1^{Stable}/p35 wing discs might be the result of two synergizing growth signals: hyper-active E2f1 and compensatory proliferation from undead cells. Our experiments cannot precisely discern the relative contribution of these two inputs, but E2f1 activity appears to make a larger contribution because E2f1$^{Stable/DBD\ Mut}$ expression does not cause dramatic overgrowth.

What might explain the 32% of en-Gal4 > E2f1^{Stable} discs that displayed a reduced posterior compartment rather than an overgrown one: the "arrest" phenotype in Fig 7? The DNA damage we observed in our eye discs experiments provides a possible answer. Perhaps early in development the "arrest" class of wing discs sustained enough genomic damage to prevent proliferation, resulting in too small a pool of cells that could respond to the hyper-

active E2f1 and undead cell signals to support disc overgrowth. Thus, the wide range of phenotypes that we observed in E2f1^{Stable}/p35 wing discs may result from multiple influences that act stochastically within the population (Figure 7E).

A cellular sensor of E2f1 hyper-accumulation

Because endogenous E2f1 is quantitatively destroyed only in S phase, the relative amount of hyper-accumulation of E2f1^{Stable} is greater during S phase than during any other stage of the cell cycle. Therefore, one possibility is that E2f1^{Stable}-induced phenotypes result from the stability of E2f1 protein in S phase, and not from general over-expression throughout the cell cycle. Our failure to detect E2f1^{Stable} induced apoptosis in G1-arrested embryonic cells is consistent with this possibility. However, another difference between these embryonic cells and wing discs cells is that the former are cell cycle arrested and the latter are continuingly proliferating during larval development. Thus, another possibility is that S phase-destruction of E2f1 modulates the levels of E2f1 in proliferating cells, and cells that fail to destroy E2f1 during S phase have an increased chance of activating apoptosis at any point in the cell cycle. In either model, S phase E2f1 destruction is not essential for proliferation per se. In marked contrast, E2f1^{Stable} expression blocks endocycle progression [57], suggesting that knocking in E2f1^{Stable} to the endogenous locus would be lethal during development, perhaps even dominant lethal.

E2f1^{Stable} induces apoptosis at least in part through expression of the pro-apoptotic gene *hid*. Surprisingly, these events still occur after expression of an E2f1^{Stable} variant that cannot bind DNA and therefore lacks the ability to stimulate transcription of replication factor genes or cell cycle progression. Instead, E2f1^{Stable} requires the ability to bind Rbf1 to induce

hid gene expression and apoptosis. Genetic disruption of Rbf1 is well known to result in increased *hid* expression [18,25,51]. We therefore propose that the inappropriate accumulation of E2f1 in S phase disrupts some aspect of Rbf1 function leading to *hid* expression and apoptosis.

Our data do not discern either the function of Rbf1 that is disrupted by $E2f1^{Stable}$ or the mechanism of *hid* induction. While the mechanism connecting Rbf1/E2f1 function and *hid* may be indirect, some studies suggest that Rbf1 and/or E2f1 could regulate *hid* directly. Su and colleagues recently demonstrated that *Drosophila* wing disc cells undergo apoptosis in response to ionizing radiation independently of p53 and that this response requires E2f1 and is triggered by *hid* expression [26]. In eye discs, loss of Rbf1 function in the MF results in apoptosis that requires E2f1 transactivation function and is accompanied by *hid* expression [18,50]. However, whether these effects represent a direct induction of *hid* by E2f1 is not clear. E2f1 binding at the *hid* locus has been observed, but the binding site is located ~1.4 kb upstream of the of the start of *hid* transcription, which is more distal than in well characterized E2F-regulated promoters [58]. When located this far upstream the *hid* E2f1 binding site fails to activate gene expression in S2 cell reporter assays [25]. *hid* is also a target of p53 [59], and so any DNA damage resulting from stabilizing E2f1 during S phase, as we observed in eye discs, may also contribute to the activation of *hid* expression via p53-mediated DNA damage response pathways.

Another possibility is that E2f1, in combination with Rbf1, plays primarily a repressive role at the *hid* locus. In this model, our result that $E2f1^{Stable}$ or $E2f1^{Stable/DBD\ Mut}$ both induce apoptosis would be explained by disruption of Rbf1/E2f1 repressive complexes at the *hid* locus causing de-repression of *hid* expression. This model has interesting caveats:

what protects the Rbf1/E2f1 complex at the *hid* locus from being disrupted by Cyclin E/Cdk2, which is active during S phase and inactivates Rbf1-mediated repression of E2f1 [60], or by $CRL4^{Cdt2}$-mediate destruction of E2f1? Recent data indicate that the dREAM/MMB complex is required for the stability of E2F/Rbf1 repressive complexes in S phase, and acts to protect these complexes from CDK-mediated phosphorylation at non-cell cycle-regulated genes [61]. While there is yet no evidence that dREAM/MMB regulates *hid* [62], this work provides precedent for gene specific Rbf1 regulation during S phase.

Finally, while *hid* might be a critical player in the response to $E2f1^{Stable}$, there are likely other mechanisms responsible for sensing and modulating the apoptotic response to E2f1 levels. For instance, Frolov and colleagues recently demonstrated that a micro-RNA, mir-11, which is located within the last intron of the *Drosophila* E2f1 gene, acts to dampen expression of pro-apoptotic E2f1 target genes following DNA damage [28]. In this way, the normal controls of *E2f1* gene expression modulate apoptosis. In addition, our transgenic constructs lack the normal *E2f1* 3' UTR, which serves as a site for suppression of E2f1 expression by pumilio translational repressor complexes [63]. Thus, we have bypassed several modes of E2f1 regulation via transgenic expression of $E2f1^{Stable}$.

Conservation of E2F regulation via different molecular mechanisms

Our finding that stabilized *Drosophila* E2f1 can induce apoptosis independently of transcription and cell cycle progression parallels previous observations made in mammalian cells, albeit with important differences. In mammalian cells, E2F1 can induce apoptosis independently of transcription and cell cycle progression, but apoptosis required E2F1 DNA binding activity, unlike in our experiments [64,65]. These studies suggested that DNA

binding by E2F1 prevented pro-apoptotic promoters from binding repressor E2F family members.

This comparison of results highlights the way similar phenotypic outcomes in different species can arise from different mechanisms. While mammalian activator E2Fs are also inhibited during S phase, they are not subject to $CRL4^{Cdt2}$-mediated, S phase-coupled destruction like *Drosophila* E2f1. Instead, mammalian activator E2Fs are inhibited by direct Cyclin A/Cdk2 phosphorylation [6,7,8], targeted for destruction by SCF^{Skp2} [9,10], and functionally antagonized by E2F7 and E2F8 [11,12]. The regulation provided by E2F7 and E2F8 is of particular note, as it is essential for mouse development [11]. These atypical E2Fs homo and hetero-dimerize and act redundantly to repress E2F1 target genes independently of pRb family proteins, thus blocking E2F1 from inducing apoptosis [11]. Moreover, the E2F7 and E2F8 genes are E2F1 targets [11], consequently creating a negative feedback loop that limits E2F1 activity after the G1/S transition. A similar negative feedback loop among factors that regulate G1/S transcription exists in yeast [66]. The analogous *Drosophila* negative feedback loop involves E2f1 inducing its own destruction by stimulating *Cyclin E* transcription, which triggers S phase [60]. Therefore, the evolution of eukaryotes has resulted in the use of different molecular mechanism to achieve negative feedback regulation of G1/S-regulated transcription, and in the case of activator E2Fs this regulation is essential for normal development.

SUPPLEMENTAL FIGURES

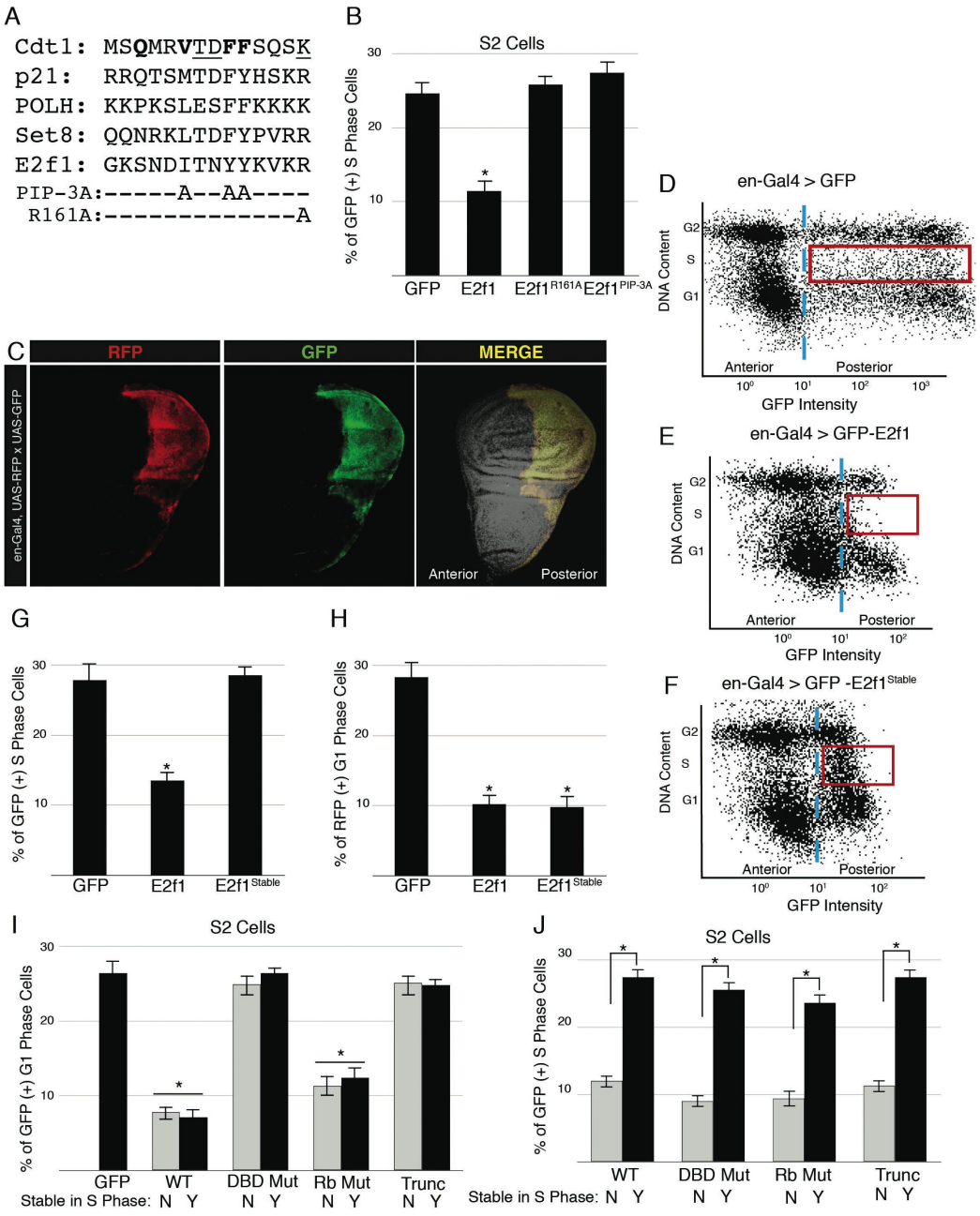

Figure S1: An *in vivo* assay for S phase-coupled E2f1 destruction A) Alignment of PIP degrons from known $CLR4^{Cdt2}$ substrates. Amino acids of the PIP box are bold and those of Cdt1 that interact with Cdt2 are underlined. E2f1 contains a PIP box located at amino acids 150-157. E2f1 also contains a basic Arg residue (R161) four amino acids downstream of the PIP box, much like the basic K+4 residue found in the Cdt1 PIP degron. Amino acid changes in $E2f1^{PIP\text{-}3A}$ and $E2f1^{R161A}$ mutants, which contain nonfunctional PIP degrons, are shown at the bottom. B) An S2 cell flow cytometry assay to quantify the number of GFP-positive cells that are in S phase. The graph indicates the percentage of GFP-positive S2 cells in S phase 200 min after heat shock expression of GFP, GFP-E2f1, $GFP\text{-}E2f1^{R161A}$ or $GFP\text{-}E2f1^{PIP\text{-}3A}$. After induction of GFP, all S phase cells in the population are GFP-positive (~25%) because GFP protein is stable throughout the S2 cell cycle. In contrast,

after induction of GFP-E2f1 expression, only ~10% of GFP-positive cells are in S phase because GFP-E2f1 is targeted by $CRL4^{Cdt2}$ for S phase destruction. The amount of GFP-positive cells in S phase after induction of $GFP\text{-}E2f1^{PIP3A}$ or $GFP\text{-}E2f1^{R161A}$ is equivalent to the amount after GFP induction, indicating that *Drosophila* E2f1 requires both a PIP box and a basic Arg residue 4 amino acids downstream of the PIP box for destruction during S phase. Here and in subsequent panels * indicates $p < 0.001$ and error bars represent the standard error of at least three independent experiments. C) Third instar larval imaginal wing disc expressing RFP and GFP with en-Gal4. D-F) Flow cytometry profile of GFP expression versus DNA content from en-Gal4 > GFP (D) en-Gal4 > GFP-E2f1 (E) or en-GAL4 > $GFP\text{-}E2f1^{Stable}$ (F) trypsin-dissociated third instar imaginal wing disc cells. For each profile, data were acquired until 10,000 total cells were detected. The red boxes illustrate a representation of the S phase cells, and the blue dotted lines indicate the threshold for categorizing a cell as GFP positive (based on GFP negative control). Note that GFP expression (D) was higher than GFP-E2f1 or $GFP\text{-}E2f1^{Stable}$ expression because the UASt promoter was used rather than UASp. G) Quantification by flow cytometry of GFP-positive S phase cells from trypsin-dissociated en-Gal4 > GFP, GFP-E2f1, or $GFP\text{-}E2f1^{Stable}$ wing discs. H) Quantification by flow cytometry of RFP-positive G1 phase cells from trypsin-dissociated, en-Gal4 wing discs expressing GFP or the indicated GFP-E2f1 fusion proteins. I) Quantification by flow cytometry of GFP-positive S2 cells in G1 phase after transient transfection of *Actin5C* promoter-driven constructs containing GFP or the indicated GFP-E2f1 fusion proteins that lack (grey) or contain (black) the PIP-3A mutation. J) Quantification by flow cytometry of GFP-positive S2 cells in S phase 200 min after heat shock expression of indicated GFP-E2f1 constructs that lack (grey) or contain (black) the PIP-3A mutation.

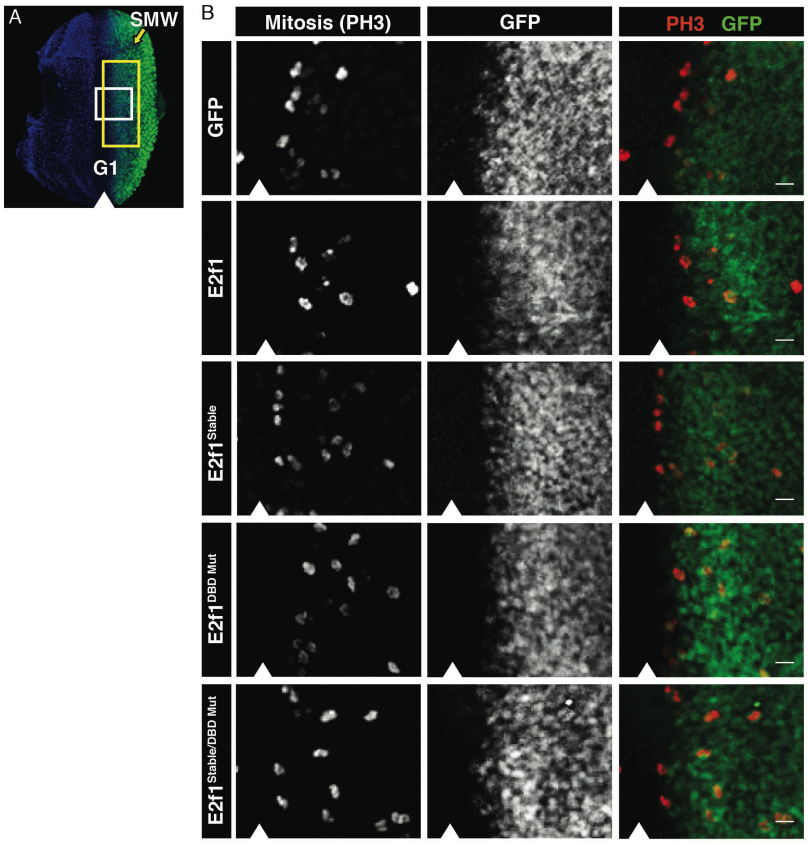

Figure S2: GMR-Gal4 > GFP-E2F1 eye discs stained with anti-PH3 A) GMR > UAS-GFP eye disc. White box indicates example of areas shown in panel B and in Figure 3. Yellow box indicates areas shown in Figure 4. B) Detection of mitosis by anti-phospho histone H3 staining (red) of GMR-Gal4 third instar larval eye imaginal discs expressing GFP or the indicated GFP-E2f1 fusion proteins (green). Arrowheads indicate the position of the MF, with anterior to the left and posterior to the right. Bars = 5mM.

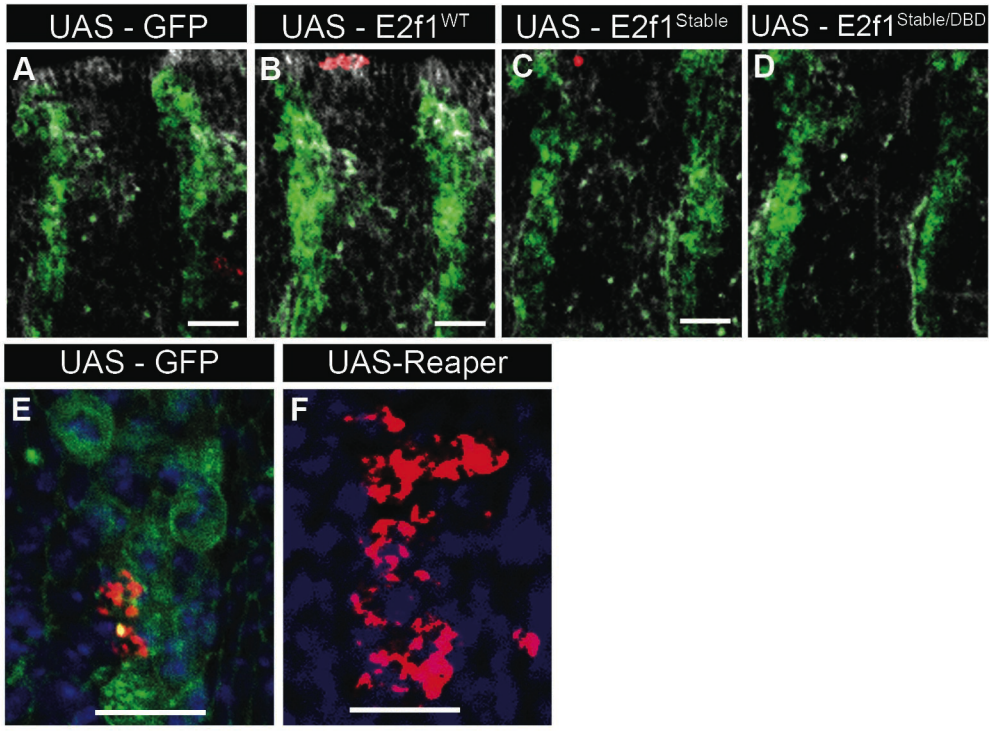

Figure S3: E2f1^{Stable} does not induce apoptosis in G_1 arrested embryonic cells. A-D) Stage 11 embryos (9-11 hours post egg laying) expressing GFP or the indicated GFP-E2f1 fusion proteins with en-Gal4. Green: GFP for transgene expression, Red: Cleaved Caspase-3, White: phospho-tyrosine for cell membrane marker, Blue: DAPI for nuclei). Epithelial cells (white) on the surface of the embryo have exited the cell cycle and are arrested in G_1. E) En-Gal4 > UAS-GFP embryo with a CC3-positive apoptotic cell (red) below the surface epithelial cells. This cell is most likely a neuronal cell and is shown as a positive control for CC3 detection. F) En-Gal4 > UAS-*reaper* embryo shown to ensure that the epidermal cells respond to pro-apoptotic signals and accumulate CC3. Bars = 10mm.

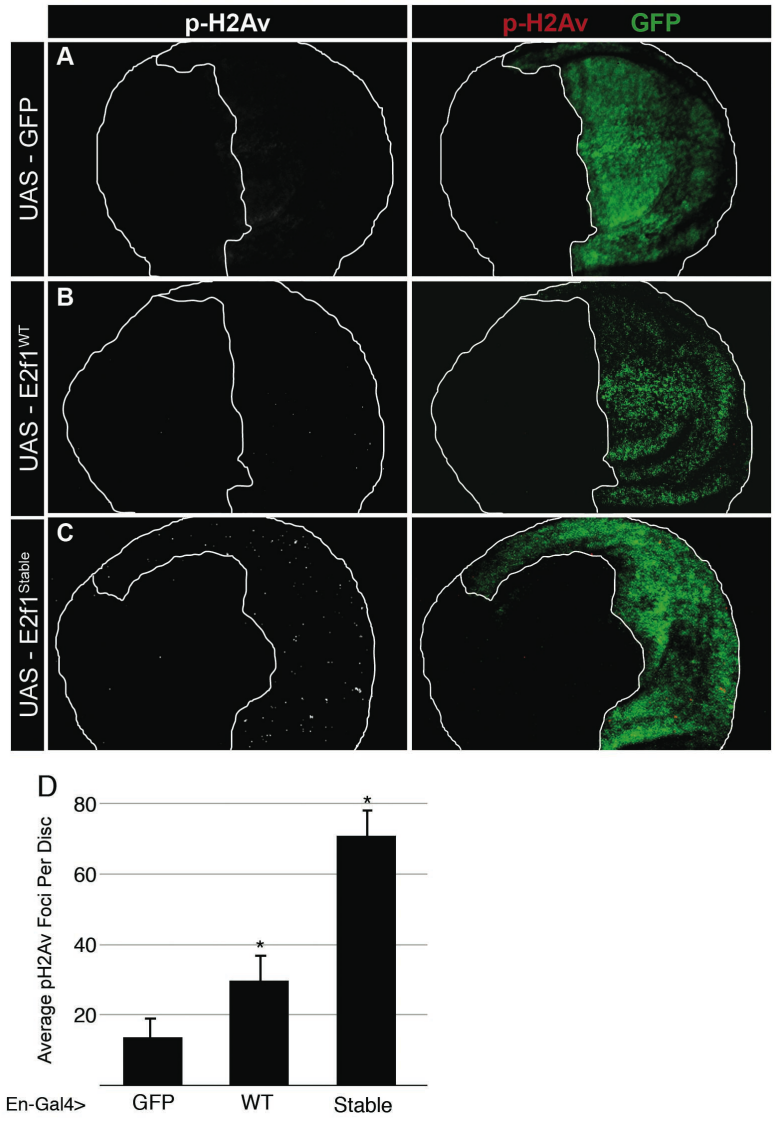

Figure S4: E2f1^{Stable} induces DNA damage in wing discs. A-C) Detection of DNA damage by anti-phospho-H2Av staining (red) in en-Gal4 third instar larval eye imaginal discs expressing GFP or the indicated GFP-E2f1 fusion proteins (green). E) Quantification of anti-phospho-H2Av staining. Foci above a calibrated threshold (ImageJ) were counted for each allele. n=10 discs for each genotype. Both E2f1 alleles had significantly more foci than UAS-GFP alone (* = $p < 0.001$).

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CHAPTER IV

ATYPICAL CELL CYCLES DURING DEVELOPMENT

PREFACE

This work was previously published as review article in Genes & Development in 2009. It was written by a former graduate student, Kate Lee, and myself, while Robert Duronio conceived the project and finalized the manuscript.

Lee, HO., Davidson, JM., and Duronio, R.J. (2009). Endoreplication: Polyploidy with Purpose. Genes & Development, 23 (21): 2461.

ABSTRACT

A great many cell types are necessary for the myriad capabilities of complex, multi-cellular organisms. One interesting aspect of this diversity of cell type is that many cells in diploid organisms are polyploid. This is called endopolyploidy and arises from cell cycles that are often characterized as "variant", but in fact are widespread throughout nature. Endopolyploidy is essential for normal development and physiology in many different organisms. Here we review how both plants and animals use variations of the cell cycle, termed collectively as endoreplication, resulting in polyploid cells to support specific aspects of development. In addition, we briefly discuss how endoreplication occurs in response to certain physiological stresses, and how it may contribute to the development of cancer. Finally, we describe the molecular mechanisms that support the onset and progression of

endoreplication.

ENDOREPLICATION BIOLOGY, CONSERVATION AND SIGNIFICANCE

Definition of endoreplication

Endopolyploidy arises from variations of the canonical G1-S-G2-M cell division cycle that replicate the genome without cell division. In this review, we use endoreplication as a general term encompassing any type of cell cycle leading to endopolyploidy. One widespread form of endoreplication is the developmentally controlled endocycle, which consists of discrete periods of S phase and G phase resulting in cells with a single polyploid nucleus (Edgar and Orr-Weaver, 2001; Lilly and Duronio, 2005). A key feature of the endocycle is that DNA content increases by clearly delineated genome doublings (Fig. 4.1A).

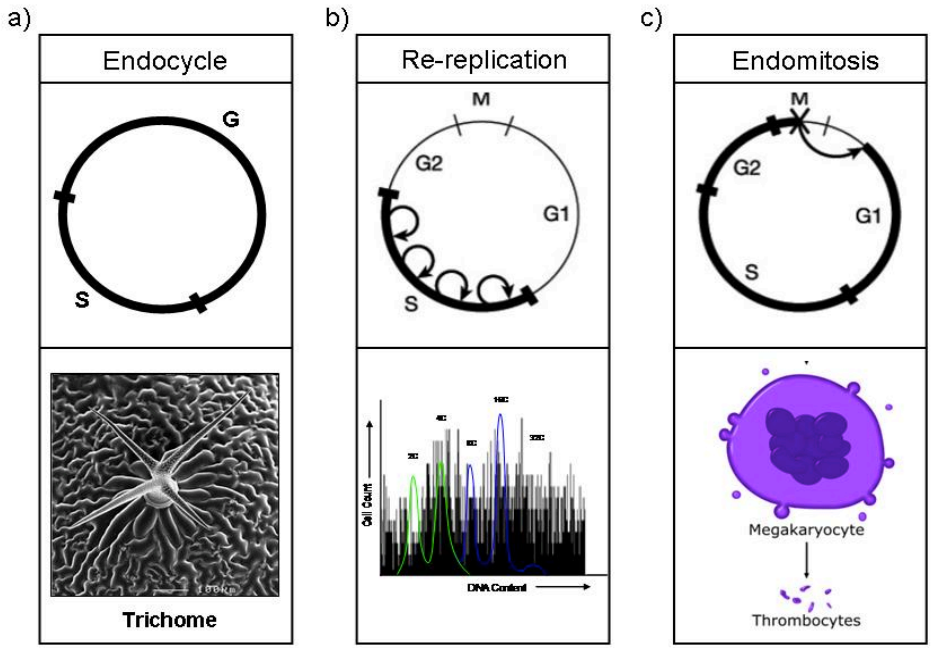

Figure 4.1: Endoreplication. A) Endocycles are defined by controlled cycles of S and G phase without cell division. Endocycling cells do not eneter mitosis, and thus do not exhibit features of mitosis such as condensed chromosomes and nuclear envelope breakdown. Trichomes arise from polyploid cells that can be found of the surface of a variety of plant tissues. B) Re-replication results from aberrant regulation in which DNA synthesis

is initiated multiple times at individual origins of replication within a single S phase. This results in an indistinct DNA content as depicted by black lines in this hypothetical FACS profile (y axis is cell number and x axis is DNA content). Green represents the diploid mitotic cell profile, with 2C and 4C peaks. Blue represents endoreplication cycles that result in distinct populations of cells with more than 4C DNA content. C) During endomitosis, cells enter mitosis and begin to condense chromosomes, but do not segregate chromosomes to daughters. Instead, they enter a G1 like state and re-enter S phase. Megakaryocytes use endomitosis upon maturation, leading to a globulated nuclear structure. Blood clot-promoting thrombocytes (or platelets) bud off of the polyploidy megakaryocytes.

This is an important distinction from the aberrant process of re-replication, which is characterized by uncontrolled, continuous re-initiation of DNA synthesis within a given S phase resulting in increases in DNA content without clearly recognizable genome doublings (Blow and Hodgson, 2002; Zhong et al., 2003). Re-replication results from perturbations to the molecular mechanisms that control the "once and only once" firing of replication origins during a normal diploid S phase, and is thought to be a source of genome instability that contributes to oncogenic transformation (Fig. 4.1B).

Another major form of endoreplication occurs through the process of endomitosis in which cells enter but do not complete mitosis (Fig. 4.1C). The best studied example occurs in 64N polyploid megakaryocytes (Ravid et al., 2002), which are responsible for producing the anucleated platelets (or thrombocytes) that mediate blood-clotting (Ebbe, 1976). Endomitosis is distinguished by the presence of early mitotic markers such as phospho-histone H3 (pH3), which marks condensed chromosomes (Hendzel et al., 1997). Endomitotic megakaryocytes reach metaphase or anaphase A, but never fully separate sister chromatids or undergo cytokinesis, resulting in globulated polyploid nuclei (Nagata et al., 1997; Vitrat et al., 1998). Endocycling cells, in contrast, do not display features of mitosis such as nuclear envelope breakdown, chromosome condensation, or pH3 staining. Thus, evolution has resulted in multiple mechanisms for achieving endopolyploidy. In the following sections we describe some of the biological functions of endopolyploidy.

Endoreplication is crucial for early development

The evasion of controls that maintain diploidy may seem like a dangerous escapade for endoreplicating cells by opening up possibilities to upset genome integrity. However, endoreplication is an essential part of normal development. Many organisms employ endoreplication as part of terminal differentiation to provide nutrients and proteins needed to support the developing egg or embryo. Some of the best studied examples include plant endosperm, *Drosophila* follicle and nurse cells, and rodent trophoblasts. The logical implication is that increasing DNA content by endoreplication is needed to sustain the mass production of proteins and high metabolic activity necessary for embryogenesis. Disrupting endoreplication in these cells often leads to embryonic lethality.

Developing plant seeds depend on endosperm tissue as an energy store before becoming self-sufficient through photosynthesis and root formation (Fig. 4.2A). Endosperm differentiation occurs soon after fertilization and is associated with a switch from a mitotic cell cycle to an endocycle. This initiation of endocycles correlates with an increase in endosperm mass and rapid synthesis of starch (Schweizer et al., 1995), suggesting that by increasing the number of individual loci, endoreplication is able to assist in maximizing mRNA and protein synthesis. The importance of endoreplication in seed development is evident after exposure to environmental stress, such as high temperature or water deficit. In these resource-limited settings, the endosperm remains primarily mitotic, and reduction in the magnitude of endoreplication leads to a smaller endosperm, unfit to support the embryo (Engelen-Eigles et al., 2001). Another important polyploid cell type in early plant development is called the suspensor cell (Fig. 4.2A).

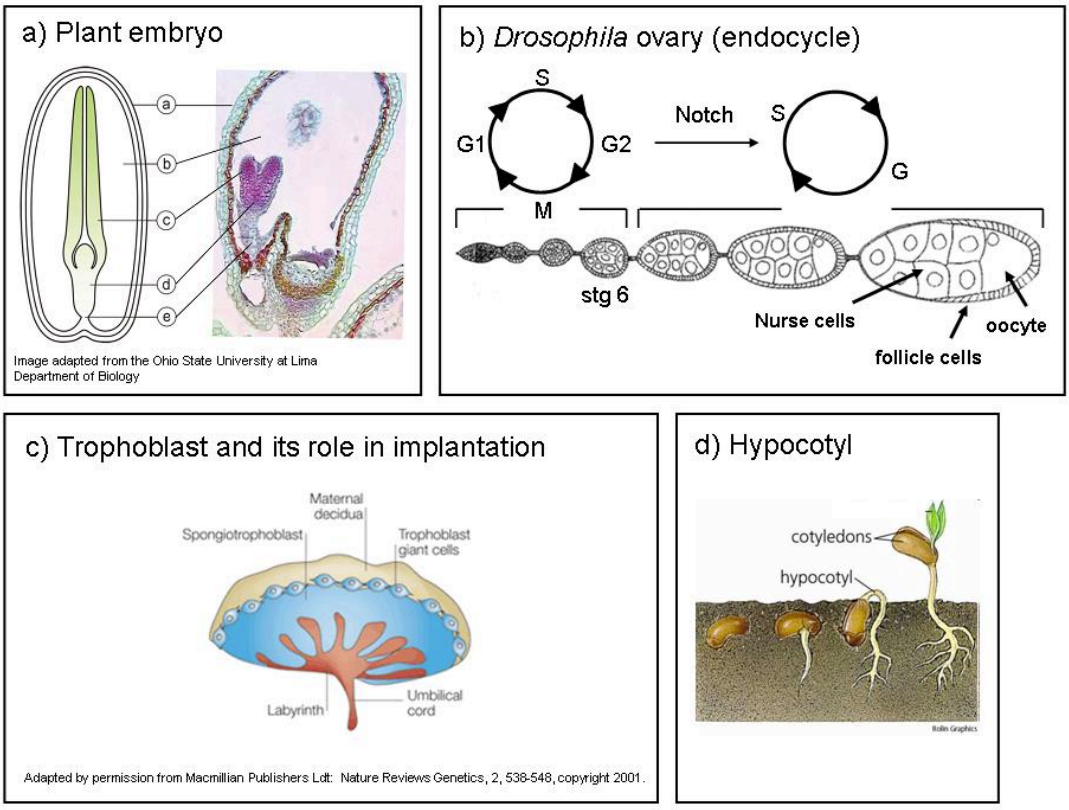

Figure 4.2: Examples of Endocycling Tissues. A) A schematic and image of section of a plant embryo. The seed coat (a) covers the endosperm (b), which surrounds and provides nutrients for the growing cotyledons (c) and hypocotyl (d) of the embryo. Suspensor cells (e) arise from asymmetric division of the fertilized egg and connect the embryo to the endosperm and are thought to be crucial in nutrient transfer. B) *Drosophila* ovaries consist of 12-15 ovarioles (one is shown) containing a series of developing egg chambers. The germarium (far left) houses germ-line and somatic stem cells that differentiate into nurse cells and oocyte, and follicle cells respectively. Follicle cells switch to endocycles mid-oogenesis in response to Notch signaling, which down regulates stimulators of mitosis like string^{cdc25} and activates inhibitors of mitosis like APC$^{fzr/cdh1}$. C) Rodent trophoblast giant cells are highly polyploid and facilitate implantation by contributing to invasion of placental tissue into the uterine wall. D)The plant hypocotyl undergoes endocycles to rapidly grow above the ground. Once the young plant reaches the sun, hypocotyl endoreplication stops.

After fertilization, a plant zygote undergoes asymmetric division to give rise to the embryo and suspensor cell (Gilbert, 2000). Suspensor cells employ endocycles to become polyploid, and provide nutrients to the embryo by bridging to the endosperm. Although a direct effect of suspensor endoreplication on embryogenesis is unknown, cultured scarlet beans with suspensor cells were twice as likely to survive as embryos without suspensor cells (Yeung and Meinke, 1993).

In *Drosophila melanogaster* females, endoreplication is essential for the production of eggs. The highly polyploid, germline-derived nurse cells form an interconnected cyst that shares cytoplasm with the oocyte, and support oogenesis by synthesizing and transferring proteins and mRNA to the growing oocyte (Fig. 4.2B). This maternal supply of gene products is essential to direct the early stages of embryogenesis, which occur in the absence of zygotic transcription (Bastock and St Johnston, 2008). Somatic follicle cells are also polyploid and envelop the developing oocyte to enable vitellogenesis and egg shell formation. Reduction of endoreplication in nurse and follicle cells causes sterility, supporting the idea that the endocycle plays a crucial role in oogenesis and early development (Lilly and Spradling, 1996; Maines et al., 2004).

Because viviparous gestations do not require the same level of self-sufficiency as that of seeds or insect eggs, there is no truly comparable mammalian tissue to that of endosperm or nurse and follicle cells. In rodents there is a specialized zygotic cell type that adopts the endocycle to establish the interface between the embryo and the mother to support embryogenesis (Zybina and Zybina, 2005). Trophoblast Giant Cells (TGCs) facilitate uteral implantation of the fertilized egg and metastasis into maternal blood vessels to allow transport of nutrients, oxygen, and immunoglobins into the embryo (Cross, 2000, 2005; Cross et al., 2002). TGCs differentiate from trophoectoderm that surround the early blastocyst. Differentiation is associated with rapid endocycling resulting in up to a 1000C DNA content (Cross, 2000). TGC endoreplication is not used to directly provide gene products to the embryo, but increased gene expression through polyploidy may supply the energy necessary for aggressive invasion into the maternal tissue (Fig. 4.2C). In addition, a significant reduction in the magnitude of endoreplication in TGCs causes embryonic lethality

(Garcia-Higuera et al., 2008; Geng et al., 2003; Parisi et al., 2003). Restoration of endocycling in these cells is sufficient to rescue embryonic viability, indicating that the endocycle also plays a crucial role in early mammalian development.

Endoreplication supports the function of differentiated cells

There are many examples of cells adopting endoreplication as part of terminal differentiation to support a specialized function. From plants to mammals, endoreplication is used to facilitate growth and to provide key functions to the adult organism, from nutrient uptake to defense. Perturbing endoreplication in these cells often causes organ malfunction and pathogenesis.

Endoreplication and growth:

Organisms can grow either via an increase in cell number or an increase in cell size, or both. Since an increase in DNA content often correlates with increased cell size, endoreplication provides an efficient strategy for growth. For instance, producing the necessary surface area of cell membrane needed for several generations of cell division has been proposed to be slower and require more energy than simply increasing the volume of a single cell (Kondorosi et al., 2000). Thus, in situations where energy sources are limiting or rapid growth is necessary, increasing cell volume without division may be more advantageous (Kondorosi et al., 2000). Endoreplication in plants most commonly occurs in tissues that develop mass quickly and have high metabolic activity (Inze and De Veylder, 2006). One example of this occurs during early growth prior to photosynthesis, when the young hypocotyl emerges from the soil (Fig. 4.2D). This rapid growth is accomplished

through endoreplication (Jakoby and Schnittger, 2004). After emergence, this early developmentally controlled endoreplication subsequently becomes impacted by the environment, as endocycles are negatively regulated by sunlight (Gendreau et al., 1998). While different than the endopolyploidy we have been discussing, it is interesting to note that the acquisition of a fully polyploid genome during the process of inbreeding or evolutionary selection may provide some plants with the advantage of a larger size and greater green mass over their diploid, subspecies counterparts (Ayala et al., 2000). Full genome polyploidy is commonly observed in cultivated plants such as coffee, watermelon, maize, potatoes, and bananas, among others. Finally, overall growth of *C. elegans* and *Drosophila* larvae is also mainly driven by endoreplication (Edgar and Orr-Weaver, 2001; Lozano et al., 2006). However, it is important to remember that endoreplication-associated growth is usually confined to specialized cell types that perform specific biological functions and is not a universal mechanism to control organism size. It has long been known that variations in mammalian body size are due to differences in cell number alone and not cell size. In fact, cells from mice and elephants have similar sizes (Wilson, 1925).

The correlation between polyploidy and cell size raises the question of whether endoreplication per se triggers growth or whether growth promotes endoreplication. The answer is likely not a unidirectional cause and effect relationship, but rather a mutual feedback between growth and endoreplication: organism growth can be mediated by, and depend upon, an increase in cell size through endoreplication, while conversely inhibition of growth leads to reduction in endoreplication. Genetic perturbations in *C. elegans* that result in reduced body size are associated with reduced endoreplication of hypodermal cells (Flemming et al., 2000). Similarly, starvation in insects reduces endoreplication (Britton and

Edgar, 1998), and nutrient deprivation through inhibition of the insulin signaling pathway also blocks endoreplication (Britton et al., 2002). In addition, mutation of the *Drosophila myc* oncogene, which in flies acts to induce growth, causes a dramatic decrease in endoreplication in both somatic and germline cells of the ovary (Maines et al., 2004). Since Myc over-expression stimulates growth and could rescue the reduction in endoreplication imposed by inhibitors of insulin signaling, it was proposed that the endoreplication defect observed in *Drosophila myc* mutants is a secondary consequence of growth arrest (Pierce et al., 2004).

Endoreplication and nutrient utilization:

Endoreplication is employed extensively in tissues reserved for nutrient uptake and storage. Plant leaves and root hairs undergo endoreplication (Kondorosi et al., 2000), as do intestinal cells in *Drosophila* and *C. elegans* (Hedgecock and White, 1985; Smith and Orr-Weaver, 1991). Endoreplication in leaves and root hairs may aid in maximizing surface area to absorb light and water. However, whether polyploidy resulting from endoreplication is necessary for efficient or effective nutrient uptake has not been specifically addressed. Polyploid cells themselves can be used as an energy source. During metamorphosis, a *Drosophila* pupae is completely isolated from an exogenous food supply, and the biomass accumulated in polyploid cells during larval feeding is recycled for the differentiation and morphogenesis of adult tissues. Similarly, polyploid plant fruit tissue is utilized as energy for early plant development.

Endoreplication and functional tissue morphology:

Endoreplication is also used by tissues that are needed to maintain organism

homeostasis. Trichomes are a specialized, branched cellular structures made by polyploid epidermal cells found on the aerial surface of many plant tissues (Fig. 4.1A). Trichomes can form irritable spines that work to deter herbivorous animals, keep frost away from other epidermal cells, or reflect ultra violet radiation in exposed areas. They can also reduce the degree of evaporation by blocking the flow of air across the surface, or enhance the collection of rain and dew (Galbraith et al., 1991; Hulskamp et al., 1999). Trichome structure is dependent on the degree of cellular polyploidy resulting from endoreplication. Mutation of the *SIAMESE* gene converts the normally unicellular trichomes of *Arabidopsis* into multicellular trichomes with reduced ploidy that sometimes have aberrant morphology (Walker et al., 2000). Thus, some tissues may grow via endoreplication because this avoids the cell shape changes associated with mitosis. The most recognized application of trichome structures are cotton fibers derived from the epidermal layer of the seed coat. These single cells differentiate through multiple rounds of endoreplication to become elongated "hair-like" structures. The extent and function of this elongation primarily depends on the plant's environment. In addition, plant root hairs allow the plants to become firmly rooted to the ground and the lack of this structure leads to instability (Menand et al., 2007).

Utilization of endoreplication for tissue regeneration after stress

Endoreplication can be employed for growth and tissue regeneration during conditions that would otherwise prevent proliferation (Weigmann et al., 1997). By bypassing the controls that maintain genomic stability through diploidy, certain tissues react to exogenous stress by utilizing endoreplication to grow and retain cell and organ function. There are clear examples of this in both plants and animals, indicating that a switch to endoreplication is a conserved method to maintain homeostasis despite dire conditions.

In *Arabidopsis*, there is a distinct correlation between response to environmental stresses and endocycle-dependent leaf area (Cookson et al., 2006). By over-expressing or mutating the gene encoding E2fe/DEL1, an atypical E2F transcription factor that acts to repress the endocycle (Vlieghe et al., 2005), Cookson et al. (2006) asked if the extent of endoreplication affected the plant's ability to respond to shade or water deficit stress. An increase in the extent of endoreduplication reduced the negative impact of water deficit on final leaf size. This suggests that adaptation via endopolyploidy can provide protection to stress and thus increase organism fitness, perhaps by maintaining tissues such as leaves that have a high photosynthetic capability. However, not all conditions were improved by endoreplication. The same study showed that increased endoreplication reduced the ability of leaves to achieve proper size in response to shade, likely because switching to an endocycle prevented the compensatory increase in cell number, and thus leaf expansion, via proliferation necessary to properly combat the reduced available light. Thus, depending on the signal and the situation, the most beneficial stress response can be achieved by endoreplication or cell proliferation.

A dramatic example of protective endoreplication in response to stress has been observed in animal cells (Lazzerini Denchi et al., 2006). In many tissues, telomere dysfunction (e.g. shortening or de-protecting) induces senescence or apoptosis (Hemann et al., 2001; Herbig et al., 2004). In contrast, hepatocytes in the liver do not apoptose in response to compromised telomeres that trigger a DNA damage response (Lazzerini Denchi et al., 2006). In addition, while loss of telomere integrity blocks hepatocyte cell division, these cells can nonetheless regenerate functional livers that were damaged by partial hepatecomy, and they did so via endoreplication. Thus, endoreplication can provide a means

to achieve necessary growth in response to exogenous stress in a situation where compromised genome integrity precludes cell proliferation. Similar stress-induced switches to endoreplication have been observed in tumor tissues responding to genotoxic insults (Ivanov et al., 2003), in damaged cardiomyoctes (Anatskaya and Vinogradov, 2007; Meckert et al., 2005), and in aging mouse hepatocytes (Funk-Keenan et al., 2008). Thus, it is interesting to speculate that stress-induced endoreplication is a general mechanism to achieve an increase in tissue mass and regain essential functions in response to compromised genomic integrity.

Endoreplication as a default program upon mitotic catastrophe in both cancer and normal cells

Endoreplication has been observed in cancer cells for many decades (Storchova and Pellman, 2004). Early studies were aimed at understanding the mechanisms by which cancer cells became polyploid. Whether endoreplication is a causative agent in oncogenic transformation or progression is also not yet entirely clear. One possibility is that polyploidization is a precursor to aneuploidy that may contribute to oncogenesis (Storchova and Pellman, 2004) (Fig. 3).

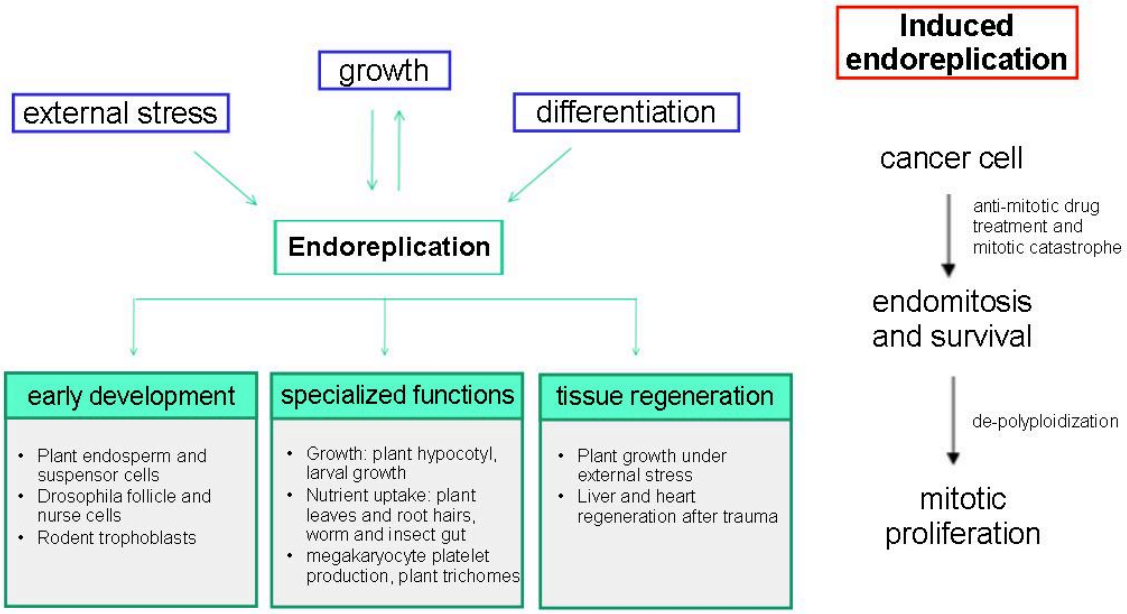

Figure 4.3: Common Uses of the Endocycle during Normal and Cancer Development

Another possibility is that cancer cells use endoreplication as a means of survival during mitotic catastrophe or genotoxic stress. For instance, some p53 mutant cancer cells undergo endoreplication rather than apoptosis upon treatment with anti-mitotic drugs such as colcemid and vinblastine [for review: (Erenpreisa et al., 2005a)]. This induces a form of endomitosis that appears for the most part to be a senescent situation. However, at low frequency some of these polyploid cancer cells can actually revert back into mitotic cell cycles via a process of genome reduction called de-polyploidization (Erenpreisa et al., 2005a; Erenpreisa et al., 2005b; Prieur-Carrillo et al., 2003; Puig et al., 2008). Human embryonic cells infected with SV-40 virus and subsequent inactivation of p53, and fibroblasts undergoing senescence, endoreplicate. These cells can also successfully de-polyploidize (Walen, 2002, 2007a, b).

Survival from anti-mitotic drug treatment by endoreplication and subsequent de-polyploidization suggests a mechanism for how cancer cells become insensitive to anti-mitotic drugs. Could this also contribute to recurrence of more aggressive cancer? Not only

can endoreplication prolong the existence of cancer cells, it may also promote the selection of additional oncogenic mutations resulting from repeated rounds of replication in a cell that might have compromised the fidelity of DNA synthesis. Consequently, de-polyploidization and re-entering the mitotic cycle after endoreplication could result in daughter cells with different genotypes, some of which might be highly cancerous.

What might be the mechanism of de-polyploidization, which seems so counterintuitive? While the mechanism is unknown, some features of genome structure and organization in cells that undergo induced endoreplication (e.g. with mitotic spindle poisons) are likely important. The genome is likely to be completely replicated during cancer cell endomitosis, and the nuclear packaging of the condensed, duplicated chromosomes may be advantageous in facilitating polyploid genomes to be separated during de-polyploidization (Erenpreisa et al., 2005a; Erenpreisa et al., 2005b). Curiously, cancer cells that undergo de-polyploidization activate meiosis specific genes (Erenpreisa et al., 2009; Ianzini et al., 2009), but how this might contribute to de-polyploidization or if the de-polyploidization process resembles in any way the reductional division of meiosis is not entirely clear (Erenpreisa et al., 2005a).

Other polyploid genomes display characteristic variations in organization and structure that likely preclude a return to mitotic proliferation. For example, unlike cancer cell endomitosis, the endocycles that generate polyploid cells during *Drosophila* development under-replicate the pericentric heterochromatin and thus do not duplicate the entire genome each endocycle S phase (Lilly and Duronio, 2005). In addition, some cells organize their polyploid genome by aligning the multiple copies of sister chromatids along their lengths, leading to giant polytene chromosomes that contain a distinct banding pattern (Dej and

Spradling, 1999). This is perhaps most famous in the *Drosophila* salivary gland, but polytene chromosomes are also observed in plant ovules, leaves, roots and some tissues of the pollen sacs (Kondorosi and Kondorosi, 2004). The functional significance for why a polyploid genome becomes polytene is not well understood. Nevertheless, polyteney coupled with incomplete replication of the whole genome, particularly centromeres, represents a terminally differentiated state that is not conducive to de-polyploidization and a return to proliferative cycles.

Certain non-cancerous cells can also be induced to undergo endoreplication upon mitotic stress, in contrast to most cells that arrest from mitotic checkpoints and/or undergo apoptosis. For over 70 years, plant biologists have used colchicine to induce polyploidy (Eigsti, 1938). Likewise, nocodazole treatment of keratinocytes also results in endoreplication (Gandarillas et al., 2000). Mammalian cells deficient of Fbw7, which encodes a component of a Cullin-RING E3 ubiquitin ligase (Koepp et al., 2001; Strohmaier et al., 2001), were shown to induce endoreplication upon exposure to spindle toxins (Finkin et al., 2008). It will be interesting to determine whether the resumption of proliferation via de-polyploidization in cells that undergo endomitosis-like endoreplication is utilized during normal development or part of normal tissue homeostasis. Intriguingly, de-polyploidization has been recently noted in hepatocytes (Duncan et al., 2009).

TRANSITION INTO ENDOREPLICATION

Given that endoreplication is a crucial component of development and disease, an understanding of the molecular controls that govern the switch from mitotic cycles to

endoreplication is important. In the following sections, we will examine some of the best characterized examples of the developmental signals controlling the onset of endoreplication.

Endocycles induced by Notch signaling

During development, endocycling cells originate from proliferating diploid cells, which undergo conversion of the cell cycle as part of their program of differentiation. Studies of follicle cells in the *Drosophila* ovary have provided the most detailed paradigm for the developmental signals that regulate this type of cell cycle transition. Follicle cells are derived from somatic stem cells and proliferate to give rise to ~650 diploid cells encapsulating the germ line cells (i.e. nurse cells and oocyte) (Bastock and St Johnston, 2008). The mitosis to endocycle transition occurs midway through oogenesis and marks the beginning of terminal differentiation of follicle cells. Subsequently, follicle cell endoreplication drives the production of proteins and mRNAs that support vitellogenesis and formation of the egg shell, or chorion.

Studies in the last decade have indicated that Notch signaling is a key regulator of the follicle cell mitotic to endocycle transition. Notch is a transmembrane receptor that binds Delta or Serrate (Jagged in vertebrates) ligands, activating cleavage of Notch's intracellular domain which enters the nucleus to regulate transcription of Notch-responsive genes [For reviews: (Gordon et al., 2008; Poellinger and Lendahl, 2008; Talora et al., 2008)]. Notch mutant follicle cells do not switch to endocycles and continue to mitotically divide and express undifferentiated markers (Deng et al., 2001; Lopez-Schier and St Johnston, 2001). Conversely, ectopic expression of Delta leads to precocious initiation of follicle cell endocycles (Jordan et al., 2006). Hedgehog (Hh) signaling antagonizes Notch by promoting

the proliferation of follicle cells, and mutations in *patched*, a negative regulator of Hh signaling, leads to mitotic cycles at stages when endocycling normally occurs (Zhang and Kalderon, 2000).

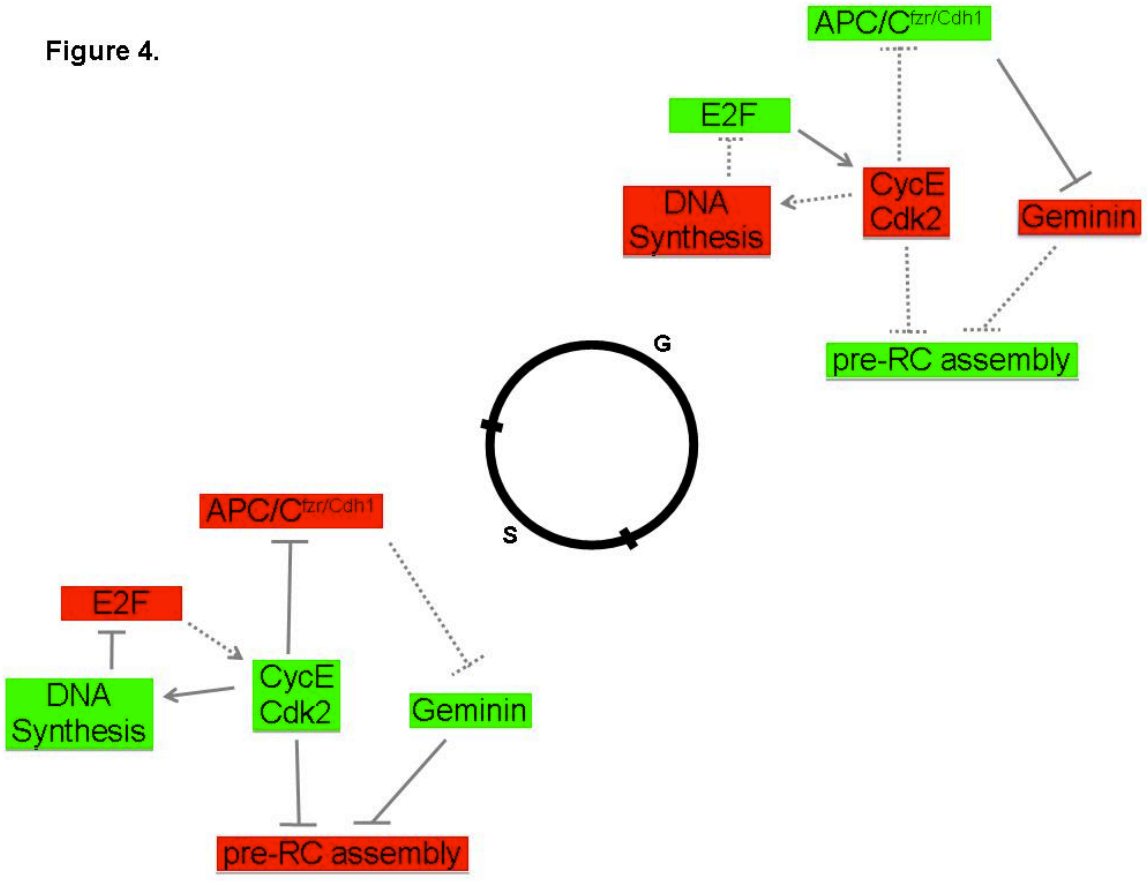

Figure 4.4: Regulation of the endocycle. A complex array of controls ensures once and only once replication during endocycle progression. The key players are shown when they are active (green, solid lines) or inactive (red, dashed lines) in either the G or S phase of the endocycle. Control of CycE/Cdk2 activity forms the core of endocycle regulation. CycE and CycE/Cdk2 activity are low during G phase when APC/Cfzr/cdh1 represses accumulation of Geminin, thereby allowing pre-RC formation. E2F1 stimulation of CycE transcription contributes to activation of CycE/Cdk2 and the initiation of DNA replication, which triggers E2F1 destruction. CycE/Cdk2 directly represses pre-RC formation and inactivates APC/Cfzr/cdh1, which allows Geminin accumulation that also inhibits pre-RC formation.

Recent studies have shed much light onto the mechanisms by which Notch signaling promotes the transition into endocycles (Fig. 4.4). In general, Notch promotes changes in gene expression resulting in the down-regulation of mitotic functions and the up-regulation

activities needed for endoreplication. Notch signaling is known to modulate three important genes in this process: It induces expression of fizzy-related (fzr/Cdh1), an activator of the Anaphase Promoting Complex/Cyclosome (APC/C) that triggers ubiquitin-mediated destruction of mitotic cyclins (Morgan, 2007), and it represses expression of the S-phase CDK-inhibitor dacapo (dap^{p27}) and $string^{cdc25}$, a phosphatase that activates Cyclin/Cdk1 complexes needed for mitosis (Deng et al., 2001; Schaeffer et al., 2004; Shcherbata et al., 2004). These Notch-induced changes in gene expression are necessary. For instance, mutations affecting *fzr/Cdh1* result in uncharacteristically small follicle cell nuclei due to a failure to switch into endocycles (Schaeffer et al., 2004). Likewise, mutation of Shaggy (sgg), the *Drosophila* GSK3 kinase, prevents Notch intracellular domain cleavage and a failure to both down regulate $string^{cdc25}$ and express endocycling follicle cell markers (Jordan et al., 2006).

Several transcription factors respond to Notch signaling to control the mitotic to endocycle transition in follicle cells. Notch activates a transcription factor called Hindsight that mediates the down regulation of the Hh pathway as well as the down regulation of the homeodomain protein Cut, which is a repressor of fzr/Cdh1 expression (Sun and Deng, 2005, 2007). Notch-mediated Hindsight expression is also crucial for down-regulating $string^{cdc25}$, which when mutated causes precocious activation of endocycles (Sun and Deng, 2005). Similarly, the absence of the zinc-finger transcription factor tramtrack (ttk), a downstream target of Notch signaling, resulted in misregulation of $string^{cdc25}$, *dacapo*, and *fzr/Cdh1* causing a failure to transition into endocycles (Jordan et al., 2006). Thus, Notch signaling facilitates the mitotic to endocycle switch by regulating transcription factors that mediate the repression of genes needed for mitosis (e.g. $string^{cdc25}$) and the activation of genes that

stimulate destruction of mitotic regulators (e.g. fzr/Cdh1). Whether the *fzr/Cdh1*, *dacapo* or *string*^{cdc25} genes are direct targets of these transcription factors is not known.

Interestingly, modulation of Notch signaling has also been implicated in the termination of follicle cell endocycles. In later stages of *Drosophila* oogenesis, follicle cells terminate endoreplication and undergo another transition in which genes needed for chorion formation become specifically amplified via re-initiation of origins of replication (Calvi and Spradling, 1999; Tower, 2004). Such gene amplification on top of endocycle-mediated polyploidization is needed to generate the gene copy number to support sufficient biosynthesis of proteins needed for eggshell production. Down-regulation of Notch signaling plays a crucial role in conjunction with ecdysone hormone signaling to promote the switch from endocycles to gene amplification (Sun et al., 2008).

Notch signaling may contribute to the transition from mitotic to endocycles in mammals as well. More than a decade ago, tissue specific Notch signaling factors were identified in trophoblast giant cells and were proposed to down-regulate the Mash-2 transcription factor, a step that is necessary for giant cell differentiation (Nakayama et al., 1997). Targeted deletion in mice of the F-box protein, Fbw7, results in elevated levels of Notch signaling and an increased number of trophoblast giant cells undergoing endoreplication (Tetzlaff et al., 2004). In addition, a requirement for Notch in megakaryocyte differentiation was recently described (Mercher et al., 2008).

Hormone-induced endoreplication in plants and animals

Notch signaling is not conserved in plants, and as plants possess some of the best characterized examples of developmentally regulated endoreplication, there is still much to learn about the signals that regulate the onset and the degree of endoreplication. Studies in plants as well as megakaryocytes in mammals suggest that hormone-induced pathways are also crucial for the transition to endoreplication.

Plants: Many genes that affect endoreplication in plant development have been identified through mutational studies (Inze and De Veylder, 2006; Sugimoto-Shirasu and Roberts, 2003). Interestingly, it appears that there are tissue-specific pathways that are responsible for endoreplication. The phytohormone gibberellin (GA) acts antagonistically to salicylic acid to initiate endocycles in trichomes and the hypocotyl (Collett et al., 2000; Joubes and Chevalier, 2000). GA signals are mediated through GIS transcription factors and zinc-finger protein 8 to upregulate the expression of GL1, a potent transcriptional activator of endocycles (Gendreau et al., 1999). Mutations in the GA pathway exhibit defects in endoreplication, leading to smaller or less branched trichome structures and hypocotyl elongation (Gendreau et al., 1998; Gendreau et al., 1999). Spy is a negative regulator of GA and its mutation causes over-endoreplication phenotypes similar to wildtype plants treated with high concentration of GA (Swain et al., 2002). In the roots, ethylene and auxin promote root hair formation and elongation. Thus, phytohormones are thought to mediate the fate determination of endocycling cells, as well as the magnitude of endoreplication. However, whether these signals actually initiate the transition into endocycles is not yet clear. It has been suggested that specific combinations of phytohormones, nutrient, and light trigger endoreplication(Kondorosi et al., 2000).

Megakaryocytes: Abnormal megakaryocyte function resulting in changes to the normal number of platelets is directly attributable to hematopoietic pathologies (Nurden, 2005). Megakaryocyte ploidy is sometimes affected in patients with thrombocytopenia (low platelet counts) and thrombocytosis (high platelet counts), suggesting that endomitosis is important for efficient platelet formation (Pang et al., 2005; Tomer et al., 1989). However, the detailed mechanisms by which megakaryocyte ploidy is regulated are still not well understood.

Thrombopoietin (TPO) is a cytokine that stimulates differentiation of megakaryocyte progenitor cells by binding its receptor, c-Mpl (Kaushansky, 2005, 2008). Injection of recombinant c-Mpl into normal mice increased platelet counts and megakaryopoiesis (Kaushansky et al., 1994). Recombinant TPO has been shown to induce megakaryocyte maturation and polyploidization *in vitro* (Kaushansky, 1995). In addition, *c-mpl* or *tpo* mutations in mice or human patients exhibit reduced polyploidy of megakaryocytes and severe thrombocytopenia (Alexander et al., 1996; Gurney et al., 1994; Ihara et al., 1999; Murone et al., 1998; Solar et al., 1998).

TPO mediates proliferative signals largely through JAK/STAT pathways (Drachman et al., 1999), while endomitosis signals seem to be mediated by ERK1/2 (Rojnuckarin et al., 1999). Megakaryocytes from mice expressing a truncated version of c-mpl did not efficiently activate ERK, leading to reduced endomitosis after TPO induction (Luoh et al., 2000). Although the mechanisms by which these signals are transduced are not well understood, evidence suggests that TPO-induced endomitosis also relies on similar downstream factors as Notch-induced endocycles. Differentiating megakaryocytes were shown to maintain high levels of the S-phase promoting G1 cyclin, CycE, and ectopic expression of CycE could

induce promegakaryocytes into endomitosis (Garcia and Cales, 1996; Garcia et al., 2000). Studies of different megakaryoblastic cell lines suggest that endomitosis is promoted by the down-regulation of cyclin B/cdk1 mitotic kinase activity, similar to what occurs in *Drosophila* endocycles (Datta et al., 1996; Kikuchi et al., 1997; Matsumura et al., 2000; Zhang et al., 1996).

THE REGULATION OF THE ENDOCYCLE

After the mitotic to endocycle transition, progression through the endocycle is coordinated by a subset of the same factors that control progression through mitotic cell cycles. These factors form a complex regulatory network that produce oscillations in the activity of cyclin dependent kinases that control DNA synthesis, resulting in alternating S and G phases leading to polyploidy.

Replication origin control via oscillations of Cyclin E/CDK2 drive the endocycle

To maintain genomic integrity, proliferating diploid cells must duplicate the entire genome once, and only once, per cell division cycle. This task is complicated by the fact that during S phase eukaryotic cells initiate DNA replication at many distinct sites in the genome (i.e. origins of replication). Highly conserved mechanisms exist to control origin initiation during S phase, and to prevent origin re-initiation, and thus inappropriate re-replication of portions of the genome, within a given S phase and the subsequent G2. This occurs through the regulated assembly of pre-replicative complexes (pre-RCs) at each origin during G1 phase. The pre-RC is a multiprotein complex consisting of the hexameric Origin Recognition Complex (ORC), Cdc6, and Cdc10 dependent transcript1 (Cdt1). These proteins recruit the

replicative DNA helicase, which is composed of the MCM2-7 complex (MCM=mini-chromosome maintenance) and the GINS complex (Labib and Gambus, 2007). Once DNA synthesis is initiated at an origin, a variety of mechanisms that act on individual pre-RC proteins, including nuclear export, inactivating modification (e.g. phosphorylation), and ubiquitin-mediated degradation, prevent pre-RCs from reassembling until the next G1 [For review: (Arias and Walter, 2007)]. Importantly, the current data suggest that, as with diploid cells, these same origin controls are operative during endocycles (Edgar and Orr-Weaver, 2001).

Progression through both cell division cycles and endocycles is directed by periodic activation and inactivation of cyclin-dependent kinases (CDKs). The last 20 years of cell cycle research has revealed an elegant molecular paradigm for S phase control in which a period of low CDK activity (e.g. during G1) is permissive for pre-RC assembly, while a period of high CDK activity (e.g. during S) both triggers the initiation of DNA synthesis and blocks the re-assembly of pre-RCs. As a result, after the completion of S phase cells must sufficiently reduce CDK activity to become competent for another round of DNA replication. In cell division cycles this happens during mitosis, when several mechanisms (e.g. cyclin destruction) lead to a period of low CDK activity during G1. In endocycles, CDK activity oscillates between high (S phase) and low (G phase) to achieve the repeated rounds of DNA replication resulting in polyploidy.

In metazoan cell division cycles, activation of Cdk2 by S phase cyclins drives entry into S phase, while activation of Cdk1 by M phase cyclins promotes entry into and progression through mitosis (Morgan, 2007). Mitotic Cdks are expressed at very low levels in endocycles (Narbonne-Reveau et al., 2008; Zielke et al., 2008), and endoreplication is

driven by periodic activation/deactivation of S phase Cdks. In mammals the Cdk requirement for S phase, including endo S phase, is provided redundantly between Cdk1 and Cdk2 (Aleem and Kaldis, 2006; Aleem et al., 2005; Santamaria et al., 2007; Ullah et al., 2008), whereas in *Drosophila* Cdk2 is essential (Lane et al., 2000). CycE function is required for endoreplication in rodent trophoblasts and megakaryocytes (Geng et al., 2003; Parisi et al., 2003), and mutation of the single *Drosophila CycE* gene blocks DNA synthesis in both proliferating and endocycling cells (Knoblich et al., 1994). Thus, CycE/Cdk2 appears to be a major Cdk regulator of the endocycle in both insects and mammals (Fig. 4).

CycE/Cdk2 promotes DNA replication in several ways (Sclafani and Holzen, 2007). For example, CycE expression can drive the chromatin loading of MCM proteins in *Drosophila* endocycling cells (Su and O'Farrell, 1998), as it does in mitotic mammalian cells that are stimulated to leave quiescence by serum addition (Coverley et al., 2002; Geng et al., 2003). Importantly, CycE/Cdk2 can also direct dissociation of pre-replication members from origins to inhibit re-loading of the MCM helicase (Arias and Walter, 2007). Thus, CycE/Cdk2 both triggers S phase and subsequently inhibits re-replication within S phase. Consequently, a prevailing model of endocycle regulation is that periodic activation, or oscillation, of CDK2 activity both promotes endocycle progression and ensures once and only once replication during each endocycle S phase. In support of this model, constitutive expression of CycE stalls endocycles in *Drosophila* salivary glands (Follette et al., 1998; Weiss et al., 1998). Interestingly, continuous CycE expression is permissive for mitotic cycles, as occurs naturally in early embryogenesis (Jackson et al., 1995; Sauer et al., 1995), and during gene amplification in *Drosophila* ovarian follicle cells (Calvi et al., 1998). These observations suggest that endocycle regulation is particularly dependent upon oscillation in

CycE/Cdk2 activity.

How is the oscillation of CycE/Cdk2 activity during an endocycle achieved? There are both transcriptional and post-transcriptional inputs. In *Drosophila* endocycles, CycE mRNA abundance oscillates during endocycles, with peak levels in late G and S phase. CycE expression in endocycling cells requires the E2F1 transcription factor (Duronio et al., 1998; Duronio and O'Farrell, 1995; Royzman et al., 1997). We will elaborate on this aspect of CycE regulation in a following section. Studies in mammals and *Drosophila* revealed that a Cullin-RING E3 ubiquitin ligase (CRL) of the SCF type is responsible for regulated CycE protein destruction (Koepp et al., 2001; Moberg et al., 2001; Strohmaier et al., 2001). *Drosophila* Archipelago (Ago; Fbw7 in mammals) is an F-box protein that acts as a substrate receptor for the SCF ubiquitin ligase by directly binding to CycE (Moberg et al., 2001). *ago* mutants fail to undergo endocycles in ovarian follicle cells, leading to small nuclei (Shcherbata et al., 2004). Thus, when CycE transcription is terminated in late endo S phase, CycE protein destruction likely contributes to a decline in CycE/Cdk2 activity necessary for the period of low CDK activity that is permissive for pre-RC assembly. CycE protein destruction may also contribute to mammalian endocycles. *Fbw7* null mutant mice display elevated levels of CycE in trophoblasts, which display defects in endoreplication (Tetzlaff et al., 2004). Similarly, mice that lack Cullin1 activity exhibit elevated levels of CycE in trophoblasts, which fail to undergo endoreplication (Tateishi et al., 2001). However, these observations may be a result of CycE over-expression rather than changes in oscillations of CycE expression, since fluctuations of neither CycE protein nor mRNA were detected in trophoblasts (Geng et al., 2003).

This last observation suggests that additional regulators contribute to oscillations in

CycE/Cdk2 activity during endocycles. Likely candidates include CDK inhibitors, or CKIs, which bind to and inhibit CDK kinase activity (Morgan, 2007). The level of the p57 CKI oscillates during rodent trophoblast giant cell endocycles (Hattori et al., 2000), with the greatest amount during G phase (Ullah et al., 2008). p57 activity was recently shown to promote endoreplication through down-regulating CDK1 (Ullah et al., 2008). Similarly, expression of the *Drosophila* CKI Dacapo, which inhibits CycE/Cdk2, oscillates in ovarian nurse cells (de Nooij et al., 2000) and follicle cells (Hong et al., 2007). Furthermore, Dacapo expression in nurse cells is stimulated by CycE, suggesting negative feedback regulation that may be important for endocycle progression (de Nooij et al., 2000). Mutation of *dap* disrupts nurse cell endoreplication, suggesting that Dap functions to enforce the period of low CycE/Cdk2 activity needed for pre-RC assembly (Hong et al., 2007).

The existence of multiple mechanisms that each contribute to oscillations in CycE/Cdk2 activity creates the potential for variations in endocycle regulation in different cell types. Some evidence from *Drosophila* supports this idea. For example, ovarian nurse cell endocycles are disrupted in *dap* mutants (Hong et al., 2007), but both endocycling ovarian follicle cells and endocycling socket and shaft cells of mechanosensory bristles do not express *dap*, and thus likely do not require Dap function (Audibert et al., 2005; Shcherbata et al., 2004). In the salivary gland, transcriptional control of CycE expression appears to be more important than in nurse cells, where CycE protein levels oscillate (Lilly and Spradling, 1996), but CycE mRNA levels do not (Royzman et al., 2002). Multiple mechanisms of CycE/Cdk2 control lead to increased robustness of endocycle progression and provide an opportunity for multiple regulatory inputs that may differentially utilized in different cell types.

Ubiquitin-mediated proteolysis promotes endocycle progression

Ubiquitin-mediated proteolysis plays an important role in both endocycle initiation and progression (Ullah et al., 2009). The key regulator is the Anaphase Promoting Complex/Cyclosome (APC/C), an E3 ubiquitin ligase best known for its role in targeting proteins (e.g. cyclins) for destruction during mitosis (Morgan, 2007). To target specific proteins for ubiquitination and destruction, the APC/C interacts with two activator proteins, fzy/Cdc20 and fzr/Cdh1, which effectively function as substrate receptors for the APC at different points in the cell cycle. $APC/C^{fzy/Cdc20}$ is active only during mitosis and triggers the metaphase to anaphase transition, while $APC/C^{fzr/Cdh1}$ is active from the metaphase/anaphase transition through the subsequent G1. One of the jobs of $APC/C^{fzr/Cdh1}$ is to prevent mitotic cyclin accumulation during G1. This helps maintain a period of low CDK activity necessary for pre-RC formation. It is this job that is important for the mitotic to endocycle transition, as first shown in *Drosophila fzr/Cdh1* mutant embryos, which inappropriately accumulate mitotic cyclins and fail to enter endocycles (Sigrist and Lehner, 1997). As discussed above, Notch signaling induces fzr/Cdh1 expression during the mitotic to endocycle transition in *Drosophila* follicle cells. Similarly, genetic inhibition of a plant ortholog of fzr/cdh1, ccs52, results in inhibition of endocycles (Cebolla et al., 1999) and mutation of mutation of mouse *fzr* blocks trophoblast giant cell endoreplication (Garcia-Higuera et al., 2008), suggesting that $APC/C^{fzr/Cdh1}$ involvement in the mitotic to endocycle transition is an evolutionarily ancient mechanism. However, from these data it was unclear whether APC/C activity is needed *during* endocycle progression.

Recent reports from two groups revealed APC/C activity is necessary to sustain endocycle progression in *Drosophila*, in part by targeting the Geminin protein for destruction

(Narbonne-Reveau et al., 2008; Zielke et al., 2008). Geminin is an inhibitor of DNA replication, and acts by binding directly to Cdt1 and preventing Cdt1 from recruiting the MCM2-7 helicase to pre-RCs (Wohlschlegel et al., 2000). In mitotic cycles, Geminin is targeted for destruction by APC/C at the metaphase-anaphase transition, and does not re-accumulate until late G1 when APC/C is inactivated by G1 Cyclin/Cdk-mediated phosphorylation of Cdh1 (McGarry and Kirschner, 1998; Zachariae et al., 1998). Genetic depletion of APC/C components in *Drosophila* follicle cells and salivary glands results in Geminin hyper-accumulation and disrupts endocycle progression, likely because pre-RC formation is inhibited (Narbonne-Reveau et al., 2008; Zielke et al., 2008). Conversely, mutation of Geminin causes premature endoreplication during early mouse embryogenesis (Gonzalez et al., 2006).

Geminin protein accumulation oscillates in unperturbed *Drosophila* endocycles, with high levels occurring during S phase and low levels during G phase (Zielke et al., 2008). This cyclic expression likely helps constrain Cdt1 activity to G phase when pre-RCs are formed, and helps prevent re-replication during endo S phase. How is cyclic Geminin accumulation achieved? Narbonne-Reveau et al. (2008) show that APC/C activity also oscillates in endocycles. They suggest that CycE/Cdk2 promotes the phosphorylation and inhibition of fzr/Cdh1, which prevents the APC/C from acting on its targets resulting in accumulation of Geminin during S phase. The model which emerges is that APC/C$^{fzr/Cdh1}$ plays a critical role in the G phase of an endocycle by stimulating pre-RC assembly in two ways: 1) targeting the Cdt1 inhibitor Geminin for destruction and 2) maintaining low levels of mitotic CDK activity. In this way APC/C$^{fzr/Cdh1}$ acts much like it does during G1 phase of a mitotic cycle, suggesting that endocycles are essentially G1-S cycles. Moreover, cycles of

APC/C$^{fzr/Cdh1}$ activity are directly, and inversely, tied to cycles of CycE/Cdk2 activity, thus forming a key component of the endocycle regulatory circuit (Fig. 4).

While inappropriate Geminin hyper-accumulation may be sufficient to block endocycles, there are likely other targets of the APC/C$^{fzr/Cdh1}$ that must be kept low for normal endocycle progression to occur. For instance, in *Drosophila* salivary glands, preventing Geminin accumulation does not relieve the block to endoreplication in conditions where APC/C$^{fzr/Cdh1}$ is held inactive by over-expression of CycE (Narbonne-Reveau et al., 2008). Mitotic cyclins are targets of APC/C$^{fzr/Cdh1}$, and Cyclin A activity can suppress endocycles in both flies and plants (Hayashi, 1996; Imai et al., 2006; Sauer et al., 1995). Thus, in the absence of APC/C$^{fzr/Cdh1}$ activity the inappropriate accumulation of Cyclin A could also contribute to endocycle arrest by ectopically activating CycA/Cdk1 and inhibiting pre-RC assembly.

Transcriptional control of the endocycle

As noted above, transcriptional controls via a variety of factors play an important role in endoreplication. Some of these factors affect the activity of CycE/Cdk2, and thus contribute to the core endocycle mechanism, while others regulate the transition into endocycles and/or contribute to the differentiated state that is permissive for endoreplication.

Modulation of endocycle progression by E2F

The E2F family of transcription factors regulates the G1-S transition in both mitotic and endocycling cells by controlling genes encoding factors necessary for DNA synthesis and S phase progression (DeGregori and Johnson, 2006; Dimova and Dyson, 2005; van den

Heuvel and Dyson, 2008). The E2F family is composed of positive and negative regulators of transcription, and both types play a role in endocycle progression in animals and plants (Boudolf et al., 2004; Duronio et al., 1998). In *Drosophila* E2f1 mutants, DNA synthesis and endocycle progression is drastically attenuated (Duronio et al., 1998; Duronio et al., 1995; Royzman et al., 1997), similar to observations made in trophoblast giant cells in a mouse mutant of DP1, the obligate binding partner of E2F (Kohn et al., 2003). Therefore, at least some E2F transcriptional targets must be important for endocycle progression, even though recent reports indicate that in *Drosophila* these targets are expressed at lower levels than in mitotic cells (B. Calvi, pers. comm.; (Zielke et al., 2008)). *Drosophila* E2f1/Dp is required for the expression of a host of replication factors during endoreplication. However, the key E2F target is the *CycE* gene, whose expression both oscillates and requires E2f1 and Dp during *Drosophila* endocycle progression (Duronio et al., 1998; Duronio and O'Farrell, 1995; Duronio et al., 1995; Royzman et al., 1997). Interestingly, *Drosophila* CycE also negatively regulates its own expression by down-regulating E2f1 activity (Duronio et al., 1995; Sauer et al., 1995). These data suggest a model whereby E2F-directed transcriptional regulation of *CycE* contributes to the oscillations of CycE/Cdk2 activity that are critical for endocycle progression (Fig. 4.4).

How might cycles of E2f1 activation and inhibition occur? Very recent work has provided new insight into the mechanism. The most well studied mode of E2F regulation is via E2F interaction with the retinoblastoma family of tumor suppressor proteins, which are conserved in both insects and plants (Inze and De Veylder, 2006; van den Heuvel and Dyson, 2008). pRb family proteins bind and inhibit E2F during periods of low CDK activity (i.e. G1). However, mutations in *Drosophila* Rbf1, which binds and inhibits E2f1, do not affect

endocycle progression (Du, 2000; Du and Dyson, 1999; Du et al., 1996), suggesting the possibility for a pRb-independent mode of regulation. Like its transcriptional targets and other regulators that we have discussed, E2f1 protein accumulation oscillates during endocycles, with high levels during G phase and low levels during S phase (Zielke et al., 2008). We recently demonstrated that E2f1 is targeted for destruction in replicating cells by a mechanism requiring a motif in the E2f1 protein called a PIP box (Shibutani et al., 2008), which interacts with PCNA bound to chromatin at replication forks (Arias and Walter, 2006; Havens and Walter, 2009; Higa et al., 2006; Hu and Xiong, 2006; Senga et al., 2006). This interaction recruits a $Cul4^{Cdt2}$ E3 ubiquitin ligase that targets E2f1 for destruction (Shibutani et al., 2008). This suggests a model in which accumulation of E2f1 during G phase drives *CycE* transcription, which activates Cdk2 and triggers entry into S phase and the subsequent destruction and inactivation of E2f1. The resulting down regulation of *CycE* transcription and destruction of CycE protein (described above) create the period of low CDK activity in the following G phase where origins are assembled in preparation for the next cycle. A predication of this model is that blocking S phase-coupled E2f1 destruction will attenuate endocycle progression. Indeed, expressing a mutant version of E2f1 lacking a functional PIP box results in continuous CycE expression and blocks the endocycle in larval salivary glands (B.A. Edgar, pers. comm.). Interestingly, the same E2f1 mutant does not block cell proliferation, even though E2f1 protein is destroyed during S phase in cell division cycles (Shibutani et al., 2008). This again illustrates that endocycles and cell division cycles contain common modes of regulation, but depend differently on these forms of regulation for cell cycle progression. In addition, because robust oscillations of CycE transcription are not observed in ovarian nurse cells (Royzman et al., 2002), it will be interesting and important to

determine if S phase-coupled E2f1 destruction is important in all endocycles.

Modulation of endocycle progression by repressor E2Fs.

Plants also contain pRb and both repressor and activator E2Fs. Tobacco pRb function modulates the extent of endoreplication, as disruption of pRb resulted in increased endoreplication (Park et al., 2005). Similarly, functional reduction of the *Arabidopsis* E2fc/DPB repressor results in higher proliferation activity, yet a severe reduction in organ size because cells are unable to switch to endoreplication-mediated growth (del Pozo et al., 2006). Thus, pRb/E2F pathways regulate a balance between proliferation and endoreduplication during development that is a critical feature of plant growth and final organ size. In *Drosophila*, the absence of the E2f1 activator results in the E2f2 repressor acting to inhibit proliferation, likely by repressing cell cycle targets of E2f1 (Frolov et al., 2001; Rasheva et al., 2006). In the absence of both E2f1 and E2f2, some endocycles are inhibited because of elevated, continuous expression of *CycE* (Weng et al., 2003).

A recently described family of atypical E2F repressors plays an important role in endocycle initiation in plants. These E2F repressors are also found in animals, and contain two DNA binding domains, do not bind to DP, and lack an obvious pRb interaction domain (Lammens et al., 2009). E2fe/DEL1 is an *Arabidopsis* atypical E2F expressed in mitotically active cells that controls the timing of endocycle onset by repressing the expression of a homolog of fzr/Cdh1 (called CCS52A2) (Lammens et al., 2008; Vlieghe et al., 2005). As in *Drosophila*, *Arabidopsis* APC/C^{Cdh1} triggers endocycle onset by triggering the destruction of mitotic cyclins and the consequent inhibition of mitotic CDK activity (Boudolf et al., 2009). Such regulation may be conserved in mammals, since human E2f7 associates with the

promoter of the Cdh1 gene (Lammens et al., 2008).

Other transcriptional inputs into endoreplication.

Transcription factors other than E2F have been implicated in the initiation and maintenance of endocycles. *Drosophila* mutants of the zinc finger transcription factor *escargot (esg)* display ectopic entry into the endocycle in normally diploid larval histoblasts (Hayashi et al., 1993). Esg acts to maintain the activity of Cdk1, which when inactivated can trigger endoreplication in normally diploid cells (Hayashi, 1996; Weigmann et al., 1997). Similarly, mSna, a murine homologue of Esg, acts to repress the mitotic to endocycle transition of trophoblast giant cells (Nakayama et al., 1998). In addition, constitutive ectopic expression of Escargot inhibits megakaryocyte endomitosis (Ballester et al., 2001). The basic helix-loop-helix (bHLH) transcription factor superfamily member, Hand1, promotes trophoblast giant cell differentiation and endoreplication, but the mechanism by which Hand1 (e.g. via transcriptional targets) acts is not known (Martindill and Riley, 2008).

Endocycle specific regulation

We have been emphasizing similarities in replication control between mitotic cycles and endocycles. As more and more is learned about endocycle regulation, these similarities of molecular mechanism may seem obvious in retrospect, as completely new mechanisms for fundamental cell biological processes like DNA replication typically do not arise during evolution. However, recent data has provided hints that there may indeed be endocycle-specific mechanisms, or at least modifications of core regulation that support specific aspects of the biology of endocycling cells.

Endocycle modulation of pre-RC assembly

Endoreplicating cells in plants and animals control and respond to the expression of pre-RC components differently than in proliferating cells. *Arabidopsis* contains two ORC1 genes which are targets of E2F and that show peaks of expression as cells enter S phase. One of these two ORC1 genes is preferentially expressed in endocycling tissues (Diaz-Trivino et al., 2005). Similarly, human CDC6 contains an endocycle-specific cis regulatory element that binds to Esg (Vilaboa et al., 2004). Pre-RC components are more stable in endoreduplicating plant cells and megakaryocytes (Bermejo et al., 2002; Castellano et al., 2001).

A recent report raised the possibility that regulation of pre-RC assembly may be different in endocycling cells (Park and Asano, 2008). *Drosophila orc1* mutants survive through larval development and the highly polyploid salivary glands of these mutants were indistinguishable from wild type. As Orc1 is a critical component of pre-RCs and is essential for DNA replication in other contexts (Bell and Dutta, 2002), one possibility is that maternally-derived Orc1 protein is sufficient to support DNA replication during larval growth, as suggested by genetic studies of other *Drosophila* Orc subunits (Pflumm and Botchan, 2001; Pinto et al., 1999). Interestingly, Park and Asano (2008) could not detect Orc1 protein in *orc1* mutant salivary glands and concluded that *Drosophila* Orc1 is dispensable for endoreplication. This is particularly surprising because Orc1 is required for cell proliferation and for gene amplification in follicle cells (Park and Asano, 2008). Moreover, other components of the pre-RC such as Cdt1 are required for endoreplication (Park and Asano, 2008). Because *Drosophila* Orc1 is degraded at mitosis by the APC/C (Araki et al., 2003; Araki et al., 2005), presumably including the last mitosis before the onset

of endocycles, there should be no Orc1 present when salivary gland cells transition to the endocycle during embryogenesis. However, embryonic salivary gland cells enter the first endoreplication S phase from G2 (Smith and Orr-Weaver, 1991), suggesting that a small amount of Orc1 synthesized during interphase from maternal transcript could be present in *orc1* mutant salivary gland cells. However, this interpretation demands that an amount of Orc1 below detection by molecular and microscopic methods is sufficient to support genome duplication to the level of 1000C over the course of larval development. A discussion of possible Orc-independent endoreplication can be found in (Asano, 2009).

Endocycle modulation of the DNA damage response

In endocycling cells, S phase is often terminated before the entire genome has been duplicated (Smith and Orr-Weaver, 1991). In *Drosophila* polyploid cells, pericentric heterochromatin is often under-replicated (Leach et al., 2000; Lilly and Spradling, 1996). After repeated endocycles this results in many stalled replication forks that trigger a DNA damage response. This damage occurs in or near the under-replicated heterochromatin where replication forks presumably stall (Hong et al., 2007; Mehrotra et al., 2008). Mitotic cells respond to damage resulting from stalled replication forks either by arresting the cell cycle or by inducing apoptosis, but endocycling cells do neither. How does the cell differentiate between the type of cycle utilized and the level of sensitivity to unreplicated, or damaged, DNA? Mehrotra et al. (2008) probed this question by inducing re-replication-mediated DNA damage with over-expression of Cdt1. In diploid cells, this treatment triggers apoptosis via p53-dependent and -independent pathways. However, despite the accumulation of DNA damage in endocycling cells in response to Cdt1 over-expression, there was no evidence of induction of apoptosis. While endocycling cells can still respond to pro-apoptotic genes and

enter apoptosis, they have a muted response to p53 activation and express pro-apoptotic genes at a lower level than cycling diploid cells (Mehrotra et al., 2008). Similarly, DNA damage induced by chromatin assembly factor-1 (CAF-1) depletion does not adversely affect endocycle progression (Klapholz et al., 2009). Thus, polyploid cells have evolved a mechanism to buffer against the DNA damage that accumulates during normal endocycle progression.

A similar situation exists in mammals. In the process of trophoblast stem cells differentiating into endocycling trophoblast giant cells, p57 expression in response to FGF4 deprivation initiates the transition to endocycles by inhibiting Cdk1, which is required to enter mitosis, while the CKI p21 suppresses expression of the checkpoint protein kinase Chk1 (Ullah et al., 2008). p21 is not required for endocycle initiation, but instead is needed to suppress the DNA damage response. Thus, this combination of regulation induces the transition into endocycles while preventing the normal cell cycle checkpoint machinery from detecting endoreplication as detrimental DNA damage.

SUMMARY

Recent research has provided new insight into the mechanisms of endoreplication and the function of polyploidization. Endoreplication is generally controlled by the same cell cycle regulators that drive the cell division cycle, particularly those that control the G1-S transition and subsequent DNA synthesis. Importantly, endoreplication is highly conserved in evolution and is employed as a form of growth by multiple cell types that perform specialized functions during the development of many plant and animal species. In each of these species, the magnitude of polyploidization varies from one cell type to another, but

little is known about how this is achieved or what function it might serve. More recently, there is increasing appreciation for how endoreplication and polyploidy contribute to stress response and pathogenesis, but much remains to be learned in this regard. Our increasing knowledge of, and ability to manipulate, cell cycle progression should provide the tools to address these interesting questions.

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CHAPTER V

DISCUSSION AND FUTURE DIRECTIONS

By utilizing established tools of Drosophila genetics in combination with known functional alleles of E2f1, we were able to address the question of the biological requirement of E2f1 S phase-coupled destruction. In this thesis, we started with the question of how E2F1 stablization can be measured both S2 cells as well as in proliferating imaginal wing discs (Chapter II). By utilizing this assay as well as generating several Drosophila alleles that disrupted known functions of E2f1 protein, we found that the stabilization of E2F1 causes apoptosis in rapidly proliferating tissues, yet this apoptosis is not due to activity of E2f1 as a transcription factor. Instead, it was only when E2f1 lacked a functional Rb binding domain that stabilization of this protein failed to induce apoptosis. Following this observation, we sought to understand the mechanism. We proposed a "sensor" model, where stabilized E2f1 may titrate Rb away from a repressive complex found distally upstream of the promoter of a pro-apoptotic gene called *hid*. We observed that *hid* transcripts increased following the expression of E2f1 alleles with intact Rb-binding domains, and could reduce apoptosis with reduction of *hid* gene dosage. We demonstrated that this sensor is present in order to maintain tissue homeostasis in rapidly proliferating tissues, such as imaginal discs, and that stabilized E2f1 may cause neoplastic growth if apoptosis is blocked (Chapter III). In addition, we overviewed the current literature surrounding the mechanism and function of atypical cell cycles on normal development and their possible contributions towards disease

(Chapter IV). Overall, this dissertation focuses on biological mechanisms required for maintaining genomic integrity, which is achieved both through careful regulation of the cell cycle, and the distinct "choice" of atypical cell cycles as required for normal development.

Regulation of Onset of E2f1 S Phase Coupled Destruction

During *Drosophila* embryogenesis, prior to the onset of zygotic transcription, E2f1 is stable during S phase with no severe consequences (Shibutani et al., 2007). However, recent evidence suggests that all the required players for the $CRL4^{Cdt2}$ ubiquitin mediated proteolysis are maternally contributed and active (C. Swanson, pers. comm). The rapid nature of the early syncitial cycles ($S\text{-}M_{1\text{-}13}$) may require E2f1 to be protected from destruction, as all E2f1 protein is maternally supplied at this point and zygotic transcription has not initiated. *E2f1* mutant embryos show impaired DNA replication when the maternal supply of E2f1 is depleted (Duronio et al., 1995).

However, the developmental signal regulating the onset of E2f1 destruction is currently unknown. The signal may be the downregulation of a maternally loaded protein or RNA that protects E2f1 from destruction, the induction of a zygotic gene that is required for the destruction of E2f1, or a post-translational modification which could protect E2f1 from degredation. It is clear that this stabilization of E2f1 in the early cycles does not activate apoptosis, raising interesting questions about what developmental context allows the culling of cells with inappropriate E2f1 levels, and where it is best to keep those cells around. One hypothesis raised is that once 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. Another is that rapidly proliferating tissues have a lower threshold for allowing cells with inappropriate E2f1 levels to persist in the population, as there are robust compensatory mechanisms to replace cells that apoptosis, and the rapid cell proliferation could allow a small population of hyper-proliferative cells to have serious consequences on the development and homeostasis of the tissue.

E2f1 Stabilization induced DNA Damage

Similarly intriguing, other $CRL4^{Cdt2}$ targets have been shown to be destroyed following UV damage (Hu et al., 2004; Jorgensen et al., 2011; Nishitani et al., 2008; Soria and Gottifredi, 2010). This seems logical, as many of the targets are required for the progression of the cell cycle and their destruction may act as an arrest mechanism to allow sufficient time for DNA repair (Barkley et al., 2007). However, it has been demonstrated that mammalian E2F1 is not destroyed following UV damage (Hofferer et al., 1999). Conversely, E2F1 has been implicated in DNA damage repair as E2FI/DNA interactions increased as well as up-regulation of specific target gene expression, such as ribonucleotide reductase (Filatov et al., 1996). Yet, our results demonstrate that E2f1 is not destroyed following UV damage (Fig 5.1).

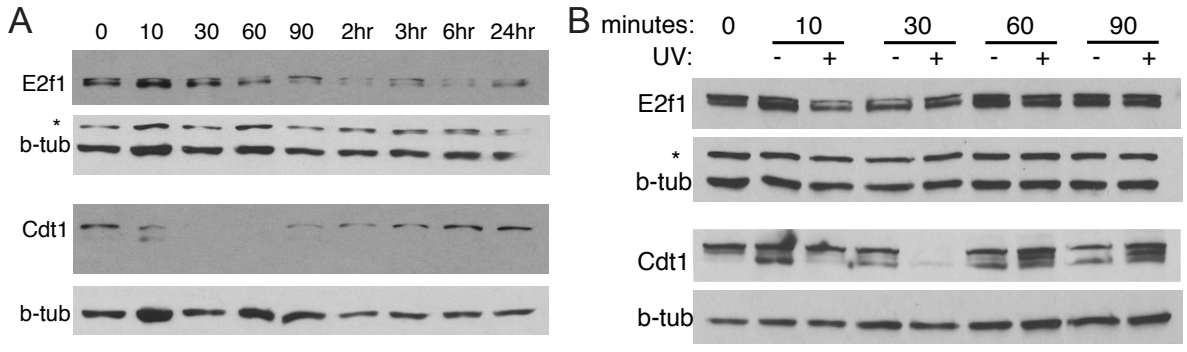

Figure 5.1: E2f1 is stabilized following UV radiation, while Cdt1 is destroyed. A) Western blot measuring levels of E2f1 and Cdt1 in S2 cells following 200uJ/cm^2 UV radiation over 24 hours. E2f1 levels remain

constant, while Cdt1 is immediately destroyed and reaccumulates hours later. beta-tubulin as loading control. B) Western blot comparing E2f1 and Cdt1 levels with or without UV radiation over 90 minute time course.

These preliminary results suggest that there may be a mechanism where *Drosophila* E2f1 can avoid ubiquitin-mediated proteolysis via the $CRL4^{Cdt2}$ mechanism in order to participate in DNA damage repair. Perhaps E2f1 is modified, not destroyed, so that cell cycle re-entry can occur rapidly after DNA damage repair, instead of necessitating the new synthesis of E2f1 protein. E2f1 consistently appears as a doublet, suggesting there maybe a post-translational modification which could be supporting this stabilization. While preliminary results suggest it is not phosphorylation, as phosphatase treatment does not result in band shifts, we have utilized SUMOsp 2.0 software to identify potential sumolaytion cites in E2f1. Sumolyation is promising, as another $CRL4^{Cdt2}$ target, Polη, is sumolayted by GEI-17 in *c.elegans* in order to prevent Cdt2 binding while Polη acts as a translesion synthetase following DNA damage (Kim and Michael, 2008). Only after Polη has bypassed the lesion and the sumolatyion has been removed, can Cdt2 target this protein for destruction, to prevent its' activity during normal DNA replication. It will be intriguing to investigate whether the mechanism which protects E2f1 from degradation in the early embryo is the same mechanism at play after DNA damage and could potentially uncover more details of the $CRL4^{Cdt2}$ dependent mechanism of proteolysis and general cell cycle regulation.

The Mechanism of *hid* Expression

Our data shown here suggest that ^{hid} gene expression is increased following the stabilization of E2f1 in S phase due to de-repression of an Rbf1 containing complex upstream of the promoter. However, the same publication that presented the presence of this repressive complex, and that *hid* expression could be induced by removing Rbf1, did not

implicate E2f1 as the transcription factor responsible for *hid* expression (Moon et al., 2008). Therefore, while it is previously known that E2f1 may drive expression of known pro-apoptotic genes directly, especially reaper, we propose this stabilized E2f1 sensor mechanism is independent of E2f1 transcriptional activity. This hypothesis is supported by the expression of a transcriptionally inert E2f1 molecule resulting in a robust apoptotic response (Fig. 3.2). It is further supported by genetic evidence that reducing hid gene dose results in less cell death (Fig 3.5). This raises interesting questions about the specific mechanism at hand. First, how is this apoptotic mechanism repressed during normal cell cycle progression? Our model predicts that stable E2f1 is able to titrate Rbf1 away from a repressive complex which also contains endogenous E2f1. But, as mentioned in the introduction, during the G_1-S transition, CyclinE/CDK2 levels accumulate in order to hyper-phosphorylate Rbf1 and prevent its' binding to E2f1 (Frolov and Dyson, 2004). How does Rbf1 at this repressive locus bypass this phosphorylation that could potentially be so deletrious (such as the cell would not want to activate hid every S phase)? Secondly, how does the repressive complex work biologically? The E2f1 binding sites to which Rbf1 protein was detected via ChIP are -1.2kb upstream of the *hid* promoter (Moon et al., 2008). And, as mentioned above, E2f1 is not the transcription factor responsible for *hid* gene expression. It will be interesting to uncover which factor is responsible for initiating *hid* expression and the regulation surrounding its activation.

It is intriguing to consider this repressive complex functioning via interactions with the known histone modifying partners known to bind Rb (Funayama and Ishikawa, 2007). It was previously reported that Rbf1 plays a critical role in condensing chromatin during mitosis (Longworth et al., 2008). However, recent observations have suggested that this

condensation role is not exclusive to mitosis. Instead, many genes are reguated at the transcriptional level based on chromatin environment regulated by Rbf1 (Longworth et al., 2012). Also, the E2F/Rb containing complex, dREAM/MMB, has been implicated in regulation of gene expression via chromatin modifications, especially in the context of differentiation (Lee et al., 2012).

It will be interesting to understand the specificity of the Rbf1/E2f1 interaction at the repressive complex upstream of *hid*. Our results demonstrate that apoptosis cannot be induced simply by titrating Rbf1 away with an E2f1 protein with only the Rb-binding domain intact (Figure 3.6). Instead, it seems there is some uncharacterized functional domain within the first 336 amino acids that is critical for the cells ability to sense the inappropriate stability of E2f1 and induce cell death. It will be interesting to investigate both the potential functional domains of E2f1 and the chromatin environment around the *hid* locus during normal cell cycle progression and following the stabilization of E2f1.

The Global Consequences of E2f1 Mis-regulation

The hypothesis that the repressive complex upstream of *hid* depends on chromatin modifications brings up interesting questions about the specificity of *hid* activation after E2f1 stabilization. It is interesting to consider that the stabilzation of E2f1 could titrate Rbf1 away from multiple repressive loci. In order to investigate this possibility, we are currently measuring global gene expression changes using high-throughput RNA sequencing. We have collected RNA from imaginal discs expressing GFP alone, GFP-E2f1^{WT}, GFP-E2f1^{Stable}, and E2f1$^{Stable/DBD}$. We generated cDNA with the appropriate adaptors and bar codes following the Illumina protocol. It will be interesting to compare changes in both E2f1

target gene expression following stabilization as well as the changes that occur after expression of the transcriptionally inert E2f1 transgene (Figure 5.2). By making pair-wise comparisons, we may be able to identify genes whose expression increases after E2f1 stabilization, but is not dependent on E2f1 transcription. The expression changes of the gene *hid* will act as our positive control. This experiment could potentially uncover multiple genes responsible for the "sensing" of inappropriate E2f1 levels in order to maintain tissue homeostasis.

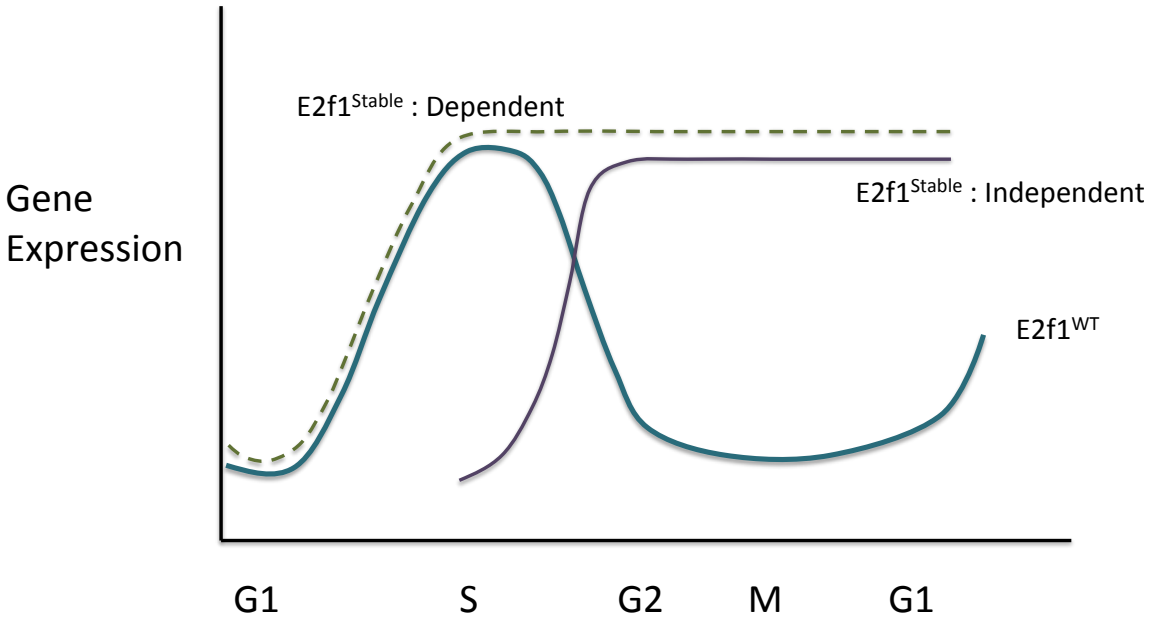

Figure 5.2: Schematic of Gene Expression Profiles During Cell Cycle

Concluding Remarks

The work presented in this thesis has advanced our understanding about E2f1 regulation in the context of development and tissue homeostasis. We showed evidence that the S phase specific stabilization of E2f1 induces apoptosis independently of its' transcriptional activitiy and uncovered a potential mechanism to carefully sense E2f1 levels in rapidly proliferative tissues in order to prevent potential neoplastic growth. By expressing

E2f1 transgenes with mutations in known functional domains, we were able to understand this mechanism requires an intact E2f1/Rbf1 interaction. We therefore proposed a mechanism where stabilized E2f1 could titrate Rbf1 away from a repressive complex upstream of a pro-apoptotic gene called *hid*. We demonstrated that this mechanism can quickly sense inappropriate stabilization of E2f1 protein and induce a robust apoptotic response. If apoptosis is not completed, stabilized E2f1 causes severe tissue overgrowth in imaginal wing discs. This supports the hypothesis that cells maintain a mechanism to quickly sense E2f1 levels and respond with programmed cell death before that cell has the potential to precociously re-enter the cell cycle or undergo re-replication stress and endanger the overall homeostasis of the tissue.

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