Cell Type-Dependent Requirement For PIP Box-Regulated Cdt1 Destruction During S Phase

Hyun Ok Lee

A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Curriculum of Genetics and Molecular Biology.

Chapel Hill
2010

Approved By:

Jean Cook
Robert Duronio
Bob Goldstein
Steve Rogers
Yue Xiong

ABSTRACT

Hyun Ok Lee: Cell Type-Dependent Requirement For PIP Box-Regulated Cdt1
Destruction
(Under the direction of Robert J. Duronio)

The development of a multi-cellular organism is driven by an increase in
cellular growth (increase in cell size) and cell proliferation (increase in cell
number through division). In order for these processes to coordinate smoothly,
appropriate genes must be expressed and terminated. The termination of gene
expression is often regulated through protein degradation mediated by the
Ubiquitin Proteasome System. In this system, target proteins are marked by poly-
ubiquitination and are shuttled to be degraded by a protease complex, the
proteasome. Substrate ubiquitination is carried out by E3 ubiquitin ligases that
each recognizes limited number of targets. My thesis work focused on a member
of a large ubiquitin ligase family, Cullin Ring Ligase 4, and its role during cell
growth and proliferation. Mammals have two closely related Cul4s, Cul4A and
Cul4B, complicating the study of CRL4 function. However, *Drosophila
melanogaster* has one Cul4, making this model organism more accessible.

During my thesis, I used previously generated alleles of *Cul4* and *Ddb1*
and demonstrated CRL4 mediated destruction of an important inhibitor of cellular
growth, TSC2/Gigas. I also studied CRL4 function in replication control using a
mutant substrate, Cdt1/Dup, that cannot be recognized by CRL4. We found that

ii

CRL4-mediated destruction of Cdt1/Dup is crucial for mitotic cell cycle progression in embryos. However, CRL4 regulation is dispensable in endocycles during oogenesis. Taken together, these results suggest tissue specific contribution of CRL4 in replication control.

ACKNOWLEDGEMENTS

Graduate school has been an amazing experience that taught me not only most of what I know about scientific procedures and thinking (aside from what I learned as an undergraduate), but also a great deal about life in general; it has been a great introduction to the world outside in the safety and comfort of education. I am grateful for those who made this opportunity possible for me: Drs. Sean Morrison, Ricardo Pardal, and Sharon Milgram to name a few.

I also thank those who made my graduate education the most it could be: my advisor, committee, teachers, and friends. First, I would like to thank my advisor Dr. Bob Duronio who has allowed me the opportunities to learn and grow in his lab. I have always admired his passion for understanding how basic biology works, and his insurmountable knowledge of…everything. Bob also has many responsibilities in the university, I respect his ability to juggle them all and still maintain his energy and optimism. My sincere hope is that I will take some of that away with me (and I think I have). Last but not least, Bob was always willing to listen, and tried his best to provide a comfortable research environment regardless of budget/time, which I have appreciated.

Secondly, I thank all my committee members who have shared their time with me: in teaching, collaborations, and encouragement. Drs. Steve Rogers and

without what I learned from and shared with them. I thank all my friends for allowing me to ask as many questions as I do and so willingly sharing their thoughts on life, love, careers and science…and thereby continuing my education outside of the lab. I owe it to them for making my Chapel Hill days so enjoyable.

I couldn't leave out special thanks to my second best friend (after my sister), Josh Currie. He has been the direct beneficiary (?) of my ups and downs during the last years of graduate school and start of my new job(s). Aside from me, Josh is likely the only person who has read all of my papers from start to end, including this thesis, multiple times. He so willingly spends countless hours on my practice talks, and by now he could give my thesis defense as well as, if not better, than I could. I truly appreciate your constant interest and support for all of my endeavors. These last years of graduate school would not have been as memorable and meaningful without the company of my favorite partner in crime.

Save the best for last…I could never express enough gratitude towards my family, who has sacrificed so much over the years for me to be here. I only hope that I can repay them a fraction of what they have given me for the years to come. They are my home away from where I physically belong; the best group of cheerleaders and mentors one could ask for; and teachers of unconditional love by example. Over the vast distance, they never failed to be there for me in joy and sadness with unceasing encouragement and prayer. Thank you.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

UPS: Ubiquitin Proteasom System

Pre-RC: Pre-Replicative Complex

RING: Really Interesting New Gene

CRL: Cullin Ring E3 Ligase

HECT: Homologous to E6-AP C-Terminus

APC/C: Anaphase Promoting Complex and Cyclosome

CDT1: Cdc10 Dependent Transcript 1

ORC: Origin Recognition Complex

CDT2: Cdc10 Dependent Transcript 2

CDC6: Cell Division Cycle 6

MCM: Mini-Chromosome Maintenance proteins

DDB1: DNA Damage Binding protein 1

CUL4: Cullin RING Ubiquitin Ligase 4

PCNA: Proliferating Cell Nuclear Antigen

CycE: Cyclin E

CDK: Cyclin-Dependent Kinases

TSC1: Tuberous Sclerosis Complex 1

TSC2: Tuberous Sclerosis Complex 2

TORC1: Target Of Rapamycin Complex 1

eIF4: Eukaryotic translation Initiation Factor 4

4E-BP1: eIF4 Binding Protein 1

GAP: GTPase-Activating Protein

Rheb: Ras Homologue Enriched in Brain

Rap1: Ras Proximate 1

InR: Insulin/IGF-1 Receptor

PTEN: Phosphatase and Tensin homologue deleted on chromosome 10

Akt: Ak Transforming (a.k.a Protein Kinase B)

S6K: 70kDa ribosomal protein S6 kinase (p70S6K)

Raptor: Regulatory Associated Protein of mTOR

LST8: Lethal with SEC13 protein 8

CHAPTER 1

INTRODUCTION

Summary

All living cells rely on timely initiation of gene expression as well as termination of gene function to drive cellular processes. One way eukaryotic cells accomplish the latter is through well-controlled protein degradation executed by the ubiquitin proteasome system (UPS). Substrates of the UPS are selectively recruited by E3 ubiquitin ligases. My thesis focused on a major E3 ubiquitin ligase family called Cullin Ring ubiquitin Ligases (CRL). More specifically, I studied a member of the CRL family, CRL4, and its role in regulating growth and replication. In this chapter, I discuss the details of the UPS components and provide brief background for regulatory mechanisms of growth and replication.

Ubiquitin Proteasome System (UPS)

Protein turn-over is essential for various cellular processes, and disrupting protein destruction can lead to diseases. The Ubiquitin Proteasome System (UPS) carries out regulated proteolysis.

Most proteins undergo post-translational modifications that affect their state: activation, localization, protection, or degradation. Two common modifications on proteins are phosphorylation and ubiquitination. Ubiquitination designates a process in which a highly conserved small protein, ubiquitin, is attached to substrates by a peptide bond (Ubiquitination is unique from phosphorylation in that substrates can be mono- or poly-ubiquitinated, which produce different consequences (Figure 1); Mono-ubiquitination modulates protein activity, poly-ubiquitination marks proteins for degradation (For review: Pawson and Scott 2005; Komander 2009; Wolek et al., 2007).

Both poly-ubiquitination and proteolysis are carried out by the Ubiquitin Proteasome System (UPS), which maintains the correct balance of protein levels through two types of protein turn-over: general destruction of damaged or misfolded proteins; or regulated destruction of cellular regulators. The UPS therefore influences most aspect of cellular metabolism and organism development including DNA repair, transcription, cell cycle progression, apoptosis, signal transduction, and stress responses. Thus, disrupting components of the UPS often leads to deleterious disorders such as cancer, neurodegenerative diseases, and diabetes (Ardley 2009; Ande et al., 2009; Lehman NL 2009, Naujokat 2009).
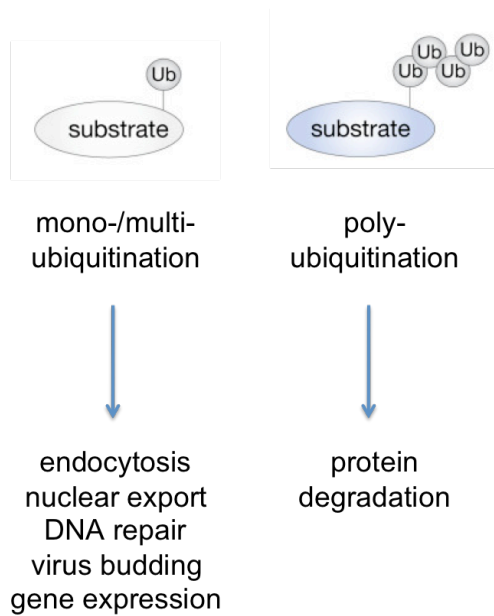
Figure 1. Protein ubiquitination.

Substrate ubiquitination can happen in multiple ways, each of which designates a specific signal. Two best-studied examples are mono (or multi)- and poly-ubiquitination. Mono-ubiquitination signals a change in activation or localization of a substrate, whereas poly-ubiquitination signals substrate degradation. (Adapted from Woelk et al. 2007)

Components of UPS: ubiquitin ligase and the proteasome. Ubiquitin ligation process is step-wise: E1-E2-E3 (-E4)

The ubiquitination process occurs in three distinct steps of ubiquitin transfer (Pickart 2001; Dye and Schulman 2007). In the initial step, ubiquitin is attached by a thioester bond to the E1 'ubiquitin activating enzyme', which then adenylates ubiquitin at the C terminus in an ATP dependent manner (Figure 2). There are eight E1 enzymes identified to date, and different E1s are thought to be present in the nucleus and the cytosol (Ye and Rape 2009). In the next step, E1 transfers the activated ubiquitin to a cysteine residue of E2 'ubiquitin conjugating enzyme'. Several dozen E2s are estimated to exist, each interacting with specific E3 enzymes (Markson et al., 2009). In the final step, E3 ubiquitin ligases bind both E2 and the substrate, and ubiquitin is transferred to the lysine residue on the substrate. E3 ligase involvement in this last ubiquitin transfer can vary in two ways; E3 may actually form an intermediate ubiquitin-E3 conjugate prior to substrate ubiquitination, or E3 merely serves as a docking site as ubiquitin transfer occurs directly from E2 to the substrate (Komander 2009). Each of several hundreds of E3 ligases recognizes a limited number of substrates, thereby conferring specificity to the ubiquitination process (DeShaies and Jazeiora 2009). In this manner, the UPS ensures the appropriate protein to be degraded at the correct time and place within the cell.
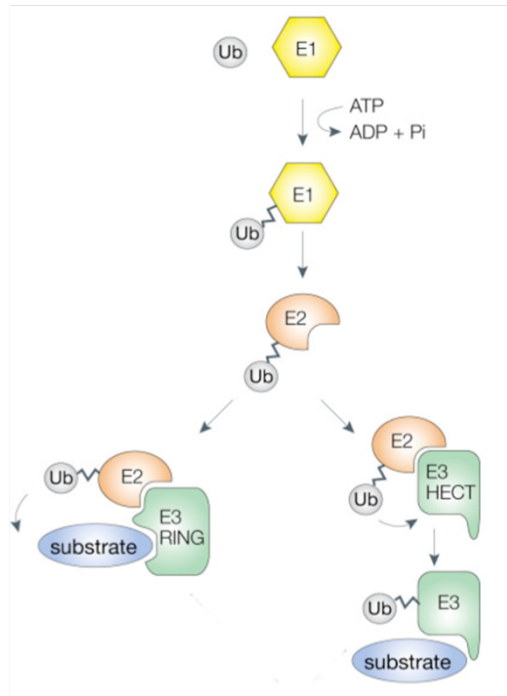
Figure 2. Steps of ubiquitination and types of ubiquitin ligases.

Ubiquitiation is a step-wise process where a small molecule ubiquitin is activated, transferred to a conjugating enzyme, and finally added to the substrate. How the last step occurs depends on the type of E3 ubiquitin ligase: RING E3s facilitate direct transfer of ubiquitin from E2 to substrate, whereas HECT E3s form a bond with ubiquitin before transferring over to the substrate. (Adapted from Woelk et al. 2007)

A four-chain ubiquitin polymer is considered necessary and sufficient for substrate recognition by the protease complex, the 26S proteasome. For the elongation of the ubiquitin chain, the mono ubiquitinated substrate sometimes requires E4 enzymes (Review: Hoppe 2009). E4 enzymes use the multiple lysine residues on the ubiquitin itself to add additional ubiquitin molecules. In this manner, the UPS ensures the appropriate protein is degraded at the correct time and place within the cell.

Types of E3 ubiquitin ligases: HECT and RING

There are two main classes of E3 ligases based on primary sequence (Figure 2): Those containing a HECT (Homologous to E6-AP C-Terminus) domain and others with a RING (Really Interesting New Gene) domain (Rotin and Kumar 2009; DeShaies and Joazeiro 2009; Jackson and Xiong 2009; Merlet et al., 2009). Importantly, these E3s also differ in their involvement in the ubiquitination process. While HECT E3s contain a cysteine that forms an obligate thioester bond with ubiquitin prior to substrate ubiquitination, RING-type E3s do not make direct contact with ubiquitin.

RING is the prominent family of E3s: APC/C and CRL

RING-domain E3 ligases constitute a prominent majority of E3s in the cell and can be divided into two groups: the APC/C (Anaphase Promoting Complex/Cyclosome) and the Cullin Ring Ligases (CRL). The APC/C is an

important regulator of mitosis progression that targets Securins and mitotic Cyclins (van Leuken et al., 2008). APC/C binds its targets through WD40 domain subunits, CDC20/Fzy and CDH1/Hct1/Fzr that recognize KEN- and D-box degradation motifs (or degrons) (Pfleger et al., 2001; Pfleger et al., 2000). Cullin subunit is composed of a family of up to six proteins that are crucial for many aspects of the cell cycle, growth, DNA repair, and chromatin remodeling (Jackson and Xiong 2009). Cullin subunits function as scaffolds to bind both the ubiquitin-loaded E2 through the RING finger protein RBX1/ROC1, and the substrate through a Cullin specific adaptor. My thesis focused on a member of CRL family, CRL4 (Figure 3). CRL consists of ROC1, Cullin4, adaptor DDB1, and recruits substrates through WD40 motif containing substrate specificity factors (DCAFs/DWDs) (Angers et al., 2006; Jin et al., 2006; He et al., 2006; Higa et al., 2006; Review: Higa and Zhang 2007). During my thesis study, I investigated the role of Cul4 in growth and replication control.
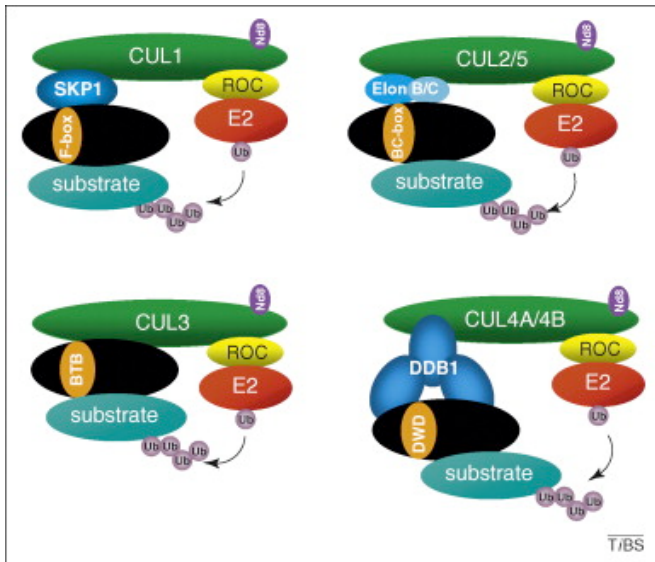
Figure 3. Cullin Ring ubiquitin Ligases (CRLs).

Cullin subunits function as a scaffold to hold both the E2 ubiquitin conjugating enzyme and the substrate. E2 binding occurs through a conserved Ring domain protein, ROC. Substrate binding occurs through a Cullin specific adaptor, and substrate specificity factor. (Jackson and Xiong 2009)

Regulation of growth and cancer by Cullin E3

Studies in the past decade identified the TSC1–TSC2 complex as the sensor and integrator of growth factors, nutrient, and stress signals to control cell growth (increase in cell size) and proliferation (increase in cell number).

Tuberous Sclerosis Complex and TSC1/2

TSC1 and TSC2 were first identified as the genes mutated in Tuberous sclerosis complex (TSC). TSC is an autosomal dominant disorder characterized by the development of benign tumors called hamartomas with an incidence of 1 in 6000 births (Crino 2006). Hamartomas affect various tissues and organs. Although hamartomas rarely progress to malignancy, they can inhibit normal tissue/organ function and cause serious symptoms or mortality. For instance, brain hamartomas often cause seizure, mental retardation, and autism (Inoki and Guan 2009; Au et al., 2008). TSC has 100% penetrance, however the clinical expression and developmental timing varies widely among the patients.

Inactivation of either *Tsc1* or *Tsc2* causes TSC, and disease-associated mutations involve nearly all exons of these two genes. Biochemical and genetic studies indicate that TSC1 and TSC2 (or hamartin and tubertin respectively) form a heterodimer and usually function as a complex (Plank et al., 1998; Hodges et al., 2001; van Slegtenhorst et al., 1997). The TSC1–TSC2 interaction appears to be important for the stability of both proteins, although TSC2 contains the enzymatic activity (Hodges et al., 2001; Benveuto 2000; Ikenoue et al., 2008).

9

TSC2 function and regulation (Figure 4)

TSC2 has a conserved GAP (GTPase-activating protein) domain in its C terminus, which functions to trigger Rheb (Ras homologue enriched in brain) GTPase activity (Figure 4) (Inoki et al., 2003; Zhang et al., 2003). Rheb-GTP hydrolysis renders it unable to activate mTOR, a potent activator of growth (Tee et al., 2003; Long et al., 2005; Bai et al., 2007). In its active state, mTOR promotes translation by phosphorylating two proteins: eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) and p70 ribosomal protein S6 kinase (S6K1) (Fingar and Blenis 2004; Hay and Sonenberg 2004; Wullschleger et al., 2006; Guertin and Sabatini 2007). S6K1 phosphorylation allows it to activate 40S ribosomal protein S6, which enhances translation of mRNAs (Burnett et al., 1998; Pearson et al., 1995). On the other hand, 4E-BP1 phosphorylation restrains its function as an inhibitor of the translation initiation factor eIF4E (Fingar et al., 2002). Thus, S6K1 and 4E-BP1 were constitutively phosphorylated in either Tsc1 or Tsc2 mutant cells (Huang and Manning 2008).

TSC2 itself is negatively regulated by phosphorylation. AKT phosphorylates TSC2 at at least three conserved sites (Manning et al., 2002; Inoki et al., 2002; Potter et al., 2002). TSC2 mutations that mimic constitutive phosphorylation reduce S6K1 inhibition, whereas mutants that cannot be phosphorylated at these sites show enhanced inhibition of S6K1. This evidence suggests that TSC2 phosphorylation by AKT inhibits its activity. Conversely,

TSC2 phosphorylation by other kinases, like AMPK, can also be activating (Inoki et al., 2003; Shaw et al., 2004).

TSC2 is a short-lived protein that is rapidly degraded in the absence of TSC1 (Chong-Kopera et al., 2006). TSC-associated mutations also show a significant decrease in TSC2 levels, suggesting proteolysis as a mechanism to downregulate TSC2. However, the mechanism of TSC2 degradation was not clear. Part of my thesis research focused on the role of CRL4 in TSC2 regulation. A detailed description of this study can be found in Chapter 2.
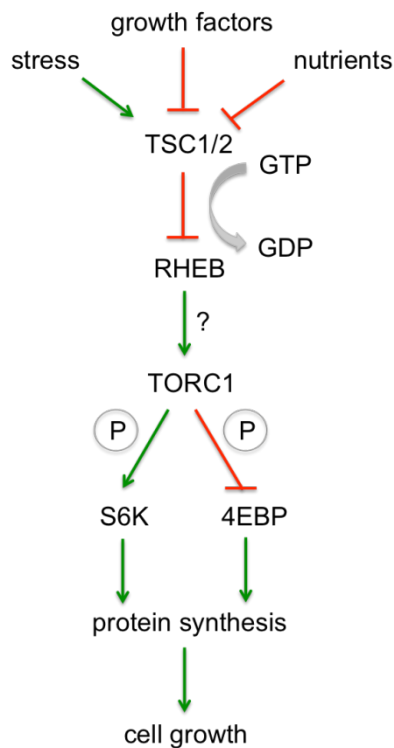
Figure 4. TSC2-TOR pathway.

TSC1/2 respond to various signals to regulate growth. Once activated, TSC1/2 stimulates Rheb GTPase activity to hydrolyze GTP to GDP. Once GDP bound, Rheb cannot activate TORC1. Active TORC1 promotes protein synthesis and subsequent cell growth by phosphorylating downstream targets S6K and 4EBP.

Regulation of replication by Cullin 4 E3

Replication vs. re-replication

Mitotic cell division produces two cells with the same genetic material. To accomplish this, the parental cell accurately duplicates its genome during the process of DNA replication using discrete locations in the genome called origins of replication. Replication is initiated at these origins, thus each origin must carefully be regulated to maintain genome integrity. Misregulation of origins leads to re-replication that causes DNA damage and genomic instability, which contribute to cancer development (Petropoulou et al., 2008). Origin regulation occurs through controlled assembly and disassembly of pre-replicative complex (pre-RC) (Figure 5).

Pre-RC and origin licensing

Pre-RCs contain the Origin Recognition Complex (ORC), Cdc6, and Cdc10 dependent transcript-1 (Cdt1) proteins that together recruit a core component of the replicative DNA helicase, the mini-chromosome maintenance complex (MCM2-7) (Figure 5). Assembly of the pre-RC constitutes 'licensing' of the replicative origin, and occurs during late metaphase to G1 (Bell and Dutta, 2002). Once DNA replication is initiated, pre-RC components are displaced and prevented from reassembling until the next G1 via multiple, often redundant regulatory mechanisms. These mechanisms include nuclear export, inhibitory phosphorylation, and ubiquitin mediated proteolysis (Arias and Walter, 2007).

Cdt1 is the rate-limiting factor of pre-RC assembly

Among the most important of these mechanisms is the negative regulation of Cdt1, as increased Cdt1 activity alone is sufficient to trigger re-replication in many situations (Zhong et al. 2003; Arias and Walter 2005, 2006; May et al. 2005; Sansam et al. 2006). Moreover, recent experiments in mice suggest that Cdt1 over-expression may promote tumor formation or progression (Arentson et al., 2002; Liontos et al., 2007; Petropoulou et al., 2008; Seo et al., 2005).

Two known mechanisms to negatively regulate Cdt1 are ubiquitin mediated proteolysis and inhibition by Geminin binding (Arias and Walter, 2007). However, how Cdt1 regulation occurs in development has not been studied. During my thesis research, I studied the mechanism and requirement of Cdt1 degradation during *Drosophila melanogaster* development. In addition, I investigated the relative contribution of Cdt1 degradation and Geminin inhibition, and tissue specificity of these regulatory mechanisms in mitotic and endocycling tissues. A detailed description of these regulatory pathways will be introduced in Chapter 3.

G1

S
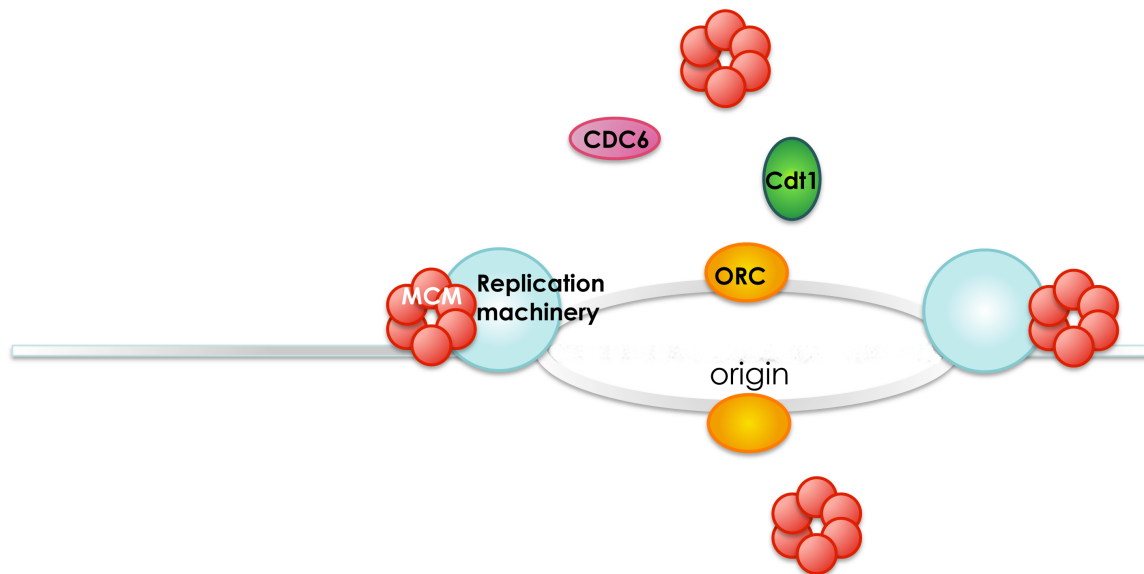
Abberant re-replication
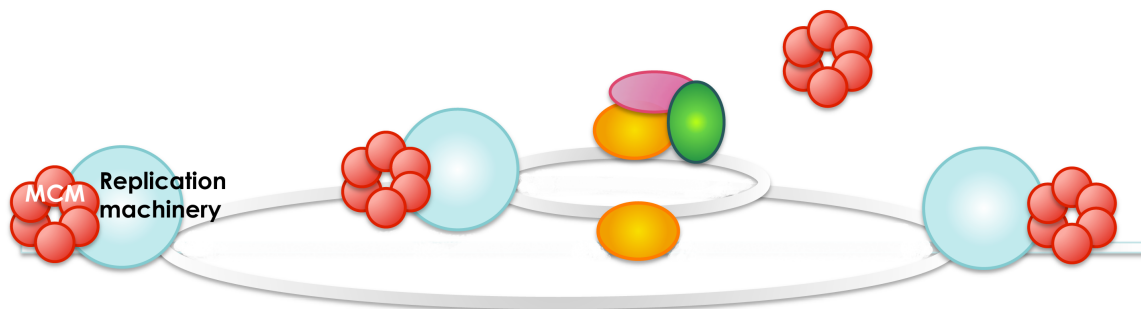
Figure 5. Pre-RC assembly and origin licensing versus re-replication

Pre-replicative complex members (pre-RC) ORC, Cdc6, Cdt1 bind the origin of replication during G1 and recruit DNA helicase MCMs. During S phase, Cdc6 and Cdt1 are displaced from the origin, and replication occurs in a bi-directional manner. However, misregulation of Cdc6 and/or Cdt1 can result in re-replication, where the same origin replicates more than once. ORC: yellow; CDC6: pink; Cdt1: Green; MCMs: red; Replication machinery: Carolina blue.

Conclusion

We determined Cul4 dependent degradation of TSC2

In a collaborative study with the Xiong lab, we showed that TSC2 and CRL4 subunits interact physically both in mammalian and *Drosophila* cells. We also found that TSC2 is stabilized when CRL4 activity is compromised by RNAi knockdown. Similar results were found using *Cul4* and *Ddb1* null fly extracts. Finally, I determined that the growth defects of *Cul4* and *Ddb1* null *Drosophila* mutants could be partially rescued by reduction of *Tsc2* gene dosage.

We found interesting evidence for tissue specific regulation of replication by Cul4 and Geminin.

I took several approaches to address the functional importance of Geminin and CRL4 regulation of *Drosophila* Cdt1, called Doubleparked (Dup), during development. I characterized *Cul4* and *Ddb1* mutants and also analyzed phenotypes caused by expression of a stabilized mutant Dup protein. Our results indicate that rapid Dup destruction during S phase is necessary for normal progression of the embryonic cell division cycle, but not for normal endocycle progression in follicle cells where inhibition by Geminin can compensate for Dup stabilization in S phase. Thus, we conclude that Dup is regulated in cell type specific manner by two distinct modes of regulation during normal animal development.

The function and determinants of a variant cell cycle

Also included in this thesis are the contents of my review article, 'Endoreplication: polyploidy with purpose' published in Genes and Development. Many organisms from plants to humans use a unique replication program called endoreplication to support specialized functions. Endoreplication program differs from mitotic cell cycles in that there is no cell division. However, each replication cycles and resulting polyploidy in endoreplicating cells is carefully regulated and should not be confused with aberrant re-replication. In Chapter 4, we discuss the examples and function of this special cell cycle program, as well as its regulators in Chapter 4.

CHAPTER 2

REGULATION OF GROWTH BY CUL4

The study described in this chapter was performed in collaboration with Drs. Jian Hu, Sima Zacharek, and Yue Xiong, who initiated this project and performed biochemical analysis. I generated Drosophila plasmids and dsRNA used in Fig 6 and 7, and produced SEM images and genetic experiments in Drosophila described in Fig 8. Although I re-wrote the chapter, the results discussed were published in Genes and Development. WD40 protein FBW5 promotes ubiquitination of tumor suppressor TSC2 by DDB1-CUL4-ROC1 ligase. 2008. 22:866-871.

Summary

The TSC1–TSC2 complex functions as a tumor suppressor by negatively regulating TORC1. TSC1-TSC2 accomplishes this regulation of TORC1 through its GAP (GTPase-activating protein) activity to inhibit the activator of TORC1, Rheb. Multiple phosphorylation events on TSC1–TSC2 modulate its activity to control protein synthesis, cell growth, and proliferation in response to growth factors, nutrient, and stress signals. TSC2 was shown to be ubiquitinated and quickly degraded in the absence of TSC1. In addition, disease-associated mutations in TSC1-TSC2 often result in a significant decrease in protein levels,

suggesting that proteolysis plays an important role in TSC1-TSC2 regulation. In this study, we determined the mechanism of TSC2 degradation. TSC2 protein binds CRL4 E3 ligase via a DDB1-binding WD40 (DWD) protein, FBW5. In addition, depletion of CRL4 components stabilized TSC2, while over-expression of CRL4 enhanced TSC2 degradation. Similarly, *Ddb1* or *Cul4* mutations in *Drosophila* result in accumulation of Gigas/TSC2 and exhibit growth defects that can be partially rescued by Gigas/Tsc2 gene dosage reduction. Taken together, our results indicate that CRL4^{FBW5} mediates TSC2 protein degradation, which is essential for growth control *in vivo*.

Introduction

      TSC1 and *TSC2* were identified as the genetic loci mutated in the tumor syndrome, Tuberous Sclerosis Complex (TSC) (Kandt 1992, van Slegtenhorst 1997), an autosomal dominant disorder characterized by the formation of benign tumors called hamartomas (review: Crino 2006). Hamartomas are benign tumors that occur in various tissues and organs including skin, lungs, and brain. While hamartomas do not progress to malignancy, they often inhibit the normal function of affected tissues, causing detrimental symptoms. Understanding the molecular function of TSC1-TSC2 complex would provide further insight into TSC disease progression.

      TSC1 and *TSC2* produce gene products hamartin and tuberin that are conserved in most eukaryotes, but do not share homology with each other or with other proteins (Huang and Manning 2008). Genetic studies of TSC1 and TSC2 in

S. pombe, D. melanogaster, and *M. musculus* suggest that these two proteins primarily function as a heterodimer in growth: TSC1 and TSC2 physically interact (Plank et al., 1998; van Slegtenhorst et al.,1997; Hodges et al., 2001); *Tsc1, Tsc2* double mutants phenocopy single mutants (Potter et al. 2001; Potter et al., 2002; Gao et al., 2001; Matsumoto et al., 2002); and the over-expression of both *Tsc1* and *Tsc2*, but not of either alone, could suppress TSC mutant phenotypes. The C terminus of TSC2 protein contains the only functional domain found in either of the TSC proteins, the GAP (GTPase Activating Protein) domain homologous to Rap1GAP (Wienecke 1995). Interestingly, mutations in this domain have been identified in TSC patients (Maheshwar 1997), suggesting that this domain contains the tumor suppressor activity of TSC2. Subsequent studies found that TSC1 and TSC2 gene products are interdependent: TSC2 provides the enzymatic activity of GAP, while TSC1 is required to stabilize the complex (Chong-Kopera et al., 2006; Hodges et al., 2001; Benvenuto 2000; Ikenoue et al., 2008). Mutations that disrupt TSC1 and TSC2 interaction are found in TSC patients (Jin et al., 1996). *Tsc1* or *Tsc2* mutants result in similar organ overgrowth phenotype due to increased cell proliferation and cell size, suggesting their importance in growth control (Tapon et al., 2001; Potter et al., 2001; Gao et al., 2001).

TSC1/2 regulate growth by inhibiting TORC1 (Target of Rapamycin-Complex 1), a potent activator of cellular growth and proliferation (Figure 4) (Goncharova et al., 2002; Jaeschke et al., 2002; Tee et al., 2002; Gao et al., 2002). TORC1 consists of TOR (Target of Rapamycin), Raptor, and LST8 that

together function as a kinase complex to activate proteins important in translation; TOR protein is the catalytic effector, whereas substrate binding occurs through Raptor. TORC1 activates proteins important in translation such as ribosomal kinases S6K1/2 and eIF4E (eukaryotic translation initiation factor 4E) (Reviews: Fingar and Blenis 2004; Hay and Sonenberg, 2004; Wullschleger et al., 2006; Guertin and Sabatini 2007). TORC1 phosphorylates S6K, which then triggers mRNA translation by phosphorylating ribosomal S6 (Burnett et al., 1998; Pearson et al., 1995) and eIF4B (Gingras et al., 2001; Mothe-Satney et al., 2000). TORC1 phosphorylation of the 4E-BPs (eIF4E-binding proteins) releases their inhibition of eIF4E, an activator of CAP-dependent translation (Fingar et al., 2002). In this manner, TORC1 promotes translation and growth.

TORC1 activities are inhibited by TSC1/2 through Rheb inactivation (Figure 4) (Tee et al., 2003). Through a yet unknown mechanism, Rheb GTPase powerfully activates TORC1 catalytic function in its GTP-bound state (Long et al. 2005; Bai et al. 2007). However, Rheb becomes inactivated by hydrolyzing its GTP to GDP, which is accelerated by the GAP activity of TSC2 (Inoki et al., 2003; Zhang et al., 2003). TSC2 activity is regulated by multiple phosphorylation events: some activating, others inactivating (Crino 2006, Huang and Manning 2008). During energy depeletion, TSC2 is directly phosphorylated by AMPK, and actively inhibits protein synthesis through TORC1 inhibition (Inoki et al., 2003; Shaw et al., 2004). TSC2 is also directly phosphorylated by Akt, which leads to TSC2 inactivation (Manning et al. 2002; Inoki et al., 2002; Potter et al., 2002). Interestingly, the TSC1-TSC2 complex also regulates Akt by inhibiting TORC2,

an activating kinase for Akt (Sarbassov et al., 2005; Huang and Manning 2009). The mechanism by which TSC2 phosphorylation causes its inactivation is unclear.
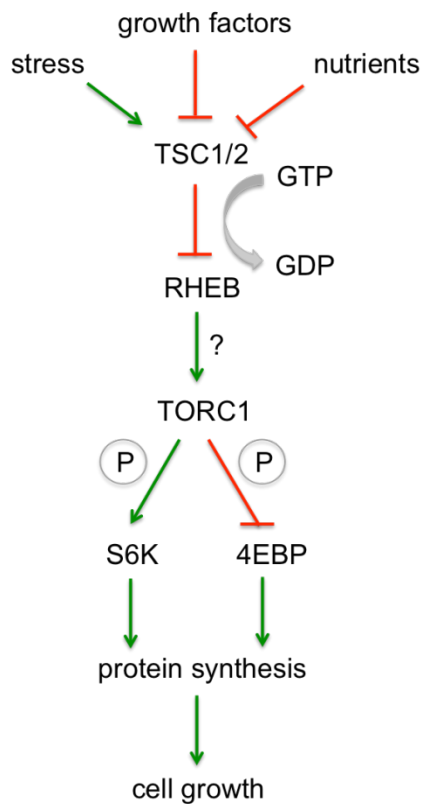
Figure 4. TSC2-TOR pathway.

TSC1/2 respond to various signals to regulate growth. Once activated, TSC1/2 stimulates Rheb GTPase activity to hydrolyze GTP to GDP. Once GDP bound, Rheb cannot activate TORC1. Active TORC1 promotes protein synthesis and subsequent cell growth by phosphorylating downstream targets S6K and 4EBP.

TSC2 is a short-lived protein that is actively ubiquitinated (Chong-Kopera et al. 2006) and unstable without TSC1. In addition, disease-associated *Tsc1/2* mutations cause a substantial decrease in the protein levels (Inoki et al. 2002; Nellist et al. 2005). Together these results suggest that protein turnover plays an important role in TSC regulation. To determine the mechanism of TSC2 destruction, we used *Drosophila* in which both the TSC mediated growth pathway and ubiquitin proteasome system are conserved.

Results

***Drosophila* Cul4 or Ddb1 mutants accumulate Gigas/TSC2**

To study the role of *Cul4* in development, we generated new alleles of this gene. Cul4 alleles, Cul4[6AP] and Cul4[11], were generated by P-element excision mutagenesis that deleted portions of the C-terminus of the Cul4 gene, 82 and 65 amino acids, respectively. The Cul4[11L] product cannot be detected by western blot analysis and therefore is null, while Cul4[6AP] produces a stable truncated protein of the predicted size. Ddb1[S026316] and Ddb1[EY01408] are publicly available P-element insertion alleles that produce no detectable protein (Figure 7B). *Cul4* and *Ddb1* mutant larvae arrest growth in the first or second instar stages, surviving for over 10 days without increasing in size (Figure 6A). This result led us to hypothesize that CRL4 plays a role in negatively regulating the growth pathway, specifically TSC2 degradation.

To test this hypothesis, we ectopically expressed tagged components of CRL4 genes in cultured S2 cells and determined their binding to the *Drosophila* TSC2 homolog, Gigas, by co-immunoprecipitation assay. As shown in Figure 6B, Flag-Gigas was readily detected in Myc-dDDB1 (lane 3), HA-dDDB1 (lane 4), and HA-Fbw5 (lane 5) immunocomplexes. Likewise, Myc- and HA-dDDB1, HA-Fbw5 complexes contained Flag-Gigas (lane 7). These results indicate that *Drosophila* TSC2, Gigas binds directly to DDB1.

If Gigas degradation is dependent on CRL4 ubiquitin ligase activity, depleting CRL4 should lead to accumulation of Gigas. To test this hypothesis, we silenced CRL4 components in S2 cells and determined the level of Gigas (Figure 7A). The specificity of the Gigas antibody was confirmed by comparing the detection of bacterially produced GST-Gigas fusion protein and endogenous Gigas that was depleted by RNAi. As predicted, silencing of dCul4, or dDdb1 all led to the accumulation of Gigas. Moreover, the level of Gigas was also significantly increased in *Cul4* and *Ddb1* mutant larval extracts (Figure 7B).
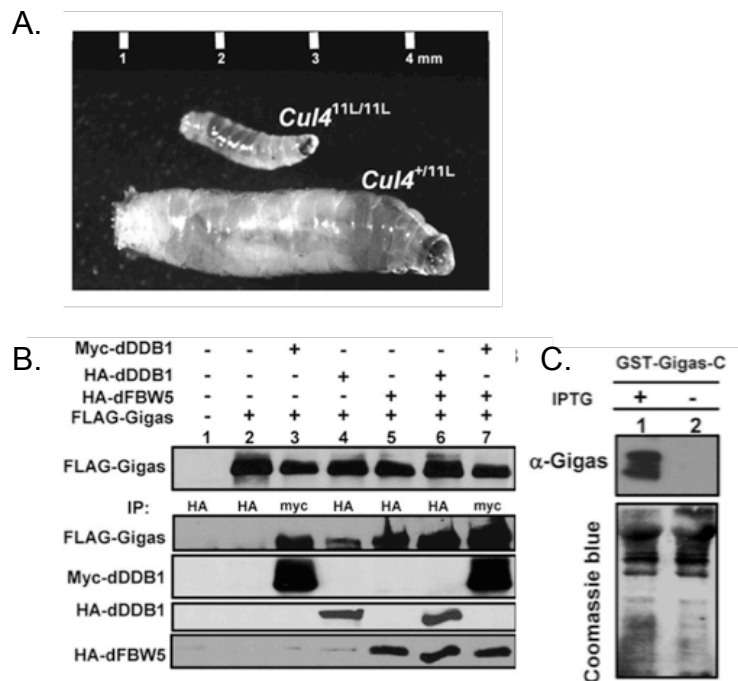
Figure 6. *Cul4* mutants are inhibited in growth. Gigas/TSC2 interact with CRL4^{FBW5}

(A) Size comparison of growth-arrested *Cul4*11L/11L homozygous mutant larvae with a normal *Cul4*11L/+ heterozygous sibling third instar larvae. (B) *Drosophila* S2 cells were transfected with plasmids expressing the indicated proteins, and protein–protein association was examined by coupled IP-Western analysis. (C) Characterization of an anti-Gigas antibody. *Escherichia coli* BL21 cells were transformed with a plasmid expressing a C-terminal portion of the Gigas protein, and were cultured in LB media with or without IPTG to induce the expression of GST-Gigas. Cells were lysed in SDS buffer followed by western analysis.

FBW5 is the specificity factor for TSC2 recruitment to CRL4

CRL4 selectively target substrates that are recruited by substrate specificity factors. Recent studies identified number of these factors that contain WD40 motifs, thus referred to as DWD (Ddb1 binding WD40 proteins) or DCAF (Ddb1- and Cul4-associated factors) proteins. (Angers et al. 2006; He et al. 2006; Higa et al. 2006; Jin et al. 2006). *Drosophila* gene CG9144 encodes a protein that exhibits 40% identity and 58% similarity to human FBW5. This protein contains N-terminal F-box and WD40 repeats that span the protein. The F-box motif functions in substrate recruitment to CRL1 (Bai et al. 1996; Feldman et al. 1997; Skowyra et al. 1997),

To determine whether TSC2 is recruited to CRL4 by FBW5, we ectopically expressed these proteins and examined their binding. TSC2 was found in FBW5 immunocomplex, suggesting that they physically interact (Figure 6B). In addition, depletion of FBW5 in S2 cells resulted in significant accumulation of TSC2. Together, these results demonstrate that the *Drosophila* CRL4^{FBW5} E3 ligase is responsible for Gigas degradation in cultured cells and animals.
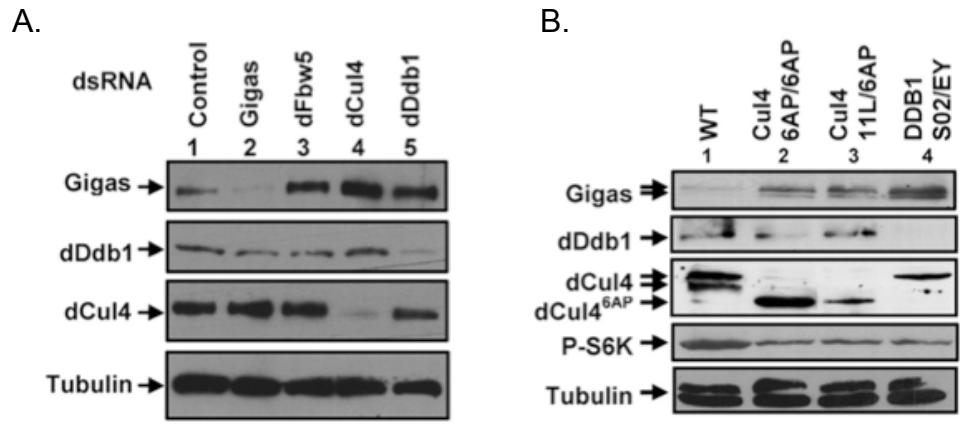
Figure 7. Accumulation of Gigas/TSC2 protein.

(A) S2 cells were treated with dsRNA targeting *Gigas*, *dFbw5*, *dCul4*, and *dDdb1* and control dsRNA for 2 days. The cells were lysed in RIPA buffer followed by Western analysis with the indicated antibodies. (B) Lysates of *Cul4* and *Ddb1* transheterozygotes were analyzed on western blot.

Loss of *Drosophila* Ddb1 results in growth defects that are alleviated by a reduction of Tsc2/Gigas

As mentioned, *Cul4* and *Ddb1* fly mutants exhibit growth arrest phenotype, which we hypothesized was due to TSC2 over-activity. If this model is correct, *Tsc2* mutation should rescue the growth defect of *Cul4* or *Ddb1* mutants. To test this hypothesis, we used existing and newly isolated Ddb1 hypomorphic alleles (Figure 8B).

We determined that the previously described *piccolo* locus was allelic to Ddb1 by complementation analysis. *piccolo* mutants are semi-lethal, and were originally characterized based on shared irregularities in bristle, wing, and tergite growth (Rushlow and Chovnick 1984; Clark and Chovnick 1985, 1986). These phenotypes are similar to growth defects observed in hypomorphic myc and heterozygous Minute mutants, which encode ribosomal proteins (Figure 8A). *pic²* encodes an X-ray-induced G21 → D substitution that results in severely reduced Ddb1 protein levels (Chapter 3). We also generated a new *Ddb1* hypomorphic allele (Ddb1^{PL12c}) by Ddb1^{EY01408} P element excision. Unlike *Ddb1* null mutants, *Ddb1$^{pic2/PL12c}$* transheterozygotes develop into viable adults, but show a significant developmental delay; These flies eclose on average 2 and 4 days later than control siblings at 25°C and 18°C, respectively (Figure 8B). In addition, these flies exhibit the *piccolo* phenotype with missing or small bristles (Figure 8A). To test whether these growth defects in *Ddb1* mutants are due to Gigas accumulation and over-activity, we reduced *gigas* gene dose by half in *Ddb1$^{pic2/PL12c}$* mutants. *gigas* and *Ddb1* double reduction significantly rescued the

late eclosion and the semi-lethality of *Ddb1$^{pic2/PL12c}$* mutants (Figure 8B), suggesting Gigas is a downstream target of DDB1 responsible for controlling eclosion rates. However, the bristle defects of *Ddb1$^{pic2/PL12c}$* were not suppressed by Gigas reduction, therefore it is possible that this phenotype is caused by misregulation of additional CRL4 substrates.

A.

B.

Genotype	25°C				18°C			
	Total eclosed flies	% expected	% observed	Mean eclosion (day)*	Total eclosed flies	% expected	% observed	Mean eclosion (day)*
Ddb1pic2/PL12C sibs	1025	67	79	3.6 ± 0.06$	756	67	96	5.2 ± 0.09$
Ddb1pic2/PL12c	278	33	21#	6.1 ± 0.09^	29	33	4#	9.6 ± 0.3$
Ddb1pic2 gig192 /Ddb1PL12c sibs	1784	67	73	4.06 ± 0.05$	1622	67	74	5.6 ± 0.08$
Ddb1pic2 gig192 /Ddb1PL12c	661	33	27#	5.5 ± 0.06^	564	33	26#	9.7 ± 0.1$

#P < 0.0001   *± = 95% confidence interval   ^P < 0.05 $P > 0.05

Figure 8. Growth defects of *Ddb1* mutants can be rescued by *gigas* reduction.

(A) *Drosophila* hypomorphic *Ddb1* mutants display small and missing macrochaete. Scanning electron micrographs of wild-type, *Minute*, and *Ddb1pic2/PL12C* mutant adults. Growth defects of *Cul4* and *Ddb1* mutants are rescued by heterozygosity of *gig (TSC2)*. (B) Total eclosed flies and mean eclosion time of *Ddb1*pic2/*Ddb1*PL12c mutants and their siblings and *Ddb1*pic2*gig*192/ *Ddb1*PL12c mutants and their siblings were measured at 18°C and 25°C.

Discussion

During this study, we found that the *Drosophila* homolog of TSC2, Gigas, is targeted by CRL4^{FBW5} for destruction. We provided four lines of supporting evidence: FBW5 and DDB1 bind Gigas *in vivo*; depleting CRL4 components by RNAi result in increased level of Gigas in *Drosophila* S2 cells; *Cul4* and *Ddb1* mutant larval extracts have increased Gigas; and the growth defect of *Ddb1* mutants can be partially rescued by reduction of *gigas* gene dosage.

Included in the original article were similar experiments performed using mammlian cell lines, suggesting that this is a conserved mechanism to control growth (Hu et al 2008). This study using mammalian cells demonstrated the ability of CRL4 to ubiquitinate TSC2 *in vitro*, which further supports our conclusion. Importantly, TSC2 was previously found to be targeted by another ubiquitin ligase, HERC1 (Chong-Kopera et al., 2006). While both HERC1 and CRL4 may regulate TSC2 in mammals, there is no obvious homolog of HERC1 in flies. This suggests that CRL4 mediated TSC2 degradation evolved earlier.

There are still many questions that remain in terms of TSC2 degradation. The TSC1/2 complex controls growth in response to various signals including growth factors, nutrients, and stress. These signals mediate activating or inactivating phosphorylation by multiple kinases: AKT, GSK3, AMPK, RSK, ERK, and MAPKAPK2 (Crino et al., 2006; Huang and Manning 2008). Whether these phosphorylation events play a role in TSC2 degradation, or the existence of another mechanism to trigger TSC2 degradation is currently unknown.

Materials and Methods

Plasmids

dDDB1, dFBW5, and Gigas were cloned using *Drosophila* cDNA library and tagged with Myc, HA, and FLAG, respectively, using the Gateway system (Invitrogen).

Antibodies, proteins, and immunological procedures

Antibodies to hemagglutinin (HA) (12CA5; Boehringer-Mannheim), Myc (9E10; NeoMarker), and Flag (M2; Sigma) were purchased commercially. Antibodies to CUL4, DDB1, ROC1, CAND1, TSC1, and TSC2 were described previously (Shumway et al. 2003; Hu et al. 2004; Zacharek et al. 2005). Polyclonal antibody to Gigas was raised against the C-terminal epitope of Gigas protein (DMDDQRGDFIKYT). Procedures for protein purification, immunoprecipitation, and immunoblotting have been described (Ohta et al. 1999; Furukawa et al. 2003; Hu et al. 2004).

RNAi

The RNAi in Drosophila S2 cells followed the protocol developed by the Jack Dixon laboratory (http://cmm.ucsd.edu/Lab_Pages/Dixon/protocols.php). The Ambion Megascript T7 kits were used to generate the dsRNAs targeting the first 500 bases of dCul4, dDdb1, and dFbw5 genes. Eighty percent confluent S2 cells cultured in 35-mm dishes were added with 5 µg of dsRNAs and incubated for 2 days. The dsRNA-treated S2 cells were lysed in RIPA buffer and applied to

Western blot analysis.

Drosophila mutants

Cul4^{11L}, Cul4^{6AP}, and Ddb1^{PL12c} alleles were generated by imprecise excision of the P element in Cul4^{EP2518} and Ddb1^{EY01408} chromosomes, respectively. The Cul4^{EP2518}, Ddb1^{pic2}, Ddb1^{S026316}, and Ddb1^{EY01408} alleles were obtained from the Bloomington Stock Center. gig^{192} was a kind gift of D.J. Pan.

Quantification of mean eclosion day and percentage of eclosed flies

DDB1^{PL12c}/TM3 Sb, Ser P[act-GFP] females were crossed to either DDB1^{pic2}/TM3 Sb, Ser P[act-GFP] or DDB1^{pic2} gigas/TM3 × DDB1^{pl12c}/TM3 males, and Ddb1 mutant and heterozygous sibling progeny were counted. For mean eclosion day calculations, the first day any fly from the culture eclosed established day 1, and the genotype of all flies was scored each day for nine and 12 successive days at 25°C and 18°C, respectively. The experiment was performed in triplicate. A 95% Confidence Interval test was used to obtain the range of the true mean of the combined data at each temperature. A two-tailed t-test assuming unequal variances was used to compare eclosion day data between different genotypes. The percentage of eclosed flies was calculated using the combined data at each temperature, and different genotypes were compared using a Fisher's exact test.

CHAPTER 3

REGULATION OF CDT1 BY GEMININ AND CUL4

The first section of this chapter (Fig 3.3 and 3.4) involving Dup regulation in S2 cells was published in The Journal of Biological Chemistry Vol. 283, NO. 37, pp. 25356–25363. This work was conducted in collaboration with Drs. Jonathan Hall and Jean Cook who contributed intellectually and conducted parallel experiments using human cancer cells. This chapter only contains Drosophila work I performed.

The rest of this chapter represents a manuscript that is currently in review for publication. I participated in the experimental design of this project with my advisor Robert Duronio, performed the majority of the experiments and wrote the manuscript. Dr. Sima Zacharek isolated new alleles of Cul4 and Ddb1 mutant lines and characterized their molecular identity (Fig 3.5). We collaborated on the imaginal disc clonal experiments (Fig3.6).

Summary

DNA synthesis-coupled proteolysis of the pre-replicative complex component Cdt1 mediated by the CRL4^{Cdt2} E3 ubiquitin ligase is thought to be an important mechanism that prevents re-replication of the genome during S phase. Many previous studies have indicated that Cdt1 over-expression can trigger re-

replication, but not whether CRL4^{Cdt2}-mediated destruction of Cdt1 is required for normal cell cycle progression. Because we could not detect defects in Dup accumulation in cells mutant for the CRL4^{Cdt2} components Cul4 and Ddb1, we analyzed a mutant *Drosophila* Cdt1 (Dup) lacking a PCNA-binding PIP box (Dup$^{\Delta PIP}$) that cannot be regulated by CRL4^{Cdt2}. Dup$^{\Delta PIP}$ is inappropriately stabilized during S phase and causes developmental defects when ectopically expressed. Dup$^{\Delta PIP}$ restores DNA synthesis to *dup* null mutant embryonic epidermal cells, but S phase appears abnormal, and these cells arrest and do not progress into mitosis, indicating that PIP box-mediated destruction of Dup is necessary for the *Drosophila* cell division cycle. In contrast, Dup$^{\Delta PIP}$ accumulation during S phase did not affect progression of the ovary follicle cell endocycle. The combination of Dup$^{\Delta PIP}$ expression and a 50% reduction of Geminin gene results in egg chamber degeneration, suggesting that Geminin inhibition can restrain Dup$^{\Delta PIP}$ activity in follicle cell endocycles.

Introduction

Accurate genome duplication during cell cycle progression requires assembly of a pre-replicative complex (pre-RC) at origins of DNA replication, followed by inhibition of pre-RC assembly after the onset of S phase. Failure to prevent pre-RC re-assembly during a given S phase has been shown to cause inappropriate re-replication, which leads to DNA damage and genomic instability that contribute to cancer (Petropoulou et al., 2008).

Pre-RCs contain the Origin Recognition Complex (ORC), Cdc6, and Cdc10 dependent transcript1 (Cdt1) proteins, which assemble at origins during late mitosis/G1 and recruit the mini-chromosome maintenance complex (MCM2-7), a core component of the replicative DNA helicase, to constitute origin licensing (Bell and Dutta, 2002). After DNA synthesis is initiated at licensed origins, pre-RC components are displaced from the chromatin and prevented from reassembling until the next G1 via multiple, often redundant regulatory mechanisms including nuclear export, inhibitory phosphorylation, and ubiquitin mediated proteolysis (Arias and Walter, 2007).

Preventing pre-RC re-assembly and re-loading of the MCM complex within S phase is crucial to prevent re-replication. Among the key mechanisms for preventing pre-RC re-assembly is negative regulation of Cdt1 in metazoans, as increased Cdt1 activity is sufficient to trigger re-replication in many situations (Zhong et al. 2003; Arias and Walter 2005, 2006; May et al. 2005; Sansam et al. 2006). Moreover, recent experiments in mice suggest that Cdt1 over-expression may promote tumor formation or progression (Arentson et al., 2002; Liontos et al., 2007; Petropoulou et al., 2008; Seo et al., 2005).

There are two known mechanisms of negative regulation of metazoan Cdt1: inhibition by binding to the protein Geminin and by regulated proteolysis (Arias and Walter, 2007).

After origins are licensed, Cdt1 is rapidly destroyed upon the onset of DNA replication via ubiquitin-mediated proteolysis (Kim and Kipreos, 2007b). Cdt1 proteolysis is controlled by the Cullin-RING family of E3 ubiquitin ligases (CRL).

Two ligases that utilize different ways of targeting Cdt1 have been described: CRL1 (aka SCF) and CRL4. Phosphorylation of the conserved cyclin binding (Cy) motif of Cdt1 by S phase Cyclin-dependent kinases (e.g. Cyclin E/Cdk2) triggers ubiquitylation by CRL1[Skp2] (Kondo et al., 2004; Li et al., 2003; Liu et al., 2004; Nishitani et al., 2006; Nishitani et al., 2001). CRL4[Cdt2] triggers replication-coupled destruction through a degron at the NH_2-terminus of Cdt1 containing a motif called a PIP (PCNA interacting polypeptide) box (Figure 9). The PIP box is thought to confer direct binding to PCNA at replication forks after the initiation of S phase, and the PIP-box containing degron recruits CRL4[Cdt2] for ubiquitylation and subsequent destruction of Cdt1 (Arias and Walter, 2006; Hall et al., 2008; Havens and Walter, 2009; Higa et al., 2006a; Higa et al., 2003; Hu et al., 2004; Hu and Xiong, 2006; Jin et al., 2006; Ralph et al., 2006; Senga et al., 2006). In human cells these pathways act redundantly, as mutations in both the PIP box and Cy domains are necessary to stabilize Cdt1 in S phase (Nishitani et al., 2006). In other situations there appears to be no redundancy between these ligases. For instance, Cul4 loss of function in *C. elegans* causes Cdt1 hyper-accumulation and re-replication (Kim and Kipreos, 2007a; Zhong et al., 2003). Cdt1 is also destroyed after DNA damage, and CRL4 depletion or mutations in the PIP box block this destruction in fission yeast, *Drosophila*, and mammalian cells (Hall et al., 2008; Higa et al., 2006a; Higa et al., 2003; Hu et al., 2004; Hu and Xiong, 2006; Ralph et al., 2006).
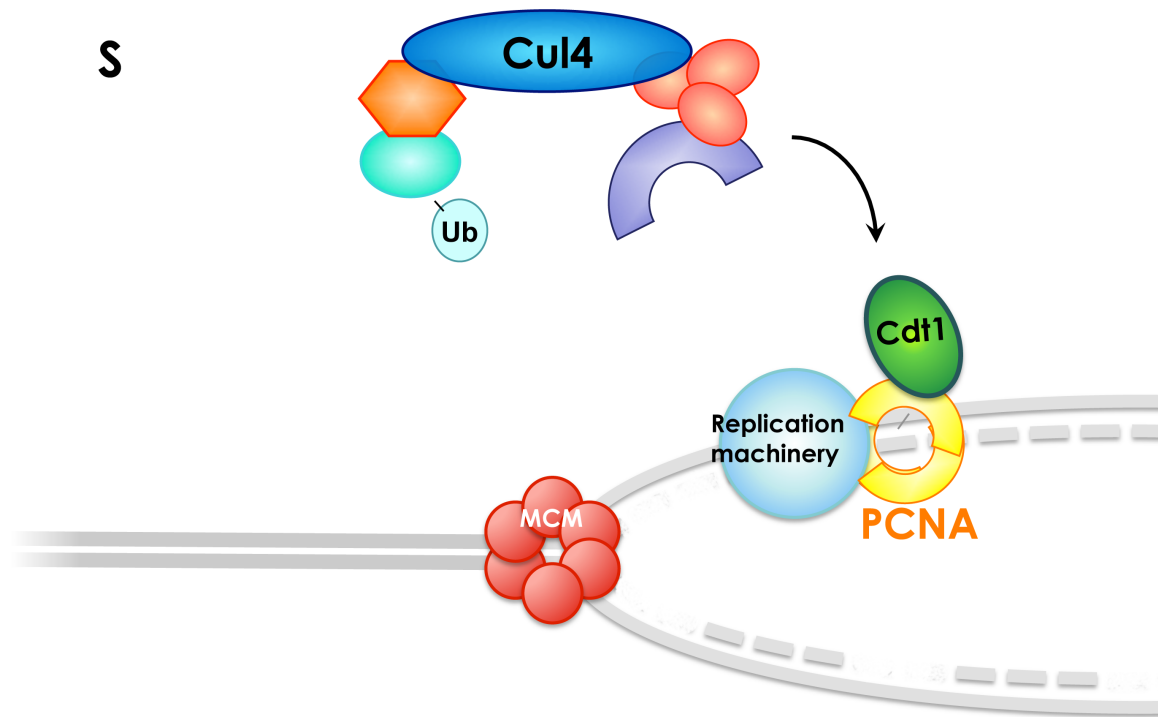
Figure 9. S phase destruction of Cdt1 by CRL4 depends on PCNA binding.

Once Cdt1 binds to PCNA on a replicating fork, Cul4 recognizes it as a substrate.

Geminin blocks the ability of Cdt1 to load the replicative helicase at origins, most likely because the Geminin and MCM2-7 binding domains of Cdt1 overlap (De Marco et al. 2009; Lee et al. 2004; Saxena et al. 2004).   Studies in mammalian and *Drosophila* cells have shown that the loss of Geminin function can cause massive re-replication, indicating that this inhibitory mechanism is required for normal genome duplication in some cell types (Hall et al., 2008) (Melixetian et al. 2004; Zhu et al. 2004).

Interestingly, re-replication induced by manipulating the levels of Cdt1 or Geminin is somewhat limited, and the extent of such re-replication varies among different cell types or organisms. This suggests that certain mechanisms exist to restrain the re-replication events once they occur (Vaziri et al. 2003; Melixetian 2004; Zhu et al. 2004; Mihaylov et al. 2002; Lui et al. 2007; Lee et al. 2007; Tatsumi et al 2006). However, the identity of these mechanisms are unclear.

The degree of redundancy or cell-type specificity between CRL1 and CRL4 ligase control and Geminin inhibition of Cdt1 during animal development is not completely understood. If Geminin is the major regulator of Cdt1 regulation in all cell types, cell cycle progression should not be affected when Cdt1 destruction is inhibited.   In order to test the significance of Cdt1 destruction during development, we studied the *Drosophila melanogaster* homolog of Cdt1, *double parked* (Dup). Dup is required to initiate DNA replication (Whittaker et al., 2000), and is degraded promptly upon S phase entry (Thomer et al., 2004). Dup contains both a Cy domain that is important for its normal function and mediates

regulation by Cyclin E/Cdk2 (Thomer et al., 2004) as well as a conserved PIP box whose function has yet to be specifically studied.

We took several approaches to address the functional importance of Geminin and CRL4 regulation of Dup during development. First we measured the re-replication of depleting the regulators of Dup in *Drosophila* S2 cells. We isolated and characterized *Cul4* and *Ddb1* mutants and also analyzed phenotypes caused by expression of a mutant Dup protein lacking the PIP box. Our results indicate that Geminin regulation of Dup acts dominantly to prevent re-replication in S2 cells. However, Cul4-mediated destruction was crucial to limit the extent of re-replication when Geminin activity is compromised (Figure 10). PIP box-dependent regulation is necessary for rapid Dup destruction during S phase and for normal progression of the embryonic cell division cycle, but not for normal endocycle progression in a cell type where Gem function can compensate for Dup stabilization in S phase. Thus, cell type specific dependencies on the different modes of Cdt1 regulation occurs during normal animal development.

Results

Regulation of Cdt1 by Geminin and Cul4 in S2 cells

Dup Is Degraded in Geminin-depleted Cells through a Cul4-dependent Mechanism

To examine the functional significance of two Dup regulatory pathways, we first used cultured *Drosophila* cells. S2 cells are derived from dissociated

early embryonic cells that provide an excellent system readily accessible for RNAi and cell cycle profiling. We hypothesized that If Geminin and Cul4 pathways function truly redundantly, depletion of either pathway would maintain Dup regulation and normal replication control. On the other hand, co-depletion of the two regulatory mechanisms would result in maximal Dup misregulation and subsequent re-replication. To test this hypothesis, dsRNA targeting the regulators of Dup, Cul4 and Geminin, was transfected into S2 cells for 96 hours, subsequently these samples were assayed for the extent of re-replication and protein levels. Interestingly, Geminin RNAi resulted in significant increase in number of cells with greater than 4C DNA content indicative of re-replication (Figure 11A), which could be suppressed by co-depletion of Dup. However, Cul4 depletion did not show any increased amount of re-replication compared to the control RNAi sample. These data suggest that Geminin inhibition is required and more important than Cul4 in the regulation of Dup activity and normal replication control.
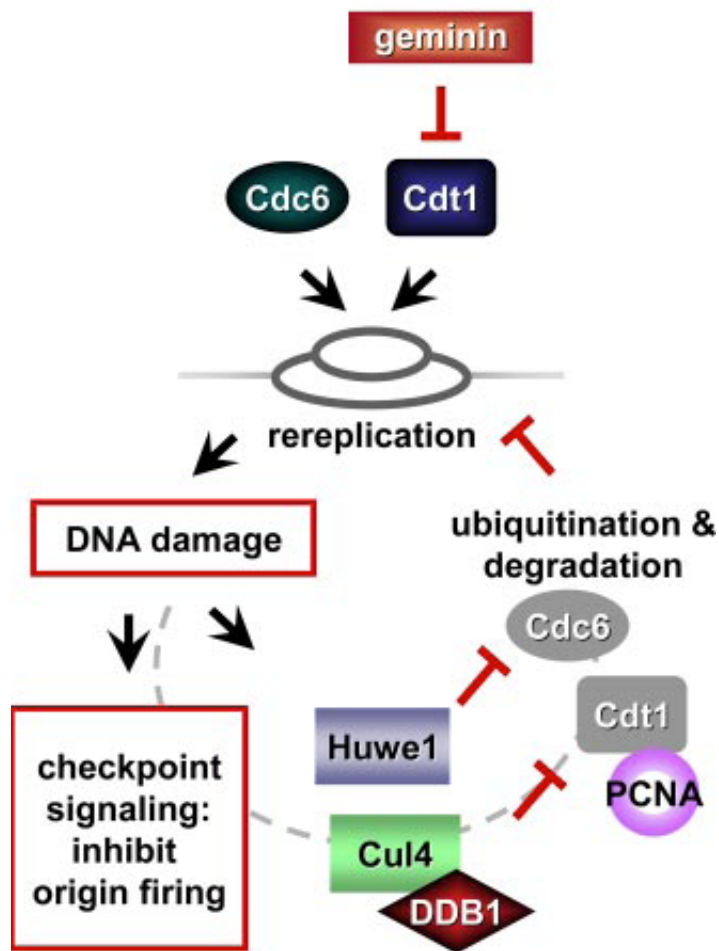
Figure 10. Re-replication limiting model

Re-replication induced by either geminin depletion (or the overproduction of Cdt1 or Cdc6) results in DNA damage. The DNA damage triggers the ubiquitination of PCNA-bound Cdt1 by Cul4-DDB1 and ubiquitination of Cdc6 by Huwe1 as well as checkpoint kinase Chk1 activation. Degradation of Cdt1 and Cdc6 prevents further rounds of re-licensing, and Chk1 activation inhibits origin firing, thus limiting the extent of re-replication. (Hall et al. 2008)

Surprisingly, western blot analysis of Dup protein levels revealed that depletion of Geminin resulted in Dup degradation. A similar phenomenon has been noted in previous studies of human or *Drosophila* cells (Mihaylove et al. 2002; Bellabini et al. 2004), however the mechanism of Dup co-depletion has not been studied. Other reports have shown that CRL4 dependent pathway targets Cdt1 for degradation upon ionizing radiation and subsequent DNA damage. Re-replication has also been shown to cause DNA damage. From this, we hypothesized that re-replication resulting from Geminin depletion activates CRL4 mediated Dup destruction (Figure 10). To test this, we co-depleted Cul4 along with Geminin. If the hypothesis is correct, depleting Cul4 should rescue Dup degradation. Indeed, co-depletion of Cul4 and Geminin partially stabilized Dup compared to Geminin depletion alone (Figure 11B). In addition, this result is specific to Cul4 as depletion of Cul1 had little effect on Dup levels in Geminin-depleted cells. Furthermore, triple depletion of Geminin, Cul1, and Cul4 showed Dup levels close to that of Geminin and Cul4 depleted cells. Together these data suggest that Cul4 is the main ligase responsible for Dup degradation upon Geminin depletion. Interestingly, Cul1-depleted cells accumulated a small amount of Dup with a slower mobility than that of Cul4 depleted cells, suggesting that Dup may be differentially modified in these two samples.

Previous studies that examined the extent of re-replication after manipulating Cdt1 or Geminin levels found that the resulting re-replication is limited. Based on our results, we hypothesized that early stages of re-replication

is sufficient to generate DNA damage and subsequent Dup degradation, inhibiting further re-replication. This model predicts, then, that preventing Dup degradation would exacerbate re-replication. To test this, we performed double knockdown of Geminin and Cul4 in a timecourse and measured the percentage of re-replicated cells (Figure 12). Depletion of Cul4 protein takes longer than that of Geminin, therefore we treated S2 cells with Cul4 dsRNA 48 or 72 hours prior to Geminin dsRNA treatment. As predicted, co-depletion of Cul4 and Geminin significantly increased the number of re-replicated cells compared to Geminin depletion alone. This increased re-replication was more prominent when Cul4 depletion was 72 hours prior to Geminin depletion. Taken together, our data suggest that Geminin inhibition is required for Dup regulation in S2 cells, and compromised Geminin inhibition is compensated by CRL4 mediated Dup degradation to limit re-replication events.
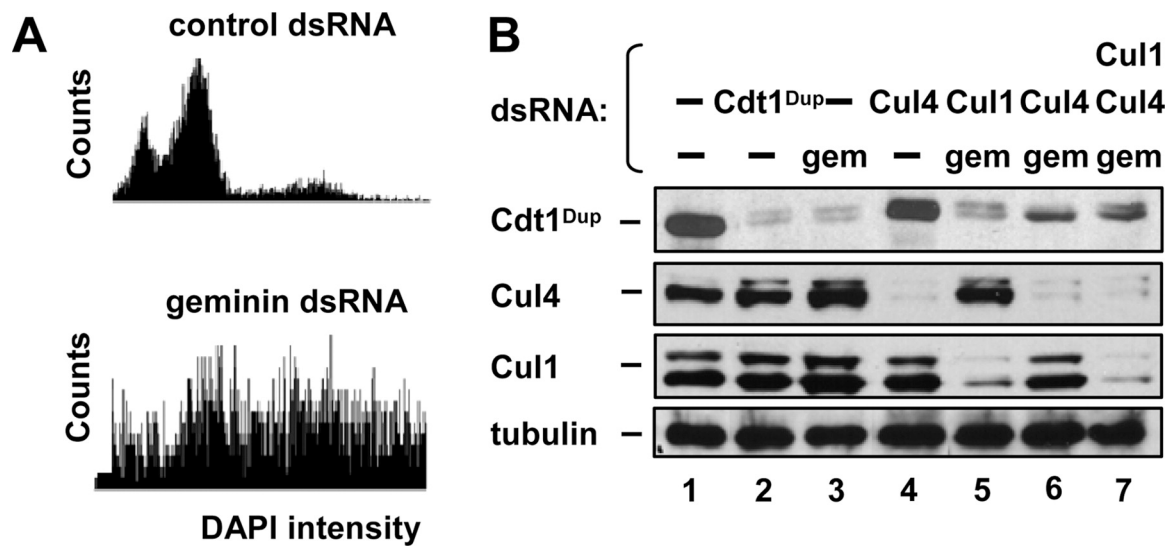
Figure 11. Geminin depletion in *Drosophila* S2 cells induces Cul4-dependent Dup degradation.

(A) S2 cells were treated with dsRNA targeting control sequence or geminin for 4 days. DNA content was determined by integrated fluorescence intensity of DAPI-stained nuclei. (B) S2 cells were transfected with the indicated dsRNA for 96 hours then evaluated for Dup, Cul4, Cul1, and tubulin by immunoblotting.
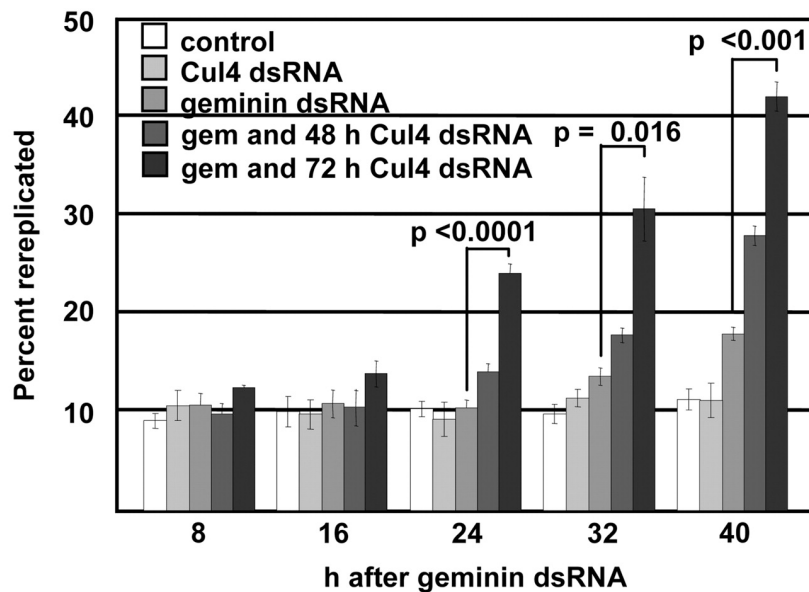
Figure 12. Cdt1 degradation limits the extent of re-replication.

Drosophila S2 cells were treated with Cul4 dsRNA every 24 hours for either 48 or 72 hours prior to treatment with geminin dsRNA. Samples were collected every 8 hours post-geminin dsRNA treatment. DNA content was measured by DAPI intensity, and the percentage of the total population harboring DNA content greater than 4C is reported. *Error bars* indicate Standard Deviation from three independent experiments.

Regulation of Cdt1 in development

Isolation and molecular characterization of _Drosophila Cul4_ and _Ddb1_ mutants.

To test whether CRL4^{Cdt2} regulates Dup accumulation during S phase, we first isolated and characterized mutations of _Cul4_ and _Ddb1_. The _Drosophila_ genome contains a single _Cul4_ gene encoding a protein that is 66% identical to human CUL4A and 64% identical to human CUL4B, and a single _Ddb1_ gene that is 61% identical to human DDB1 (BLASTp). We previously reported the isolation of new mutant alleles of these genes in a study describing the negative regulation of the TSC2 tumor suppressor by the CLR4^{Fbw5} E3 ubiquitin ligase (Hu _et al._, 2008). Here we present results from the molecular characterization of these mutant alleles that did not appear in the Hu et al., (2008) study, followed by an analysis of the effect of these mutations on cell proliferation and Dup accumulation.

We obtained two publicly available P-element insertion alleles of _Drosophila Cul4_: KG02900 located in the 5'UTR and EP2518 located in the 3'UTR (Fig. 13A). Homozygous _Cul4^{EP2518}_ flies are viable, and _Cul4^{KG02900}_ causes recessive lethality that was reverted after precise excision of the KG02900 P-element. We isolated additional _Cul4_ alleles by mobilizing the EP2518 P-element and screening for excision mutations that failed to complement the lethality of _Cul4^{KG02900}_. Three different _Cul4_ mutant alleles were identified from 400 independent excision events: _Cul4^{6AP}_, _Cul4^{11L}_, and _Cul4^{11R}_

(Fig. 13A). All three *Cul4* excision mutants arrested during development as first instar larvae, either as homozygotes, in trans to each other, or over a deficiency (*Df(2R)CA53*) that deletes *Cul4*. The *Cul4^{KG02900}* allele is less severe, and *Cul4^{KG02900}/Df(2R)CA53* mutants arrest as second instar larvae. Strikingly, although *Cul4* mutants display early developmental arrest, they do not die and can survive for at least a week without growing (Hu *et al.*, 2008).

We generated an antibody specifically recognizing the NH$_2$-terminus of fly Cul4 and detected full length Cul4 and neddylated Cul4 in cultured S2 cells and wild type first instar larvae (Fig. 13B, lanes 1, 2), but not in *Cul4^{11L}*, *Cul4^{11R}*,or *Cul4^{6AP}* mutant larvae (Fig. 13B, lanes 4-7). *Cul4^{KG02900}* mutants expressed reduced levels of full length Cul4, although the ratio of neddylated to unneddylated Cul4 was increased relative to wild type larvae (Fig. 13B, lane 3). Sequencing of the breakpoints of each excision mutant predicts open reading frames encoding a C-terminal deletion of 18 residues in *Cul4^{11L}*, 65 residues in *Cul4^{6AP}* and 82 residues in *Cul4^{11R}* (Fig. 13A). Truncated proteins corresponding to the predicted molecular weights were detected in both *Cul4^{11R}* and *Cul4^{6AP}* mutants as a single species (Fig. 13B, lanes 6, 7), whose stability may be partly attributable to an inability to be neddylated (Wu *et al.*, 2005). The *Cul4^{11L}* allele produced very little if any protein as assessed by western blot, and is likely null (Fig. 13B, lanes 4, 5). The end of the predicted open reading frame of *Cul4^{11L}* contains P element sequence encoding 54 amino acids at the COOH terminus that may destabilize the protein. In contrast, the *Cul4^{11R}* and *Cul4^{6AP}* alleles encode only 3 and 4 additional P-element-derived COOH-terminal amino acids,

respectively. All three truncation mutants retain the Roc1a binding site, but lack a highly conserved C-terminal domain that is also required for the function of *Drosophila* Cul3 (Mistry *et al.*, 2004).) *Cul4^6AP* and *Cul4^11R* also lack the NEDD8 conjugation site (K767) and are predicted to produce nonfunctional protein that may lack the ability to productively interact with the E2 (Kleiger *et al.*, 2009). These data suggest that NEDD8 conjugation and/or the C-terminal domain is essential for Cul4 function *in vivo* (Kipreos *et al.*, 1996; Feldman *et al.*, 1997; Furukawa *et al.*, 2000; Furukawa *et al.*, 2002; Liu *et al.*, 2002).

Co-immunoprecipitation analysis using cultured S2 cells demonstrated that *Drosophila* Cul4 and Ddb1 physically interact either when ectopically expressed (Fig. 13C) or as endogenous proteins (Fig. 13D). The publicly available *Ddb1^EY01408* allele contains a P element insertion in the 5'UTR of *Ddb1* (Fig. 13A), and causes developmental arrest early during the second larval instar when homozygous or when placed in trans with deficiencies *Df(3R)Exel6167* or *Df(3R)ry^75*. Precise excision of the EY01408 P-element reverted the lethality of *Ddb1^EY01408*. These data indicate that *Ddb1* is an essential gene as previously reported (Takata *et al.*, 2004; Lin *et al.*, 2009). We isolated multiple additional *Ddb1* alleles with a range of severity resulting from imprecise repair of EY01408 excision events. The most severe *Ddb1^EY01408* excision alleles caused second instar lethality, while the least severe resulted in adult flies with reduced viability and fertility that displayed growth defects including missing and thin thoracic bristles (Hu *et al.*, 2008).
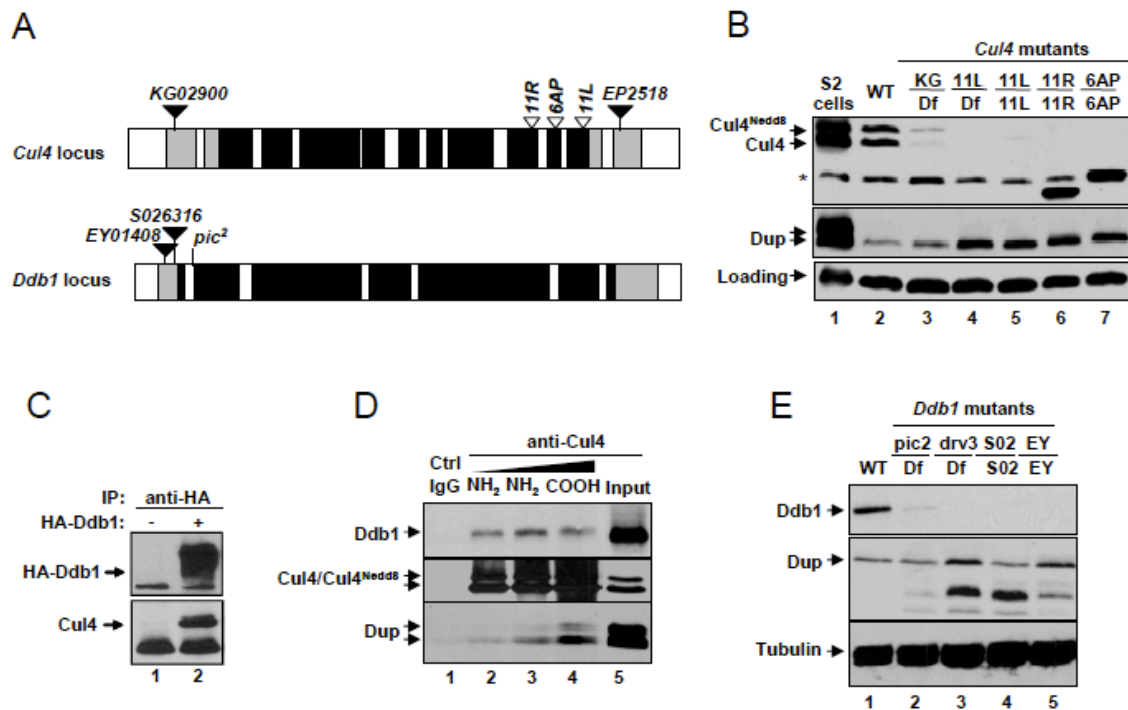
Figure 13. Analysis of *Drosophila Cul4* and *Ddb1* mutants

(A) The *Drosophila Cul4* locus is located on chromosome 2R at 44B1 and contains 12 exons (black and grey boxes). The P-elements *KG02900* and *EP2518* are located in the 5' UTR and 3' UTR, respectively (grey boxes). Open arrowheads indicate the breakpoints within the open reading frame (black boxes) of P-element excision alleles *Cul4^{11R}*, *Cul4^{6AP}* and *Cul4^{11L}*. The *Drosophila Ddb1/piccolo* locus is located on chromosome 3R at 87D10 and contains 7 exons. The P-elements *EY01408* or *SO26316* are located in the 5' UTR, and the *pic^2* missense mutation is located at the 5' end of exon 2. (B) S2 cells or 1st instar larvae of the indicated genotypes (Df = *Df(2R)CA53*) were homogenized and analyzed by western blot with anti-Cul4 or anti-Dup antibodies. The asterisk indicates a cross-reacting protein, which co-migrates with the truncated Cul4^{6AP} protein. (C) HA-Ddb1 was ectopically expressed in S2 cells, immunoprecipitated,

and analyzed by western blot using anti-Cul4 and anti-HA antibodies.   (D)

Extracts from S2 cells were immunoprecipitated with increasing concentrations of

anti-Cul4 antibodies specific for the NH_2- or COOH-terminus and analyzed by

western blot using anti-Ddb1, anti-Cul4, or anti-Dup antibodies. (E) 2nd instar

larvae of the indicated genotypes (Df = *Df(3R)ry75*) were homogenized and

analyzed by western blot with anti-Ddb1, anti-Dup, or anti-Tubulin antibodies.

Several lower molecular weight Dup species hyper-accumulated in the mutants.

The morphological phenotypes in the *Ddb1* hypomorphs helped us to establish that *Ddb1* is allelic to the *piccolo* (*pic*) locus (Hu *et al.*, 2008), which was originally defined by 40 X-ray and EMS-induced mutations (Hilliker *et al.*, 1980), some of which result in viable flies that display defects in bristle, wing, and tergite growth (Hilliker *et al.*, 1980; Rushlow and Chovnick, 1984; Clark and Chovnick, 1986; Deak *et al.*, 1997). We obtained flies carrying $pic^{SO26316}$, pic^{Drv3}, and pic^2 alleles, and found that they caused 2nd ($pic^{SO26316}$ and pic^{Drv3}) or 3rd (pic^2) instar lethality, and failed to complement the lethality caused by $Ddb1^{EY01408}$. Pic^{Drv3} resulted from an X-ray induced rearrangement, leaving a large segment of genomic DNA inserted within the *Ddb1* locus (Scott *et al.*, 1983; Clark and Chovnick, 1986). Publicly available sequence flanking the SO26316 P-element insertion corresponds to the 5' UTR of *Ddb1* (Flybase ID FBrf0125057)(Deak *et al.*, 1997). By western blot analysis, pic^{Drv3}, $pic^{SO26316}$, and $Ddb1^{EY01408}$ appear to be *Ddb1* null alleles (Fig. 13E, lanes 3-5). As we previously reported (Hu *et al.*, 2008), the pic^2 X-ray allele contains an Asp substitution for the well conserved Gly21 positioned at a turn in propeller A of Ddb1 (Li *et al.*, 2006). This missense mutation may destabilize the protein because pic^2 mutants express reduced amounts of Ddb1 (Fig. 13E, lane 2), consistent with this allele being a hypomorph. The pic^2 allele combined with other weak $Ddb1^{EY01408}$ excision alleles (i.e., $Ddb1^{PL12C}$) results in viable flies that are piccolo in phenotype (Hu *et al.*, 2008).

Melanotic masses were previously observed in *Drosophila* larvae in which *Ddb1* was silenced by RNAi (Takata *et al.*, 2004). We also observed melanotic masses in *Ddb1* mutant larvae, as well as in hypomorphic *Ddb1* mutant adults and *Cul4*$^{11L/KG02900}$ mutant larvae. Melanotic masses are thought to result from abnormal hemocyte development that elicits an auto-immune response (Rizki and Rizki, 1983; Dearolf, 1998) suggesting that CRL4 may be involved in hemocyte development.

Cul4 and Ddb1 mutant cells proliferate poorly

To assess the effect of *Cul4* or *Ddb1* disruption on cell proliferation and to generate mutant cells for analysis of Dup expression, we generated mutant imaginal disc clones via FLP-FRT-mediated mitotic recombination (Xu and Rubin, 1993). Mitotic recombination was induced in first instar larvae, and the resulting clones were analyzed as adjacent groups of GFP positive and GFP negative cells (i.e. twin spots) in wing and eye-antennal discs dissected from third instar larvae. Wild type controls yielded twin spot clones that were roughly equal in size (Fig. 14A). The area of *Ddb1* mutant cell clones was on average 4 times smaller than wild type, indicating that the growth of *Ddb1* mutant cells is defective (Fig. 14A). In contrast to the *Ddb1* clones, *Cul4* mutant clones were undetectable when generated in first instar larvae and analyzed during third instar. When mitotic recombination was induced at late second instar, however, small *Cul4* mutant clones were visible (Fig. 14D). These results suggest that *Cul4* mutant cells proliferate poorly and are consequently eliminated from the

disc epithelium by cell-cell competition, a well known phenomenon in *Drosophila* whereby faster growing cells actively induce apoptosis in adjacent slower growing cells during larval development (Adachi-Yamada and O'Connor, 2004).
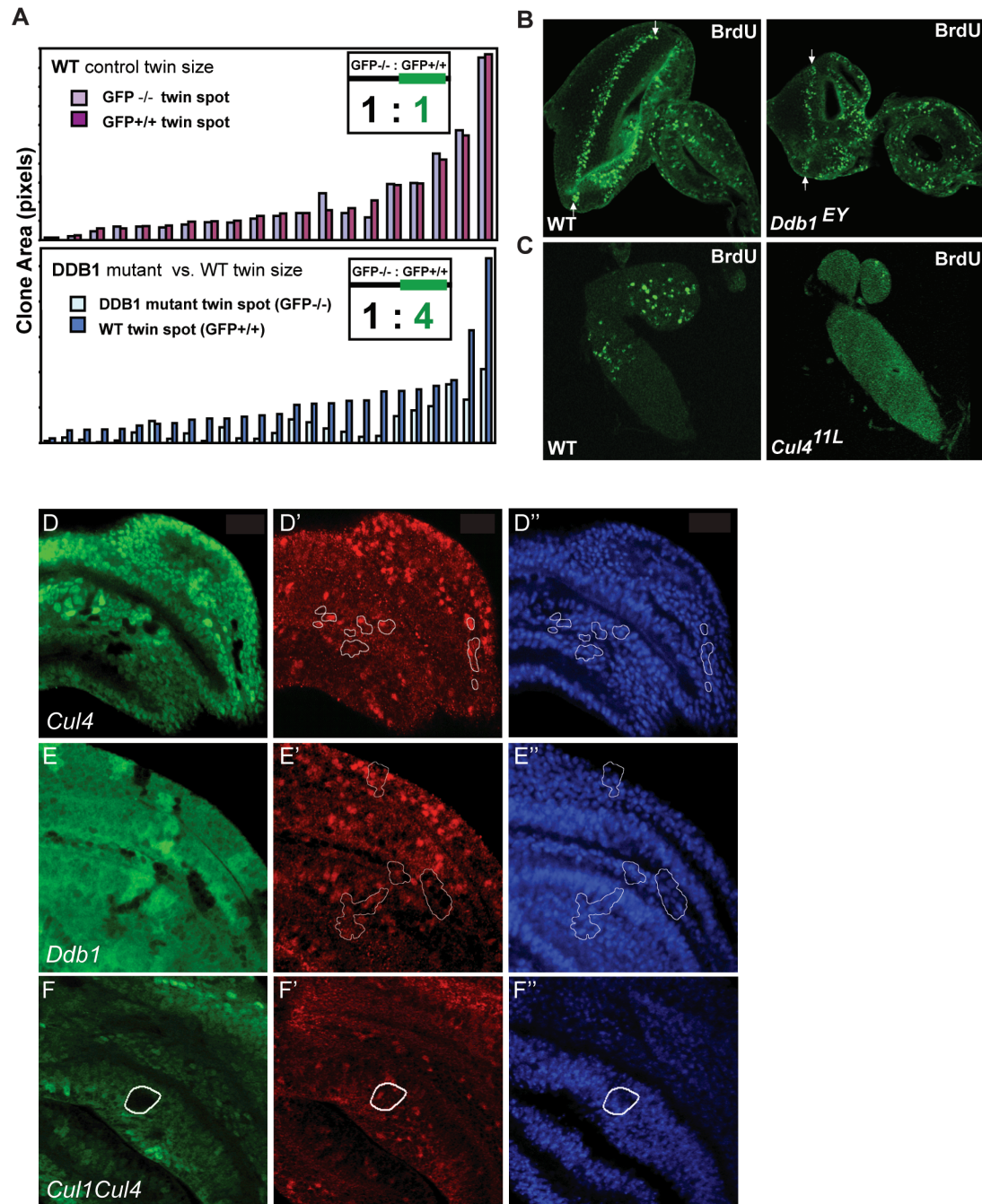
Figure 14. Analysis of *Cul4* and *Ddb1* mutant imaginal disc clones.

(A) Histogram of the size measured in pixel area of twin spot clones analyzed in imaginal discs of third instar larvae. Twin spots are ordered on the X axis by GFP$^+$ clone size. (B) BrdU-labeled eye imaginal discs dissected from wild type or *Ddb1^{pic2}/ Df(3R)CA53* 3rd instar larvae. Arrows indicate BrdU incorporation in the

synchronous S phase of the second mitotic wave. (C) 1st instar brain lobes labeled dissected from wild type or $Cul4^{11L}$ mutant larvae and labeled with BrdU. (D-F) Wing imaginal discs containing *Cul4* (D), *Ddb1* (E), or *Cul1Cul4* (F) mutant clones generated during 2nd instar and analyzed one day later. In each case, clones containing cells with or without Dup staining are outlined.

These results are essentially indistinguishable to the *Cul4* and *Ddb1* mutant cell clone analysis recently described by Lin et al. (2009). In addition, disruption of *pcu4* or *ddb1* in fission yeast causes proliferation defects (Osaka *et al.*, 2000; Zolezzi *et al.*, 2002; Bondar *et al.*, 2003; Liu *et al.*, 2003), as does mutation of mouse Ddb1 (Cang *et al.*, 2006) and (Liu *et al.*, 2009).

Developmental defects consistent with reduced growth and proliferation were also apparent in tissues dissected from *Cul4* or *Ddb1* mutant larvae. Hypomorphic *Ddb1* mutant animals (pic^2/$Df(3R)ry^{75}$) develop until the third larval instar, but contain imaginal discs that are smaller in size relative to wild type (Fig. 14B, bottom). Eye imaginal discs from these animals displayed a reduced and irregular pattern of BrdU incorporation within the second mitotic wave, a group of cells just posterior to a wave of differentiation that sweeps across the eye disc epithelium and synchronously enter a final mitotic cell division cycle prior to differentiating (Fig. 14B, arrows). Similarly, the CNS dissected from *Cul4* null mutant first instar larvae contained very few if any BrdU positive cells compared to wild type controls (Fig. 14C). These data indicate that Cul4 and Ddb1 are necessary for normal cell proliferation in *Drosophila*.

Cdt1^{Dup} does not hyper-accumulate in *Cul4* or *Ddb1* mutant imaginal cells.

Using S2 cell extracts, we detected Dup in Cul4 immunoprecipitates (Fig. 13D), suggesting that a CRL4 E3 ubiquitin ligase may act to regulate the abundance of Cdt1 in *Drosophila* as occurs in other species (Higa *et al.*, 2003; Hu *et al.*, 2004; Ralph *et al.*, 2006; Kim and Kipreos, 2007a). Consistent with this

possibility, western analysis of extracts made from whole first instar larvae indicated an elevated level of Dup in *Cul4* or *Ddb1* mutants relative to wild type controls (Figs 13B, E). To more specifically test whether Dup is regulated by CRL4 during cell proliferation, we measured Dup levels by immunostaining wing imaginal discs containing *Cul4[11L]* or *Ddb1[EY01408]* mutant clones (Fig. 14D). Other proteins have previously been shown to inappropriately accumulate in mitotic clones mutant for components of CRL E3 ubiquitin ligases (Jiang and Struhl, 1998; Noureddine *et al.*, 2002; Ou *et al.*, 2002). In wild type imaginal cells, Dup is primarily nuclear and most abundant in G1, and then rapidly destroyed as cells enter S phase (Thomer *et al.*, 2004). We could not detect Dup hyper-accumulation in *Cul4* or *Ddb1* mutant cells (Fig. 14D, E), nor did we observe an overlap between Dup staining and BrdU incorporation, as would be expected if CRL4 was required for destruction of Dup during S phase. This result was not due to redundancy between CRL4 and CRL1 ligases, as was observed in human cells (Nishitani *et al.*, 2006), because *Cul4 Cul1* double mutant cells also failed to show evidence of Dup misregulation (Fig. 14F). Similar results were obtained with *Cul1* single mutant clones.

While one interpretation of this clonal analysis is that CRL4 does not regulate Dup, there are several caveats to consider. Most importantly, because CRL4 complexes regulate the degradation of many substrates, phenotypic pleiotropy may have masked our ability to detect alterations to the normal accumulation of Dup. For instance, G1 arrest is known to occur after RNAi depletion of Cul4 in cultured S2 cells (Rogers *et al.*, 2002; Bjorklund *et al.*, 2006;

60

Higa *et al.*, 2006b; Li *et al.*, 2006; Rogers and Rogers, 2008). G1 arrest, which is consistent with the proliferation defect we observed, would preclude our ability to detect inappropriate Dup accumulation during S phase. The few BrdU positive cells in *Cul4* and *Ddb1* mutant clones may not have yet been sufficiently depleted of Cul4 and Cul1 protein to observe an effect on Dup. Likewise, the hyper-accumulation of Dup in *Cul4* and *Ddb1* mutant whole larval extracts may result from an increase in the number of G1 arrested cells throughout the animal (Fig. 14C). For these reasons we developed an alternative strategy to specifically test the requirement for CRL4^{Cdt2} regulation of Dup during the cell cycle.

PIP box deletion blocks Dup degradation at the onset of S phase.

To specifically test the contribution of CRL4-dependent Dup regulation to S phase and cell cycle progression *in vivo*, we generated a mutant version of Dup (Dup$^{\Delta PIP}$) lacking the NH$_2$-terminal PIP box (Fig. 15 A,B). Previous studies have shown that mutating the PIP box abolishes CRL4 binding to Cdt1 (Arias and Walter, 2006; Higa *et al.*, 2006a; Hu and Xiong, 2006; Senga *et al.*, 2006). Both full-length wild type Dup (Dup^{FL}) and Dup$^{\Delta PIP}$ were tagged with GFP at their COOH-termini and expressed using various ubiquitous or tissue-specific Gal4 drivers. Ubiquitous Dup$^{\Delta PIP}$ expression using *da*-Gal4 and *act*-Gal4 caused embryonic lethality, while animals expressing Dup^{FL} with the same drivers developed until adulthood. Eye-specific expression of Dup$^{\Delta PIP}$ using GMR-Gal4 resulted in massive tissue malformation whereas Dup^{FL} caused mildly rough eyes (Fig. 15C). These data indicate that Dup$^{\Delta PIP}$ behaves distinctly from Dup^{FL},

and suggest that our Dup^{FL} transgenes do not produce the level of over-expression previously shown to cause re-replication after heat shock production of wild type Dup (Thomer *et al.*, 2004).

One possibility for the severe developmental defects observed after Dup$^{\Delta PIP}$ expression is disruption to cell cycle progression because of stabilization of Dup during S phase, which may cause re-replication and DNA damage that results in cell cycle arrest or cell death. To determine whether or not Dup$^{\Delta PIP}$ is degraded correctly at the onset of S phase, we expressed Dup^{FL} and Dup$^{\Delta PIP}$ in alternating segments of the embryo using *paired (prd)*-GAL4 and detected S phase cells with BrdU pulse labeling and exogenous Dup with anti-GFP antibodies. We did not detect Dup^{FL} staining in BrdU positive cells, indicating that Dup^{FL} is correctly degraded very early in S phase (Fig. 15D). In contrast, 48% of S phase cells within the *prd*-GAL4 expressing domains also expressed Dup$^{\Delta PIP}$, indicating that the PIP motif is required for Dup destruction at the onset of S phase (Fig. 15E, open arrows). In addition, because approximately half of the S phase cells lack Dup$^{\Delta PIP}$, these data also suggest the presence of another mechanism for Dup destruction during S phase.

A

PIP box
phosphorylation sites
Geminin binding
MCM binding
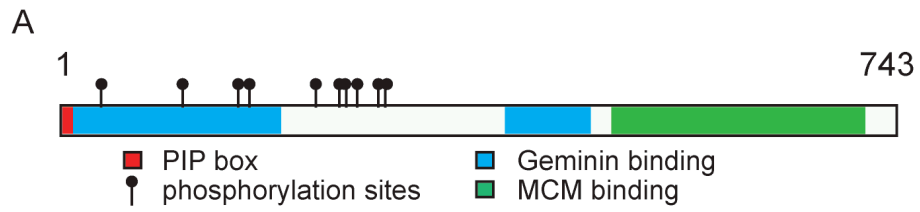
B

PIP box : Q   I/L/M/V   Y/F Y/F

		Q			V	T	D	F	F			R		
H. sapiens	M E	Q	R R	V	T	D	F	F	A R	R	R	P G
M. musculus	M A	Q	S R	V	T	D	F	Y	A C	R	R	P G
X. laevis	M S	Q	M R	V	T	D	F	F	A Q	S	K	R G
D. melanogaster	M A	Q	P S	V	A	A	F	F	T N	R	K	R A
C. elegans	T P	Q	T A	V	T	D	F	F	K T	T	K	P R

C   C'   C''
WT   Dup^FL   Dup^ΔPIP

D Dup^FL   D'   D''   D'''
E Dup^ΔPIP   E'   E''   E'''
F Dup^10A   F'   F''   F'''
G Dup^ΔPIP10A   G'   G''   G'''
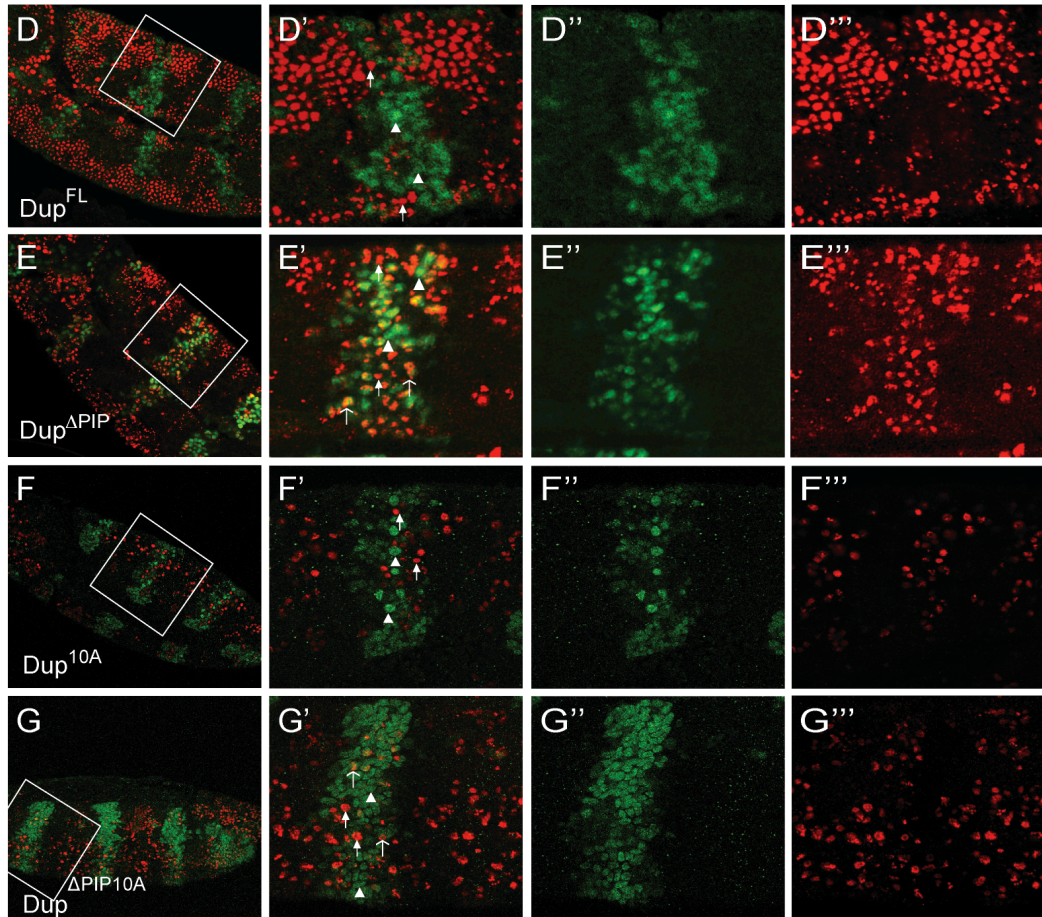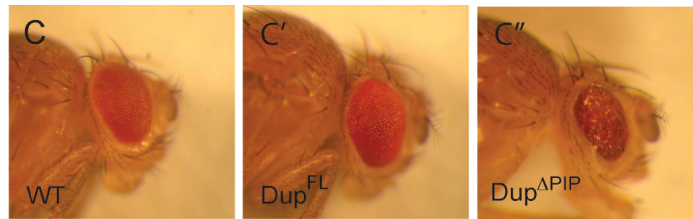
Figure 15. Stabilization of Dup during S phase after deletion of the PIP box.

(A) Schematic of the *Drosophila* Dup protein. The 10 potential CycE/Cdk2 phosphorylation sites are S37, S111, T158, S168, S226, S249, T256, T264, S285, and S291. Gem and MCM binding domains taken from (Lee *et al.*, 2004; Saxena *et al.*, 2004). (B) Alignment of the Cdt1 CRL4^{Cdt2} degron from several species. Highly conserved residues within the PIP box are located in the black boxes, and the conserved residues necessary for PIP degron function are boxed in green. The red box indicates the residues deleted in Dup$^{\Delta PIP}$. (C) Images of a WT adult eye (C) and eyes expressing Dup^{FL} (C') or Dup$^{\Delta PIP}$ (C'') driven by GMR-Gal4. (D-G) Confocal micrographs of proliferating embryonic epidermal cells expressing the indicated Dup-GFP transgenes using the *paired (prd)*-Gal4 driver. Dup-GFP is visualized by staining with anti-GFP antibodies (green; D''-G'') and S phase cells are marked by BrdU incorporation (red; D'''-G''')). Closed arrows indicate BrdU positive cells, arrowheads indicate Dup-GFP expressing cells, and open arrows indicated BrdU positive cells also expressing Dup-GFP. The rectangles indicate the area of the higher magnification images shown in D'-G'.

Thomer et al. (2004) showed that a Dup mutant containing 10 potential CycE/Cdk2 phosphorylation sites (Fig. 15A) changed to alanine (Dup^{10A}) was somewhat more stable than wild type Dup after heat shock-induced ectopic production. To test whether the 10A mutations would augment the stability of Dup$^{\Delta PIP}$ in the embryo, we generated UAS-Dup^{10A}-GFP and UAS-Dup$^{\Delta PIP/10A}$-GFP transgenes and expressed them with *prd*-GAL4. Dup^{10A} was degraded normally during S phase as we could not detect cells that were positive for both BrdU and GFP (Fig. 15F). The same observation was made by Thomer et al. (2004) in ovarian follicle cells. Similar to our observations using Dup$^{\Delta PIP}$, approximately 45% of BrdU positive cells in the *prd*-GAL4 stripe also contained Dup$^{\Delta PIP/10A}$. These data indicate that the 10 potential CycE/Cdk2 phosphorylation sites do not account for the residual destruction of Dup$^{\Delta PIP}$ during embryonic S phase, and raise the possibility of a regulatory mechanism in addition to CRL-type E3 ligases for degrading Dup during S phase. Alternatively, Dup$^{\Delta PIP}$ may still be recognized by CRL4^{Cdt2}, but much more poorly than WT, resulting in a slower destruction during S phase.

Dup$^{\Delta PIP}$ supports DNA replication but not completion of the cell division cycle

Many studies have reported that over-expression of Cdt1 leads to re-replication (Zhong *et al.*, 2003; Arias and Walter, 2005; May *et al.*, 2005; Arias and Walter, 2006; Sansam *et al.*, 2006). However, these studies did not directly test whether PIP-dependent destruction of Cdt1 is required for normal cell cycle

progression *in vivo*. Moreover, the reported redundancy between CRL1 and CRL4 for S phase destruction of human Cdt1, and inhibition of Cdt1 by Geminin, raise the possibility that CRL4-mediated destruction of Cdt1 may not be essential for cell cycle progression. We therefore determined if Dup^{FL}-GFP and Dup$^{\Delta PIP}$-GFP could rescue the lack of S phase and consequent cell cycle arrest in *dup* null mutant embryos. *Dup^{a1}* mutant embryos develop normally through the first 15 cell cycles, presumably due to maternal stores of Dup protein, but fail to incorporate BrdU in S phase of the 16th cell cycle (Fig. 16A) (Whittaker *et al.*, 2000). Both Dup^{FL} and Dup$^{\Delta PIP}$ expression driven by *prd*-Gal4 restored BrdU incorporation in *dup* null epidermal cells, indicating that these transgenic proteins were capable of assembling pre-RC complexes and supporting the initiation of DNA replication (Fig. 16A). However, close inspection revealed an unusual BrdU incorporation pattern in Dup$^{\Delta PIP}$ expressing cells: the staining appeared less uniform and more punctuate than when Dup^{FL} was expressed (Fig 16B).
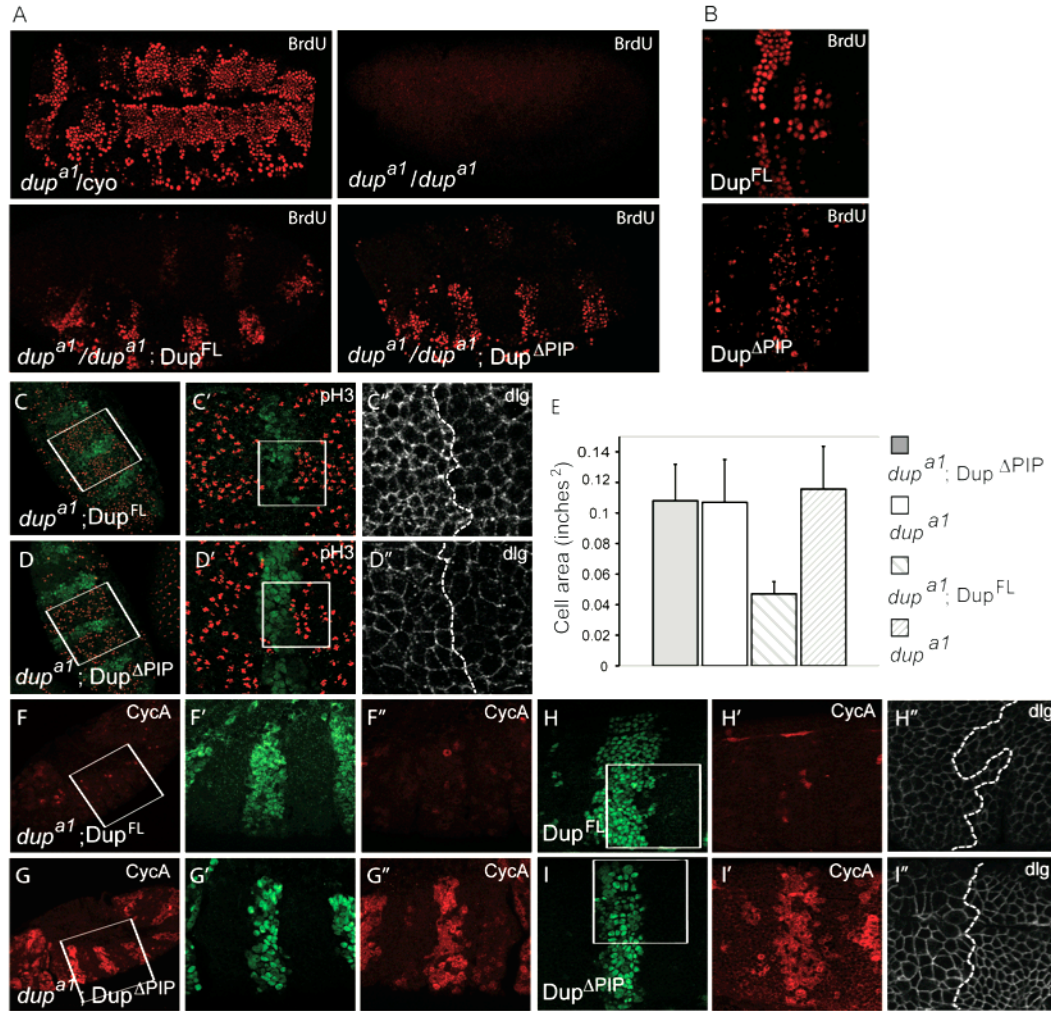
Figure 16. Molecular complementation analysis of the *dup* mutant phenotype

(A) BrdU labeling (red) of *dup^a1*/CyO control and *dup^a1* homozygous mutant embryos with and without *prd*-Gal4 expression of Dup^FL or Dup^ΔPIP. Note the restoration of BrdU incorporation in the prd-Gal4 pattern in the *dup^a1* mutant embryos. (B) Higher magnification images of the BrdU incorporation pattern after *prd*-Gal4 expression of Dup^FL and Dup^ΔPIP in *dup^a1* homozygous mutant embryos. (C-D) Anti-pH3 (red C, C', D, D') and discs large (white C'', D'') staining of *dup* null embryos expressing Dup^FL-GFP (green C, C') or Dup^ΔPIP-GFP (green D, D') with *prd*-Gal4. The area of the merged image in C'-D' is indicated by the box in C, D. The area of the Discs large panel is indicated by the box in C'-D'. Note the

67

40% (E) smaller size of the Dup^{FL}-expressing cells on the left side of the C''
panel while the Dup$^{\Delta PIP}$-expressing cells are similar in size to control (D'', E). (E)
Quantification of cell area in Dup^{FL} or Dup$^{\Delta PIP}$-expressing cells compared to that
of their *dup* null neighbors. Error bars indicate standard deviation. (F-G) Dup^{FL}-
GFP (F) or Dup$^{\Delta PIP}$-GFP (G) -expressing *dup^{a1}* null cells stained with anti-CycA
(red) and GFP (green). (H-I) Anti- GFP (green, H, I) and anti-CycA (red, H', I')
staining of Dup^{FL}-GFP (H) or Dup$^{\Delta PIP}$-GFP (I) -expressing WT cells. H'' and I''
show dlg staining. Note the larger cell size in the left side of panel I'', indicating
cell cycle arrest caused by Dup$^{\Delta PIP}$ expression.

We therefore asked if *dup* null cells expressing Dup$^{\Delta PIP}$ could complete mitosis and divide, which would be indicative of normal completion of S phase. A curious feature of the *dup* mutant phenotype is that while the epidermal cells fail to undergo S16 they nonetheless enter and arrest in mitosis with condensed chromosomes that can be detected with anti-phospho histone H3 (pH3) antibodies (Whittaker *et al.*, 2000). The entry into and arrest in mitosis likely occurs because of an inability to activate a checkpoint response to aberrant or incomplete replication (Kelly *et al.*, 1993; Piatti *et al.*, 1995). We hypothesized that if Dup^{FL} or Dup$^{\Delta PIP}$ expression could support a complete cell cycle, then this aberrant accumulation of pH3-positive cells throughout the epidermis would be eliminated. Indeed, both Dup^{FL} and Dup$^{\Delta PIP}$ expression eliminated pH3 staining in *prd*-GAL4 stripes (Fig. 16C', D'). However, this result would be obtained from a normal S phase and completion of mitosis, <u>OR</u> if Dup^{FL} and Dup$^{\Delta PIP}$ expression caused an aberrant S phase that triggered a checkpoint response and the cells arrested in interphase *prior* to entry into mitosis.

To distinguish between these two possibilities, we assessed whether cell division occurred by first examining cell size. Each epidermal cell division during *Drosophila* embryogenesis results in a reduction in cell size (Lehner and O'Farrell, 1989). Thus, if the Dup transgenes were able to support progression through mitosis and cell division, then the cells will be smaller than the *dup* null neighbors. To assess cell size, embryos were stained for the membrane protein Discs large (Dlg) and the size of the cells within and outside the domain of Dup

transgene expression was quantified. While Dup^{FL} expressing cells were approximately half the size of their *dup* mutant neighbors (Fig. 16C'', 4E), Dup$^{\Delta PIP}$ expressing cells remained the same size as their neighbors (Fig. 16D'', 4E). This finding suggests that Dup^{FL} can rescue the *dup* null cell phenotype and support completion of the cell cycle while Dup$^{\Delta PIP}$-expressing *dup* null cells remain in interphase and do not enter mitosis. To test this assertion, we detected Cyclin A protein, which should accumulate in cells arrested in interphase of cycle 16 but not in cells that divide and enter the following G1 phase of cycle 17 (Lehner and O'Farrell, 1989). The Dup$^{\Delta PIP}$ expressing cells accumulate high levels of Cyclin A (Fig. 16G), while the Dup^{FL} cells do not (Fig. 16F). Together these data indicate that Dup^{FL} transgenic protein provides normal Dup function and rescues the replication and cell cycle defect of *dup* null cells, while Dup$^{\Delta PIP}$ does not.

Why do Dup$^{\Delta PIP}$ expressing cells fail to enter mitosis? One possibility is that these cells re-replicate, due to the failure to degrade Dup, resulting in DNA damage that induces a cell cycle checkpoint. However, we were unable to detect a difference in γ-H2aV staining between Dup$^{\Delta PIP}$ expressing and non-expressing cells, suggesting that either Dup$^{\Delta PIP}$ does not induce re-replication or that the level of re-replication induced DNA damage is low enough not to be detected by the γ-H2aV antibody. In addition, we did not detect BrdU incorporation in *dup* mutant cells expressing Dup$^{\Delta PIP}$ at the time when the neighboring *dup* mutant cells arrest in mitosis 16, indicating that Dup$^{\Delta PIP}$ does not induce continuous re-replication. We found no difference in cleaved Caspase-3 staining within and outside of the Dup$^{\Delta PIP}$ transgene expression domain, suggesting that Dup$^{\Delta PIP}$

70

expressing cells do not apoptose. Taken together, our data suggest that *dup* mutant epidermal cells expressing Dup$^{\Delta PIP}$ enter but do not complete S phase of cell cycle 16 and arrest in interphase prior to mitosis.

Dup$^{\Delta PIP}$ causes cell cycle arrest in a wild type background

Our data indicate that Dup$^{\Delta PIP}$ cannot support cell division in a *dup* null background. Since endogenous Dup is promptly degraded at the onset of S phase, ectopic expression of Dup$^{\Delta PIP}$ in a WT background should create a situation in which Dup$^{\Delta PIP}$ is the only active Dup present in S phase. If the cell cycle arrest we see in *dup* null embryos is due to having active Dup in S phase, Dup$^{\Delta PIP}$ expression in WT embryos should also cause the cells to arrest in interphase. This prediction was confirmed by the presence of large undivided, Cyclin A-positive cells expressing Dup$^{\Delta PIP}$ (Fig. 16I). In contrast, these phenotypes did not arise after Dup^{FL} expression in WT embryos (Fig. 16H). The Dup$^{\Delta PIP}$-expressing cells are not simply delayed in cell cycle progression, as anti-pH3 staining does not reveal mitosis in later embryonic stages (not shown). Together, our data indicate that stabilization of Dup in S phase causes cell cycle arrest.

Follicle cell endocycle progression is not affected by Dup$^{\Delta PIP}$

Much of animal and plant growth and development rely on endoreplication, the process by which cells in certain tissues become polyploid as part of their terminal differentiation program (Lee *et al.*, 2009).

Endoreplication in *Drosophila* occurs via endocycles, which consist of alternating S and G phases without cell division. Current models of replication control in endocycles suggest that individual origins of DNA replication fire once and only once as they do in mitotic cycles and that cycles of low (G phase) and high (S phase) CDK activity permit and prevent pre-RC assembly, respectively. Follicle cells of the *Drosophila* ovary become 16C polyploid via developmentally controlled endocycles that occur between stages 6-9 of oogenesis (Lilly and Duronio, 2005). To test the requirement for Dup degradation in endocycle progression, we expressed Dup^{FL} and Dup$^{\Delta PIP}$ in endocycling follicle cells using c323a-Gal4, which drives expression in all follicle cells of stages 8-14 (Fig. 17A). More follicle cells expressed Dup$^{\Delta PIP}$ than Dup^{FL}, suggesting that Dup$^{\Delta PIP}$ was stabilized (Fig. 17B, C). We then determined whether Dup degradation during endo S phase is PIP-box dependent by quantifying the number of BrdU pulse-labeled S phase cells that also express Dup^{FL} or Dup$^{\Delta PIP}$. We found that 43% of endocycle S phase cells retained Dup$^{\Delta PIP}$ (Fig. 5E'', open arrows), while Dup^{FL} is degraded at the onset of endocycle S phase (Fig. 17D''). The pattern of BrdU incorporation was similar between Dup^{FL} and Dup$^{\Delta PIP}$ -expressing follicle cells, and 87.4% and 86.4%, respectively, of eggs laid by these mothers hatched into viable larvae. These data suggest that Dup$^{\Delta PIP}$ expression does not adversely affect follicle cell endocycle progression.
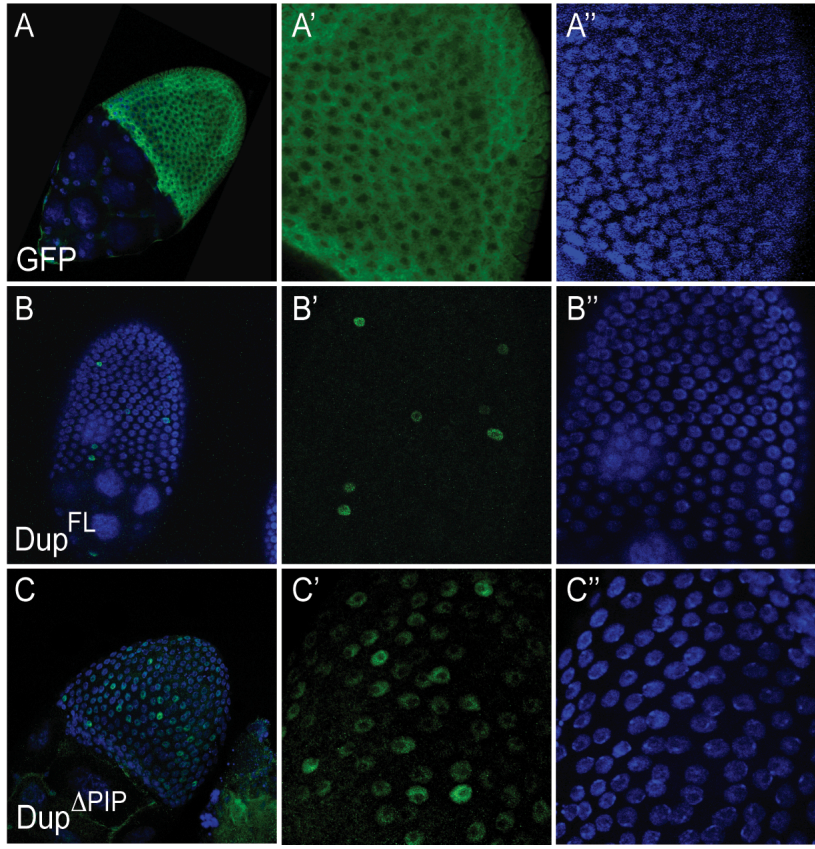
Figure 17. Analysis of Dup^{FL} and Dup ^{ΔPIP} expression in follicle cells.

(A-C) Confocal images of follicle cells from stage 9 egg chambers expressing GFP (A), Dup^{FL}-GFP (B) or Dup$^{\Delta PIP}$-GFP (C) using the c323a-Gal4 driver and stained with anti-GFP (green) and DAPI (blue).
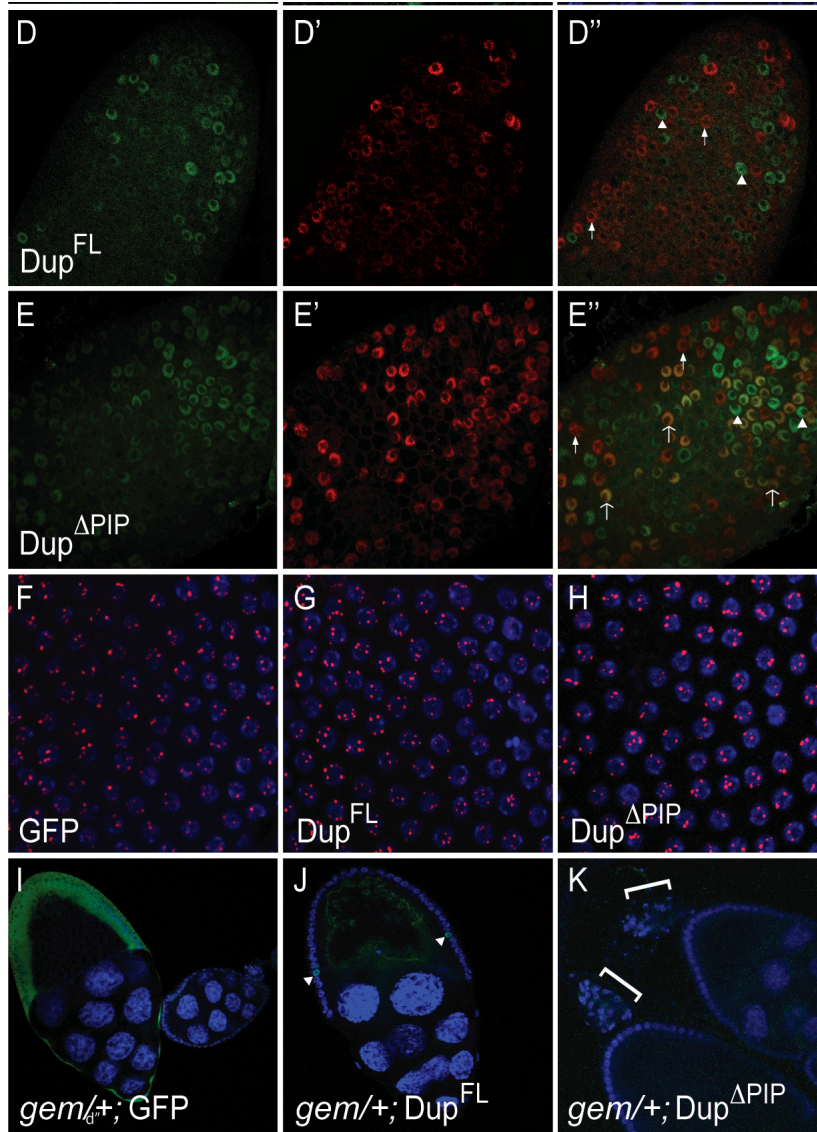
Figure 17 continued

(D-E) Confocal images of follicle cells from stage 9 egg chambers expressing Dup^{FL}-GFP (D) or Dup$^{\Delta PIP}$-GFP (E) with c323a-Gal4 and stained with anti-BrdU (red) and anti-GFP (green). Arrows and arrowheads as in Figure 3. (F-H) Confocal images of follicle cells undergoing chorion gene amplification expressing GFP (F), Dup^{FL}-GFP (G), or Dup$^{\Delta PIP}$-GFP (H) with c323a-Gal4 and stained with anti-BrdU (red) and DAPI (blue). (I-K) gem$^{l(2)03202}$/CyO follicle cells

74

from stage 9 expressing GFP (I), Dup^{FL}-GFP (J), or Dup$^{\Delta PIP}$-GFP (K) stained with anti-GFP antibodies (green) and DAPI (blue). The arrowheads in panel J indicate two cells expressing Dup^{FL}-GFP. The brackets in panel K indicate degenerated stage 9 egg chambers.

Follicle cell gene amplification is not inhibited by Dup$^{\Delta PIP}$

Beginning in stage 10A and after the completion of endoreplication, several specific follicle cell loci begin a program of gene amplification that increases the copy number, and thus the biosynthetic capacity, of genes encoding proteins necessary for chorion synthesis and vitellogenesis (Calvi and Spradling, 1999; Tower, 2004; Claycomb and Orr-Weaver, 2005). Gene amplification occurs by repeated firing of specific origins of replication while the remainder of the origins throughout the genome stays quiescent. This phenomenon can be detected as distinct foci of BrdU incorporation within each follicle cell nucleus (Fig. 17F). While the precise mechanism of this regulation is unknown, it likely involves cycles of pre-RC assembly/disassembly since virtually all the known pre-RC components, including Dup, are required for gene amplification (Tower, 2004). To determine whether PIP-mediated regulation of Dup was required for this process, we examined BrdU incorporation in stage 10A follicle cells expressing Dup^{FL} or Dup$^{\Delta PIP}$. Our results indicate that this pattern of BrdU incorporation is largely unaffected by Dup^{FL} (Fig. 17G), while expression of Dup$^{\Delta PIP}$ caused slightly enlarged BrdU foci (Fig. 5H) as previously described for an allele of Dup lacking the first 46% of the protein (including the PIP box) (Thomer *et al.*, 2004). Importantly, no ectopic BrdU incorporation throughout the nucleus was observed, indicating that the normal inactivation of genomic replication is retained in the presence of Dup$^{\Delta PIP}$. Ectopic Brdu incorporation occurs in these cells mutant for Cul4 or Ddb1 (Lin *et al.*, 2009).

76

Geminin function restrains Dup$^{\Delta PIP}$ activity in follicle cells

Our findings indicate that the absence of PIP box-dependent degradation of Dup does not adversely affect follicle cell endocycles and gene amplification. Because this result is different than what we obtained in mitotic embryonic cells, we asked whether Geminin function acts to restrain Dup$^{\Delta PIP}$ activity in endocycling follicle cells. To test this hypothesis, we reduced the gene dose of *geminin* in half together with c323a-Gal4-driven expression of Dup^{FL} or Dup$^{\Delta PIP}$ and compared the results to wild type and *geminin* heterozygote ovaries. While ovaries from *geminin*/+ heterozygous control flies and *geminin*/+ flies expressing Dup^{FL} appeared wild type (Fig. 17I and J, respectively), *geminin*/+ flies expressing Dup$^{\Delta PIP}$ contained ovaries lacking normal stage 9 and older egg chambers due to massive degeneration (Fig. 17K). This phenotype occurred soon after the initiation of Dup$^{\Delta PIP}$ expression around stage 8-9. These results suggest that Geminin and PIP-mediated destruction cooperate to control Dup activity during follicle cell endocycles.

Discussion

Although several regulatory mechanisms of Cdt1 have been described, how they work together and when they are required in different tissues during animal development is not well understood. Here we show that regulation of *Drosophila* Dup via an NH$_2$-terminal PIP box is required for progression through the cell division cycle in embryonic epidermal cells but is dispensable for progression

through follicle cell endocycles.

Geminin and Cul4 together limit the extent of re-replication

In this study, we found that *Drosophila* Cdt1 homolog, Dup is degraded via CRL4 dependent mechanism in the event of Geminin depletion and subsequent DNA re-replication. Similar results were obtained using various human cancer cells (HCT116, HeLa, and NHF1), which suggest that this mechanism to degrade Cdt1 when Geminin activity is compromised is conserved. In addition, another member of pre-RC, Cdc6, is also degraded upon re-replication by Huwe1 ubiquitin ligases in human cells (Hall et al. 2008). This indicates a redundant mechanism exists to regulate further origin licensing event as cells undergo re-replication. *Drosophila* has a conserved HUWE1 protein, CG8184. However, whether this mechanism is conserved in flies is not yet known.

Interestingly, Cul4 depletion in S2 cells did not induce re-replication, unlike DDB1 depletion in human cells. Two possible explanations are, 1) Cul4 depletion results in a stronger epistatic event resulting in G1 arrest, 2) CRL4 mediated degradation pathway is not essential in S2 cells. A p27 homolog, Dacapo plays an important role in G1-S transition as an inhibitor of S phase CDKs. Dacapo is also targeted by CRL4 to be degraded (Higa et al. 2006), thus it is possible that G1 arrest is a result of Dacapo accumulation. Co-depletion of Cul4 and Dacapo could alleviate the G1 arrest, allowing cells to undergo DNA replication and re-replication (Data not shown, Higa et al. 2006). However, current results cannot distinguish between these possibilities.

Our results suggest another mechanism of Dup regulation, perhaps phosphorylation. Dup exists as a doublet in most of our experiments and Cul4 depletion only affected the faster migrating band. Thomer et al. (2004) showed that CycE phosphorylates the slower migrating Dup band, although we did not determine if this was the case with our doublets. In mammlian cells, Cdt1 is regulated both by CRL4 and CRL1, and CRL1 binding requires Cdt1 phosphorylation (Nishitani et al., 2006; Kondo et al. 2004). Therefore, it is possible that this mechanism is conserved in flies. Our data does not show detectable change in Dup doublets upon Cul1 depletion, however. Importantly, Cul4 and Geminin dsRNA treatment could not completely rescue Dup levels. This may be due to the reported down-regulation of Dup mRNA in Geminin depleted S2 cells (Mihaylov et al. 2002).

PIP box-dependent degradation of Dup

Our results indicate that deletion of the PIP box prevents the rapid destruction of Dup at the beginning of S phase. Prior to discovery of the PIP degron/CRL4 mechanism of replication-coupled proteolysis, Thomer et al. (2004) reported a similar result with a mutant version of Dup lacking the NH_2-terminal 46% of the protein, including the PIP box. Thus, our results suggest that the Thomer et al. (2004) observation is due to deletion of the PIP degron. Biochemical and genetic experiments from a number of species suggest that the PIP degron recruits proteins to chromatin-bound PCNA at replication forks during S phase . These proteins are subsequently ubiquitylated by CRL4[Cdt2] and proteolyzed (Arias and Walter, 2006; Higa *et al.*, 2006a; Hu and Xiong, 2006;

Senga *et al.*, 2006; Abbas *et al.*, 2008; Kim and Michael, 2008; Kim *et al.*, 2008; Nishitani *et al.*, 2008; Shibutani *et al.*, 2008; Havens and Walter, 2009). Although we did not detect hyper-accumulation of Dup in imaginal cells mutant for components of CRL4^{Cdt2}, the PIP degron mechanism is conserved in *Drosophila* (Shibutani *et al.*, 2008) and CRL4^{Cdt2} is required for Dup destruction after DNA damage in cultured S2 cells (Higa *et al.*, 2006a). As discussed above, phenotypic pleiotropy resulting from abrogation of CRL4^{Cdt2} function may have masked our ability to detect effects on Dup protein.

Interestingly, deletion of the PIP box resulted in inappropriate Dup accumulation in only about half of S phase cells, indicating that Dup was not completely stabilized by inactivation of the PIP box-dependent destruction mechanism. This result suggests the possibility of a mechanism in addition to the PIP-degron/CRL4 mechanism of inducing Cdt1 degradation during S phase. Cdk-directed phosphorylation triggers CRL1-mediated destruction of mammalian Cdt1 (Li *et al.*, 2003; Liu *et al.*, 2004; Takeda *et al.*, 2005; Nishitani *et al.*, 2006). However, mutating the 10 previously described Cyclin E/Cdk2 phosphorylation sites in the NH$_2$-terminus of Dup did not alter the stability of Dup$^{\Delta PIP}$ or Dup during S phase in both mitotic and endocycling cells (Fig. 15 and Thomer et al. (2004)). We also did not detect Dup hyper-accumulation in cells lacking Cul1 or both Cul1 and Cul4. While it is possible that there are additional Cdk phosphorylation sites remaining on Dup^{10A}, these results suggest that Cyclin E/Cdk2-dependent phosphorylation and CRL1 ubiquitylation of Cdt1 are not major mechanisms of Dup destruction during S phase in *Drosophila*. It has been recently proposed

80

that CRL1-dependent regulation of Cdt1 arose in higher metazoans (Kim and Kipreos, 2007b).

A requirement for Dup degradation in mitotic cycles

By using rescue of *dup* embryonic mutant phenotypes as an assay, our data clearly demonstrate that Dup$^{\Delta PIP}$ is unable to support progression through the cell division cycle. Similarly, Dup$^{\Delta PIP}$ expression in WT embryos caused cell cycle arrest in interphase. In these experiments there was no evidence for massive re-replication, as occurs in other cell types after over-expression of Cdt1 or depletion of Cdt1 regulatory mechanisms (e.g. CRL4 or Gem) (Arias and Walter, 2007). We also did not detect extensive DNA damage or apoptosis. We propose that the near physiological levels of Dup$^{\Delta PIP}$ expression achieved in our experiments, as suggested by our ability to phenotypically rescue *dup* mutant cells using transgenic wild type Dup, causes a small number of replication origins to re-initiate. This situation results in a low level of DNA damage that activates a checkpoint and arrests cells in interphase. Alternatively, Dup$^{\Delta PIP}$ may block DNA synthesis more directly, as a recent study reported that excess Cdt1 prevents nascent DNA strand elongation (Tsuyama *et al.*, 2009).

Mechanisms of Dup regulation in endocycling cells

Previous data showed that heat-shock driven over-expression of Dup in endocycling follicle cells cause re-replication (Thomer *et al.*, 2004), and that Cul4 mutant follicle cells hyper-accumulate Dup and exhibit replication defects during

gene amplification (Lin *et al.*, 2009). We found that Gal4-driven expression of Dup$^{\Delta PIP}$ does not adversely affect progression through follicle cell endocycles. In addition, we did not find obvious defects in endocycle S phase or chorion gene amplification. As in the embryo, we propose that the lack of massive re-replication seen with Dup$^{\Delta PIP}$ is due to lower expression levels than that obtained by Thomer et al. (2004). Also, small level of DNA damage might not disrupt the endocycle (Mehrotra *et al.*, 2008). Finally, our data suggest that the replication defects seen in *Cul4* mutant cells by Lin et al. (2009) may be due to misregulation of another CRL4 target.

Several observations suggest the possibility that Cdt1 is regulated in a cell-type specific manner. In *Drosophila* S2 cells and mammalian cells, RNAi against Gem but not Cul1 or Cul4 results in re-replication (Melixetian *et al.*, 2004; Zhu *et al.*, 2004; Hall *et al.*, 2008). In contrast, *Drosophila* Gem is not required for proliferation of imaginal discs or endoreplication in salivary glands (Quinn *et al.*, 2001). Null mutations of *C. elegans Cul4* or *Ddb1* caused over-replication only in seam cells (Zhong *et al.*, 2003; Kim and Kipreos, 2007a). Finally, ectopic expression of *Arabidopsis* Cdt1 induced over-replication only in endocycling cells (Castellano Mdel *et al.*, 2004). The basis for these cell type differences is not known.

We showed that reduction of Gem gene dose in combination with Dup$^{\Delta PIP}$ expression in follicle cells causes massive deterioration of egg chambers during oogenesis. This suggests that Dup inhibition by Gem can compensate for the loss of PIP-mediated destruction of Dup in this cell type. In proliferating

embryonic epidermal cells loss of PIP-mediated Dup destruction was sufficient to block the cell cycle, suggesting that Gem activity is unable to provide compensatory inhibition of Dup in this situation. Cell type specific differences in Gem expression or activity could explain why cells are differently sensitive to stabilized Dup. For instance, the *C. elegans* Gem homolog, GMN-1, is expressed at higher levels in the germ line (Yanagi *et al.*, 2005), suggesting that this tissue might be buffered against disruption of Dup destruction as we observed in *Drosophila* follicle cells. (May *et al.*, 2005) reported that in some cell types Gem levels increase concomitantly with increased levels of Dup after DNA replication is compromised. Determining the mechanisms by which certain cell types are more sensitive to mis-regulation of Cdt1 destruction than others will add to our understanding of replication control in developing organisms.

Materials and Methods

Fly stocks. Stocks carrying *Cul4* mutant alleles *EP2518* and *KG02900, Ddb1/piccolo* mutant alleles *EY01408*, *pic^2*, *pic^{drv3}* were obtained from the Bloomington Stock Center. The *Ddb1/pic^{SO26316}* line was obtained from the Szeged Stock Center. *gem$^{l(2)k03202}$* was a gift from Helena Richardson (Quinn *et al.*, 2001).

P-element excision-mediated mutagenesis. The *EP2518* P-element in the 3' UTR of *Cul4* was mobilized by crossing to *w-; Sb/CyO; Dr, Δ 2-3/TM6* flies. Resulting mosaic males were crossed to *Pin^{88k}/Cyo* flies, and three EP2518 excision

events were identified from ~400 w^- progeny as novel *Cul4* mutant alleles by a failure to complement $Cul4^{KG02900}$. The breakpoints of $Cul4^{6AP}$, $Cul4^{11L}$, and $Cul4^{11R}$ were determined by sequencing. Note that in Hu et al. (2008) the amount of truncation in $Cul4^{11R}$ allele was incorrectly indicated as that of $Cul4^{11L}$. The *EY01408* P-element in the 5' UTR of *Ddb1* was similarly mobilized, and resulting w^- progeny were tested for complementation with the $Ddb1^{S026316}$ allele.

Mitotic recombination and clonal analysis. Mitotic recombination was carried out using the FLP/FRT technique (Xu and Rubin, 1993) using *hs-FLP; FRT42B Ubi-GFP/ FRT42B* $Cul4^{11L}$, or *hs-FLP; FRT82B Ubi-GFP/ FRT82B* $Ddb1^{EY01408}$, or *hs-FLP; FRT42D Ubi-GFP/ FRT42D* $Cul1^{EX}$. Larvae were heat-shocked for 45 minutes at 37 °C, 48-80 hours after egg deposition, and dissected as third instar larvae.

Transgenic flies. Dup^{FL}, $Dup^{\Delta PIP}$, Dup^{10A}, and $Dup^{\Delta PIP10A}$ cDNAs were cloned into pENTR (Invitrogen) and recombined into a Gateway compatible set of UASp vectors that permitted a COOH-terminal GFP fusion and provided by Terence Murphy (http://www.ciwemb.edu/labs/murphy/Gateway%20vectors.html). The Dup^{10A} open reading frame (Thomer *et al.*, 2004) was kindly provided by Brian Calvi (Indiana University). Transgenic flies were generated by Rainbow Transgenic Flies Inc. and BestGene Inc. The *prd-* (Treisman *et al.*, 1991), c323a- (Manseau *et al.*, 1997), and GMR- (Moses and Rubin, 1991) Gal4 driver lines were obtained from the Bloomington Stock Center.

dup^{a1} rescue. Staged embryo collections from *dup^{a1}/+;prd*-gal4/+ and *dup^{a1}/+;* UAS-Dup-GFP/+ parents were fixed and stained with various combinations of antibodies (see below). *dup^{a1}/dup^{a1};prd*-Gal4/UAS-Dup-GFP embryos were identified by a combination of GFP expression and the *dup* mutant phenotype, which is obvious with DAPI staining of nuclei. Relative cell size was determined as the product of cell width and cell height, measured in pixels using Photoshop from images of anti-Dlg staining.

*Geminin reduction schemes. gem$^{l(2)k03202}$/*CyO; UAS-Dup-GFP/+ females were crossed with 323a-Gal4 or *Sco/CyO*; 323a-Gal4/+ males. Ovaries from all female progeny from the 323a-Gal4 cross or *Cy* female progeny from the *Sco/CyO*; 323a-Gal4/+ cross were dissected and analyzed.

Antibodies. A synthetic peptide (MSAAKKYKPMDTTELHEN) derived from the NH$_2$-terminus of *Drosophila* Cul4 was coupled to KLH and used to generate antibodies in rabbits (Pocono Farms) that were subsequently affinity purified (Hu *et al.*, 2004). A COOH-terminal anti-Cul4 antibody was a gift from Dr. Hui Zhang (Yale University). Mouse antibodies generated using a GST fusion protein containing the NH$_2$-terminal 2/3 of human Ddb1 (Zymed) were used to recognize *Drosophila* Ddb1. Guinea pig anti-Dup was kindly provided by Dr. Terry Orr-Weaver (MIT, MA) (Whittaker *et al.*, 2000), and mouse anti-*Drosophila* Cyclin A was obtained from the Developmental Studies Hybridoma Bank (University of

Iowa). Mouse anti-HA (12CA5, NeoMarkers), mouse anti-tubulin (NeoMarkers) mouse anti-BrdU (BD Biosciences), and rabbit anti-GFP (Abcam) were obtained commercially. Antibody to *Drosophila* Cul1 (ZL18) was purchased from Zymed Laboratories Inc., and antibodies to both *Drosophila* tubulin were purchased from Sigma. Dup antibody was a gift from T. Orr-Weaver, and anti-DDB1 was a gift from Y. Xiong.

S2 cell culture, transfection, and RNAi.

Drosophila S2 cells were grown in F-900 II SFM serum-free medium (Invitrogen) and treated with 20 ug of dsRNA/ml, as previously described (Rogers et al., 2002). Primers for generating dsRNA: cul-1 5'-CTGCTCAACGCAGACCG and 5'-TGTCCTGCAGTTGCTGG, cul-4 5'-TTGGCCAAACGATTACTTGTGGG and 5'-GAGAAGATTATGGCTCAGCG, geminin 5'-ATGTCTTCGAGCGCTGCC and 5'-GGCGTTGACCTTGTCCTCG, Dup 5'-ACAAACCGCAAACGCGCCG and 5'-CCAGCACTGCCTTGAGTTCC, control (pBluescript SK sequence) 5'-ATGGATAAGTTGTCGATCG and 5'- ACCAGGTTCACATGCTTGCG. For pull-down studies, S2 cells were cultured in Schneider's/10% FBS at 25 °C, and were transfected using Effectene (Qiagen).

Cell cycle analysis

Drosophila cells were transfected then plated in concanavalin A-coated 24-well glass bottom dishes (MatTek) for 1 h prior to fixation as described (22), stained with DAPI at 5 _g/ml, and scanned with either an IC100 Image Cytometer

(Beckman Coulter) or an Array Scan VTI (Cellomics) equipped with a 20_ 0.5NAobjective and an ORCA-ER cooledCCDcamera. Images of _5,000 cells per well were acquired and analyzed using CytoShop v2.1 (Beckman-Coulter) or vHCS View (Cellomics). Integrated fluorescence intensity measurements were determined from unsaturated images. *p* values were determined using an unpaired Student's *t* test.

Western blot analysis. S2 cells were cultured in Schneider's/10% FBS at 25 °C, and were transfected using Effectene (Qiagen). Larval and cell lysates were made in RIPA (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1mM EDTA, 0.1% SDS, 0.1% Triton X-100, 0.5% sodium deoxycholate), supplemented with 1 mM DTT, 1 mM PMSF, 1 m M sodium vanadate, 2 µg/ml aprotinin, 2 µg/ml leupeptin, 10 µg/ml trypsin inhibitor, and 150 µg/ml benzamidine, and cleared by high speed centrifugation. Larval lysate were further clarified through 0.65 micrometer centrifugal low binding durapore membrane filters (Ultrafree-MC, Millipore). Lysates were resolved by SDS-PAGE and analyzed by Western blot.

Immunohistochemistry. Dissected larval tissues were fixed in 4% formaldehyde/ PBS-T for 20 minutes and blocked in 5% NGS for 1 hour. Dissected larvae were incubated with 10 uM BrdU in Schneider's media for 1 hour prior to fixation. Embryos were BrdU labeled as described (Shibutani *et al.*, 2008), and fixed in 5% formaldehyde. For BrdU and GFP co-staining, embryos were stained for GFP and fixed again in 5% formaldehyde, prior to 2N HCl treatment and anti-BrdU

staining. Ovaries were incubated with 1mg/ml BrdU for 45 minutes, fixed in 5% formaldehyde/PBS and permeabilized in 0.5% Triton-X for 30 min. To expose BrdU epitope, dissected ovaries were treated with 30u/ul DNaseI (Fermentas). Stained tissues were analyzed using a Zeiss 510 confocal microscope.

CHAPTER 4

ENDOREPLICATION: POLYPLOIDY WITH PURPOSE

This chapter was written as a review article in collaboration with Jean Davidson

under the direction of Dr. Robert Duronio. Jean Davidson wrote the sections on

tissue regeneration upon stress and regulation of endocycle by E2F, and I wrote

the remainder of the chapter. This work was published in Genes and

Development.

Summary

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 that 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

1. 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. 18A). 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 (Fig. 18B) (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 cancer.

90

Another major form of endoreplication occurs through the process of endomitosis in which cells enter but do not complete mitosis (Fig. 18C). The best studied example occurs in 64N polyploid megakaryocytes (Ravid et al. 2002), which are responsible for producing the anucleated thrombocytes (or platelets) 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.
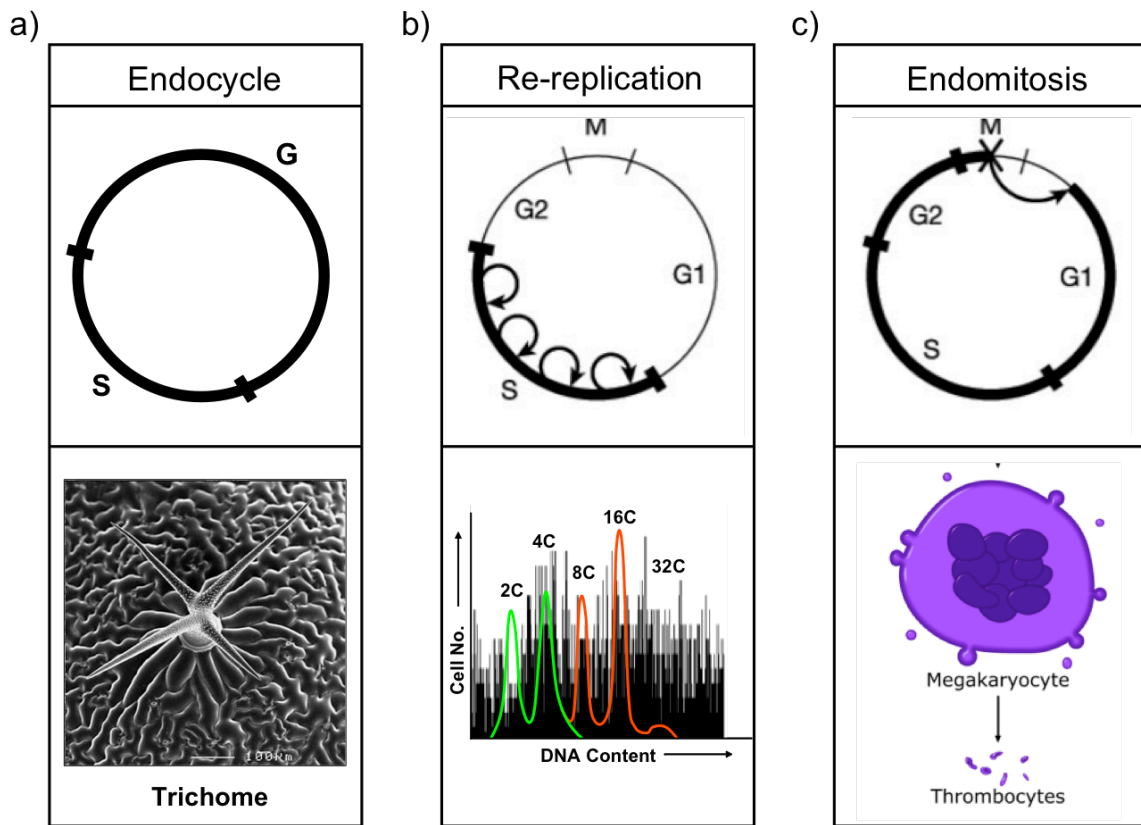
Figure 18. Endoreplication

(A) Endocycles are defined as cell cycles consisting of S and G phase without cell division. Endocycling cells do not enter 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 on 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 cycle profile, with 2C and 4C peaks. Red represents endoreplication cycles that result in distinct populations of cells with more than a 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 polyploid megakaryocytes.

2. 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. 19A). Endosperm formation occurs soon after fertilization and is associated with a switch from a mitotic cell cycle to an endocycle (Grafi and Larkins 1995; Leiva-Neto et al. 2004). 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. However, a 50% reduction in the mean DNA content of polyploid maize endosperm cells had very little affect on the accumulation of starch and the accumulation of storage proteins and their mRNAs (Leiva-Neto et al. 2004). The authors therefore

suggest that endosperm polyploidy may simply provide a mechanism to store nucleotides for use during embryogenesis and germination.

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. 19A). 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. 19B). 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 promote placenta development and establish the interface between the embryo and the mother that supports embryogenesis (Zybina and Zybina 2005). Trophoblast Giant Cells (TGCs) are extraembryonic cells that facilitate uteral implantation of the fertilized egg and metastasis into maternal blood vessels to allow transport of nutrients, oxygen, and immunoglobins into the embryo (Fig. 19C) (Cross 2000; Cross et al. 2002; Cross 2005). 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.   In addition, a significant reduction in the magnitude of endoreplication in TGCs causes embryonic lethality (Geng et al. 2003; Parisi et al. 2003; Garcia-Higuera et al. 2008). Restoration of endoreplication in these cells is sufficient to rescue embryonic viability, indicating that endoreplication plays a crucial role in early mammalian development.
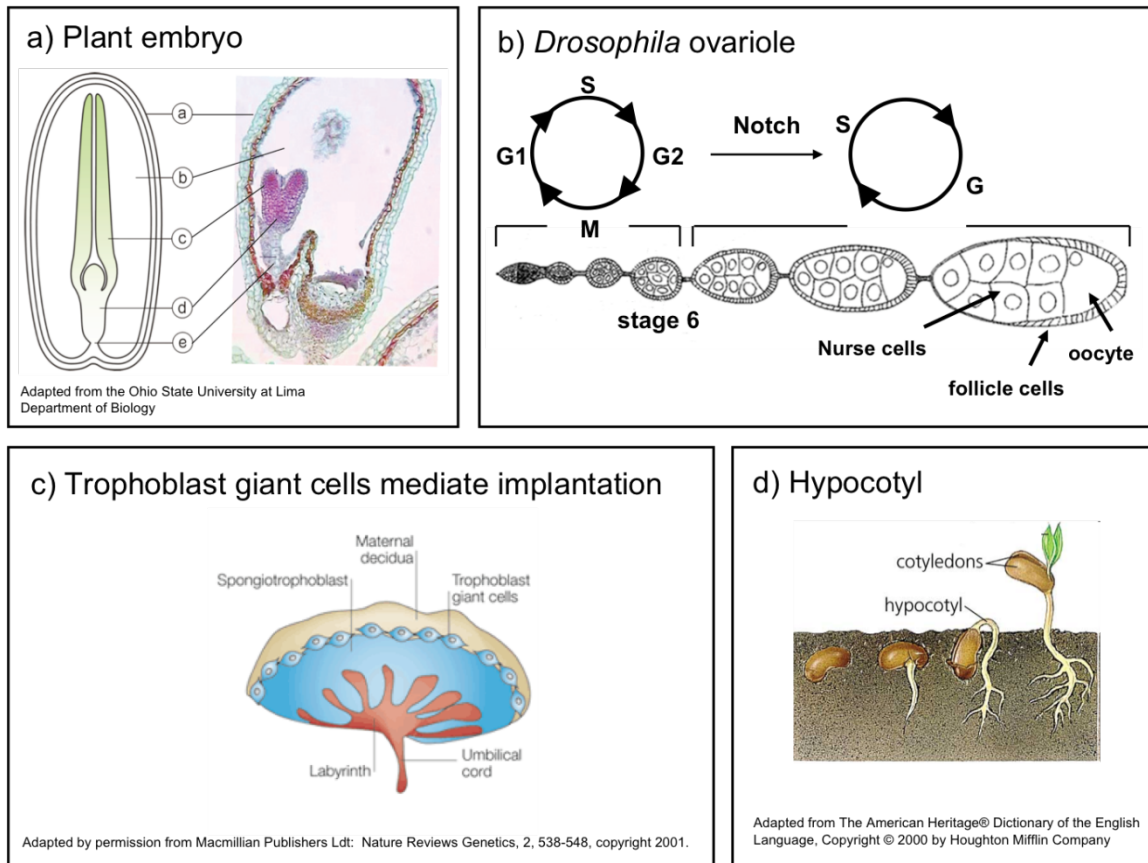
Figure 19. Examples of endocycling tissues

(A) A schematic and image of a 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 plus 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 embryo implantation by contributing to invasion 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.

3. 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. 19D). 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 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 (Edgar and Nijhout 2004). 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; Micchelli and Perrimon 2006; Ohlstein and Spradling 2006). 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. 18A). 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 ene 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).
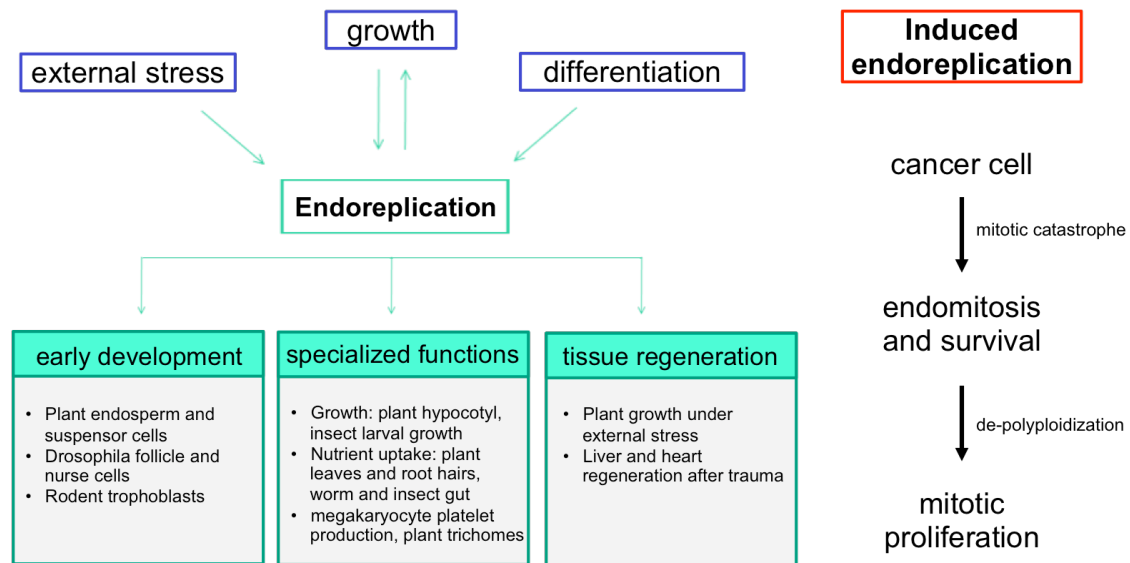
Figure 20. Examples of the endoreplication during normal and cancer development.

4. 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 (Meckert et al. 2005; Anatskaya and Vinogradov 2007), 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.

5. 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 entirely clear. One possibility is that polyploidization is a precursor to aneuploidy that may contribute to oncogenesis (Storchova and Pellman 2004) (Fig. 20). 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 (Prieur-Carrillo et al. 2003; Erenpreisa et al. 2005a; Erenpreisa et al. 2005b; 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; Walen 2007a; Walen 2007b).

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

106

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) may be 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, polyteny 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. Polyploidy in differentiated cell types could also provide an advantage relative to diploid cells because the multiple gene copies may increase buffering against random, gene inactivating mutations (e.g. by exogenous DNA damaging agents).

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).

108

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.

1. 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) (Fig. 19B) (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).

Recent studies have shed much light onto the mechanisms by which Notch signaling promotes the transition into endocycles (Fig. 21). 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$^{p21/p27}$) 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; Sun and Deng 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}, dap$^{p21/p27}$, 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, dap$^{p21/p27}$ 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; Claycomb et al. 2004; Tower

111

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).

2. Hormone-induced endoreplication in plants and animals

Plants provide many examples of developmentally regulated endoreplication. Because Notch signaling is not conserved in plants (Wigge and Weigel 2001), other signals must have evolved to regulate the onset and degree of endoreplication. Studies in plants as well as megakaryocytes in mammals suggest that hormone-mediated pathways are also crucial for the transition to endoreplication.

112

Plants: Many genes that affect endoreplication in plant development have been identified through mutational studies (Sugimoto-Shirasu and Roberts 2003; Inze and De Veylder 2006). 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 (Tomer et al. 1989; Pang et al. 2005). 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; Kaushansky 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 (Gurney et al. 1994; Alexander et al. 1996; Murone et al. 1998; Solar et al. 1998; Ihara et al. 1999).

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

114

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; Zhang et al. 1996; Kikuchi et al. 1997; Matsumura et al. 2000).

THE REGULATION OF ENDOCYCLE PROGRESSION

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.

1. 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 multi-protein 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 G1 cyclins (e.g. Cyclin E) drives entry into S phase, while activation of Cdk1 by M phase cyclins (e.g. Cyclin B) 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 (Follette et al. 1998; Weiss et al. 1998). In mammals the Cdk requirement for S phase, including endo S phase, is provided redundantly between Cdk1 and Cdk2 (Aleem et al. 2005; Aleem and Kaldis 2006; Santamaria et al. 2007; Ullah et al. 2008), whereas in *Drosophila* Cdk2 is essential (Lane et al. 2000). Cyclin E (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. 20).

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 abundance oscillates during endocycles, with peak levels in late G and S phase (Lilly and Spradling 1996). CycE gene expression in endocycling cells requires the E2F1 transcription factor (Duronio and O'Farrell 1995; Royzman et al. 1997; Duronio et al. 1998). 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). Similarly, mutation of *minus*, which likely encodes a novel substrate recognition subunit of SCF, results in hyperaccumulation of CycE and a block to endoreplication (Szuplewski et al. 2009). 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). 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 in this cell type (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 (Shcherbata et al. 2004; Audibert et al. 2005). 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 be differentially utilized in different cell types.
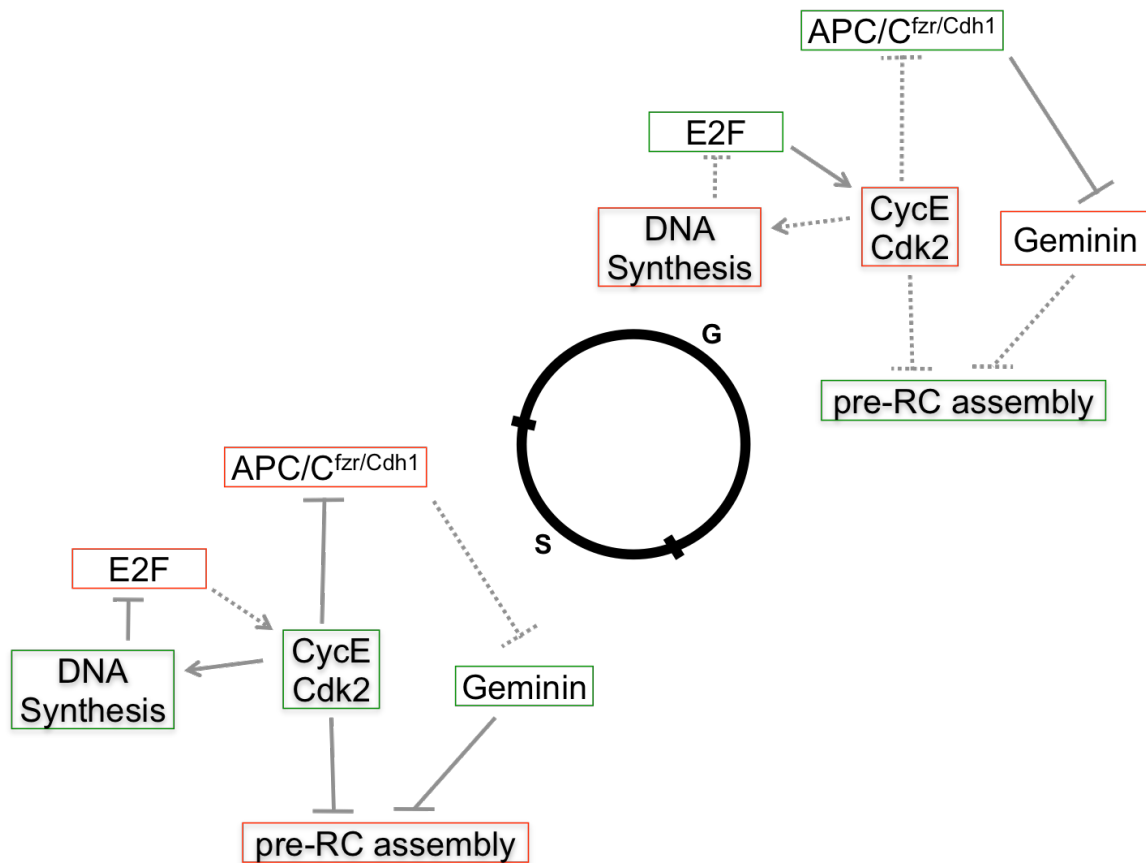
Figure 21. Regulation of the *Drosophila* endocycle.

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/C$^{fzr/cdh1}$ represses accumulation of Geminin, thereby allowing pre-RC formation. E2F 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/C$^{fzr/cdh1}$, which allows Geminin accumulation that also inhibits pre-RC formation.

2. 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 proteins, fzy/Cdc20 and fzr/Cdh1, which function as activators 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 functions 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. This function 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 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 defects during very early mouse embryogenesis that are consistent with either inappropriate endoreplication or re-replication (Gonzalez et al. 2006; Hara 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 could help constrain Cdt1 activity to G phase when pre-RCs are formed, and thus may prevent re-replication during endo S phase. How is cyclic Geminin accumulation achieved? By monitoring the levels of the APC/C target Orc1, Narbonne-Reveau et al. (2008) show that APC/C activity also oscillates in endocycles. They and Zielke et al. (2008) 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. 21).

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 (Sauer et al. 1995; Hayashi 1996; Imai et al. 2006). Thus, in the absence of APC/C$^{fzr/Cdh1}$ activity the inappropriate accumulation of Cyclin A, even in cells with very little CycA mRNA (Zielke et al. 2008), could also contribute to endocycle arrest by ectopically activating CycA-dependent Cdks and inhibiting pre-RC assembly.

3. 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 (Dimova and Dyson 2005; DeGregori and Johnson 2006; 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 (Duronio et al. 1998; Boudolf et al. 2004). In *Drosophila* E2f1 mutants, DNA synthesis and endocycle progression is drastically attenuated (Duronio et al. 1995; Royzman et al. 1997; Duronio et al. 1998), 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 E2f1 target is the CycE gene, whose expression both oscillates and requires E2f1 and Dp during *Drosophila* endocycle progression (Duronio and O'Farrell 1995; Duronio et al. 1995;

125

Royzman et al. 1997; Duronio et al. 1998). 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. 21).

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 et al. 1996; Du and Dyson 1999; Du 2000), 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; Higa et al. 2006; Hu and Xiong 2006; Senga et al. 2006; Havens and Walter 2009). 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 (Edgar and Nijhout 2004). 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). E2f2 mutant salivary gland cells have reduced ploidy, and 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) (Vlieghe et al. 2005; Lammens et al. 2008). 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

128

ectopic entry into the endocycle in normally diploid larval histoblasts (Hayashi et al. 1993). Ectopic Esg expression can also block endoreplication (Fuse et al. 1994). 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).

4. 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 regulatory mechanims, 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 (Castellano et al. 2001; Bermejo et al. 2002).

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 (Pinto et al. 1999; Pflumm and Botchan 2001). 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. Another possibility is that Orc1 function in the salivary gland is provided by another of the Orc subunits. 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 (Lilly and Spradling 1996; Leach et al. 2000). 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.

CONCLUSION

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 variation 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.

ACKNOWLEDGEMENTS

CHAPTER 5

DISCUSSION AND FUTURE DIRECTIONS

DNA replication is tightly controlled to initiate once and only once during each S phase, in order to ensure accurate duplication of the genome. This regulation occurs at each replicative origin by requiring organization of pre-replicative complexes to make the origins competent for replication. Cdt1 is a member of this complex whose regulation is essential for preventing re-replication. There are two important regulators of Cdt1: inhibition by Geminin binding during S-M phases; and destruction mediated by CRL at the onset of S phase (Feng and Kipreos 2003; Diffley 2004; DePamphilis 2003). Compromising either of these regulatory mechanisms can cause Cdt1 hyper-activity and subsequent re-replication (Arias and Walter 2007). However, how Cdt1 regulation is coordinated by Geminin and S phase degradation in a developing organism was not understood. During my thesis work, I aimed to elucidate the relative contribution or redundancy of these regulatory mechanisms using *Drosophila* as a model organism.

I started with the question of Geminin and CRL4 redundancy in regulating Cdt1 activity, which lead to the discovery of re-replication induced Cdt1 degradation mediated by CRL4 (Chapter 3). In addition, I found that while

Geminin is the major regulator of Cdt1 activity in S2 cells, timely S phase destruction of Cdt1 is required for mitotic cell cycle progression during *Drosophila* embryogenesis. Interestingly, stabilizing Cdt1 in S phase did not affect endocycle progression during ovary development, indicating that the requirement for Cdt1 degradation is cell type or cell cycle program specific. We further showed that upon stabilization of Cdt1 the ability of these ovary cells to progress normally through endocycles and develop depends on Geminin activity. During the course of this study, we found that absence of *Cul4* and *DDB1* function causes growth defects in developing larvae and proliferating imaginal disc cells. In our attempt to understand the role of CRL4 in growth, we discovered that CRL4 targets a potent inhibitor of growth, TSC2, for degradation, and thereby promotes growth (Chapter 2).

CRL4 in replication control – S2 cells

The *Drosophila* homolog of Cdt1, Doubleparked (Dup), is an essential protein for replication. *dup* null mutants develop until mid-embryogenesis, however once the maternal supply is depleted, *dup* null embryos arrest in S-phase 16 due to the inability to replicate DNA (Whittaker et al. 2000). Interestingly, these never-replicated cells continue to enter mitosis and arrest again. A similar phenotype occurs in yeast Cdt1 mutants (Hofmann and Beach 1994), indicating that unlike perturbations in replication or DNA damage, un-replicated DNA does not induce a checkpoint that prevents entry into mitosis.

In S2 cells, Geminin is the major inhibitor of Dup activity (Chapter 3): While Geminin depletion causes massive re-replication, Cul4 RNAi does not. The lack of re-replication in Cul4 RNAi samples is likely due to pleitropic effects that cause G1 arrest. However, in the event of Geminin depletion, degradation of Dup by CRL4 was crucial to limit the extent of re-replication. Experiments using mammalian cells demonstrated similar results, indicating that cells have developed a double-layered security system to monitor and regulate Cdt1/Dup activity. In other words, Cdt1 is auto-regulated: excessive Cdt1 activity triggers its own destruction.

This Cdt1 security system could be especially important in cells that have a disrupted normal Cdt1-Geminin ratio (Xouri et al. 2007; Tada 2007; Saxena and Dutta 2005). If there is not enough Geminin to inhibit Cdt1, Geminin's ability to inhibit Cdt1 is compromised and these cells risk the danger of re-replication. By quickly destroying Cdt1, cells can restrict re-replication. The model predicts that cells that lack re-replication induced DNA damage checkpoint activation cannot trigger Cdt1 destruction by CRL4, and thus would not be protected at the loss of Geminin activity.

Why would limiting re-replication, instead of inducing apoptosis, be advantageous to the cell? A recent study showed that proliferating Hela cells have re-replication events (Dorn and Cook 2008), suggesting that a small number of mistakes occur in origin control normally. These mistakes could be repaired while cells are arrested in a DNA damage-induced checkpoint. In this way, cells could manage small amounts of re-replication but must prevent further

damage that could be fatal by degrading Cdt1/Dup. This suggests that there are much more Geminin than Cdt1 around normally in the cell, since stabilizing Cdt1 often does not cause re-replication.

During this study, I also found that Cul4 depletion causes G1 arrest in S2 cells, suggesting that Cul4 regulates an additional substrate that is required for the G1-S transition. Also importantly, this G1 arrest may have precluded S phase Dup accumulation and subsequent re-replication. To answer these questions I turned to S phase CDK. CycE/CDK2 maintains many aspects of progression into and during S phase, which is antagonized by the CDK inhibitor Dacapo (Swanhart 2005). Since Dacapo was previously reported to be a substrate of Cul4, I tested whether the G1 arrest upon Cul4 knock-down was Dacapo-dependent by co-depletion of Cul4 and Dacapo. Indeed, this released the G1 arrest (also shown by Higa et al. 2006). However, releasing the G1 arrest still did not significantly increase the level of re-replication or Dup accumulation (data not shown). Together, the results strengthen the conclusion that CRL4 mediated Dup degradation is not an essential mechanism in S2 cells; and suggests that there must be another Dup degradation mechanism.

I tested the possibility of a redundant mechanism in Dup degradation by screening for additional ubiquitin ligases that target Dup. CRL1^{Skp2} has also been reported to degrade Cdt1 during S phase in mammalian cells (Nishtani et al 2006, Kondo et al. 2004, Li et al 2003). However, Cul1 depletion alone or along with Cul4 depletion did not significantly increase Dup levels (Fig 9). In addition, dsRNA treatment against *Drosophila* Cullins 1-5 did not show a significant

increase in Dup levels, suggesting that there is no one dominant Cullin responsible for Dup degradation in S2 cells. Importantly, APC^{Cdh1} was recently shown to interact with Cdt1 and mediate its destruction in mammalian cells (Sugimoto et al. 2008). Future screens that include additional E3 ligases, APC/C and HECT E3s, will test whether the APC/C mechanism is conserved and may elucidate novel regulators of Cdt1/Dup. In addition, another member of the pre-RC, Cdc6, is also degraded upon re-replication by Huwe1 ubiquitin ligases in human cells (Hall et al. 2008). This indicates that a redundant mechanism exists to regulate further origin licensing event as cells undergo re-replication. *Drosophila* has a conserved HUWE1 protein, CG8184. However, whether this mechanism is conserved in flies is not yet known.

The best way to test whether Dup degradation is essential for replication control would be to make stable versions of Dup and measure DNA replication profiles. I did not test this possibility in S2 cells, but rather in flies, as described in the following section.

CRL4 in replication control – Flies

The interpretation of RNAi data is complicated by the fact that RNAi is not a null situation. In other words, depleting Cullins and measuring Dup levels may be limited such that the remaining Cullin activity may sufficiently degrade Dup. To study the effect of having no Cullin function, I used previously generated null alleles of *Cul4* and *Ddb1,* an adaptor of Cul4 required for substrate recruitment.

Interestingly, *Cul4* and *Ddb1* mutant animals exhibited different growth defects with Cul4 mutants showing increased severity. While *Cul4* mutants died as 1st instar larvae, *Ddb1* mutants often survived through 2nd instar larvae. One may suspect that the observed differences between *Cul4* and *Ddb1* mutants are due to: 1) higher stability of DDB1 protein in comparison to that of Cul4; or 2) higher maternal load of DDB1 versus Cul4. We tested the first possibility by measuring the half-life of DDB1 and Cul4, and found that the DDB1 protein half-life is shorter than that of Cul4. Secondly, both *Cul4* and *Ddb1* mutant larvae lack detectable protein at the same larval stages. Thus it is unlikely that differences in Cul4 and Ddb1 mutants are simply due to the differences in protein availability.

In addition to differences in growth defects, *Cul4* mutant imaginal disc clones had significantly reduced rate of proliferation compared to *Ddb1* mutant cells. Similarly, Shibutani et al. (2008) found that while Cul4 RNAi stabilized E2F1, DDB1 RNAi did not. These results suggest the intriguing possibility that Cul4 has additional functions that exclude DDB1, although studies to date have established DDB1 as the sole adaptor for Cul4 (Jackson and Xiong 2009). Conversely, Cul4 independent functions of DDB1 have been identified: DDB1 associates with HECT ubiquitin ligase family without Cul4 or Roc proteins (Maddika 2009). In this complex, DDB1 interacts with two other proteins EED and VPRBP to mark a microtubule severing enzyme, Katanin for degradation. Importantly, a protein that has high sequence homology to DDB1 named SAP130 (Spliceosome-associated protein 130) was found to associate with Cul4 as well as Cul1 and 2 (Menon et al. 2008). Thus it seems possible that other proteins

139

could compensate for DDB1 function by serving as the adaptor for Cul4. There is one other protein that shares the same three-propeller structure as DDB1: CPSF160 (Cleavage and Polyadenylation Specificity Factor 160) (Li and Zheng 2006). Both SAP130 and CPSF160 are highly conserved in flies (CG 13900 and CG10110, respectively), therefore it will be interesting to test whether these proteins function in a complex with CRL4 as an adaptor, in regulating Dup stability.

S phase specific degradation of Dup is required in mitotic cells

To test the significance of Cdt1/Dup degradation in development directly, I engineered a mutant Dup that cannot be recognized by CRL4 (Chapter 3). In order for Dup to be recognized by CRL4, it must first bind PCNA through a conserved PCNA Interacting Peptide (PIP) motif in its N terminus (Arias and Walter 2006, Hu and Xiong 2006, Senga 2006, Higa 2006). Deleting the PIP motif did not affect Dup function in initiating DNA replication, but stabilized Dup in S phase. This stabilization, however, was only partial, further suggesting the possibility of an additional player in Dup degradation. A previous report indicated that CycE dependent phosphorylation increases steady-state levels of Dup (Thomer et al. 2004). However, a Dup mutant with changes in all 10 potential CycE phosphorylation sites to non-phosphorylatable alanines was degraded correctly at the onset of S phase. In addition, Dup mutated in both PIP motif and 10 phosphorylation sites also was not more stabilized than the PIP mutation alone. These results suggest that CycE dependent degradation does not play a

significant role in Dup regulation in developing flies, or that additional CycE phosphorylation sites exist. In mammalian cells, Dup is targeted by CRL1 in a phosphorylation-dependent manner (Nishitani et al. 2006). Interestingly, phosphorylation dependent Dup degradation mechanism was recently suggested to have evolved in higher eukaryotes (Kim and Kipreos 2007b). Our data supports this hypothesis. What causes degradation of Dup^{PIP} remains to be answered.

The consequence of replacing endogenous Cdt1/Dup with stabilized Cdt1/Dup has not been studied. I could rescue the replication defect of *dup* mutants using Dup^{PIP} in embryos, but these cells arrested in interphase. We determined this on three accounts: Dup^{PIP} expressing cells did not condense chromosomes, did not divide, and stained highly positive for cytoplasmic CycA. This study provides the first evidence that Dup^{PIP} can support DNA synthesis, but not the completion of the cell division cycle.

What could be the mechanism of this interphase arrest? One possibility is that stabilization of Dup^{PIP} in S phase leads to re-replication that triggers DNA damage checkpoint. Initiating replication multiple times at each origin can cause the replication forks to run into each other causing fork stalling and DNA break (reviews: de Bruin and Wittenberg 2009, Cook 2009, Willis and Rhind 2009). Another possibility is that instead of inducing re-replication, Dup^{PIP} could not support normal replication and the state of incomplete replication triggered activation of DNA replication checkpoint and arrest cells in G2/M. The resulting DNA damage activates the DNA dammage checkpoint, which manifest in multiple

ways: histone modifications, delay in the cell cycle progression, and sometimes apoptosis. However, I did not find evidence supporting activation of DNA damage checkpoint: 1) There was no increase in γ-H2AV staining. However, we cannot exclude the possibility that re-replication induced DNA damage is not detected using this antibody. 2) If the cells are simply delayed in the cell cycle progression, these cells eventually will progress into mitosis. However, I did not find any slow-dividing populations in later developmental stages, indicating that DUP^{PIP} cells are not simply delayed in the cell cycle. 3) There was no increase in cleaved-Caspase 3 in Dup^{PIP} expressing cells, indicating that they are not undergoing apoptosis.

Interestingly, Dup^{PIP} expression in wildtype embryos also blocked cells in interphase and remained undivided for multiple rounds of the cell cycle. This indicates that the Dup^{PIP} expression acts dominantly in interphase arrest and that cells are likely not simply arrested due to the inability to complete replication.

One way to directly test this checkpoint-G2/M arrest hypothesis is to inhibit with drugs or genetically reduce DNA damage checkpoint kinases, ATM/ATR. If the DNA damage checkpoint is responsible for blocking the cell cycle, relieving the checkpoint should allow cell cycle progression. Understanding the mechanisms involved in cell cycle arrest upon stabilization of Cdt1/Dup will better equip us to explore the consequences of Dup dependent re-replication in developing organisms.

Geminin inhibition rules in cells that do not divide

142

Stabilizing Dup in S phase prevented entry into mitosis, which led us to the question, what happens in a cell type that does not undergo mitosis? *Drosophila* oogenesis provides a good model to address this, as it contains cells undergoing endocycles; endocycles consist of alternating S and G phases without cell division (Lilly and Duronio 2005, Lee et al 2009). Intriguingly, Dup^{PIP} stabilization in ovary follicle cells did not block endocycle progression, suggesting that excess Dup in S phase only affects cells that are programmed to divide. In these cells, Geminin inhibition plays an important role in regulating stabilized Cdt1/Dup, and this regulation is required for normal oogenesis (Chapter 3).

A recent study showed that dividing cells are more sensitive to re-replication induced apoptosis than endocycling cells (Mehrotra 2008). One might speculate that accurate duplication of the genome is more important for cells that are programmed to divide to ensure that genome integrity is maintained in the daughter cells. Thus a mitotic cell may rely more on irreversible degradation of Cdt1/Dup to control the origins. However, in cells that do not divide, small amounts of re-replication do not pose a risk in passing on genetic instability and therefore might be tolerated. Furthermore, since endocycling cells replicate after one gap phase, their origins may need to be more poised to replicate than those of mitotic cells. Thus, using Geminin inhibition may provide a quick and reversible way to regulate Cdt1 in endocycling cells.

Studies have shown that cells in early development, like stem cells, have increased genome plasticity: they often overlook mistakes in replication and are less prone to inducing checkpoint activation. Thus early embryonic cells may

143

regulate Cdt1 similarly to endocycles. Interestingly, S2 cells are derived from early embryos and also rely more heavily on Geminin inhibition rather than Cdt1/Dup degradation (Chapter 3). In addition, since differentiated cells are no longer replicating, they may also be expected to tolerate Dup stability. Surprisingly, when I expressed Dup^{PIP} behind the morphogenetic furrow of the eye imaginal disc, cells that have differentiated exhibited a substantial increase in caspase3 activation (data not shown). This preliminary result suggests a curious possibility that differentiated cells are more sensitive to Dup stability. Differentiated cells are in quiescent phase in terms of cell cycle progression, and the potential outcome of re-initiating replication might be so deleterious that they developed cell death as a mechanism to control for that. Further studies will provide valuable insight into Dup regulation in proliferation versus differentiated cells.

If endocycles rely on Geminin inhibition of Dup, compromising Geminin activity should severely disable endocycle and ovary development. Indeed, I found that expressing Geminin shRNA in endocycling ovary cells completely demolished the tissue (Fig 12), suggesting that Dup degradation mechanism was not sufficient to support the endocycle. Similarly, this hypothesis (endocycles rely on Geminin inhibition) predicts that overwhelming Geminin inhibition by expressing high levels of Dup should also result in inhibition of normal endocycle progression. Unfortunately, I could not test this carefully, since there are limited number of truly ovary-specific drivers and using strong drivers cause early development lethality. However, expressing high levels of Dup^{PIP} in another

144

endocycling tissue, salivary glands, caused inhibition of endocycle progression. These cells exhibited reduced cell and nuclei sizes, suggesting that they have undergone limited rounds of replication, resulting in insufficient increase in ploidy (personal communication, N. Zielke). Salivary gland specific driver used here (*patched*-Gal4) expresses Dup^{PIP} in much higher levels than that used in ovaries. In fact, expressing Dup^{PIP} with this driver cause early larval lethality, which was overcome by simultaneous expression of apoptosis inhibitors. This supports the hypothesis that endocycling progression can be stalled from expressing Dup^{PIP} at high levels that overwhelms Geminin's ability to inhibit excess Dup. However, the best way to test this hypothesis directly would be to design Dup that cannot bind Geminin and test its effect in endocycling tissues. To do this, the regions of Dup that are required to bind Geminin must first be narrowed down, as the most recent Geminin binding region studied in *Xenopus* spans majority of Cdt1 protein (Sexana et al. 2004; Lee et al. 2004).

Cul4 in growth control

In an attempt to understand the growth defects associated with *Cul4* and *Ddb1* mutants, we discovered a novel target of CRL4, TSC2. TSC2 functions as an inhibitor of TORC1, a potent activator of cellular growth and proliferation (Huang and Manning 2008). What triggers TSC2 degaradation by Cul4? The rate of TSC2 turn-over is high, which may be a mechanism to reset various modifications on TSC2. TSC2 receives signals from multiple pathways that regulate cellular growth, through activating or inactivating phosphorylation events

145

(Crino 2006). In order to remain sensitive to multiple stimuli that affect growth, cells must be able to quickly re-modify TSC2. CRL4 mechanism may act as part of the recycling system to constantly degrade TSC2, or perhaps modified forms of TSC2. On the other hand, CRL4 may act directly downstream of one of the growth promoting pathways to inhibit TSC2.

While the slow-growth defects of *Ddb1* mutants could be alleviated by reducing *gigas*, the fly TSC2 homolog, their bristle defects were not rescued. Missing and thin bristle phenotypes observed in *Ddb1* mutants are often associated with growth. However, it is possible that instead, defects in bristle cell formation cause this phenotype. *Drosophila* bristles arise from a single precursor cell, which divides twice to form four types of cells (review: Tilney and De Rosier 2005). The initial asymmetric division determines the progeny's neural or structural fate; socket and bristle cells compose the visible structures. Bristle cell undergoes endocycles to become polyploid, which may aid in formation of strong actin bundle shaft. Thus bristle defect seen in *Ddb1* mutants may be due to perturbations in bristle cell fate specification, endocycle, or actin organization. Identifying additional targets of Cul4 in these pathways could elucidate the mechanism by which bristle defects occur.

Concluding Remarks

The results of this study demonstrate that the contribution of Geminin inhibition and Dup degradation varies depending on cell type. More specifically, cells that undergo mitotic cell cycles require timely destruction of Dup upon S

phase for normal cell cycle progression, while endocycling cells that are programmed not to divide do not require Dup degradation. Cdt1 over-activity has been proposed as a contributing factor to tumorigenesis, thus delineating the mechanism that arrests cells with excess Dup could provide valuable insight into preventing tumor growth. In addition, we found a novel substrate of Cul4, TSC2, which is required for normal growth control.

REFERENCES

Abbas, T., U. Sivaprasad, et al. (2008). "PCNA-dependent regulation of p21 ubiquitylation and degradation via the CRL4Cdt2 ubiquitin ligase complex." Genes Dev 22(18): 2496-506.

Adachi-Yamada, T. and M. B. O'Connor (2004). "Mechanisms for removal of developmentally abnormal cells: cell competition and morphogenetic apoptosis." J Biochem 136(1): 13-7.

Aggarwal, P., M. D. Lessie, et al. (2007). "Nuclear accumulation of cyclin D1 during S phase inhibits Cul4-dependent Cdt1 proteolysis and triggers p53-dependent DNA rereplication." Genes Dev 21(22): 2908-22.

Alexander, W. S., A. W. Roberts, et al. (1996). "Deficiencies in progenitor cells of multiple hematopoietic lineages and defective megakaryocytopoiesis in mice lacking the thrombopoietic receptor c-Mpl." Blood 87(6): 2162-70.

Anatskaya, O. V. and A. E. Vinogradov (2007). "Genome multiplication as adaptation to tissue survival: evidence from gene expression in mammalian heart and liver." Genomics 89(1): 70-80.

Ande, S. R., J. Chen, et al. (2009). "The ubiquitin pathway: an emerging drug target in cancer therapy." Eur J Pharmacol 625(1-3): 199-205.

Angchaisuksiri, P., P. L. Carlson, et al. (1994). "Replication and endoreplication in developing megakaryocytes in vitro." Exp Hematol 22(7): 546-50.

Angers, S., T. Li, et al. (2006). "Molecular architecture and assembly of the DDB1-CUL4A ubiquitin ligase machinery." Nature 443(7111): 590-3.

Araki, M., R. P. Wharton, et al. (2003). "Degradation of origin recognition complex large subunit by the anaphase-promoting complex in Drosophila." Embo J 22(22): 6115-26.

Araki, M., H. Yu, et al. (2005). "A novel motif governs APC-dependent degradation of Drosophila ORC1 in vivo." Genes Dev 19(20): 2458-65.

Ardley, H. C. (2009). "Ring finger ubiquitin protein ligases and their implication to the pathogenesis of human diseases." Curr Pharm Des 15(31): 3697-715.

Arentson, E., P. Faloon, et al. (2002). "Oncogenic potential of the DNA replication licensing protein CDT1." Oncogene 21(8): 1150-8.

Arias, E. E. and J. C. Walter (2004). "Initiation of DNA replication in Xenopus egg extracts." Front Biosci 9: 3029-45.

Arias, E. E. and J. C. Walter (2005). "Replication-dependent destruction of Cdt1 limits DNA replication to a single round per cell cycle in Xenopus egg extracts." Genes Dev 19(1): 114-26.

Arias, E. E. and J. C. Walter (2006). "PCNA functions as a molecular platform to trigger Cdt1 destruction and prevent re-replication." Nat Cell Biol 8(1): 84-90.

Arias, E. E. and J. C. Walter (2007). "Strength in numbers: preventing rereplication via multiple mechanisms in eukaryotic cells." Genes Dev 21(5): 497-518.

Asano, M. (2009). "Endoreplication: The advantage to initiating DNA replication without the ORC?" Fly (Austin) 3(2).

Asano, M. and R. P. Wharton (1999). "E2F mediates developmental and cell cycle regulation of ORC1 in Drosophila." Embo J 18(9): 2435-48.

Au, K. S., C. H. Ward, et al. (2008). "Tuberous sclerosis complex: disease modifiers and treatments." Curr Opin Pediatr 20(6): 628-33.

Axelrod, J. D., K. Matsuno, et al. (1996). "Interaction between Wingless and Notch signaling pathways mediated by dishevelled." Science 271(5257): 1826-32.

Ayala, F. J., W. M. Fitch, et al. (2000). "Variation and evolution in plants and microorganisms: toward a new synthesis 50 years after Stebbins." Proc Natl Acad Sci U S A 97(13): 6941-4.

Bai, X., D. Ma, et al. (2007). "Rheb activates mTOR by antagonizing its endogenous inhibitor, FKBP38." Science 318(5852): 977-80.

Balasubramanian, M. K., D. McCollum, et al. (1998). "Isolation and characterization of new fission yeast cytokinesis mutants." Genetics 149(3): 1265-75.

Ballabeni, A., M. Melixetian, et al. (2004). "Human geminin promotes pre-RC formation and DNA replication by stabilizing CDT1 in mitosis." Embo J 23(15): 3122-32.

Ballabeni, A., R. Zamponi, et al. (2009). "Human CDT1 associates with CDC7 and recruits CDC45 to chromatin during S phase." J Biol Chem 284(5): 3028-36.

Ballester, A., J. Frampton, et al. (2001). "Heterologous expression of the transcriptional regulator escargot inhibits megakaryocytic endomitosis." J Biol Chem 276(46): 43413-8.

Baroux, C., P. Fransz, et al. (2004). "Nuclear fusions contribute to polyploidization of the gigantic nuclei in the chalazal endosperm of Arabidopsis." Planta 220(1): 38-46.

Bastock, R. and D. St Johnston (2008). "Drosophila oogenesis." Curr Biol 18(23): R1082-7.

Bell, S. P. and A. Dutta (2002). "DNA replication in eukaryotic cells." Annu Rev Biochem 71: 333-74.

Benjamin, J. M., S. J. Torke, et al. (2004). "Geminin has dimerization, Cdt1-binding, and destruction domains that are required for biological activity." J Biol Chem 279(44): 45957-68.

Benvenuto, G., S. Li, et al. (2000). "The tuberous sclerosis-1 (TSC1) gene product hamartin suppresses cell growth and augments the expression of the TSC2 product tuberin by inhibiting its ubiquitination." Oncogene 19(54): 6306-16.

Bermejo, R., N. Vilaboa, et al. (2002). "Regulation of CDC6, geminin, and CDT1 in human cells that undergo polyploidization." Mol Biol Cell 13(11): 3989-4000.

Biswas, N., V. Sanchez, et al. (2003). "Human cytomegalovirus infection leads to accumulation of geminin and inhibition of the licensing of cellular DNA replication." J Virol 77(4): 2369-76.

Bjorklund, M., M. Taipale, et al. (2006). "Identification of pathways regulating cell size and cell-cycle progression by RNAi." Nature 439(7079): 1009-13.

Blow, J. J. and A. Dutta (2005). "Preventing re-replication of chromosomal DNA." Nat Rev Mol Cell Biol 6(6): 476.

Blow, J. J. and P. J. Gillespie (2008). "Replication licensing and cancer [mdash] a fatal entanglement?" Nat Rev Cancer 8(10): 799.

Blow, J. J. and B. Hodgson (2002). "Replication licensing -- Origin licensing: defining the proliferative state?" Trends in Cell Biology 12(2): 72.

Blow, J. J. and S. Tada (2000). "Cell cycle. A new check on issuing the licence." Nature 404(6778): 560-1.

Bondar, T., E. V. Mirkin, et al. (2003). "Schizosaccharomyces pombe Ddb1 is functionally linked to the replication checkpoint pathway." J Biol Chem 278(39): 37006-14.

Boos, A., A. Lee, et al. (2006). "Subcellular translocation signals regulate Geminin activity during embryonic development." Biol Cell 98(6): 363-75.

Borel, F., F. B. Lacroix, et al. (2002). "Prolonged arrest of mammalian cells at the G1/S boundary results in permanent S phase stasis." J Cell Sci 115(Pt 14): 2829-38.

Botchan, M. and M. Levine (2004). "A genome analysis of endoreplication in the Drosophila ovary." Dev Cell 6(1): 4-5.

Boudolf, V., T. Lammens, et al. (2009). "CDKB1;1 forms a functional complex with CYCA2;3 to suppress endocycle onset." Plant Physiol.

Boudolf, V., K. Vlieghe, et al. (2004). "The plant-specific cyclin-dependent kinase CDKB1;1 and transcription factor E2Fa-DPa control the balance of mitotically dividing and endoreduplicating cells in Arabidopsis." Plant Cell 16(10): 2683-92.

Britton, J. S. and B. A. Edgar (1998). "Environmental control of the cell cycle in Drosophila: nutrition activates mitotic and endoreplicative cells by distinct mechanisms." Development 125(11): 2149-58.

Britton, J. S., W. K. Lockwood, et al. (2002). "Drosophila's insulin/PI3-kinase pathway coordinates cellular metabolism with nutritional conditions." Dev Cell 2(2): 239-49.

Burnett, P. E., R. K. Barrow, et al. (1998). "RAFT1 phosphorylation of the translational regulators p70 S6 kinase and 4E-BP1." Proc Natl Acad Sci U S A 95(4): 1432-7.

Calvi, B. R., B. A. Byrnes, et al. (2007). "Conservation of epigenetic regulation, ORC binding and developmental timing of DNA replication origins in the genus Drosophila." Genetics 177(3): 1291-301.

Calvi, B. R., M. A. Lilly, et al. (1998). "Cell cycle control of chorion gene amplification." Genes Dev 12(5): 734-44.

Calvi, B. R. and A. C. Spradling (1999). "Chorion gene amplification in Drosophila: A model for metazoan origins of DNA replication and S-phase control." Methods 18(3): 407-17.

Cang, Y., J. Zhang, et al. (2006). "Deletion of DDB1 in mouse brain and lens leads to p53-dependent elimination of proliferating cells." Cell 127(5): 929-40.

Caro, E., B. Desvoyes, et al. (2008). "Endoreduplication control during plant development." SEB Exp Biol Ser 59: 167-87.

Caspi, N., I. Levin, et al. (2008). "A mutation in the tomato DDB1 gene affects cell and chloroplast compartment size and CDT1 transcript." Plant Signal Behav 3(9): 641-9.

Castellano Mdel, M., M. B. Boniotti, et al. (2004). "DNA replication licensing affects cell proliferation or endoreplication in a cell type-specific manner." Plant Cell 16(9): 2380-93.

Castellano, M. M., J. C. del Pozo, et al. (2001). "Expression and stability of Arabidopsis CDC6 are associated with endoreplication." Plant Cell 13(12): 2671-86.

Cebolla, A., J. M. Vinardell, et al. (1999). "The mitotic inhibitor ccs52 is required for endoreduplication and ploidy-dependent cell enlargement in plants." Embo J 18(16): 4476-84.

Chandhok, N. S. and D. Pellman (2009). "A little CIN may cost a lot: revisiting aneuploidy and cancer." Curr Opin Genet Dev 19(1): 74-81.

Chandrasekaran, V. and S. K. Beckendorf (2005). "Tec29 controls actin remodeling and endoreplication during invagination of the Drosophila embryonic salivary glands." Development 132(15): 3515-24.

Chen, C., Y. Liu, et al. (2008). "TSC-mTOR maintains quiescence and function of hematopoietic stem cells by repressing mitochondrial biogenesis and reactive oxygen species." J Exp Med 205(10): 2397-408.

Chen, S., M. A. de Vries, et al. (2007). "Orc6 is required for dynamic recruitment of Cdt1 during repeated Mcm2-7 loading." Genes Dev 21(22): 2897-907.

Chong-Kopera, H., K. Inoki, et al. (2006). "TSC1 stabilizes TSC2 by inhibiting the interaction between TSC2 and the HERC1 ubiquitin ligase." J Biol Chem 281(13): 8313-6.

Chou, D. M., P. Petersen, et al. (2002). "Protein phosphatase 2A regulates binding of Cdc45 to the prereplication complex." J Biol Chem 277(43): 40520-7.

Christensen, T. W. and B. K. Tye (2003). "Drosophila MCM10 interacts with members of the prereplication complex and is required for proper chromosome condensation." Mol Biol Cell 14(6): 2206-15.

Churchman, M. L., M. L. Brown, et al. (2006). "SIAMESE, a plant-specific cell cycle regulator, controls endoreplication onset in Arabidopsis thaliana." Plant Cell 18(11): 3145-57.

Clark, S. H. and A. Chovnick (1986). "Studies of normal and position-affected expression of rosy region genes in Drosophila melanogaster." Genetics 114(3): 819-40.

Clark, S. H., A. J. Hilliker, et al. (1986). "Genetic analysis of the right (3') end of the rosy locus in Drosophila melanogaster." Genet Res 47(2): 109-16.

Claycomb, J. M., D. M. MacAlpine, et al. (2002). "Visualization of replication initiation and elongation in Drosophila." J Cell Biol 159(2): 225-36.

Claycomb, J. M. and T. L. Orr-Weaver (2005). "Developmental gene amplification: insights into DNA replication and gene expression." Trends Genet 21(3): 149-62.

Collett, C. E., N. P. Harberd, et al. (2000). "Hormonal interactions in the control of Arabidopsis hypocotyl elongation." Plant Physiol 124(2): 553-62.

Cook, J. G. (2009). "Replication licensing and the DNA damage checkpoint." Front Biosci 14: 5013-30.

Cook, J. G., D. A. Chasse, et al. (2004). "The regulated association of Cdt1 with minichromosome maintenance proteins and Cdc6 in mammalian cells." J Biol Chem 279(10): 9625-33.

Cookson, S. J., A. Radziejwoski, et al. (2006). "Cell and leaf size plasticity in Arabidopsis: what is the role of endoreduplication?" Plant Cell Environ 29(7): 1273-1283.

Cortes, F., S. Mateos, et al. (2004). "Toward a comprehensive model for induced endoreduplication." Life Sci 76(2): 121-35.

Cross, J. C. (2000). "Genetic insights into trophoblast differentiation and placental morphogenesis." Semin Cell Dev Biol 11(2): 105-13.

Cross, J. C. (2005). "How to make a placenta: mechanisms of trophoblast cell differentiation in mice--a review." Placenta 26 Suppl A: S3-9.

Cross, J. C., M. Hemberger, et al. (2002). "Trophoblast functions, angiogenesis and remodeling of the maternal vasculature in the placenta." Mol Cell Endocrinol 187(1-2): 207-12.

D'Urso, G., B. Grallert, et al. (1995). "DNA polymerase alpha, a component of the replication initiation complex, is essential for the checkpoint coupling S phase to mitosis in fission yeast." J Cell Sci 108 (Pt 9): 3109-18.

Damiens, E., B. Baratte, et al. (2001). "Anti-mitotic properties of indirubin-3'-monoxime, a CDK/GSK-3 inhibitor: induction of endoreplication following prophase arrest." Oncogene 20(29): 3786-97.

Datta, N. S., J. L. Williams, et al. (1996). "Novel alterations in CDK1/cyclin B1 kinase complex formation occur during the acquisition of a polyploid DNA content." Mol Biol Cell 7(2): 209-23.

Davidson, I. F., A. Li, et al. (2006). "Deregulated replication licensing causes DNA fragmentation consistent with head-to-tail fork collision." Mol Cell 24(3): 433-43.

de Bruin, R. A., T. I. Kalashnikova, et al. (2006). "Constraining G1-specific transcription to late G1 phase: the MBF-associated corepressor Nrm1 acts via negative feedback." Mol Cell 23(4): 483-96.

De Marco, V., P. J. Gillespie, et al. (2009). "Quaternary structure of the human Cdt1-Geminin complex regulates DNA replication licensing." Proc Natl Acad Sci U S A 106(47): 19807-12.

de Nooij, J. C., K. H. Graber, et al. (2000). "Expression of the cyclin-dependent kinase inhibitor Dacapo is regulated by cyclin E." Mech Dev 97(1-2): 73-83.

Deak, P., M. M. Omar, et al. (1997). "P-element insertion alleles of essential genes on the third chromosome of Drosophila melanogaster: correlation of physical and cytogenetic maps in chromosomal region 86E-87F." Genetics 147(4): 1697-722.

Dearolf, C. R. (1998). "Fruit fly "leukemia"." Biochim Biophys Acta 1377(1): M13-23.

Debili, N., E. Hegyi, et al. (1991). "In vitro effects of hematopoietic growth factors on the proliferation, endoreplication, and maturation of human megakaryocytes." Blood 77(11): 2326-38.

DeGregori, J. and D. G. Johnson (2006). "Distinct and Overlapping Roles for E2F Family Members in Transcription, Proliferation and Apoptosis." Curr Mol Med 6(7): 739-48.

Dej, K. J. and A. C. Spradling (1999). "The endocycle controls nurse cell polytene chromosome structure during Drosophila oogenesis." Development 126(2): 293-303.

del Pozo, J. C., S. Diaz-Trivino, et al. (2006). "The balance between cell division and endoreplication depends on E2FC-DPB, transcription factors regulated by the ubiquitin-SCFSKP2A pathway in Arabidopsis." Plant Cell 18(9): 2224-35.

Deng, W. M., C. Althauser, et al. (2001). "Notch-Delta signaling induces a transition from mitotic cell cycle to endocycle in Drosophila follicle cells." Development 128(23): 4737-46.

Deshaies, R. J. and C. A. Joazeiro (2009). "RING domain E3 ubiquitin ligases." Annu Rev Biochem 78: 399-434.

Desvoyes, B., E. Ramirez-Parra, et al. (2006). "Cell type-specific role of the retinoblastoma/E2F pathway during Arabidopsis leaf development." Plant Physiol 140(1): 67-80.

Diaz-Trivino, S., M. del Mar Castellano, et al. (2005). "The genes encoding Arabidopsis ORC subunits are E2F targets and the two ORC1 genes are differently expressed in proliferating and endoreplicating cells." Nucleic Acids Res 33(17): 5404-14.

Dimitrova, D. S., T. A. Prokhorova, et al. (2002). "Mammalian nuclei become licensed for DNA replication during late telophase." J Cell Sci 115(Pt 1): 51-9.

Dimova, D. K. and N. J. Dyson (2005). "The E2F transcriptional network: old acquaintances with new faces." Oncogene 24(17): 2810-26.

Dominguez-Sola, D., C. Y. Ying, et al. (2007). "Non-transcriptional control of DNA replication by c-Myc." Nature 448(7152): 445-51.

Dorn, E. S., P. D. Chastain, 2nd, et al. (2009). "Analysis of re-replication from deregulated origin licensing by DNA fiber spreading." Nucleic Acids Res 37(1): 60-9.

Drachman, J. G., K. M. Millett, et al. (1999). "Thrombopoietin signal transduction requires functional JAK2, not TYK2." J Biol Chem 274(19): 13480-4.

Drury, L. S. and J. F. Diffley (2009). "Factors affecting the diversity of DNA replication licensing control in eukaryotes." Curr Biol 19(6): 530-5.

Du, W. (2000). "Suppression of the rbf null mutants by a de2f1 allele that lacks transactivation domain." Development 127(2): 367-379.

Du, W. (2003). "Endocycle and E2F-dependent transcriptional activation and repression." Cell Cycle 2(6): 515-6.

Du, W. and N. Dyson (1999). "The role of RBF in the introduction of G1 regulation during Drosophila embryogenesis." Embo J 18(4): 916-925.

Du, W., M. Vidal, et al. (1996). "RBF, a novel RB-related gene that regulates E2F activity and interacts with cyclin E in Drosophila." Genes Dev 10(10): 1206-1218.

Dudas, J., B. Saile, et al. (2003). "Endoreplication and polyploidy in primary culture of rat hepatic stellate cells." Cell Tissue Res 313(3): 301-11.

Duncan, A. W., R. D. Hickey, et al. (2009). "Ploidy reductions in murine fusion-derived hepatocytes." PLoS Genet 5(2): e1000385.

Duronio, R. J., P. C. Bonnette, et al. (1998). "Mutations of the Drosophila dDP, dE2F, and cyclin E genes reveal distinct roles for the E2F-DP transcription factor and cyclin E during the G1-S transition." Mol Cell Biol 18(1): 141-51.

Duronio, R. J., A. Brook, et al. (1996). "E2F-induced S phase requires cyclin E." Genes Dev 10(19): 2505-13.

Duronio, R. J. and P. H. O'Farrell (1995). "Developmental control of the G1 to S transition in Drosophila: cyclin Eis a limiting downstream target of E2F." Genes Dev 9(12): 1456-68.

Duronio, R. J., P. H. O'Farrell, et al. (1995). "The transcription factor E2F is required for S phase during Drosophila embryogenesis." Genes Dev 9(12): 1445-55.

Dye, B. T. and B. A. Schulman (2007). "Structural mechanisms underlying posttranslational modification by ubiquitin-like proteins." Annu Rev Biophys Biomol Struct 36: 131-50.

Ebbe, S. (1976). "Biology of megakaryocytes." Prog Hemost Thromb 3: 211-29.

Edgar, B. A. and T. L. Orr-Weaver (2001). "Endoreplication cell cycles: more for less." Cell 105(3): 297-306.

Edwards, M. C., A. V. Tutter, et al. (2002). "MCM2-7 complexes bind chromatin in a distributed pattern surrounding the origin recognition complex in Xenopus egg extracts." J Biol Chem 277(36): 33049-57.

Eigsti, O. J. (1938). "A Cytological Study of Colchicine Effects in the Induction of Polyploidy in Plants." Proc Natl Acad Sci U S A 24(2): 56-63.

El-Hashash, A. H., P. Esbrit, et al. (2005). "PTHrP promotes murine secondary trophoblast giant cell differentiation through induction of endocycle, upregulation

of giant-cell-promoting transcription factors and suppression of other trophoblast cell types." Differentiation 73(4): 154-74.

Engelen-Eigles, G., G. Holden, et al. (2006). "The Effect of Temperature, Photoperiod, and Light Quality on Gluconasturtiin Concentration in Watercress (Nasturtium officinale R. Br.)." Journal of Agricultural and Food Chemistry 54(2): 328-334.

Engelen-Eigles, G., R. J. Jones, et al. (2001). "DNA Endoreduplication in Maize Endosperm Cells is Reduced by High Temperature During the Mitotic Phase." Crop Sci 41(4): 1114-1121.

Erenpreisa, J. and M. S. Cragg (2007). "Cancer: a matter of life cycle?" Cell Biol Int 31(12): 1507-10.

Erenpreisa, J., M. S. Cragg, et al. (2009). "The role of meiotic cohesin REC8 in chromosome segregation in gamma irradiation-induced endopolyploid tumour cells." Exp Cell Res.

Erenpreisa, J., M. Kalejs, et al. (2005). "Mitotic catastrophe and endomitosis in tumour cells: an evolutionary key to a molecular solution." Cell Biol Int 29(12): 1012-8.

Erenpreisa, J., M. Kalejs, et al. (2005). "Segregation of genomes in polyploid tumour cells following mitotic catastrophe." Cell Biol Int 29(12): 1005-11.

Espinosa, L., J. Ingles-Esteve, et al. (2003). "Phosphorylation by glycogen synthase kinase-3 beta down-regulates Notch activity, a link for Notch and Wnt pathways." J Biol Chem 278(34): 32227-35.

Evrin, C., P. Clarke, et al. (2009). "A double-hexameric MCM2-7 complex is loaded onto origin DNA during licensing of eukaryotic DNA replication." Proc Natl Acad Sci U S A 106(48): 20240-5.

Eward, K. L., E. C. Obermann, et al. (2004). "DNA replication licensing in somatic and germ cells." J Cell Sci 117(Pt 24): 5875-86.

Feger, G., H. Vaessin, et al. (1995). "dpa, a member of the MCM family, is required for mitotic DNA replication but not endoreplication in Drosophila." Embo J 14(21): 5387-98.

Feldman, R. M., C. C. Correll, et al. (1997). "A complex of Cdc4p, Skp1p, and Cdc53p/cullin catalyzes ubiquitination of the phosphorylated CDK inhibitor Sic1p." Cell 91(2): 221-30.

Feng, H. and E. T. Kipreos (2003). "Preventing DNA re-replication--divergent safeguards in yeast and metazoa." Cell Cycle 2(5): 431-4.

Ferenbach, A., A. Li, et al. (2005). "Functional domains of the Xenopus replication licensing factor Cdt1." Nucleic Acids Res 33(1): 316-24.

Fingar, D. C., C. J. Richardson, et al. (2004). "mTOR controls cell cycle progression through its cell growth effectors S6K1 and 4E-BP1/eukaryotic translation initiation factor 4E." Mol Cell Biol 24(1): 200-16.

Fingar, D. C., S. Salama, et al. (2002). "Mammalian cell size is controlled by mTOR and its downstream targets S6K1 and 4EBP1/eIF4E." Genes Dev 16(12): 1472-87.

Finkin, S., Y. Aylon, et al. (2008). "Fbw7 regulates the activity of endoreduplication mediators and the p53 pathway to prevent drug-induced polyploidy." Oncogene 27(32): 4411-21.

Flemming, A. J., Z. Z. Shen, et al. (2000). "Somatic polyploidization and cellular proliferation drive body size evolution in nematodes." Proc Natl Acad Sci U S A 97(10): 5285-90.

Follette, P. J., R. J. Duronio, et al. (1998). "Fluctuations in cyclin E levels are required for multiple rounds of endocycle S phase in Drosophila." Curr Biol 8(4): 235-8.

Foltz, D. R., M. C. Santiago, et al. (2002). "Glycogen synthase kinase-3beta modulates notch signaling and stability." Curr Biol 12(12): 1006-11.

Francis, D. (2007). "The plant cell cycle--15 years on." New Phytol 174(2): 261-78.

Frolov, M. V., D. S. Huen, et al. (2001). "Functional antagonism between E2F family members." Genes Dev 15(16): 2146-60.

Frolov, M. V., N. S. Moon, et al. (2005). "dDP is needed for normal cell proliferation." Mol Cell Biol 25(8): 3027-39.

Fujita, M. (2006). "Cdt1 revisited: complex and tight regulation during the cell cycle and consequences of deregulation in mammalian cells." Cell Div 1: 22.

Funk-Keenan, J., F. Haire, et al. (2008). "Hepatic endopolyploidy as a cellular consequence of age-specific selection for rate of development in mice." J Exp Zoolog B Mol Dev Evol 310(5): 385-97.

Furukawa, M., T. Ohta, et al. (2002). "Activation of UBC5 ubiquitin-conjugating enzyme by the RING finger of ROC1 and assembly of active ubiquitin ligases by all cullins." J Biol Chem 277(18): 15758-65.

Furukawa, M., Y. Zhang, et al. (2000). "The CUL1 C-terminal sequence and ROC1 are required for efficient nuclear accumulation, NEDD8 modification, and ubiquitin ligase activity of CUL1." Mol Cell Biol 20(21): 8185-97.

Fuse, N., S. Hirose, et al. (1994). "Diploidy of Drosophila imaginal cells is maintained by a transcriptional repressor encoded by escargot." Genes Dev 8(19): 2270-81.

Galbraith, D. W., K. R. Harkins, et al. (1991). "Systemic Endopolyploidy in Arabidopsis thaliana." Plant Physiol. 96(3): 985-989.

Gandarillas, A., D. Davies, et al. (2000). "Normal and c-Myc-promoted human keratinocyte differentiation both occur via a novel cell cycle involving cellular growth and endoreplication." Oncogene 19(29): 3278-89.

Ganier, O. and M. Mechali (2008). "New cell or new cycle?" Genes & Development 22(21): 2908-2913.

Gao, J., H. X. Ma, et al. (2006). "The association of polymorphisms of CDT1 and GMNN gene with the risk of breast cancer in Chinese women: a case-control analysis." Zhonghua Yi Xue Yi Chuan Xue Za Zhi 23(5): 544-7.

Gao, X. and D. Pan (2001). "TSC1 and TSC2 tumor suppressors antagonize insulin signaling in cell growth." Genes Dev 15(11): 1383-92.

Garcia, P. and C. Cales (1996). "Endoreplication in megakaryoblastic cell lines is accompanied by sustained expression of G1/S cyclins and downregulation of cdc25C." Oncogene 13(4): 695-703.

Garcia, P., J. Frampton, et al. (2000). "Ectopic expression of cyclin E allows non-endomitotic megakaryoblastic K562 cells to establish re-replication cycles." Oncogene 19(14): 1820-33.

Garner, M., S. van Kreeveld, et al. (2001). "mei-41 and bub1 block mitosis at two distinct steps in response to incomplete DNA replication in Drosophila embryos." Curr Biol 11(20): 1595-9.

Gendreau, E., H. Hofte, et al. (1998). "Phytochrome controls the number of endoreduplication cycles in the Arabidopsis thaliana hypocotyl." Plant J 13(2): 221-30.

Gendreau, E., V. Orbovic, et al. (1999). "Gibberellin and ethylene control endoreduplication levels in the Arabidopsis thaliana hypocotyl." Planta 209(4): 513-6.

Geng, Y., Q. Yu, et al. (2003). "Cyclin E ablation in the mouse." Cell 114(4): 431-43.

Gilbert, S. F. (2000). Developmental Biology, Sinauer Associates Inc.

Gillespie, P. J., A. Li, et al. (2001). "Reconstitution of licensed replication origins on Xenopus sperm nuclei using purified proteins." BMC Biochem 2: 15.

Gingras, A. C., B. Raught, et al. (2001). "Regulation of translation initiation by FRAP/mTOR." Genes Dev 15(7): 807-26.

Glozak, M. A. and E. Seto (2009). "Acetylation/deacetylation modulates the stability of DNA replication licensing factor Cdt1." J Biol Chem 284(17): 11446-53.

Goldman, S. J. (1995). "Preclinical biology of interleukin 11: a multifunctional hematopoietic cytokine with potent thrombopoietic activity." Stem Cells 13(5): 462-71.

Gonzalez, M. A., K. E. Tachibana, et al. (2006). "Geminin is essential to prevent endoreduplication and to form pluripotent cells during mammalian development." Genes Dev 20(14): 1880-4.

Gonzalez, M. A., K. E. Tachibana, et al. (2004). "Geminin predicts adverse clinical outcome in breast cancer by reflecting cell-cycle progression." J Pathol 204(2): 121-30.

Gordon, W. R., K. L. Arnett, et al. (2008). "The molecular logic of Notch signaling--a structural and biochemical perspective." J Cell Sci 121(Pt 19): 3109-19.

Green, C. M. (2006). "One ring to rule them all? Another cellular responsibility for PCNA." Trends Mol Med 12(10): 455-8.

Guertin, D. A. and D. M. Sabatini (2007). "Defining the role of mTOR in cancer." Cancer Cell 12(1): 9-22.

Guo, B., J. Romero, et al. (2005). "High levels of Cdc7 and Dbf4 proteins can arrest cell-cycle progression." Eur J Cell Biol 84(12): 927-38.

Gurney, A. L., K. Carver-Moore, et al. (1994). "Thrombocytopenia in c-mpl-deficient mice." Science 265(5177): 1445-7.

Hall, J. R., H. O. Lee, et al. (2008). "Cdt1 and Cdc6 are destabilized by rereplication-induced DNA damage." J Biol Chem 283(37): 25356-63.

Hammond, M. P. and C. D. Laird (1985). "Chromosome structure and DNA replication in nurse and follicle cells of Drosophila melanogaster." Chromosoma 91(3-4): 267-78.

Hara, K., K. I. Nakayama, et al. (2006). "Geminin is essential for the development of preimplantation mouse embryos." Genes Cells 11(11): 1281-93.

Hase, Y., S. Fujioka, et al. (2005). "Ectopic endoreduplication caused by sterol alteration results in serrated petals in Arabidopsis." J Exp Bot 56(414): 1263-8.

Hattori, N., T. C. Davies, et al. (2000). "Periodic expression of the cyclin-dependent kinase inhibitor p57(Kip2) in trophoblast giant cells defines a G2-like gap phase of the endocycle." Mol Biol Cell 11(3): 1037-45.

Havens, C. G. and J. C. Walter (2009). "Docking of a specialized PIP Box onto chromatin-bound PCNA creates a degron for the ubiquitin ligase CRL4Cdt2." Mol Cell 35(1): 93-104.

Hay, N. and N. Sonenberg (2004). "Upstream and downstream of mTOR." Genes Dev 18(16): 1926-45.

Hayashi, S. (1996). "A Cdc2 dependent checkpoint maintains diploidy in Drosophila." Development 122(4): 1051-8.

Hayashi, S. (1996). "[Drosophila imaginal development and the escargot gene]." Tanpakushitsu Kakusan Koso 41(8 Suppl): 1095-103.

Hayashi, S., S. Hirose, et al. (1993). "Control of imaginal cell development by the escargot gene of Drosophila." Development 118(1): 105-15.

He, Y. J., C. M. McCall, et al. (2006). "DDB1 functions as a linker to recruit receptor WD40 proteins to CUL4-ROC1 ubiquitin ligases." Genes Dev 20(21): 2949-54.

Hedgecock, E. M. and J. G. White (1985). "Polyploid tissues in the nematode Caenorhabditis elegans." Dev Biol 107(1): 128-33.

Hegyi, E., S. Navarro, et al. (1990). "Regulation of human megakaryocytopoiesis: analysis of proliferation, ploidy and maturation in liquid cultures." Int J Cell Cloning 8(4): 236-44.

Hemann, M. T., K. L. Rudolph, et al. (2001). "Telomere dysfunction triggers developmentally regulated germ cell apoptosis." Mol Biol Cell 12(7): 2023-30.

Hendzel, M. J., Y. Wei, et al. (1997). "Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation." Chromosoma 106(6): 348-60.

Herbig, U., W. A. Jobling, et al. (2004). "Telomere shortening triggers senescence of human cells through a pathway involving ATM, p53, and p21(CIP1), but not p16(INK4a)." Mol Cell 14(4): 501-13.

Higa, L. A., D. Banks, et al. (2006). "L2DTL/CDT2 interacts with the CUL4/DDB1 complex and PCNA and regulates CDT1 proteolysis in response to DNA damage." Cell Cycle 5(15): 1675-80.

Higa, L. A., I. S. Mihaylov, et al. (2003). "Radiation-mediated proteolysis of CDT1 by CUL4-ROC1 and CSN complexes constitutes a new checkpoint." Nat Cell Biol 5(11): 1008-15.

Higa, L. A., M. Wu, et al. (2006). "CUL4-DDB1 ubiquitin ligase interacts with multiple WD40-repeat proteins and regulates histone methylation." Nat Cell Biol 8(11): 1277-83.

Higa, L. A., X. Yang, et al. (2006). "Involvement of CUL4 ubiquitin E3 ligases in regulating CDK inhibitors Dacapo/p27Kip1 and cyclin E degradation." Cell Cycle 5(1): 71-7.

Higa, L. A. and H. Zhang (2007). "Stealing the spotlight: CUL4-DDB1 ubiquitin ligase docks WD40-repeat proteins to destroy." Cell Div 2: 5.

Hilliker, A. J. and A. Chovnick (1981). "Further observations on intragenic recombination in Drosophila melanogaster." Genet Res 38(3): 281-96.

Hilliker, A. J., S. H. Clark, et al. (1980). "Cytogenetic analysis of the chromosomal region immediately adjacent to the rosy locus in Drosophila melanogaster." Genetics 95(1): 95-110.

Hirose, F., M. Yamaguchi, et al. (1999). "Targeted expression of the DNA binding domain of DRE-binding factor, a Drosophila transcription factor, attenuates DNA replication of the salivary gland and eye imaginal disc." Mol Cell Biol 19(9): 6020-8.

Hochegger, H., D. Dejsuphong, et al. (2007). "An essential role for Cdk1 in S phase control is revealed via chemical genetics in vertebrate cells." J Cell Biol 178(2): 257-68.

Hodges, A. K., S. Li, et al. (2001). "Pathological mutations in TSC1 and TSC2 disrupt the interaction between hamartin and tuberin." Hum Mol Genet 10(25): 2899-905.

Hodgson, B., A. Li, et al. (2002). "Geminin becomes activated as an inhibitor of Cdt1/RLF-B following nuclear import." Curr Biol 12(8): 678-83.

Hofmann, J. F. and D. Beach (1994). "cdt1 is an essential target of the Cdc10/Sct1 transcription factor: requirement for DNA replication and inhibition of mitosis." Embo J 13(2): 425-34.

Hong, A., K. Narbonne-Reveau, et al. (2007). "The cyclin-dependent kinase inhibitor Dacapo promotes replication licensing during Drosophila endocycles." Embo J 26(8): 2071-82.

Hoppe, T. (2005). "Multiubiquitylation by E4 enzymes: 'one size' doesn't fit all." Trends Biochem Sci 30(4): 183-7.

Hu, J., C. M. McCall, et al. (2004). "Targeted ubiquitination of CDT1 by the DDB1-CUL4A-ROC1 ligase in response to DNA damage." Nat Cell Biol 6(10): 1003-9.

Hu, J. and Y. Xiong (2006). "An evolutionarily conserved function of proliferating cell nuclear antigen for Cdt1 degradation by the Cul4-Ddb1 ubiquitin ligase in response to DNA damage." J Biol Chem 281(7): 3753-6.

Hu, J., S. Zacharek, et al. (2008). "WD40 protein FBW5 promotes ubiquitination of tumor suppressor TSC2 by DDB1-CUL4-ROC1 ligase." Genes Dev 22(7): 866-71.

Huang, J. and B. D. Manning (2008). "The TSC1-TSC2 complex: a molecular switchboard controlling cell growth." Biochem J 412(2): 179-90.

Huang, J. and B. D. Manning (2009). "A complex interplay between Akt, TSC2 and the two mTOR complexes." Biochem Soc Trans 37(Pt 1): 217-22.

Hulskamp, M., A. Schnittger, et al. (1999). "Pattern formation and cell differentiation: trichomes in Arabidopsis as a genetic model system." Int Rev Cytol 186: 147-78.

Ianzini, F., E. A. Kosmacek, et al. (2009). "Activation of Meiosis-Specific Genes Is Associated with Depolyploidization of Human Tumor Cells following Radiation-Induced Mitotic Catastrophe." Cancer Res 69(6): 2296-2304.

Ihara, K., E. Ishii, et al. (1999). "Identification of mutations in the c-mpl gene in congenital amegakaryocytic thrombocytopenia." Proc Natl Acad Sci U S A 96(6): 3132-6.

Iida, T. and M. A. Lilly (2004). "missing oocyte encodes a highly conserved nuclear protein required for the maintenance of the meiotic cycle and oocyte identity in Drosophila." Development 131(5): 1029-39.

Imai, K. K., Y. Ohashi, et al. (2006). "The A-type cyclin CYCA2;3 is a key regulator of ploidy levels in Arabidopsis endoreduplication." Plant Cell 18(2): 382-96.

Inoki, K. and K. L. Guan (2009). "Tuberous sclerosis complex, implication from a rare genetic disease to common cancer treatment." Hum Mol Genet 18(R1): R94-100.

Inoki, K., Y. Li, et al. (2003). "Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling." Genes Dev 17(15): 1829-34.

Inoki, K., Y. Li, et al. (2002). "TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling." Nat Cell Biol 4(9): 648-57.

Inze, D. and L. De Veylder (2006). "Cell cycle regulation in plant development." Annu Rev Genet 40: 77-105.

Inze, D. and L. D. Veylder (2006). "Cell Cycle Regulation in Plant Development." Annu Rev Genet.

Ivanov, A., M. S. Cragg, et al. (2003). "Endopolyploid cells produced after severe genotoxic damage have the potential to repair DNA double strand breaks." J Cell Sci 116(Pt 20): 4095-106.

Jackson, P. K., S. Chevalier, et al. (1995). "Early events in DNA replication require cyclin E and are blocked by p21CIP1." J Cell Biol 130(4): 755-769.

Jackson, S. and Y. Xiong (2009). "CRL4s: the CUL4-RING E3 ubiquitin ligases." Trends Biochem Sci 34(11): 562-70.

Jakoby, M. and A. Schnittger (2004). "Cell cycle and differentiation." Current Opinion in Plant Biology 7(6): 661.

Jiang, J. and G. Struhl (1998). "Regulation of the Hedgehog and Wingless signalling pathways by the F-box/WD40-repeat protein Slimb." Nature 391(6666): 493-6.

Jin, J., E. E. Arias, et al. (2006). "A family of diverse Cul4-Ddb1-interacting proteins includes Cdt2, which is required for S phase destruction of the replication factor Cdt1." Mol Cell 23(5): 709-21.

Jordan, K. C., V. Schaeffer, et al. (2006). "Notch signaling through tramtrack bypasses the mitosis promoting activity of the JNK pathway in the mitotic-to-endocycle transition of Drosophila follicle cells." BMC Dev Biol 6: 16.

Joubes, J. and C. Chevalier (2000). "Endoreduplication in higher plants." Plant Mol Biol 43(5-6): 735-45.

Kandt, R. S., J. L. Haines, et al. (1992). "Linkage of an important gene locus for tuberous sclerosis to a chromosome 16 marker for polycystic kidney disease." Nat Genet 2(1): 37-41.

Karakaidos, P., S. Taraviras, et al. (2004). "Overexpression of the replication licensing regulators hCdt1 and hCdc6 characterizes a subset of non-small-cell lung carcinomas: synergistic effect with mutant p53 on tumor growth and chromosomal instability--evidence of E2F-1 transcriptional control over hCdt1." Am J Pathol 165(4): 1351-65.

Kaushansky, K. (1995). "Thrombopoietin: basic biology, clinical promise." Int J Hematol 62(1): 7-15.

Kaushansky, K. (2005). "The molecular mechanisms that control thrombopoiesis." J Clin Invest 115(12): 3339-47.

Kaushansky, K. (2008). "Historical review: megakaryopoiesis and thrombopoiesis." Blood 111(3): 981-6.

Kaushansky, K., S. Lok, et al. (1994). "Promotion of megakaryocyte progenitor expansion and differentiation by the c-Mpl ligand thrombopoietin." Nature 369(6481): 568-71.

Kawasaki, Y., H. D. Kim, et al. (2006). "Reconstitution of Saccharomyces cerevisiae prereplicative complex assembly in vitro." Genes Cells 11(7): 745-56.

Kelly, T. J., G. S. Martin, et al. (1993). "The fission yeast cdc18+ gene product couples S phase to START and mitosis." Cell 74(2): 371-82.

Kerns, S. L., S. J. Torke, et al. (2007). "Geminin prevents rereplication during xenopus development." J Biol Chem 282(8): 5514-21.

Khayrutdinov, B. I., W. J. Bae, et al. (2009). "Structure of the Cdt1 C-terminal domain: conservation of the winged helix fold in replication licensing factors." Protein Sci 18(11): 2252-64.

Kikuchi, J., Y. Furukawa, et al. (1997). "Polyploidization and functional maturation are two distinct processes during megakaryocytic differentiation: involvement of cyclin-dependent kinase inhibitor p21 in polyploidization." Blood 89(11): 3980-90.

Kim, J., H. Feng, et al. (2007). "C. elegans CUL-4 prevents rereplication by promoting the nuclear export of CDC-6 via a CKI-1-dependent pathway." Curr Biol 17(11): 966-72.

Kim, J. and E. T. Kipreos (2008). "Control of the Cdc6 replication licensing factor in metazoa: the role of nuclear export and the CUL4 ubiquitin ligase." Cell Cycle 7(2): 146-50.

Kim, K. J., V. Tran, et al. (2009). "Mechanism of an Endocycle." submitted.

Kim, S. H. and W. M. Michael (2008). "Regulated proteolysis of DNA polymerase eta during the DNA-damage response in C. elegans." Mol Cell 32(6): 757-66.

Kim, Y. and E. T. Kipreos (2007). "The Caenorhabditis elegans replication licensing factor CDT-1 is targeted for degradation by the CUL-4/DDB-1 complex." Mol Cell Biol 27(4): 1394-406.

Kim, Y. and E. T. Kipreos (2007). "Cdt1 degradation to prevent DNA re-replication: conserved and non-conserved pathways." Cell Div 2: 18.

Kim, Y., N. G. Starostina, et al. (2008). "The CRL4Cdt2 ubiquitin ligase targets the degradation of p21Cip1 to control replication licensing." Genes Dev 22(18): 2507-19.

Kipreos, E. T., L. E. Lander, et al. (1996). "cul-1 is required for cell cycle exit in C. elegans and identifies a novel gene family." Cell 85(6): 829-39.

Klapholz, B., B. H. Dietrich, et al. (2009). "CAF-1 is required for efficient replication of euchromatic DNA in Drosophila larval endocycling cells." Chromosoma 118(2): 235-48.

Kleiger, G., A. Saha, et al. (2009). "Rapid E2-E3 assembly and disassembly enable processive ubiquitylation of cullin-RING ubiquitin ligase substrates." Cell 139(5): 957-68.

Knoblich, J. A., K. Sauer, et al. (1994). "Cyclin E controls S phase progression and its down-regulation during Drosophila embryogenesis is required for the arrest of cell proliferation." Cell 77(1): 107-120.

Koepp, D. M., L. K. Schaefer, et al. (2001). "Phosphorylation-dependent ubiquitination of cyclin E by the SCFFbw7 ubiquitin ligase." Science 294(5540): 173-7.

Kohn, M. J., R. T. Bronson, et al. (2003). "Dp1 is required for extra-embryonic development." Development 130(7): 1295-305.

Kolesnikova, T. D., I. V. Makunin, et al. (2005). "Functional dissection of the Suppressor of UnderReplication protein of Drosophila melanogaster: identification of domains influencing chromosome binding and DNA replication." Genetica 124(2-3): 187-200.

Komander, D. (2009). "The emerging complexity of protein ubiquitination." Biochem Soc Trans 37(Pt 5): 937-53.

Kondo, T., M. Kobayashi, et al. (2004). "Rapid degradation of Cdt1 upon UV-induced DNA damage is mediated by SCFSkp2 complex." J Biol Chem 279(26): 27315-9.

Kondorosi, E. and A. Kondorosi (2004). "Endoreduplication and activation of the anaphase-promoting complex during symbiotic cell development." FEBS Lett 567(1): 152-7.

Kondorosi, E., F. Roudier, et al. (2000). "Plant cell-size control: growing by ploidy?" Current Opinion in Plant Biology 3(6): 488.

Kulartz, M. and R. Knippers (2004). "The replicative regulator protein geminin on chromatin in the HeLa cell cycle." J Biol Chem 279(40): 41686-94.

Kuran, H. (1999). "Senescence vs. changes in nuclear DNA, protein and dry mass contents in leaf mesophyll of two species differing in occurrence of endomitotic polyploidy." Folia Histochem Cytobiol 37(1): 39-47.

Kwong, Y. L., E. P. Robertson, et al. (1993). "Dual-color flow cytometric analysis of megakaryocytic DNA ploidy in the investigation of blastic phase chronic myelogenous leukemia with rearrangement of 3q26." Cancer 71(12): 3882-7.

Lammens, T., V. Boudolf, et al. (2008). "Atypical E2F activity restrains APC/CCCS52A2 function obligatory for endocycle onset." Proc Natl Acad Sci U S A 105(38): 14721-6.

Lammens, T., J. Li, et al. (2009). "Atypical E2Fs: new players in the E2F transcription factor family." Trends Cell Biol 19(3): 111-8.

Lanzotti, D. J., J. M. Kupsco, et al. (2004). "string(cdc25) and cyclin E are required for patterned histone expression at different stages of Drosophila embryonic development." Dev Biol 274(1): 82-93.

Lau, E., T. Tsuji, et al. (2007). "The role of pre-replicative complex (pre-RC) components in oncogenesis." FASEB J. 21(14): 3786-3794.

Lazzerini Denchi, E., G. Celli, et al. (2006). "Hepatocytes with extensive telomere deprotection and fusion remain viable and regenerate liver mass through endoreduplication." Genes Dev 20(19): 2648-53.

Leach, T. J., H. L. Chotkowski, et al. (2000). "Replication of heterochromatin and structure of polytene chromosomes." Mol Cell Biol 20(17): 6308-16.

Lee, C., B. Hong, et al. (2004). "Structural basis for inhibition of the replication licensing factor Cdt1 by geminin." Nature 430(7002): 913-7.

Lee, H. O., J. M. Davidson, et al. (2009). "Endoreplication: polyploidy with purpose." Genes Dev 23(21): 2461-77.

Lehman, N. L. (2009). "The ubiquitin proteasome system in neuropathology." Acta Neuropathol 118(3): 329-47.

Lehner, C. F. and P. H. O'Farrell (1989). "Expression and function of Drosophila cyclin A during embryonic cell cycle progression." Cell 56(6): 957-68.

Lehner, C. F. and P. H. O'Farrell (1990). "The roles of Drosophila cyclins A and B in mitotic control." Cell 61(3): 535-47.

Lei, M. and B. K. Tye (2001). "Initiating DNA synthesis: from recruiting to activating the MCM complex." J Cell Sci 114(Pt 8): 1447-54.

Leiva-Neto, J. T., G. Grafi, et al. (2004). "A dominant negative mutant of cyclin-dependent kinase A reduces endoreduplication but not cell size or gene expression in maize endosperm." Plant Cell 16(7): 1854-69.

Lendahl, U. (2008). "[Dedifferentiation of specialized cells. New way open for cell therapy and regenerative medicine]." Lakartidningen 105(20): 1492-4.

Li, A. and J. J. Blow (2005). "Cdt1 downregulation by proteolysis and geminin inhibition prevents DNA re-replication in Xenopus." Embo J 24(2): 395-404.

Li, J., C. Ran, et al. (2008). "Synergistic function of E2F7 and E2F8 is essential for cell survival and embryonic development." Dev Cell 14(1): 62-75.

Li, T., X. Chen, et al. (2006). "Structure of DDB1 in complex with a paramyxovirus V protein: viral hijack of a propeller cluster in ubiquitin ligase." Cell 124(1): 105-17.

Li, X., Q. Zhao, et al. (2003). "The SCF(Skp2) ubiquitin ligase complex interacts with the human replication licensing factor Cdt1 and regulates Cdt1 degradation." J Biol Chem 278(33): 30854-8.

Lilly, M. A. and R. J. Duronio (2005). "New insights into cell cycle control from the Drosophila endocycle." Oncogene 24(17): 2765-75.

Lilly, M. A. and A. C. Spradling (1996). "The Drosophila endocycle is controlled by Cyclin E and lacks a checkpoint ensuring S-phase completion." Genes Dev 10(19): 2514-26.

Lin, H. C., J. T. Wu, et al. (2009). "Cul4 and DDB1 regulate Orc2 localization, BrdU incorporation and Dup stability during gene amplification in Drosophila follicle cells." J Cell Sci 122(Pt 14): 2393-401.

Liontos, M., M. Koutsami, et al. (2007). "Deregulated overexpression of hCdt1 and hCdc6 promotes malignant behavior." Cancer Res 67(22): 10899-909.

Liu, C., K. A. Powell, et al. (2003). "Cop9/signalosome subunits and Pcu4 regulate ribonucleotide reductase by both checkpoint-dependent and -independent mechanisms." Genes Dev 17(9): 1130-40.

Liu, E., A. Y. Lee, et al. (2007). "The ATR-mediated S phase checkpoint prevents rereplication in mammalian cells when licensing control is disrupted." J Cell Biol 179(4): 643-57.

Liu, E., X. Li, et al. (2004). "Cyclin-dependent kinases phosphorylate human Cdt1 and induce its degradation." J Biol Chem 279(17): 17283-8.

Liu, J., M. Furukawa, et al. (2002). "NEDD8 modification of CUL1 dissociates p120(CAND1), an inhibitor of CUL1-SKP1 binding and SCF ligases." Mol Cell 10(6): 1511-8.

Liu, L., S. Lee, et al. (2009). "CUL4A abrogation augments DNA damage response and protection against skin carcinogenesis." Mol Cell 34(4): 451-60.

Long, X., S. Ortiz-Vega, et al. (2005). "Rheb binding to mammalian target of rapamycin (mTOR) is regulated by amino acid sufficiency." J Biol Chem 280(25): 23433-6.

Lopez-Schier, H. and D. St Johnston (2001). "Delta signaling from the germ line controls the proliferation and differentiation of the somatic follicle cells during Drosophila oogenesis." Genes Dev 15(11): 1393-405.

Lorson, M. A., H. R. Horvitz, et al. (2000). "LIN-5 Is a Novel Component of the Spindle Apparatus Required for Chromosome Segregation and Cleavage Plane Specification in Caenorhabditis elegans." J. Cell Biol. 148(1): 73-86.

Lovejoy, C. A., K. Lock, et al. (2006). "DDB1 maintains genome integrity through regulation of Cdt1." Mol Cell Biol 26(21): 7977-90.

Lozano, E., A. G. Saez, et al. (2006). "Regulation of growth by ploidy in Caenorhabditis elegans." Curr Biol 16(5): 493-8.

Luoh, S. M., E. Stefanich, et al. (2000). "Role of the distal half of the c-Mpl intracellular domain in control of platelet production by thrombopoietin in vivo." Mol Cell Biol 20(2): 507-15.

Lutzmann, M., D. Maiorano, et al. (2006). "A Cdt1-geminin complex licenses chromatin for DNA replication and prevents rereplication during S phase in Xenopus." Embo J 25(24): 5764-74.

Lutzmann, M. and M. Mechali (2008). "MCM9 binds Cdt1 and is required for the assembly of prereplication complexes." Mol Cell 31(2): 190-200.

Lygerou, Z. and P. Nurse (2000). "Cell cycle. License withheld--geminin blocks DNA replication." Science 290(5500): 2271-3.

Machida, Y. J. and A. Dutta (2007). "The APC/C inhibitor, Emi1, is essential for prevention of rereplication." Genes Dev 21(2): 184-94.

Maddika, S. and J. Chen (2009). "Protein kinase DYRK2 is a scaffold that facilitates assembly of an E3 ligase." Nat Cell Biol 11(4): 409-19.

Maheshwar, M. M., J. P. Cheadle, et al. (1997). "The GAP-related domain of tuberin, the product of the TSC2 gene, is a target for missense mutations in tuberous sclerosis." Hum Mol Genet 6(11): 1991-6.

Maines, J. Z., L. M. Stevens, et al. (2004). "Drosophila dMyc is required for ovary cell growth and endoreplication." Development 131(4): 775-86.

Maiorano, D., L. Krasinska, et al. (2005). "Recombinant Cdt1 induces rereplication of G2 nuclei in Xenopus egg extracts." Curr Biol 15(2): 146-53.

Maiorano, D., J. Moreau, et al. (2000). "XCDT1 is required for the assembly of pre-replicative complexes in Xenopus laevis." Nature 404(6778): 622-5.

Maiorano, D., W. Rul, et al. (2004). "Cell cycle regulation of the licensing activity of Cdt1 in Xenopus laevis." Exp Cell Res 295(1): 138-49.

Manning, J., K. Beutler, et al. (2002). "Upregulation of renal BSC1 and TSC in prenatally programmed hypertension." Am J Physiol Renal Physiol 283(1): F202-6.

Manseau, L., A. Baradaran, et al. (1997). "GAL4 enhancer traps expressed in the embryo, larval brain, imaginal discs, and ovary of Drosophila." Dev Dyn 209(3): 310-22.

Marcotte, L. and P. B. Crino (2006). "The neurobiology of the tuberous sclerosis complex." Neuromolecular Med 8(4): 531-46.

Markson, G., C. Kiel, et al. (2009). "Analysis of the human E2 ubiquitin conjugating enzyme protein interaction network." Genome Res 19(10): 1905-11.

Martindill, D. M. and P. R. Riley (2008). "Cell cycle switch to endocycle: the nucleolus lends a hand." Cell Cycle 7(1): 17-23.

Martindill, D. M., C. A. Risebro, et al. (2007). "Nucleolar release of Hand1 acts as a molecular switch to determine cell fate." Nat Cell Biol 9(10): 1131-41.

Masuda, H. P., G. B. Ramos, et al. (2004). "Genome based identification and analysis of the pre-replicative complex of Arabidopsis thaliana." FEBS Lett 574(1-3): 192-202.

Matsumura, I., H. Tanaka, et al. (2000). "Increased D-type cyclin expression together with decreased cdc2 activity confers megakaryocytic differentiation of a human thrombopoietin-dependent hematopoietic cell line." J Biol Chem 275(8): 5553-9.

May, N. R., M. Thomer, et al. (2005). "Levels of the origin-binding protein Double parked and its inhibitor Geminin increase in response to replication stress." J Cell Sci 118(Pt 18): 4207-17.

McGarry, T. J. and M. W. Kirschner (1998). "Geminin, an inhibitor of DNA replication, is degraded during mitosis." Cell 93(6): 1043-53.

Meckert, P. C., H. G. Rivello, et al. (2005). "Endomitosis and polyploidization of myocardial cells in the periphery of human acute myocardial infarction." Cardiovasc Res 67(1): 116-23.

Mehrotra, S., S. B. Maqbool, et al. (2008). "Endocycling cells do not apoptose in response to DNA rereplication genotoxic stress." Genes Dev 22(22): 3158-71.

Melixetian, M., A. Ballabeni, et al. (2004). "Loss of Geminin induces rereplication in the presence of functional p53." J Cell Biol 165(4): 473-82.

Menand, B., K. Yi, et al. (2007). "An ancient mechanism controls the development of cells with a rooting function in land plants." Science 316(5830): 1477-80.

Mendez, J. (2003). "Cell proliferation without cyclin E-CDK2." Cell 114(4): 398-9.

Menon, S., T. Tsuge, et al. (2008). "Association of SAP130/SF3b-3 with Cullin-RING ubiquitin ligase complexes and its regulation by the COP9 signalosome." BMC Biochem 9: 1.

Mercher, T., M. G. Cornejo, et al. (2008). "Notch signaling specifies megakaryocyte development from hematopoietic stem cells." Cell Stem Cell 3(3): 314-26.

Merlet, J., J. Burger, et al. (2009). "Regulation of cullin-RING E3 ubiquitin-ligases by neddylation and dimerization." Cell Mol Life Sci 66(11-12): 1924-38.

Michel, J. J. and Y. Xiong (1998). "Human CUL-1, but not other cullin family members, selectively interacts with SKP1 to form a complex with SKP2 and cyclin A." Cell Growth Differ 9(6): 435-49.

Mihaylov, I. S., T. Kondo, et al. (2002). "Control of DNA replication and chromosome ploidy by geminin and cyclin A." Mol Cell Biol 22(6): 1868-80.

Mingo-Sion, A. M., P. M. Marietta, et al. (2004). "Inhibition of JNK reduces G2/M transit independent of p53, leading to endoreduplication, decreased proliferation, and apoptosis in breast cancer cells." Oncogene 23(2): 596-604.

Miotto, B. and K. Struhl (2008). "HBO1 histone acetylase is a coactivator of the replication licensing factor Cdt1." Genes Dev 22(19): 2633-8.

Mistry, H., B. A. Wilson, et al. (2004). "Cullin-3 regulates pattern formation, external sensory organ development and cell survival during Drosophila development." Mech Dev 121(12): 1495-507.

Moberg, K. H., D. W. Bell, et al. (2001). "Archipelago regulates Cyclin E levels in Drosophila and is mutated in human cancer cell lines." Nature 413(6853): 311-6.

Morgan, D. O. (2007). The Cell Cycle: Principles of Control. London, New Science Press Ltd.

172

Moses, K. and G. M. Rubin (1991). "Glass encodes a site-specific DNA-binding protein that is regulated in response to positional signals in the developing Drosophila eye." Genes Dev 5(4): 583-93.

Mothe-Satney, I., D. Yang, et al. (2000). "Multiple mechanisms control phosphorylation of PHAS-I in five (S/T)P sites that govern translational repression." Mol Cell Biol 20(10): 3558-67.

Muris, D. F., T. Verschoor, et al. (2002). "Constitutive active GTPases Rac and Cdc42 are associated with endoreplication in PAE cells." Eur J Cancer 38(13): 1775-82.

Murone, M., D. A. Carpenter, et al. (1998). "Hematopoietic deficiencies in c-mpl and TPO knockout mice." Stem Cells 16(1): 1-6.

Nagata, Y., Y. Muro, et al. (1997). "Thrombopoietin-induced polyploidization of bone marrow megakaryocytes is due to a unique regulatory mechanism in late mitosis." J Cell Biol 139(2): 449-57.

Nagl, W. (1978). "Electron microscopic observations of a possible tubulin synthesis-polymerization complex in oocytes of a pond-skater." Cytobios 21(83-84): 165-70.

Nakayama, H., Y. Liu, et al. (1997). "Developmental restriction of Mash-2 expression in trophoblast correlates with potential activation of the notch-2 pathway." Dev Genet 21(1): 21-30.

Nakayama, H., I. C. Scott, et al. (1998). "The transition to endoreduplication in trophoblast giant cells is regulated by the mSNA zinc finger transcription factor." Dev Biol 199(1): 150-63.

Narasimhachar, Y. and M. Coue (2009). "Geminin stabilizes Cdt1 during meiosis in Xenopus oocytes." J Biol Chem 284(40): 27235-42.

Narbonne-Reveau, K. and M. Lilly (2009). "The Cyclin-dependent kinase inhibitor Dacapo promotes genomic stability during premeiotic S phase." Mol Biol Cell 20(7): 1960-9.

Narbonne-Reveau, K., S. Senger, et al. (2008). "APC/CFzr/Cdh1 promotes cell cycle progression during the Drosophila endocycle." Development 135(8): 1451-61.

Naujokat, C. (2009). "Role of ubiquitin ligases in neural stem and progenitor cells." Arch Immunol Ther Exp (Warsz) 57(3): 177-88.

Navarro, S., N. Debili, et al. (1991). "Interleukin-6 and its receptor are expressed by human megakaryocytes: in vitro effects on proliferation and endoreplication." Blood 77(3): 461-71.

Neben, T. Y., J. Loebelenz, et al. (1993). "Recombinant human interleukin-11 stimulates megakaryocytopoiesis and increases peripheral platelets in normal and splenectomized mice." Blood 81(4): 901-8.

Nellist, M., O. Sancak, et al. (2005). "Distinct effects of single amino-acid changes to tuberin on the function of the tuberin-hamartin complex." Eur J Hum Genet 13(1): 59-68.

Nevis, K. R., M. Cordeiro-Stone, et al. (2009). "Origin licensing and p53 status regulate Cdk2 activity during G(1)." Cell Cycle 8(12): 1952-63.

Nevzorova, Y. A., D. Tschaharganeh, et al. (2009). "Aberrant Cell Cycle Progression and Endoreplication in Regenerating Livers of Mice That Lack a Single E-Type Cyclin." Gastroenterology.

Nishitani, H. and Z. Lygerou (2002). "Control of DNA replication licensing in a cell cycle." Genes Cells 7(6): 523-34.

Nishitani, H., Z. Lygerou, et al. (2004). "Proteolysis of DNA replication licensing factor Cdt1 in S-phase is performed independently of geminin through its N-terminal region." J Biol Chem 279(29): 30807-16.

Nishitani, H., Z. Lygerou, et al. (2000). "The Cdt1 protein is required to license DNA for replication in fission yeast." Nature 404(6778): 625-8.

Nishitani, H., Y. Shiomi, et al. (2008). "CDK inhibitor p21 is degraded by a proliferating cell nuclear antigen-coupled Cul4-DDB1Cdt2 pathway during S phase and after UV irradiation." J Biol Chem 283(43): 29045-52.

Nishitani, H., N. Sugimoto, et al. (2006). "Two E3 ubiquitin ligases, SCF-Skp2 and DDB1-Cul4, target human Cdt1 for proteolysis." Embo J 25(5): 1126-36.

Nishitani, H., S. Taraviras, et al. (2001). "The human licensing factor for DNA replication Cdt1 accumulates in G1 and is destabilized after initiation of S-phase." J Biol Chem 276(48): 44905-11.

Noureddine, M. A., T. D. Donaldson, et al. (2002). "Drosophila Roc1a encodes a RING-H2 protein with a unique function in processing the Hh signal transducer Ci by the SCF E3 ubiquitin ligase." Dev Cell 2(6): 757-70.

Nurden, A. T. (2005). "Qualitative disorders of platelets and megakaryocytes." J Thromb Haemost 3(8): 1773-82.

O'Connell, B. C. and J. W. Harper (2007). "Ubiquitin proteasome system (UPS): what can chromatin do for you?" Curr Opin Cell Biol 19(2): 206-14.

Osaka, F., M. Saeki, et al. (2000). "Covalent modifier NEDD8 is essential for SCF ubiquitin-ligase in fission yeast." Embo J 19(13): 3475-84.

Ou, C. Y., Y. F. Lin, et al. (2002). "Distinct protein degradation mechanisms mediated by Cul1 and Cul3 controlling Ci stability in Drosophila eye development." Genes Dev 16(18): 2403-14.

Ouellet, J. and R. Roy (2007). "The lin-35/Rb and RNAi pathways cooperate to regulate a key cell cycle transition in C. elegans." BMC Dev Biol 7: 38.

Pan, H., Y. Deng, et al. (2006). "Progesterone blocks estrogen-induced DNA synthesis through the inhibition of replication licensing." Proc Natl Acad Sci U S A 103(38): 14021-6.

Pang, L., M. J. Weiss, et al. (2005). "Megakaryocyte biology and related disorders." J Clin Invest 115(12): 3332-8.

Paoletti, F., A. Mocali, et al. (1996). "Megakaryocyte-like increase in ploidy of Friend's erythroleukemia cells induced to endoreplication by colcemid." Exp Hematol 24(12): 1441-8.

Parisi, T., A. R. Beck, et al. (2003). "Cyclins E1 and E2 are required for endoreplication in placental trophoblast giant cells." Embo J 22(18): 4794-803.

Park, J. A., J. W. Ahn, et al. (2005). "Retinoblastoma protein regulates cell proliferation, differentiation, and endoreduplication in plants." Plant J 42(2): 153-63.

Park, S. Y. and M. Asano (2008). "The origin recognition complex is dispensable for endoreplication in Drosophila." Proc Natl Acad Sci U S A 105(34): 12343-8.

Pawson, T. and J. D. Scott (2005). "Protein phosphorylation in signaling--50 years and counting." Trends Biochem Sci 30(6): 286-90.

Pearson, R. B., P. B. Dennis, et al. (1995). "The principal target of rapamycin-induced p70s6k inactivation is a novel phosphorylation site within a conserved hydrophobic domain." Embo J 14(21): 5279-87.

Pelizon, C., F. d'Adda di Fagagna, et al. (2002). "Human replication protein Cdc6 is selectively cleaved by caspase 3 during apoptosis." EMBO Rep 3(8): 780-4.

Petropoulou, C., P. Kotantaki, et al. (2008). "Cdt1 and Geminin in cancer: markers or triggers of malignant transformation?" Front Biosci 13: 4485-94.

Pfleger, C. M. and M. W. Kirschner (2000). "The KEN box: an APC recognition signal distinct from the D box targeted by Cdh1." Genes Dev 14(6): 655-65.

Pfleger, C. M., E. Lee, et al. (2001). "Substrate recognition by the Cdc20 and Cdh1 components of the anaphase-promoting complex." Genes Dev 15(18): 2396-407.

Pflumm, M. F. and M. R. Botchan (2001). "Orc mutants arrest in metaphase with abnormally condensed chromosomes." Development 128(9): 1697-707.

Piatti, S., C. Lengauer, et al. (1995). "Cdc6 is an unstable protein whose de novo synthesis in G1 is important for the onset of S phase and for preventing a 'reductional' anaphase in the budding yeast Saccharomyces cerevisiae." Embo J 14(15): 3788-99.

Pickart, C. M. (2001). "Mechanisms underlying ubiquitination." Annu Rev Biochem 70: 503-33.

Pierce, S. B., C. Yost, et al. (2008). "Drosophila growth and development in the absence of dMyc and dMnt." Dev Biol 315(2): 303-16.

Pierce, S. B., C. Yost, et al. (2004). "dMyc is required for larval growth and endoreplication in Drosophila." Development 131(10): 2317-27.

Pinto, S., D. G. Quintana, et al. (1999). "latheo encodes a subunit of the origin recognition complex and disrupts neuronal proliferation and adult olfactory memory when mutant." Neuron 23(1): 45-54.

Pinyol, M., I. Salaverria, et al. (2006). "Unbalanced expression of licensing DNA replication factors occurs in a subset of mantle cell lymphomas with genomic instability." Int J Cancer 119(12): 2768-74.

Pitulescu, M., M. Kessel, et al. (2005). "The regulation of embryonic patterning and DNA replication by geminin." Cell Mol Life Sci 62(13): 1425-33.

Plank, T. L., R. S. Yeung, et al. (1998). "Hamartin, the product of the tuberous sclerosis 1 (TSC1) gene, interacts with tuberin and appears to be localized to cytoplasmic vesicles." Cancer Res 58(21): 4766-70.

Poellinger, L. and U. Lendahl (2008). "Modulating Notch signaling by pathway-intrinsic and pathway-extrinsic mechanisms." Curr Opin Genet Dev 18(5): 449-54.

Polesello, C. and N. Tapon (2007). "Salvador-warts-hippo signaling promotes Drosophila posterior follicle cell maturation downstream of notch." Curr Biol 17(21): 1864-70.

Potter, C. J., L. G. Pedraza, et al. (2002). "Akt regulates growth by directly phosphorylating Tsc2." Nat Cell Biol 4(9): 658-65.

Prasanth, S. G., J. Mendez, et al. (2004). "Dynamics of pre-replication complex proteins during the cell division cycle." Philos Trans R Soc Lond B Biol Sci 359(1441): 7-16.

Prieur-Carrillo, G., K. Chu, et al. (2003). "Computerized video time-lapse (CVTL) analysis of the fate of giant cells produced by X-irradiating EJ30 human bladder carcinoma cells." Radiat Res 159(6): 705-12.

Puig, P. E., M. N. Guilly, et al. (2008). "Tumor cells can escape DNA-damaging cisplatin through DNA endoreduplication and reversible polyploidy." Cell Biol Int 32(9): 1031-43.

Purdy, A., L. Uyetake, et al. (2005). "Regulation of mitosis in response to damaged or incompletely replicated DNA require different levels of Grapes (Drosophila Chk1)." J Cell Sci 118(Pt 15): 3305-15.

Quinn, L. M., A. Herr, et al. (2001). "The Drosophila Geminin homolog: roles for Geminin in limiting DNA replication, in anaphase and in neurogenesis." Genes Dev 15(20): 2741-54.

Rakotomalala, L., L. Studach, et al. (2008). "Hepatitis B virus X protein increases the Cdt1-to-geminin ratio inducing DNA re-replication and polyploidy." J Biol Chem 283(42): 28729-40.

Ralph, E., E. Boye, et al. (2006). "DNA damage induces Cdt1 proteolysis in fission yeast through a pathway dependent on Cdt2 and Ddb1." EMBO Rep 7(11): 1134-9.

Ramirez-Parra, E., B. Desvoyes, et al. (2005). "Balance between cell division and differentiation during plant development." Int J Dev Biol 49(5-6): 467-77.

Ramirez-Parra, E. and C. Gutierrez (2007). "E2F regulates FASCIATA1, a chromatin assembly gene whose loss switches on the endocycle and activates gene expression by changing the epigenetic status." Plant Physiol 144(1): 105-20.

Randell, J. C., J. L. Bowers, et al. (2006). "Sequential ATP hydrolysis by Cdc6 and ORC directs loading of the Mcm2-7 helicase." Mol Cell 21(1): 29-39.

Rasheva, V. I., D. Knight, et al. (2006). "Specific role of the SR protein splicing factor B52 in cell cycle control in Drosophila." Mol Cell Biol 26(9): 3468-77.

Ravid, K., J. Lu, et al. (2002). "Roads to polyploidy: the megakaryocyte example." J Cell Physiol 190(1): 7-20.

Reddy, K. L., M. K. Rovani, et al. (2006). "The Drosophila Par domain protein I gene, Pdp1, is a regulator of larval growth, mitosis and endoreplication." Dev Biol 289(1): 100-14.

Reiterer, G. and A. Yen (2006). "Inhibition of the janus kinase family increases extracellular signal-regulated kinase 1/2 phosphorylation and causes endoreduplication." Cancer Res 66(18): 9083-9.

Remus, D., F. Beuron, et al. (2009). "Concerted loading of Mcm2-7 double hexamers around DNA during DNA replication origin licensing." Cell 139(4): 719-30.

Rialland, M., F. Sola, et al. (2002). "Essential role of human CDT1 in DNA replication and chromatin licensing." J Cell Sci 115(Pt 7): 1435-40.

Rizki, T. M. and R. M. Rizki (1983). "Blood cell surface changes in Drosophila mutants with melanotic tumors." Science 220(4592): 73-5.

Rogers, S. L. and G. C. Rogers (2008). "Culture of Drosophila S2 cells and their use for RNAi-mediated loss-of-function studies and immunofluorescence microscopy." Nat Protoc 3(4): 606-11.

Rogers, S. L., G. C. Rogers, et al. (2002). "Drosophila EB1 is important for proper assembly, dynamics, and positioning of the mitotic spindle." J Cell Biol 158(5): 873-84.

Rojnuckarin, P., J. G. Drachman, et al. (1999). "Thrombopoietin-induced activation of the mitogen-activated protein kinase (MAPK) pathway in normal megakaryocytes: role in endomitosis." Blood 94(4): 1273-82.

Rotin, D. and S. Kumar (2009). "Physiological functions of the HECT family of ubiquitin ligases." Nat Rev Mol Cell Biol 10(6): 398-409.

Royzman, I., R. J. Austin, et al. (1999). "ORC localization in Drosophila follicle cells and the effects of mutations in dE2F and dDP." Genes Dev 13(7): 827-40.

Royzman, I., A. Hayashi-Hagihara, et al. (2002). "The E2F cell cycle regulator is required for Drosophila nurse cell DNA replication and apoptosis." Mech Dev 119(2): 225-37.

Royzman, I. and T. L. Orr-Weaver (1998). "S phase and differential DNA replication during Drosophila oogenesis." Genes Cells 3(12): 767-76.

Royzman, I., A. J. Whittaker, et al. (1997). "Mutations in Drosophila DP and E2F distinguish G1-S progression from an associated transcriptional program." Genes Dev 11(15): 1999-2011.

Rushlow, C. A. and A. Chovnick (1984). "Heterochromatic position effect at the rosy locus of Drosophila melanogaster: cytological, genetic and biochemical characterization." Genetics 108(3): 589-602.

Sakaue-Sawano, A., H. Kurokawa, et al. (2008). "Visualizing spatiotemporal dynamics of multicellular cell-cycle progression." Cell 132(3): 487-98.

Sansam, C. L., J. L. Shepard, et al. (2006). "DTL/CDT2 is essential for both CDT1 regulation and the early G2/M checkpoint." Genes Dev 20(22): 3117-29.

Sarbassov, D. D., D. A. Guertin, et al. (2005). "Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex." Science 307(5712): 1098-101.

Sauer, K., J. A. Knoblich, et al. (1995). "Distinct modes of cyclin E/cdc2c kinase regulation and S-phase control in mitotic and endoreduplication cycles of Drosophila embryogenesis." Genes Dev 9(11): 1327-39.

Saxena, S. and A. Dutta (2003). "Geminin and p53: deterrents to rereplication in human cancer cells." Cell Cycle 2(4): 283-6.

Saxena, S. and A. Dutta (2005). "Geminin-Cdt1 balance is critical for genetic stability." Mutat Res 569(1-2): 111-21.

Saxena, S., P. Yuan, et al. (2004). "A dimerized coiled-coil domain and an adjoining part of geminin interact with two sites on Cdt1 for replication inhibition." Mol Cell 15(2): 245-58.

Schaeffer, V., C. Althauser, et al. (2004). "Notch-dependent Fizzy-related/Hec1/Cdh1 expression is required for the mitotic-to-endocycle transition in Drosophila follicle cells." Curr Biol 14(7): 630-6.

Schalet, A., R. P. Kernaghan, et al. (1964). "Structural And Phenotypic Definition Of The Rosy Cistron In Drosophila Melanogaster." Genetics 50: 1261-8.

Schnittger, A., C. Weinl, et al. (2003). "Misexpression of the cyclin-dependent kinase inhibitor ICK1/KRP1 in single-celled Arabidopsis trichomes reduces endoreduplication and cell size and induces cell death." Plant Cell 15(2): 303-15.

Schweizer, L., G. L. Yerk-Davis, et al. (1995). "Dynamics of maize endosperm development and DNA endoreduplication." Proc Natl Acad Sci U S A 92(15): 7070-4.

Scott, M. P., A. J. Weiner, et al. (1983). "The molecular organization of the Antennapedia locus of Drosophila." Cell 35(3 Pt 2): 763-76.

Senga, T., U. Sivaprasad, et al. (2006). "PCNA is a cofactor for Cdt1 degradation by CUL4/DDB1-mediated N-terminal ubiquitination." J Biol Chem 281(10): 6246-52.

Seo, J., Y. S. Chung, et al. (2005). "Cdt1 transgenic mice develop lymphoblastic lymphoma in the absence of p53." Oncogene 24(55): 8176-86.

Sharova, N. P. and E. B. Abramova (2002). "Initiation of DNA replication in eukaryotes is an intriguing cascade of protein interactions." Biochemistry (Mosc) 67(11): 1217-23.

Shaw, R. J., N. Bardeesy, et al. (2004). "The LKB1 tumor suppressor negatively regulates mTOR signaling." Cancer Cell 6(1): 91-9.

Shaw, R. J., M. Kosmatka, et al. (2004). "The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress." Proc Natl Acad Sci U S A 101(10): 3329-35.

Shcherbata, H. R., C. Althauser, et al. (2004). "The mitotic-to-endocycle switch in Drosophila follicle cells is executed by Notch-dependent regulation of G1/S, G2/M and M/G1 cell-cycle transitions." Development 131(13): 3169-81.

Shibutani, S. T., A. F. de la Cruz, et al. (2008). "Intrinsic negative cell cycle regulation provided by PIP box- and Cul4Cdt2-mediated destruction of E2f1 during S phase." Dev Cell 15(6): 890-900.

Shreeram, S. and J. J. Blow (2003). "The role of the replication licensing system in cell proliferation and cancer." Prog Cell Cycle Res 5: 287-93.

Shreeram, S., A. Sparks, et al. (2002). "Cell type-specific responses of human cells to inhibition of replication licensing." Oncogene 21(43): 6624-32.

Sigrist, S. J. and C. F. Lehner (1997). "Drosophila fizzy-related down-regulates mitotic cyclins and is required for cell proliferation arrest and entry into endocycles." Cell 90(4): 671-81.

Sivaprasad, U., Y. J. Machida, et al. (2007). "APC/C--the master controller of origin licensing?" Cell Div 2: 8.

Skowyra, D., K. L. Craig, et al. (1997). "F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex." Cell 91(2): 209-19.

Smith, A. V. and T. L. Orr-Weaver (1991). "The regulation of the cell cycle during Drosophila embryogenesis: the transition to polyteny." Development 112(4): 997-1008.

Solar, G. P., W. G. Kerr, et al. (1998). "Role of c-mpl in early hematopoiesis." Blood 92(1): 4-10.

Soloveva, V. and D. I. Linzer (2004). "Differentiation of placental trophoblast giant cells requires downregulation of p53 and Rb." Placenta 25(1): 29-36.

Speck, C. and B. Stillman (2007). "Cdc6 ATPase activity regulates ORC x Cdc6 stability and the selection of specific DNA sequences as origins of DNA replication." J Biol Chem 282(16): 11705-14.

Spella, M., O. Britz, et al. (2007). "Licensing regulators Geminin and Cdt1 identify progenitor cells of the mouse CNS in a specific phase of the cell cycle." Neuroscience 147(2): 373-87.

Srinivasan, S. V., C. N. Mayhew, et al. (2007). "RB loss promotes aberrant ploidy by deregulating levels and activity of DNA replication factors." J Biol Chem 282(33): 23867-77.

St. Johnston, H. n. L. p.-S. a. D. (2001). "Delta signaling from the germ line controls the proliferation and differentiation of the somatic follicle cells during Drosophila oogenesis." Genes & Development 15(11): 1393-1405.

Steinbeck, R. G. (1997). "Atypical mitoses in lesions of the oral mucosa: a new interpretation of their impact upon tumorigenesis." Oral Oncol 33(2): 110-8.

Steinbeck, R. G. (1997). "Proliferation and DNA aneuploidy in mild dysplasia imply early steps of cervical carcinogenesis." Acta Oncol 36(1): 3-12.

Stern, B., G. Ried, et al. (1993). "Genetic analysis of the Drosophila cdc2 homolog." Development 117(1): 219-32.

Storchova, Z. and D. Pellman (2004). "From polyploidy to aneuploidy, genome instability and cancer." Nat Rev Mol Cell Biol 5(1): 45-54.

Strohmaier, H., C. H. Spruck, et al. (2001). "Human F-box protein hCdc4 targets cyclin E for proteolysis and is mutated in a breast cancer cell line." Nature 413(6853): 316-22.

Su, T. T. and P. H. O'Farrell (1998). "Chromosome association of minichromosome maintenance proteins in Drosophila endoreplication cycles." J Cell Biol 140(3): 451-60.

Sugimoto-Shirasu, K. and K. Roberts (2003). ""Big it up": endoreduplication and cell-size control in plants." Current Opinion in Plant Biology 6(6): 544.

Sugimoto, N., I. Kitabayashi, et al. (2008). "Identification of novel human Cdt1-binding proteins by a proteomics approach: proteolytic regulation by APC/CCdh1." Mol Biol Cell 19(3): 1007-21.

Sugimoto, N., Y. Tatsumi, et al. (2004). "Cdt1 phosphorylation by cyclin A-dependent kinases negatively regulates its function without affecting geminin binding." J Biol Chem 279(19): 19691-7.

Sugimoto, N., K. Yoshida, et al. (2009). "Redundant and differential regulation of multiple licensing factors ensures prevention of re-replication in normal human cells." J Cell Sci 122(Pt 8): 1184-91.

Sugiyama, M., A. Sakaue-Sawano, et al. (2009). "Illuminating cell-cycle progression in the developing zebrafish embryo." Proc Natl Acad Sci U S A.

Sun, J. and W. M. Deng (2005). "Notch-dependent downregulation of the homeodomain gene cut is required for the mitotic cycle/endocycle switch and cell differentiation in Drosophila follicle cells." Development 132(19): 4299-308.

Sun, J. and W. M. Deng (2007). "Hindsight mediates the role of notch in suppressing hedgehog signaling and cell proliferation." Dev Cell 12(3): 431-42.

Sun, J., L. Smith, et al. (2008). "Regulation of the endocycle/gene amplification switch by Notch and ecdysone signaling." J Cell Biol 182(5): 885-96.

Swain, S. M., T. S. Tseng, et al. (2002). "SPINDLY is a nuclear-localized repressor of gibberellin signal transduction expressed throughout the plant." Plant Physiol 129(2): 605-15.

Tachibana, K. E., M. A. Gonzalez, et al. (2005). "Depletion of licensing inhibitor geminin causes centrosome overduplication and mitotic defects." EMBO Rep 6(11): 1052-7.

Tada, S. (2007). "Cdt1 and geminin: role during cell cycle progression and DNA damage in higher eukaryotes." Front Biosci 12: 1629-41.

Tada, S., A. Li, et al. (2001). "Repression of origin assembly in metaphase depends on inhibition of RLF-B/Cdt1 by geminin." Nat Cell Biol 3(2): 107-13.

Takahashi, T., E. Ohara, et al. (2003). "Multiple ORC-binding sites are required for efficient MCM loading and origin firing in fission yeast." Embo J 22(4): 964-74.

Takata, K., H. Yoshida, et al. (2004). "Drosophila damaged DNA-binding protein 1 is an essential factor for development." Genetics 168(2): 855-65.

Takeda, D. Y., J. D. Parvin, et al. (2005). "Degradation of Cdt1 during S phase is Skp2-independent and is required for efficient progression of mammalian cells through S phase." J Biol Chem 280(24): 23416-23.

Talora, C., A. F. Campese, et al. (2008). "Notch signaling and diseases: an evolutionary journey from a simple beginning to complex outcomes." Biochim Biophys Acta 1782(9): 489-97.

Tanaka, S. and J. F. Diffley (2002). "Interdependent nuclear accumulation of budding yeast Cdt1 and Mcm2-7 during G1 phase." Nat Cell Biol 4(3): 198-207.

Tapon, N., K. H. Moberg, et al. (2001). "The coupling of cell growth to the cell cycle." Curr Opin Cell Biol 13(6): 731-7.

Tateishi, K., M. Omata, et al. (2001). "The NEDD8 system is essential for cell cycle progression and morphogenetic pathway in mice." J Cell Biol 155(4): 571-9.

Tatsumi, Y., N. Sugimoto, et al. (2006). "Deregulation of Cdt1 induces chromosomal damage without rereplication and leads to chromosomal instability." J Cell Sci 119(Pt 15): 3128-40.

Tee, A. R., R. Anjum, et al. (2003). "Inactivation of the tuberous sclerosis complex-1 and -2 gene products occurs by phosphoinositide 3-kinase/Akt-dependent and -independent phosphorylation of tuberin." J Biol Chem 278(39): 37288-96.

Tee, A. R., B. D. Manning, et al. (2003). "Tuberous sclerosis complex gene products, Tuberin and Hamartin, control mTOR signaling by acting as a GTPase-activating protein complex toward Rheb." Curr Biol 13(15): 1259-68.

Teer, J. K. and A. Dutta (2008). "Human Cdt1 lacking the evolutionarily conserved region that interacts with MCM2-7 is capable of inducing re-replication." J Biol Chem 283(11): 6817-25.

Tetzlaff, M. T., W. Yu, et al. (2004). "Defective cardiovascular development and elevated cyclin E and Notch proteins in mice lacking the Fbw7 F-box protein." Proceedings of the National Academy of Sciences of the United States of America 101(10): 3338-3345.

Thomer, M., N. R. May, et al. (2004). "Drosophila double-parked is sufficient to induce re-replication during development and is regulated by cyclin E/CDK2." Development 131(19): 4807-18.

Tomer, A., P. Friese, et al. (1989). "Flow cytometric analysis of megakaryocytes from patients with abnormal platelet counts." Blood 74(2): 594-601.

Tower, J. (2004). "Developmental gene amplification and origin regulation." Annu Rev Genet 38: 273-304.

Treisman, J., E. Harris, et al. (1991). "The paired box encodes a second DNA-binding domain in the paired homeo domain protein." Genes Dev 5(4): 594-604.

Tripathi, B. J., R. C. Tripathi, et al. (1989). "Hydrocortisone-induced DNA endoreplication in human trabecular cells in vitro." Exp Eye Res 49(2): 259-70.

Tsuyama, T., S. Tada, et al. (2005). "Licensing for DNA replication requires a strict sequential assembly of Cdc6 and Cdt1 onto chromatin in Xenopus egg extracts." Nucleic Acids Res 33(2): 765-75.

Tsuyama, T., S. Watanabe, et al. (2009). "Repression of nascent strand elongation by deregulated Cdt1 during DNA replication in Xenopus egg extracts." Mol Biol Cell 20(3): 937-47.

Ullah, Z., M. J. Kohn, et al. (2008). "Differentiation of trophoblast stem cells into giant cells is triggered by p57/Kip2 inhibition of CDK1 activity." Genes Dev 22(21): 3024-36.

Ullah, Z., C. Y. Lee, et al. (2009). "Developmentally programmed endoreduplication in animals." Cell Cycle 8(10): 1501-9.

van den Heuvel, S. and N. J. Dyson (2008). "Conserved functions of the pRB and E2F families." Nat Rev Mol Cell Biol 9(9): 713-24.

van Leuken, R., L. Clijsters, et al. (2008). "To cell cycle, swing the APC/C." Biochim Biophys Acta 1786(1): 49-59.

van Slegtenhorst, M., R. de Hoogt, et al. (1997). "Identification of the tuberous sclerosis gene TSC1 on chromosome 9q34." Science 277(5327): 805-8.

Vaziri, C., S. Saxena, et al. (2003). "A p53-dependent checkpoint pathway prevents rereplication." Mol Cell 11(4): 997-1008.

Verkest, A., C. L. Manes, et al. (2005). "The cyclin-dependent kinase inhibitor KRP2 controls the onset of the endoreduplication cycle during Arabidopsis leaf

development through inhibition of mitotic CDKA;1 kinase complexes." Plant Cell 17(6): 1723-36.

Vidwans, S. J., P. J. DiGregorio, et al. (2002). "Sister chromatids fail to separate during an induced endoreplication cycle in Drosophila embryos." Curr Biol 12(10): 829-33.

Vilaboa, N., R. Bermejo, et al. (2004). "A novel E2 box-GATA element modulates Cdc6 transcription during human cells polyploidization." Nucleic Acids Res 32(21): 6454-67.

Vitrat, N., K. Cohen-Solal, et al. (1998). "Endomitosis of human megakaryocytes are due to abortive mitosis." Blood 91(10): 3711-23.

Vlieghe, K., V. Boudolf, et al. (2005). "The DP-E2F-like gene DEL1 controls the endocycle in Arabidopsis thaliana." Curr Biol 15(1): 59-63.

Waga, S. and A. Zembutsu (2006). "Dynamics of DNA binding of replication initiation proteins during de novo formation of pre-replicative complexes in Xenopus egg extracts." J Biol Chem 281(16): 10926-34.

Walen, K. H. (2002). "The origin of transformed cells. studies of spontaneous and induced cell transformation in cell cultures from marsupials, a snail, and human amniocytes." Cancer Genet Cytogenet 133(1): 45-54.

Walen, K. H. (2007). "Bipolar genome reductional division of human near-senescent, polyploid fibroblast cells." Cancer Genet Cytogenet 173(1): 43-50.

Walen, K. H. (2007). "Origin of diplochromosomal polyploidy in near-senescent fibroblast cultures: heterochromatin, telomeres and chromosomal instability (CIN)." Cell Biol Int 31(12): 1447-55.

Walker, J. D., D. G. Oppenheimer, et al. (2000). "SIAMESE, a gene controlling the endoreduplication cell cycle in Arabidopsis thaliana trichomes." Development 127(18): 3931-40.

Waning, D. L., B. Li, et al. (2008). "Cul4A is required for hematopoietic cell viability and its deficiency leads to apoptosis." Blood 112(2): 320-9.

Weigmann, K., S. M. Cohen, et al. (1997). "Cell cycle progression, growth and patterning in imaginal discs despite inhibition of cell division after inactivation of Drosophila Cdc2 kinase." Development 124(18): 3555-3563.

Weinl, C., S. Marquardt, et al. (2005). "Novel functions of plant cyclin-dependent kinase inhibitors, ICK1/KRP1, can act non-cell-autonomously and inhibit entry into mitosis." Plant Cell 17(6): 1704-22.

Weiss, A., A. Herzig, et al. (1998). "Continuous Cyclin E expression inhibits progression through endoreduplication cycles in Drosophila." Curr Biol 8(4): 239-42.

Weng, L., C. Zhu, et al. (2003). "Critical role of active repression by E2F and Rb proteins in endoreplication during Drosophila development." Embo J 22(15): 3865-75.

Wharton, S. B., S. Hibberd, et al. (2004). "DNA replication licensing and cell cycle kinetics of oligodendroglial tumours." Br J Cancer 91(2): 262-9.

Whittaker, A. J., I. Royzman, et al. (2000). "Drosophila double parked: a conserved, essential replication protein that colocalizes with the origin recognition complex and links DNA replication with mitosis and the down-regulation of S phase transcripts." Genes Dev 14(14): 1765-76.

Wiebusch, L., R. Uecker, et al. (2003). "Human cytomegalovirus prevents replication licensing by inhibiting MCM loading onto chromatin." EMBO Rep 4(1): 42-6.

Wienecke, R., A. Konig, et al. (1995). "Identification of tuberin, the tuberous sclerosis-2 product. Tuberin possesses specific Rap1GAP activity." J Biol Chem 270(27): 16409-14.

Willis, N. and N. Rhind (2009). "Regulation of DNA replication by the S-phase DNA damage checkpoint." Cell Div 4: 13.

Wilson, L. B. (1925). "Microscopic Examination Of Fresh Tissue." Ann Surg 81(4): 863-8.

Woelk, T., S. Sigismund, et al. (2007). "The ubiquitination code: a signalling problem." Cell Div 2: 11.

Wohlschlegel, J. A., B. T. Dwyer, et al. (2000). "Inhibition of eukaryotic DNA replication by geminin binding to Cdt1." Science 290(5500): 2309-12.

Wu, J. T., H. C. Lin, et al. (2005). "Neddylation and deneddylation regulate Cul1 and Cul3 protein accumulation." Nat Cell Biol 7(10): 1014-20.

Wullschleger, B., L. Kuhn-Nentwig, et al. (2004). "CSTX-13, a highly synergistically acting two-chain neurotoxic enhancer in the venom of the spider Cupiennius salei (Ctenidae)." Proc Natl Acad Sci U S A 101(31): 11251-6.

Xouri, G., M. Dimaki, et al. (2007). "Cdt1 interactions in the licensing process: a model for dynamic spatiotemporal control of licensing." Cell Cycle 6(13): 1549-52.

Xouri, G., Z. Lygerou, et al. (2004). "Cdt1 and geminin are down-regulated upon cell cycle exit and are over-expressed in cancer-derived cell lines." Eur J Biochem 271(16): 3368-78.

Xouri, G., A. Squire, et al. (2007). "Cdt1 associates dynamically with chromatin throughout G1 and recruits Geminin onto chromatin." Embo J 26(5): 1303-14.

Xu, T. and G. M. Rubin (1993). "Analysis of genetic mosaics in developing and adult Drosophila tissues." Development 117(4): 1223-37.

Yanagi, K., T. Mizuno, et al. (2005). "Caenorhabditis elegans geminin homologue participates in cell cycle regulation and germ line development." J Biol Chem 280(20): 19689-94.

Yanagi, K., T. Mizuno, et al. (2002). "Mouse geminin inhibits not only Cdt1-MCM6 interactions but also a novel intrinsic Cdt1 DNA binding activity." J Biol Chem 277(43): 40871-80.

Yanow, S. K., Z. Lygerou, et al. (2001). "Expression of Cdc18/Cdc6 and Cdt1 during G2 phase induces initiation of DNA replication." Embo J 20(17): 4648-56.

Ye, Y. and M. Rape (2009). "Building ubiquitin chains: E2 enzymes at work." Nat Rev Mol Cell Biol 10(11): 755-64.

Yeung, E. C. and D. W. Meinke (1993). "Embryogenesis in Angiosperms: Development of the Suspensor." Plant Cell 5(10): 1371-1381.

Yoshida, K. and I. Inoue (2004). "Regulation of Geminin and Cdt1 expression by E2F transcription factors." Oncogene 23(21): 3802-12.

Yoshida, K., N. Oyaizu, et al. (2004). "The destruction box of human Geminin is critical for proliferation and tumor growth in human colon cancer cells." Oncogene 23(1): 58-70.

Yoshida, K., H. Takisawa, et al. (2005). "Intrinsic nuclear import activity of geminin is essential to prevent re-initiation of DNA replication in Xenopus eggs." Genes Cells 10(1): 63-73.

Yoshizumi, T., Y. Tsumoto, et al. (2006). "Increased level of polyploidy1, a conserved repressor of CYCLINA2 transcription, controls endoreduplication in Arabidopsis." Plant Cell 18(10): 2452-68.

You, Z. and H. Masai (2008). "Cdt1 forms a complex with the minichromosome maintenance protein (MCM) and activates its helicase activity." J Biol Chem 283(36): 24469-77.

Zachariae, W., M. Schwab, et al. (1998). "Control of cyclin ubiquitination by CDK-regulated binding of Hct1 to the anaphase promoting complex." Science 282(5394): 1721-4.

Zanet, J., S. Pibre, et al. (2005). "Endogenous Myc controls mammalian epidermal cell size, hyperproliferation, endoreplication and stem cell amplification." J Cell Sci 118(Pt 8): 1693-704.

Zhang, Y., S. Feng, et al. (2008). "Arabidopsis DDB1-CUL4 ASSOCIATED FACTOR1 forms a nuclear E3 ubiquitin ligase with DDB1 and CUL4 that is involved in multiple plant developmental processes." Plant Cell 20(6): 1437-55.

Zhang, Y., X. Gao, et al. (2003). "Rheb is a direct target of the tuberous sclerosis tumour suppressor proteins." Nat Cell Biol 5(6): 578-81.

Zhang, Y. and D. Kalderon (2000). "Regulation of cell proliferation and patterning in Drosophila oogenesis by Hedgehog signaling." Development 127(10): 2165-76.

Zhang, Y., Z. Wang, et al. (1996). "The cell cycle in polyploid megakaryocytes is associated with reduced activity of cyclin B1-dependent cdc2 kinase." J Biol Chem 271(8): 4266-72.

Zhong, W., H. Feng, et al. (2003). "CUL-4 ubiquitin ligase maintains genome stability by restraining DNA-replication licensing." Nature 423(6942): 885-9.

Zhu, W., Y. Chen, et al. (2004). "Rereplication by depletion of geminin is seen regardless of p53 status and activates a G2/M checkpoint." Mol Cell Biol 24(16): 7140-50.

Zielke, N., S. Querings, et al. (2008). "The anaphase-promoting complex/cyclosome (APC/C) is required for rereplication control in endoreplication cycles." Genes Dev 22(12): 1690-703.

Zolezzi, F., J. Fuss, et al. (2002). "Characterization of a Schizosaccharomyces pombe strain deleted for a sequence homologue of the human damaged DNA binding 1 (DDB1) gene." J Biol Chem 277(43): 41183-91.

Zybina, E. V. (1981). "[Electron microscopic study of endopolyploid nuclei in rat trophoblast giant cells. III. Nucleolus and its fibrillary center at different stages of the endoreplication cycle]." Tsitologiia 23(1): 5-11.

Zybina, T. G. and E. V. Zybina (2005). "Cell reproduction and genome multiplication in the proliferative and invasive trophoblast cell populations of mammalian placenta." Cell Biol Int 29(12): 1071-83.