Re-replication in the Absence of Replication Licensing Mechanisms

in *Drosophila Melanogaster*

by

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor of Philosophy in the Department of
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ABSTRACT

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Abstract

To ensure genomic integrity, the genome must be accurately duplicated once and only once per cell division. DNA replication is tightly regulated by replication licensing mechanisms which ensure that origins only initiate replication once per cell cycle. Disruption of replication licensing mechanisms may lead to re-replication and genomic instability.

DNA licensing involves two steps including the assembly of the pre-replicative complex at origins in G1 and the activation of pre-RC in S-phase. Cdt1, also known as Double-parked (Dup) in Drosophila melanogaster, is a key regulator of the assembly of pre-RC and its activity is strictly limited to G1 by multiple mechanisms including Cul4Ddb1 mediated proteolysis and inhibitory binding by geminin. Previous studies have indicated that when the balance between Cdt1 and geminin is disrupted, re-replication occurs but the genome is only partially re-replicated. The exact sequences that are re-replicated and the mechanisms contributing to partial re-replication are unknown. To address these two questions, I assayed the genomic consequences of deregulating the replication licensing mechanisms by either RNAi depletion of geminin or Dup over-expression in cultured Drosophila Kc167 cells. In agreement with previously reported re-replication studies, I found that not all sequences were sensitive to geminin depletion or Dup over-expression. Microarray analysis and quantitative PCR revealed that
heterochromatic sequences were preferentially re-replicated when Dup was deregulated either by geminin depletion or Dup over-expression. The preferential re-activation of heterochromatic replication origins was unexpected because these origins are typically the last sequences to be duplicated during a normal S-phase.

In the case of geminin depletion, immunofluorescence studies indicated that the re-replication of heterochromatin was regulated not at the level of pre-RC activation, but rather at the level of pre-RC formation. Unlike the global assembly of the pre-RC that occurs throughout the genome in G1, in the absence of geminin, limited pre-RC assembly was restricted to the heterochromatin. Elevated cyclin A-CDK activity during S-phase could be one mechanism that prevents pre-RC reassembly at euchromatin when geminin is absent. These results suggest that there are chromatin and cell cycle specific controls that regulate the re-assembly of the pre-RC outside of G1.

In contrast to the specific re-replication of heterochromatin when geminin is absent, re-replication induced by Dup over-expression is not restricted to heterochromatin but rather includes re-activation of origins throughout the genome, although there is a slight preference for heterochromatin when re-replication is initiated. Surprisingly, Dup over-expression in G2 arrested cells result in a complete endoreduplication. In contrast to the ordered replication of euchromatin and heterochromatin during early and late S-phase respectively, endoreduplication induced by Dup over-expression does not exhibit any temporal order of replication initiation.
from these two types of chromatin, suggesting replication timing program may be uncoupled from local chromatin environment. Taken together, these findings suggest that the maintenance of proper levels of Dup protein is critical for genome integrity.
To my father and mother, who have given me life and endless love.
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List of Abbreviations

pre-RC  Pre-replicative complex
Mb      Million base pair
bp      Base pair
CDK     Cyclin-dependent kinase
DDK     Dbf4-dependent kinase
ORC     Origin recognition complex
Dup     Double-parked
MCM     Minichromosome maintenance complex
RNAi    RNA interference
PCR     Polymerase chain reaction
qPCR    Quantitative polymerase chain reaction
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1. Introduction

The precise genomic duplication during each cell division is a fundamental requirement to maintain genomic integrity for all organisms. A challenge faced by eukaryotic cells is that they have to replicate their large genomes in a confined part of the cell cycle, S-phase, which only lasts a short period of time. For example, the fruit fly Drosophila Melanogaster has to duplicate a genome of approximately 160Mbp in 6 to 7 hours. In order to efficiently replicate the genome, DNA replication initiates not from a single site but from multiple loci along each chromosome, which are known as origins of replication. It is essential that origins only initiate DNA replication once during S-phase to ensure accurate and faithful duplication of genetic information. Once an origin initiates in S-phase, re-initiation from replicated sequences is prohibited. This inhibition must be maintained throughout the cell cycle until the subsequent S-phase.

Studies in the last two decades have led to a model for preventing origin re-initiation in which origin selection and activation are separated into two distinct phases of the cell cycle (Arias and Walter 2007). The first step, the selection of potential origins, occurs during G1 phase when cyclin-dependent kinase activities are low. Potential origins are marked by the pre-replicative complex (pre-RC) (see section 1.2 for more details). The process of pre-RC formation at all potential origins is termed origin licensing. Despite all origins being licensed by the pre-RC during G1, no origin can
initiate DNA replication in this phase of the cell cycle. During the second step, which is restricted to S-phase, licensed origins initiate replication according to a spatial and temporal program. Once initiated, origins are no longer bound by the pre-RC and the re-assembly of pre-RC is inhibited throughout S, G2 and M phase. Studies from yeast to mammalian systems have demonstrated that there are multiple redundant mechanisms to ensure that pre-RC assembly only occur once during each cell cycle. Disruption of these mechanisms may lead to more than one round of initiation from a subset of origins, termed re-replication, and potentially genomic instability.

In this thesis, I investigate the genomic consequences of disrupting re-replication prevention mechanisms in *Drosophila* tissue culture cells. I use the current chapter to provide an overview on DNA replication licensing and activation mechanisms. In the following two chapters, I describe my observations on sequence information and other features of re-replicated segments of the *Drosophila* genome, and characteristics of DNA replication in G2 phase of the cell cycle. In chapter 4, I summarize the major finding of my research and discuss how these findings contribute to our current understanding of DNA replication regulation.

### 1.1 Origins of DNA Replication

Origins of DNA replication are particular sequences or loci in the genome where DNA replication is initiated (Figure 1). Eukaryotic cells initiate DNA replication from
multiple origins on each chromosome during S-phase of the cell cycle. Origins of replication are regulated both at the level of utilization and the time of initiation during S-phase. Although there are many potential origins which are capable to initiate replication (“fire”), the number of origins that actually fire varies among cell cycles. Not all origins activate during each cell cycle even within the same cell population. Efficient origins fire in most cells in a population while inefficient origins fire only in a subpopulation of cells (Mechali 2010). For origins that actually fire in the same cell cycle, the time when they initiate DNA synthesis follows a strictly regulated timing program. When DNA replication is initiated at an origin, a pair of bidirectional replication forks will move away from the origin (Figure 1). When a replication fork encounters another fork, both forks will terminate and the segment of DNA between the two origins where these two forks originated from has completed replication (Fachinetti et al. 2010). Any origin that does not fire will be passively replicated by nearby origins. Origins that initiate replication are active origins and origins that are passively replicated are dormant origins.
Figure 1: Origins of replication.
DNA replication initiates from origins distributed along each chromosome. Origins fire at distinct times during S-phase of the cell cycle. A pair of bidirectional replication forks moves away from each fired origin and terminate when they encounter other forks. Active origins initiate DNA replication and dormant origins are passively replicated.

1.2.1 Yeast Origins of Replication

Origins of DNA replication are best understood in the budding yeast *S. cerevisiae*. In *S. cerevisiae*, origins of replication were first identified as short autonomous replication sequences (ARS, 80-120bp) which were required for the autonomous replication of an extrachromosomal plasmid (Stinchcomb et al. 1979). Mutational studies of the cis-acting sequence elements necessary for plasmid inheritance identified multiple short sequence elements (10-12 bp) that contributed to an ARS’s (Figure 2) (Van Houten
and Newlon 1990; Marahrens and Stillman 1992; Huang and Kowalski 1996). One conserved element between different origins is termed the ARS consensus sequence (ACS). The ACS is a degenerate T-rich motif (Figure 2) which is necessary for the binding of an essential replication protein complex called the origin recognition complex (ORC). Not all ACS elements function as replication origins and therefore the presence of an ACS is not sufficient to predict a functional origin of replication (Celniker et al. 1984; Breier et al. 2004). In fact, there are thousands of ACSs but only several hundreds of them have been identified as functional origins of replication in S. cerevisiae (Eaton et al. 2010). The majority of ACS matches do not function as ORC binding sites.

Figure 2: Origin of replication in S. cerevisiae.
(A) Sequence elements that are essential for ARS function. The ARS is about 80-120bp and consists of an A element with an 11-bp ACS and 2-3 additional poorly conserved B elements. The A and B1 elements are binding sites for the origin recognition complex (ORC), while the other B elements act as enhancers of origin efficiency. (Sclafani and Holzen, 2008, Cell Cycle Regulation of DNA Replication)
(B) Consensus sequence of ACS (A/TTTTAT/CA/GTTTA/T)
Plasmid maintenance assays have also been used to identify origins of replication in the fission yeast, *S. pombe*. In contrast to the short origins in *S. cerevisiae*, origins of replication in *S. pombe* are much larger, usually 0.5-1kb. Furthermore, mutagenesis studies suggested that there is no consensus sequence shared among origins in *S. pombe* (Clyne and Kelly 1995; Dubey et al. 1996; Segurado et al. 2003; Dai et al. 2005). Despite their relatively large size and lack of consensus sequence, DNA replication does initiate at defined regions in the fission yeast genome (Dubey et al. 1996; Gomez and Antequera 1999). The fission yeast origins are long AT-rich stretches of sequence found in intergenic regions of the genome (Figure 3) (Gomez and Antequera 1999). The differences between replication origins in the budding yeast and the fission yeast suggest that the mechanisms of origin specification may differ in simpler organisms.

![Figure 3: Origin of replication in S.pombe.](image)

The fission yeast *S. pombe* origins of replication are inter-genic. They consist of long stretches of asymmetric A:T-rich sequences which includes at least one autonomously replicating sequence (ARS) element.
1.2.2 Metazoan Origins of Replication

In higher eukaryotes, origins of replication are poorly defined compared to yeast replication origins. In differentiated cells, DNA replication initiates from unique sites in the genome, evidenced by reproducible replication profiles as revealed by in situ mapping of replication initiation sites by labeling newly synthesized DNA with nucleotide analogs (Goldman et al. 1984). In contrast, early embryonic origins have a high density of random initiation sites, likely due to the necessity of copying the genome is a short amount of time (Figure 4). One of the reasons that may contribute to the differences between embryonic and somatic replication programs is the lack of transcription in embryos. In contrast to the random but dense origins in embryos, DNA replication initiates from specific sites in the genome of somatic cells and these origins have an inter origin distance at about 50-250kb (Rampakakis et al. 2009). The identification and analysis of origins in multicellular organisms has proven to be difficult. Plasmid maintenance assays do not work well for identifying origins in metazoan (Krysan and Calos 1991), probably due to the increased genome complexity of higher eukaryotes. Unlike the conserved ACS sequence observed in S. cerevisiae, replication initiation in metazoans (Mechali and Kearsey 1984; Krysan and Calos 1991; MacAlpine et al. 2010) does not appear to require a defined consensus sequence.
Figure 4: Origins of replication during the cell cycle.

(A) In differentiated cells (somatic cells), origins of replication are defined at the origin decision point (ODP) in G1 phase of the cell cycle.

(B) Early embryonic division of Drosophila and Xenopus consists of overlapping S and M phase, lacking significant G1 and G2 phases. DNA replication appears to initiate from closely spaced random origins.

Adapted from (Mechali 2010).

Different origin mapping approaches employed for the identification of metazoan origins (Vassilev and DePamphilis 1992) have revealed that replication origins in metazoa belong to two classes (Figure 5). The first class of origins initiates replication from specific sites in the genome. Examples include the Drosophila chorion locus (Orr-Weaver et al. 1989; Lu and Tower 1997), human lamin B2 origin (Abdurashidova et al. 2000; Paixao et al. 2004) and the human β-globin locus (Kitsberg et al. 1993; Wang et al. 2004). In contrast, the second class of origins appears to consist of a primary origin flanked by multiple secondary origins over a broad initiation zone which exhibits
multiple initiation events. Examples of origins in this second class have been observed at the Chinese hamster DHFR (dihydrofolate reductase) locus (Dijkwel et al. 2002) and the human rRNA locus (Gencheva et al. 1996). The lack of a clear consensus sequence and the presence of relatively large initiation zones in metazoans suggest that origin specification may be influenced by other chromosomal features besides primary sequence.

Figure 5: Metazoan origins of replication.

One example from each of the two classes of replication origins are shown here. At the human β-globin locus, replication initiates within an 8 kb initiation region. At the Chinese hamster DHFR locus, replication initiation events occur throughout a 55 kb intergenic zone with several high-frequency initiation sites (Ori-β, Ori-β' and Ori-γ). Adapted from (Aladjem 2007).

1.2 DNA Replication Licensing

To ensure that each origin of DNA replication is only activated once during S phase, eukaryotic cells employ a tightly regulated origin licensing system to mark
sequences that are eligible to initiate replication during S phase (Bell and Dutta 2002). Failure to properly license replication origins will result in either re-initiation of DNA replication or inefficient and incomplete replication, which may contribute to genomic instability, cell death or abnormal growth. This licensing system ensures that the assembly of replication factors at replication origins is separated from replication initiation by strictly limiting each process to distinct phases of the cell cycle (Arias and Walter 2007) and therefore ensuring that no DNA sequence is ever replicated twice in a given S-phase.

Although the mechanisms that specify replication origins in yeast and metazoans are different, the protein factors that bind to origins of replication are conserved in all eukaryotes. An origin is licensed for replication by the stepwise association of several replication proteins in G1 phase of the cell cycle. The first step in licensing is the binding of the origin recognition complex (ORC) to origins in an ATP-dependent manner. ORC is an essential heterohexameric protein complex required for the initiation of DNA replication (Bell and Dutta 2002). The ORC subunits Orc1-Orc5 are conserved from yeast to metazoans and Orc6 is well conserved between fission yeast and metazoans (Duncker et al. 2009). ORC was initially discovered and purified from fractioned extracts of \textit{S. cerevisiae} as a heterohexameric complex that bound and protected the ACS from digestion with DNase I (Bell and Stillman 1992). In contrast, ORC purified from higher eukaryotes has a limited ability to recognize specific DNA sequences \textit{in vitro} (Vashee et
al. 2003) but has increased affinity to negatively supercoiled DNA (Remus et al. 2004).

Despite this apparent lack of sequence specificity, ORC does not associate with chromatin randomly but localizes instead to specific loci in the genomes of higher eukaryotes (Austin et al. 1999; Bielinsky et al. 2001; Ladenburger et al. 2002; Zellner et al. 2007; Karnani et al. 2010; MacAlpine et al. 2010). Once ORC binds to potential origins of DNA replication in the genome, it will utilize the energy from ATP hydrolysis to recruit two other replication proteins Cdc6 and Cdt1 (Bowers et al. 2004).

The loading of Cdc6 and Cdt1 is required for the recruitment of the replicative helicase Mcm2-7 complex (minichromosome maintenance proteins) (Figure 6). Cdc6 was first identified in yeast (Zhou et al. 1989) and homologues have been found in higher eukaryotes. Cdc6 is required for pre-RC formation and in the absence of Cdc6 no pre-RC can be formed. Cdt1 is another protein required to load the Mcm2-7 helicase. Cdt1 was initially found in fission yeast (Nishitani et al. 2000) and is conserved in eukaryotes. The association of Cdt1 with origins is required for the recruitment of Mcm2-7 onto the DNA through reiterative rounds of ATP-hydrolysis (Bowers et al. 2004) to form the pre-RC.

Loading the Mcm2-7 complex is the final step in the assembly of pre-RC. MCM proteins were originally identified in a yeast genetic screen for mutants that were defective for the maintenance of mini-chromosomes (Maine et al. 1984) and were later shown to be essential for DNA replication (Sinha et al. 1986; Tye 1999). Once the Mcm2-7 complex has been recruited to origins, ORC and Cd6 are no longer needed for origin
firing suggesting that ORC and Cdc6 act primarily to load Mcm2-7 complex (Rowles et al. 1999; Walter and Newport 2000). The Mcm2-7 complex has long been thought to be the replicative helicase despite the lack of biochemical evidence of helicase activity (Forsburg 2004). Recent studies, however, have shown that the Mcm2-7 complex is indeed the replicative helicase (Moyer et al. 2006; Bochman and Schwacha 2008; Bochman and Schwacha 2009). Although it is known that Mcm2-7 complex is recruited to origins after other pre-RC components, relatively little is known about the biochemical mechanisms of Mcm2-7 loading. Two recent studies using purified MCM proteins in reconstituted pre-RC assembly systems suggest that Mcm2-7 is loaded as a double hexamer onto origins (Evrin et al. 2009; Remus et al. 2009), but whether the complex travel along the chromatin as a double hexamer or single hexamer remains unclear. Theoretically, a replication origin needs at most two Mcm2-7 complexes to unwind DNA ahead of the replication forks. However, the number of Mcm2-7 complexes outnumbers the amount of ORC bound to origins by many fold. Depending on the organism, there are 10-40 Mcm2-7 complexes for each ORC associated with chromatin (Forsburg 2004; Randell et al. 2005; Takahashi et al. 2005). The role of excessive Mcm2-7 complexes remains unclear but recent reports suggest these excess MCM proteins allow the activation of dormant origins during replication stress (Ibarra et al. 2008).
The process of pre-RC assembly is complete when the Mcm2-7 complex is loaded onto the origin. The Mcm2-7 complexes are only recruited to replication origins during G1 and they remain tightly association with chromatin until origin activation. All potential origins that are licensed by the pre-RC remain inactive until the cell cycle enters S-phase. By restricting origin licensing to G1 phase of the cells cycle, origins cannot become competent to initiate DNA replication during any other time in the mitotic cell cycle. Therefore, genome integrity is preserved through only one round of replication.

**Figure 6: The formation of pre-RC.**

The origin recognition complex (ORC) binds to and marks all potential origin of replication in late M or early G1 phase. In G1 phase, ORC recruit Cdc6 and Cdt1 to origins, which facilitate the loading of the helicase Mcm2-7 complex to origins.
1.3 Initiation of DNA Replication and Origin Activation

All potential origins of replication are licensed by the assembly of the pre-RCs in G1 of the cell cycle. When the cell cycle enters S-phase, origins that have been licensed are activated at distinct times according to a temporal program. Before DNA replication initiates from an origin, the pre-RC is converted to a pre-initiation complex (pre-IC). An increase of the activities of S-phase cyclin dependent kinases (CDKs) and Cdc7-Ddb4 dependent kinases (DDKs) contributes to the formation of the pre-IC, which involves phosphorylation events and recruitment of a number of initiation proteins (Sclafani and Holzen 2007).

1.3.1 CDK and DDK

Two different protein kinases, cyclin dependent kinases (CDKs) and Cdc7-Ddb4 dependent kinases (DDKs), are required for the activation of replication origins during S-phase (Sclafani and Holzen 2007; Labib 2010). CDKs ensure the progression through the cell cycle including the initiation of DNA replication. In S. cerevisiae, CDK activity is low in G1 allowing the assembly of pre-RC and is gradually increased throughout S-phase. Fission yeast expresses a single CDK, Cdc2 (Fisher and Nurse 1996). Higher eukaryotes however have different CDKs for different stages of the cell cycle and CDK2 is present during S-phase. Another kinase that is required for replication initiation is Cdc7, together with its catalytic partner, Dbf4. The heterodimer is known as Dbf4-
dependent kinase (DDK). Both CDK and DDK are subjected to cell cycle regulation (Labib 2010).

1.3.2 Pre-initiation Complex and Helicase Activation

The activation of CDK and DDK causes multiple phosphorylation events and the loading of at least 6 additional factors and complexes onto the pre-RC, including Sld2, Sld3, Dpb11, Cdc45, GINS (Go, Ichi, Nii and San) and Mcm10 to form the pre-initiation complex (pre-IC) (Sclafani and Holzen 2007; Labib 2010). The formation of pre-IC leads to subsequent helicase activation, DNA unwinding and polymerase recruitment (Figure 7).

Mcm10 is the earliest initiation factor that binds to the pre-RC and the formation of pre-RC is a prerequisite for Mcm10 to associate with chromatin (Heller et al. 2011). It has been suggested that Mcm10 functions in multiple steps in the initiation of DNA replication, including the chromatin loading of Cdc45 (Izumi et al. 2000; Sawyer et al. 2004), phosphorylation of Mcm2-7 by DDK (Lee et al. 2003) and the maintenance of DNA polymerase- α at the replication fork during elongation (Gregan et al. 2003; Ricke and Bielinsky 2004).

Before the helicase can be activated, other factors besides Mcm10 have to be recruited to the pre-IC. Sld2 and Sld3 are two of these factors. First identified in yeast, Sld2 and Sld3 are both substrates of CDK (Tanaka et al. 2007; Zegerman and Diffley 2007).
Phosphorylated Sld2 and Sld3 can bind to Dpb11 through interaction with BRCT domains of Dpb11. The association between phosphorylated Sld2 and Dpb11 is essential for the recruitment of Cdc45 and the GINS complex (Labib and Gambus 2007).

The loading of Cdc45 and GINS is required for both the activation of the MCM helicase and the establishment of a productive replication fork (Tercero et al. 2000). A complex of Mcm2-7, Cdc45 and GINS was purified from *Drosophila* embryo extracts (Moyer et al. 2006), which showed ATP-dependent helicase activity *in vitro*, suggesting the helicase activity of Mcm2–7 complex can be activated by the binding of Cdc45 and GINS. The recruitment of Cdc45 to the pre-IC requires CDK and DDK activities (Dolan et al. 2004). Recent studies showed that DDK phosphorylation of Mcm4 facilitate the chromatin loading of Cdc45 (Masai et al. 2006; Sheu and Stillman 2010). Consistent with its function as the helicase activator, Cdc45 does not associate with all origins simultaneously but only bind to origins that are about to fire. For example, Cdc45 loads to early and late origins at specific times during S-phase in the budding yeast (Zou and Stillman 2000). It has also been suggested that Cdc45 facilitates the loading of polymerase α (Aparicio et al. 1999). Therefore, Cdc45 is required both for the initiation and the maintenance of the replication fork.
Figure 7: Formation of pre-initiation complex and initiation of DNA replication.

The pre-replication complex (pre-RC) is assembled onto origins in G1. Activation of the pre-RC and initiation of replication require the activities of CDK (cyclin-dependent kinase) and DDK (DBF4-dependent kinase; CDC7 kinase). CDK and DDK lead to the recruitment of additional factors including Mcm10, Sld2, Sld3, Dpb11, Cdc45 and the GINS complex to pre-RC bound origins to form the pre-initiation complex (pre-IC). The formation of pre-IC leads to the recruitment of DNA polymerases and other factors.
1.4 Replication Timing Program

The activation of origins of replication follows a tightly regulated temporal program. Although the exact relationship between DNA replication, transcription and chromatin structure remains unclear, early studies on DNA replication demonstrated that accessible and transcriptionally active euchromatin replicates early during S-phase, whereas silenced heterochromatin replicates late (Hsu et al. 1964; Lyon 1968; Stambrook and Flickinger 1970). It has therefore been proposed that active transcription positively correlates with early replication and that the replication timing program may, in part, maintain epigenetic modifications. Indeed, transcriptional changes during development are accompanied by changes of replication timing. For example, the differentiation from embryonic stem cells to specialized cell types is accompanied by global changes in the timing program (Hiratani et al. 2008; Desprat et al. 2009) and there is a change in replication timing at the human β-globin locus during erythroid differentiation (Epner et al. 1988).

Recent genome-wide approaches have confirmed early observations on the correlation between transcriptional activity and replication timing at a much higher resolution. These studies found that transcriptionally active regions of the genome are typically replicated before regions that have a lower transcriptional activity in a variety of organisms like Drosophila (Schübeler et al. 2002; MacAlpine et al. 2004; Schwartz and Ahmad 2005; Schwaiger et al. 2009), human (Ryba et al. 2010), and mouse cells (Farkash-
Amar et al. 2008; Hiratani et al. 2008; Hiratani et al. 2010). However, the resolution of replication timing is not at the level of individual origins but rather over large chromosomal domains. The correlation between replication timing and transcription is also demonstrated at the level of large chromatin domains rather than at the level of single loci. For example, in mammalian cells, the size of replicating domains ranges from hundreds of kilobases to megabases (Hiratani et al. 2008). These results suggest that replication timing over a large domain is more related to local chromatin structure rather than the transcriptional status of a specific gene.

Recent studies examining the correlation between chromatin structure and replication timing have suggested that chromatin environments permissive for transcription also promote the early activation of replication origins. Using data generated by the ENCODE and modENCODE projects, a positive correlation between early activating origins and the activating chromatin marks such as H3K4me2 and H3K4me3 and H3/H4Ac has been reported (Figure 8). Whereas late replicating origins exhibit a strong correlation with H3K27Me3 marked chromatin in mammalians (Consortium 2007; Karnani et al. 2007; Karnani et al. 2010) and Drosophila (Eaton et al. 2011). The precise mechanism by which chromatin structure is correlated with replication timing is still under investigation.
Figure 8: Replication timing during S-phase.

Active transcription usually associated with activating markers like H3K4me and H3/H4Ac corresponds to early replication during S-phase. And repressive heterochromatin marked by H3K9me and H3K27me is usually replicated late during S-phase.
1.5 Re-replication and Strategies to Prevent Re-replication

To maintain genomic stability, re-initiation of DNA replication in the same cell cycle (re-replication) is prevented via multiple mechanisms. Although the temporal separation of the loading of helicase (pre-RC assembly) and the activation of helicase appears to be universal among eukaryotes, specific regulation mechanisms preventing re-replication vary between organisms. Despite this variation, all mechanisms involve regulating the stability, activity, or localization of pre-RC components (Table 1) (Blow and Dutta 2005; Arias and Walter 2007).

In *S. cerevisiae* and *S. pombe*, pre-RC re-assembly is prevented primarily by CDK phosphorylation of pre-RC components, which leads to either degradation or nuclear export of pre-RC proteins. In *S. cerevisiae*, all six ORC subunits remain associated with the chromatin throughout the cell cycle. Phosphorylation of Orc2 and Orc6 by CDK leads to inactivation of ORC even though the exact mechanism is unclear (Nguyen et al. 2001). Cdc6 is inhibited by CDK at three levels, including degradation and direct interaction with CDK which are promoted by phosphorylation (Drury et al. 2000; Mimura et al. 2004), and transcription inhibition (Moll et al. 1991). Finally, CDKs also promote the nuclear exclusion of Mcm2-7 and Cdt1 in the budding yeast (Labib et al. 1999; Nguyen et al. 2000; Liku et al. 2005).
Higher eukaryotic cells have at least two other CDK-independent mechanisms to prevent re-replication: proteosome mediated degradation of Cdt1 or by the inhibitory binding of geminin to Cdt1 (Fujita 2006). The proteolysis of Cdt1 during S-phase is replication-coupled (Thomer et al. 2004; Arias and Walter 2006). Following origin activation, Cdt1 binds to the replication processivity clamp, PCNA, which leads to Cdt1 ubiquitylation by the Cul4\(^\text{Ddb1}\) E3 ubiquitin ligase and degradation by the proteasome (Arias and Walter 2006; Jin et al. 2006; Senga et al. 2006). Geminin is present only in higher eukaryotes and its expression is limited to the S, G2 and M phases in proliferating metazoan cells (Melixetian and Helin 2004). Geminin binds and sequesters Cdt1 in an inactive complex that cannot recruit the Mcm2-7 complexes thereby suppressing origin licensing (Wohlschlegel et al. 2000; Tada et al. 2001). The degradation of geminin in late mitosis releases Cdt1 from geminin and allows the reformation of the pre-RC in the subsequent G1 (McGarry and Kirschner 1998).

Deregulation of these re-replication preventing mechanisms may result in re-initiation of DNA replication even though the exact mechanisms that need to be disrupted in order for the detection of re-replication vary among organisms. For example, in budding yeast, simultaneous mutation of multiple pre-RC components (ORC, Cdc6, MCM) is required to override the licensing control mechanisms and the detection of re-replication (Nguyen et al. 2001). It has been shown that re-replication can be induced by Cdc6 overexpression in fission yeast (Nishitani et al. 2000; Yanow et al.
In *C. elegans*, *Drosophila* and mammalian systems, the disruption of Cdt1 downregulation is sufficient for re-replication, including Cdt1 overexpression, geminin depletion and impairment of the Cul4Ddb1 pathway (Figure 9) (Mihaylov et al. 2002; Vaziri et al. 2003; Zhong et al. 2003; Melixetian et al. 2004).

Re-replication may ultimately lead to genome instability and affect the viability of cells. Double-strand breaks and DNA damage checkpoint activation have been reported both in yeast (Green and Li 2005) and metazoans (Vaziri et al. 2003; Melixetian et al. 2004; Zhu et al. 2004) re-replication. Furthermore, a recent report suggested that gene amplification can be a long term genomic consequences of re-replication (Green et al. 2010). The exact mechanisms of how re-replication causes DNA damage and contributes to gene amplification are unknown. A common feature shared among reported re-replication is that re-replication is incomplete, with the extent of re-replication varying widely among different systems. There appears to be additional mechanisms to prevent re-replication from spreading to every origin in the genome after re-replication occurs.
Table 1: Mechanisms to prevent re-replication in different organisms.

<table>
<thead>
<tr>
<th></th>
<th>S. cerevisae</th>
<th>S. pombe</th>
<th>C. elegans</th>
<th>Drosophila</th>
<th>Mammalian</th>
</tr>
</thead>
<tbody>
<tr>
<td>ORC</td>
<td>CDK phosphorylation; Clb5 binding of Orc6 subunit</td>
<td>Phosphorylation and CDK binding</td>
<td>N/A</td>
<td>N/A</td>
<td>CDK inhibition of ORC binding</td>
</tr>
<tr>
<td>Cdc6</td>
<td>Degradation; Nuclear export; Inhibition of transcription; Inhibitory binding of Clb2</td>
<td>Degradation</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Cdt1</td>
<td>Nuclear export</td>
<td>Degradation</td>
<td>Degradation; Geminin</td>
<td>Degradation; Geminin</td>
<td>Degradation; Geminin</td>
</tr>
<tr>
<td>Mcm2-7</td>
<td>Nuclear export</td>
<td>N/A</td>
<td>N/A</td>
<td>Disassociation from chromatin</td>
<td>Disassociation from chromatin</td>
</tr>
</tbody>
</table>
Figure 9: Re-assembly of pre-RC when mechanisms downregulating Cdt1 are impaired.

When geminin is depleted or the Cul4Ddb1 pathway is compromised, excess Cdt1 may lead to re-assembly of the pre-RC during the same cell cycle.
2. Re-replication of Heterochromatin Induced by Geminin Depletion

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2.1 Introduction

2.1.1 Drosophila Homolog of Geminin

The Drosophila homolog of geminin inhibits DNA replication in Xenopus extracts by preventing Mcm2-7 loading and forms a complex with Drosophila Cdt1 (Dup) in vivo (Quinn et al., 2001). The Drosophila geminin is present in cycling cells and is degraded at metaphase to anaphase transition. Deficiency in geminin causes re-replication both during embryogenesis and in oogenesis (Quinn et al. 2001), and in cultured SD2 cells (Mihaylov et al. 2002).

2.1.2 Non-integral Re-replication of the Genome

The mechanisms preventing re-replication do not have equal effectiveness in all organisms. Regardless of the exact mechanism(s) that prevent re-replication, a common feature among eukaryotes is that not all sequences are equally susceptible to re-initiation of DNA replication. In human cancer cell lines, re-replication induced by Cdt1 overexpression or Cdt1/Cdc6 co-overexpression occurs in early replicating regions of
the euchromatin (Vaziri et al. 2003), whereas genome-wide studies in both *S. cerevisiae* and *S. pombe* have shown that re-initiating origins of replication are distributed throughout the genome with increased re-replication at subtelomeric sequences (Green et al. 2006; Tanny et al. 2006; Mickle et al. 2007). These studies also suggest the intrinsic ability of an origin to re-initiate DNA replication is regulated both at the level of pre-RC assembly and activation. Specifically, not all origins re-assemble a pre-RC in the absence of DNA replication licensing mechanisms, and furthermore, not all replication origins that re-assemble a pre-RC are competent to re-initiate DNA replication (Tanny et al. 2006).

The deregulation of replication licensing leads to DNA damage, checkpoint activation, aneuploidy and genomic instability (Vaziri et al. 2003; Melixetian et al. 2004; Zhu et al. 2004; Green and Li 2005). I sought to better understand how origins of replication are selected and activated in the absence of replication licensing controls. Specifically, I have depleted geminin from *Drosophila* Kc167 cells and assessed the consequences of re-replication using genome-wide approaches. I found that the pericentromeric heterochromatin was preferentially re-replicated in the absence of geminin. Re-replication of the heterochromatin was due to the dynamic re-assembly of a limited number of pre-RCs whose assembly was restricted to the heterochromatin. These results provide insights into how cell cycle controls and chromatin environment facilitate the selection and regulation of origins of replication.
2.2 Results

2.2.1 Geminin Depletion Induces DNA Re-replication

The increase in DNA content observed in the absence of geminin in both *Drosophila* (Mihaylov et al. 2002) and human cell lines (Melixetian et al. 2004) does not follow precise genome-unit integer steps, but rather represents a broad continuum of DNA content greater than 4N. The non-integer increase of DNA copy number observed in geminin depleted cells suggests that certain sequences may have a differential capacity to re-initiate DNA replication. I sought to characterize the susceptibility of unique sequences in the *Drosophila* genome to re-initiate DNA replication in the absence of geminin.

RNA interference (RNAi) was used to reduce the expression of geminin in *Drosophila* Kc167 cells. Asynchronous cells were treated with dsRNA targeting either geminin or a non-specific control sequence derived from the plasmid pUC119. Geminin protein levels were depleted in a time dependent manner in the cells treated with geminin dsRNA (Figure 10). In contrast, geminin levels were not perturbed by treatment with pUC dsRNA. As previously reported (Mihaylov et al. 2002; Hall et al. 2008), I observed a decrease in the stability of Dup, the *Drosophila* Cdt1 homolog, in the absence of geminin(Figure 10). Consistent with published data (Mihaylov et al. 2002), flow cytometry of DNA content revealed that geminin deficiency resulted in the
inappropriate re-replication of the genome. After only 24 hours of treatment with
geminin dsRNA, a large population of cells (>50%) contained more than 4N DNA
content and by 48 hours of treatment, many of the cells had a DNA content greater than
8N (Figure 11A).

Figure 10: Geminin depletion by RNAi results in loss of both geminin and Dup
protein levels.

Cells were treated with dsRNA targeting a non-specific control (pUC) or geminin for 24
or 48 hours. The abundance of geminin and Dup was assessed by immunoblotting.
Acetylated H4 was used as a loading control.
Figure 11: Geminin depletion results in increased ploidy.

(A) Histogram of DNA content determined by flow cytometry for cells treated with nonspecific pUC control dsRNA for 24 (gray; solid outline) and 48 hours (gray; dashed outline) or geminin dsRNA for 24 (blue; solid outline) and 48 (green; dashed outline) hours.

(B) Boxplot of the relative copy number of DNA from geminin depleted cells at 48 hours versus DNA from control cells for individual array probes grouped by chromosome. The heterochromatic 4th chromosome is at a significantly elevated copy number \( p<1\times10^{-16} \).

(C) The relative copy number for array probes following 48 hours of geminin depletion (black) as a function of chromosomal position for the left and right arms of *Drosophila* chromosome 2. The relative copy number comparing two independent populations of control cells is shown in gray. Transposon density (fraction of sequence covered by transposable elements) is indicated by the red histogram at the bottom of the plot.
2.2.2 Pericentromeric Heterochromatin Is Preferentially Re-replicated

To identify sequences prone to re-replication in geminin depleted Kc167 cells, I utilized comparative genomic hybridization (CGH) to directly measure the relative change in DNA copy number generated as a result of re-replication. In this assay, DNA from independent biological replicates was harvested from either geminin or pUC dsRNA treated cells, differentially labeled with Cy5 and Cy3 conjugated dUTP, and hybridized to custom genomic tiling microarrays containing unique probes spanning the sequenced Drosophila euchromatin. The log ratio of Cy5 to Cy3 signal served as a proxy for the relative copy number difference between geminin depleted and control cells.

Geminin depletion resulted in strikingly elevated levels of the fourth chromosome (Figure 11B). The Drosophila fourth chromosome is unique because of its small size, high transposon density and heterochromatic nature (Riddle et al. 2009). Given the distinct chromatin environment of the fourth chromosome, I sought to more closely examine copy number throughout the remaining Drosophila chromosomes. I analyzed the relative copy number of DNA from geminin depleted cells versus control DNA across each of the chromosomes by plotting the moving average of DNA copy number as a function of chromosome position. The relative copy number for the large majority of euchromatic sequences along the chromosome arms of geminin depleted cells was constant and equal to one. However, as the sequences approach the pericentromeric heterochromatin on chromosome 2, 3 and X, the copy number increased
sharply, indicating that DNA sequences in these regions have re-replicated (Figure 11C and Figure 12, black).

**Figure 12: Heterochromatin is preferentially re-replicated during geminin depletion.**

The copy number of all unique sequences in the *Drosophila* genome was determined by comparative genomic hybridization (CGH) using genomic tiling microarrays. Relative DNA copy number following 48 hours of treatment with geminin (black) or non-specific control dsRNA (gray) for each of the *Drosophila* chromosomes. Transposon density (fraction of sequence covered by transposable elements) is shown in red.
In *Drosophila*, repressive chromatin is marked by the trimethylation of histone H3 on lysine residue 9 (H3K9me3) in the constitutive pericentromeric heterochromatin and by trimethylation of histone H3 on lysine 27 (H3K27me3) at polycomb repressed sequences (Fischle et al. 2003; Ebert et al. 2006). The elevated copy number I observe for chromosome 4 and the sequences proximal to the heterochromatin for each of the remaining chromosomes suggests that the constitutive heterochromatin is being preferentially re-replicated in the absence of geminin. These sequences also exhibit an increased density of transposable elements (Figure 11C and Figure 12, red). I examined the extent of re-replication for sequences marked by either H3K9me3 or H3K27me3 using data generated by the modENCODE consortium (Celniker et al. 2009; Riddle et al. 2010). These genomic datasets describe the genome-wide location of lysine trimethylation on histone H3 using nearly identical growth conditions (including serum) as my own experiments. I found that re-replication in the absence of geminin was specific for the constitutive heterochromatin marked by H3K9me3 (Figure 13).
Figure 13: Sequences marked by H3K9me3 but not H3K27me3 are re-replicated.

Boxplots of the relative copy number for sequences marked by H3K9me3 or H3K27me3 following geminin depletion by RNAi. The genome-wide mapping of H3K9me3 and H3K27me3 was performed in Drosophila S2 cells by the modENCODE consortium (see reference 27, 28). Broad peaks of enrichment for H3K9me3 and H3K27me3 were defined (see GEO submission GSE20781 and GSE20794 for details) and the relative copy number for each mark in control and geminin depleted cells was analyzed by boxplots. The increase in copy number for sequences marked by H3K9me3 in geminin depleted cells was significant ($p<2\times10^{-16}$). Although there are a few specific differences in chromatin marks between the different Drosophila cell lines, the bulk genome-wide distributions are very similar across Drosophila cell lines, justifying the comparison of re-replication in Kc167 cells to chromatin marks in S2 cells. We also observe similar heterochromatin specific re-replication patterns in Drosophila S2 cells depleted for geminin (data not shown).
Because the heterochromatic sequences and those proximal to heterochromatin have a reduced sequence complexity, I wanted to ensure that the increase in DNA copy number was not an artifact of the array hybridization. As a control, I hybridized independent samples of control DNA versus itself. The relative copy number of the control DNA was constant across each of the chromosome arms (Figure 11C and Figure 12, gray). To account for any potential variation in the copy number estimates due to the asynchronous cell cycle distribution of the control cells, I also compared by CGH analysis DNA from geminin depleted cells to cells arrested in G2/M by colcemid treatment. This analysis revealed a copy number increase for heterochromatic sequences that was indistinguishable from my prior results (Figure 14). Finally, to further validate our copy number estimation from the genomic microarrays, I tested seven different loci throughout the euchromatin and heterochromatin of chromosome 3R by quantitative PCR (qPCR). The qPCR results confirmed the heterochromatin specific 2 – 2.5 fold increase in DNA content number I observed by the genomic tiling arrays (Figure 15).
Figure 14: Heterochromatin is preferentially re-replicated during geminin depletion compared to cells arrested with G2 DNA content.

(A) FACS analysis of cells treated with colcemid (0.5µg/ml) in the presence (middle) or absence (left) of geminin dsRNA for 48 hours. The overlay is depicted in the right panel.

(B) The relative copy number for array probes following 48 hours of geminin depletion (black) as a function of chromosomal position for the left arm of Drosophila chromosome 2. The relative copy number comparing two independent populations of colcemid treated control cells is shown in gray.
Figure 15: Validation of copy number at seven loci following a 48h RNAi depletion of geminin.

Seven loci were tested by qPCR using primer pairs designed to unique sequences in the heterochromatin and euchromatin of the right arm of chromosome 3. The copy number at each site was normalized to the copy number of site 7 which is a unique euchromatic sequence that is distant from heterochromatin. There is a 1.5 – 2.5 fold increase of DNA content for loci both in and proximal to the pericentric heterochromatin.

Genomic analysis indicated that there was increased copy number of sequences proximal to the pericentromeric heterochromatin; however, the initial genomic tiling arrays that I used lacked representative probes from this region of the genome. To further identify which regions of the genome were being re-replicated in the absence of geminin, I assessed the cytological location of active replication by immunofluorescence. Actively replicating sequences were labeled by the incorporation of 5-bromo-2-deoxyuridine (BrdU). BrdU was added to the medium for a 4 hour window at 18 hours
post RNAi treatment, at which time re-replication is clearly detectable by FACS in
geminin depleted cells (data not shown). The cells treated with pUC dsRNA exhibited
multiple distinct patterns of BrdU incorporation, including: no BrdU incorporation,
BrdU incorporation all over the nucleus, and BrdU incorporation localized to a small
region at the nuclear periphery (Figure 16A). These distinct patterns of BrdU
incorporation were classified as either 'none', 'global', or 'local', respectively (Figure
16B). These patterns likely represent cells in different stages of the cell cycle. For
example, cells with no BrdU staining (none) were likely in G2-M-G1 of the cell
cycle, cells with limited BrdU in a confined region at the nuclear periphery (local) were
in late S-phase, and finally those cells with BrdU incorporation throughout the nucleus
(global) represent cells in early to mid S-phase (Dimitrova and Gilbert 1999). In contrast,
almost 90% of BrdU staining geminin depleted cells exhibited the 'local' BrdU
incorporation pattern in a small region of the nucleus (Figure 16A and 16B). These
results were consistent with re-replication being confined to a specific region of the
genome.
Figure 16: Re-replication is specific to pericentromeric heterochromatin.

(A) Fluorescence microscopy of cells after a 4 hour BrdU pulse at 18 hours post RNAi treatment with control (pUC) or geminin dsRNA. BrdU labeled sequences were detected with a rat anti-BrdU antibody (red) and DNA was counterstained with DAPI (blue). BrdU staining patterns were classified as 'global' (arrow), 'local' (arrowhead) or 'none'.

(B) Distribution of the different BrdU incorporation patterns. At least 200 cells were counted from three independent experiments. The distribution of BrdU incorporation patterns was significantly different between control cells and geminin depleted cells (p<1x10^-16).

(C) Immunofluorescence of control and geminin depleted cells following a 30 min pulse of EdU at 24 hours post RNAi. EdU and HP1 were detected with Alexa Fluor 488-
Figure 16 (Continued)

azide (green) and rabbit anti-HP1 antibody (red), respectively, and DNA was
counterstained with DAPI (blue).

(D) Quantification of EdU and HP1 localization patterns in control and geminin
depleted cells with error bars indicating standard error. At least 200 cells were
counted in three independent experiments. The distribution of EdU/HP1 localization
patterns was significantly different between control cells and geminin depleted cells
(p<1x10^-16).

The 'local' pattern of BrdU incorporation in the nucleus, the detection of
increased copy number near the pericentromeric heterochromatin and the increased
copy number for sequences marked by H3K9me3 in geminin depleted cells suggested
that the pericentromeric heterochromatin was being preferentially re-replicated in the
absence of geminin. In Drosophila, the pericentromeric heterochromatin is enriched for
HP1, a heterochromatic protein that interacts with H3K9me3 (Ebert et al. 2006). To
confirm that re-replication was localized to the heterochromatin, I used
immunofluorescence to simultaneously detect newly synthesized DNA and HP1 (Figure
16C). Control and geminin depleted cells were pulse labeled with 5-ethyl-2-
deoxyuridine (EdU), for 30 minutes at either 24 or 48 hours post RNAi. EdU was used
for these experiments because unlike BrdU, the denaturation of DNA was not required
for detection. In control cells I observe that EdU is either specifically co-localized with
HP1 (actively replicating heterochromatin) or that it is excluded from the HP1 staining
regions of genome (actively replicating euchromatin) (Figure 16C and 16D). In contrast, geminin depleted cells undergoing re-replication almost exclusively exhibit co-localization of EdU and HP1, consistent with the preferential re-replication of the heterochromatin. Together with the array data, these results show that the majority of DNA synthesis occurring in geminin depleted cells is restricted to heterochromatic regions of the Drosophila genome.

2.2.3 Re-replication Is Not Coupled With Late Replication

Origins of replication are activated in S phase with a characteristic timing and efficiency. Chromatin structure is thought to play a role in establishing the temporal order of origin activation (Donaldson 2005). For example, active transcription and histone acetylation have been positively correlated with early replication in a variety of model organisms (Schübeler et al. 2002; Vogelauer et al. 2002; Birney et al. 2007; Calvi et al. 2007; Knott et al. 2008). Similarly, poorly transcribed regions of the genome, often marked by repressive chromatin modifications, are typically the last sequences to be duplicated in S-phase (Schübeler et al. 2002; MacAlpine et al. 2004). My genomic and cytological results are paradoxical because they suggest that in the absence of geminin, the initiation of DNA replication is most efficient in the pericentromeric heterochromatin marked by H3K9 methylation and HP1. These findings imply that either late replicating sequences are more prone to re-replication or that the observed re-replication is a function of chromatin environment.
To determine if susceptibility to re-replication was a function of late replicating sequences or a heterochromatic chromatin environment, I examined the extent of re-replication and the relative time of replication evident in heterochromatic sequences using a custom array design (MacAlpine et al. 2010) that, in addition to the euchromatic sequences, contained all of the unique *Drosophila* heterochromatic sequences (Hoskins et al. 2007). DNA was isolated from control and geminin depleted cells and hybridized to the new genomic arrays. Statistical analysis of the relative probe levels between control and geminin depleted cells revealed a clear increase in copy number for those probes located in the annotated heterochromatin (Figure 17A; p<1x10^{-16}). Analysis of the data relative to chromosome position revealed a constant one and a half to two-fold increase in ploidy in the heterochromatin (red) relative to the euchromatin (black), with a shallow transition from heterochromatin to euchromatin, as shown for a representative region of the right arm of chromosome 2 (Figure 17B). Importantly, as my previous array data indicated, re-replication and the increase in DNA copy number was limited to the heterochromatin and adjacent sequences.

The normal mitotic replication timing of the heterochromatin was examined by differentially labeling early and late replicating sequences from synchronized cells (MacAlpine et al. 2004; MacAlpine et al. 2010). Analysis of global replication timing values from either euchromatic or heterochromatic sequences revealed that the heterochromatin replicated significantly later than the euchromatin (Figure 17C; p<1x10^{-16}}
I did not detect the presence of efficient clusters of mitotic replication origins in the heterochromatin which would be evident as a continuum of enriched probes culminating in a clear and defined peak, as shown for the right arm of chromosome 2 (Figure 17D). Importantly, late replication does not appear to be a determinant of re-replication as there are many late replicating regions in the euchromatin that do not appear to re-replicate as shown for a representative region of the genome (Figure 17B and 17D). These data argue that susceptibility to re-initiation of DNA replication is distinct from the mechanisms that regulate replication timing.
Figure 17: Re-replication is dependent on chromatin environment not time of replication.

(A) Boxplot of the relative copy number between geminin depleted and control cells for array probes in the euchromatin or heterochromatin. The heterochromatin is at a significantly higher copy number (p<1x10^-16).

(B) The relative copy number for array probes in the vicinity of pericentromeric heterochromatin on the right arm of chromosome 2 following geminin RNAi treatment for 48 hours (euchromatin, black; heterochromatin, red).

(C) Boxplot of the relative time of replication for sequences in the euchromatin or heterochromatin from normal mitotic cells. The heterochromatin is significantly later replicating than the euchromatin (p<1x10^-16).

(D) Replication timing values as a function of chromosomal position for unique sequence probes in the vicinity of the pericentric heterochromatin on the right arm of chromosome 2 (euchromatin, black; heterochromatin, red).
2.2.4 Limited pre-RC Assembly in the Absence of Geminin

Prior to initiation of DNA replication, the pre-RC must be assembled at origins of replication. Typically, low CDK levels and the absence of geminin allow for the global assembly of pre-RCs in G1 (Arias and Walter 2007). I hypothesized that re-replication in the absence of geminin may be limited to the heterochromatin by restricting either pre-RC assembly or activation to these sequences. For example, in the absence of geminin the re-assembly of the pre-RC may occur almost exclusively in the heterochromatin. Alternatively, there may be a global re-assembly of the pre-RC throughout the Drosophila genome, but initiation of DNA replication is strictly limited to the heterochromatin. To differentiate between these two models, I assessed the re-loading of MCMs onto the DNA by chromatin fractionation and immunofluorescence.
Figure 18: Limited pre-RC re-assembly occurs in the absence of geminin.

(A) Chromatin from control RNAi (pUC), G1/S arrested (HU), and geminin depleted re-replicating cells (Gem) was fractionated biochemically and immunoblotted for MCMs and ORC2. Levels of ORC and MCMs are also shown for whole cell extracts (WCE).

(B) Fraction of control or geminin depleted cells exhibiting active DNA synthesis (EdU incorporation), with error bars indicating standard error. DNA synthesis was determined by pulsing the cells for 30 minutes with EdU at the given time points. At least 200 cells were counted in three independent experiments.

(C) Time course of the fraction of control (open diamond) or geminin depleted (open square) cells exhibiting nuclear MCM localization following RNAi treatment for the indicated duration, with error bars indicating standard error. At least 200 cells were counted in three independent experiments.
I first examined the global levels of chromatin-associated Mcm2-7 complex by chromatin fractionation. Chromatin was prepared from pUC dsRNA treated cells (pUC), cells arrested with hydroxyurea (HU), and cells treated with geminin dsRNA (Gem) for 24 hours. ORC remained associated with the chromatin fraction in all samples. In contrast, Mcm2-7 association with chromatin was only observed in pUC RNAi cells and cells arrested at the G1/S border by treatment with HU. A marked reduction in Mcm2-7 levels was observed on the chromatin prepared from geminin depleted cells while total Mcm2-7 levels were not affected by geminin depletion (Figure 18A). Despite the reduction in chromatin-associated Mcm2-7 levels in the geminin depleted cells, a significant number of cells (60%-85%) exhibited active DNA synthesis as measured by a 30 minute EdU pulse (Figure 18B). Thus, cells undergoing re-replication are able to synthesize DNA with a reduced complement of MCM proteins.

To gain further insights into the mechanism of Mcm2-7 reloading, I examined Mcm2-7 loading and DNA synthesis at the single cell level. The association of Mcm2-7 with chromatin was assessed by immunofluorescence of cells treated with a mild detergent and salt prior to fixation. The mild salt extraction ensures that only the loaded and active Mcm2-7 remain bound to the chromatin (Claycomb et al. 2002; Randell et al. 2005). I found that the fraction of Mcm2-7 positive staining cells gradually decreased during geminin RNAi treatment. At 24 hours following geminin depletion, less than 20% of the cells had detectable Mcm2-7 staining (Figure 18C); however, more than 80%
of the cells exhibited active DNA synthesis. Even after 48 hours of geminin depletion, nearly 70% of the cells exhibited DNA synthesis following a 30 minute EdU pulse (Figure 18B). I consistently observed that the number of geminin depleted cells that were actively synthesizing DNA was much higher than the number of cells exhibiting detectable staining for chromatin-associated Mcm2-7. Together, these data suggest that in the absence of geminin, pre-RC assembly is inefficient and that minimal levels of Mcm2-7 re-assembly are sufficient for un-regulated DNA replication.

2.2.5 pre-RC Re-assembly at Heterochromatin

The low levels of chromatin-associated Mcm2-7 which are sufficient for un-licensed re-initiation of DNA replication in the absence of geminin made it difficult to assess whether the re-assembly of the pre-RC was limited to the heterochromatin or occurred throughout the Drosophila genome. I hypothesized that during re-replication the re-assembly of the pre-RC might be labile with the Mcm2-7 exhibiting a short half life on the DNA. In order to extend the half-life of the Mcm2-7 complex on chromatin, I used aphidicolin to inhibit DNA polymerase and arrest any active replication forks. DNA synthesis was inhibited in both control and geminin depleted cells at 24 hours post RNAi treatment by the addition of aphidicolin (Figure 19A). The majority of control cells arrest with an early S-phase FACS profile upon exposure to aphidicolin. Similarly, aphidicolin inhibited additional re-replication in the absence of geminin and the cells
arrest with a DNA content profile indistinguishable from that of cells depleted of
geminin for 24 hours.

**Figure 19: MCMs are preferentially loaded onto heterochromatin in the absence of
geminin.**

(A) Flow cytometry analysis of DNA content for cells treated with nonspecific pUC or
geminin RNAi for 24 hours (dotted outline; gray), 48 hours (solid outline; gray) or 48
hours with aphidicolin (APH) added at 24 hours (solid outline; blue).

(B) Quantification of MCM localization patterns. MCM staining was classified into 3
types: type I - MCM localization throughout the nucleus, type II - MCM localization
to a small portion of the nucleus (heterochromatin), and type III - no significant
MCM localization observed. The numbers under each staining pattern represent the
percentage of nuclei with that particular pattern. At least 200 cells were counted in
three independent experiments. The distribution of MCM localization patterns in
geminin depleted cells treated with aphidicolin was significantly different from
control cells treated with aphidicolin (p<1x10^-16) and similarly, geminin depleted
cells were significantly different from geminin depleted cells treated with aphidicolin (p<1x10^-16).

(C) Low and high magnification examples of type I (arrow) and II (arrow head) MCM staining patterns in control and geminin depleted cells treated with aphidicolin. The MCMs, HP1 and DAPI are shown in green, red and blue, respectively. The type II pattern of MCM localization in geminin depleted cells often exhibited heterogeneous staining with faint signal over the entire HP1 region (open arrowhead) or partial overlap with HP1 (open arrow).

In normally dividing cells, the chromatin association of the Mcm2-7 complex changes thoughout the cell cycle (Kimura et al. 1994; Krude et al. 1996; Liang and Stillman 1997; Su and O'Farrell 1997). During G1, the Mcm2-7 complex is loaded onto the chromatin to form the pre-RC and to license replication origins for entry into S-phase. As DNA replication progresses through S-phase, the Mcm2-7 complex is displaced from the DNA by the passage of the replication fork (Kimura et al. 1994; Krude et al. 1996). Thus, during G1 and early to mid S-phase the Mcm2-7 complex is localized throughout the entire nucleus, and by late S-phase they only remain associated with late replicating sequences. I classified the untreated control cells into three categories based on their Mcm2-7 localization patterns (Figure 19B). In 37% of the control cells, the Mcm2-7 localized throughout the nucleus with increased staining at the heterochromatic region marked by HP1(type I). I interpret these cells to be in G1 and early to mid S phase. In contrast, 18% of cells appeared to be in late S-phase with Mcm2-
7staining limited to heterochromatin (type II) while the remaining 46% of cells were devoid of nuclear Mcm2-7 staining (type III) and likely in G2 or M phase. The distributions of cells in each phase of the cell cycle were consistent with the FACS profiles (Figure 19A). Treatment with aphidicolin altered the cell cycle distribution of control cells with the majority of cells accumulating at the G1/S transition and exhibiting the type I staining pattern (Figure 19B).

Treatment of geminin depleted cells with aphidicolin inhibited further re-replication and resulted in a clear increase in chromatin-associated Mcm2-7 levels as evidenced by the increase in the number of cells with positive MCM staining (type I and II, from 19% to 63%). Unlike control cells where the majority of Mcm2-7 were localized throughout the nucleus upon exposure to aphidicolin (Figure 19C, arrow), the predominant localization pattern in the geminin depleted cells was at the heterochromatin, co-incident with HP1 staining (Figure 19B and 19C). In contrast to the strong uniform heterochromatic staining I observe in control cells and a few untreated geminin depleted cells, aphidicolin treatment of geminin depleted cells often resulted in a weak and heterogeneous staining of Mcm2-7 throughout the heterochromatin. For example, there was light staining localized with a subfraction of HP1 (Figure 19C, open arrow) as well as faint and diffuse staining which completely colocalized with HP1 (Figure 19C, open arrowhead). This weak and relatively heterogenous staining was only
detected in geminin depleted cells treated with aphidicolin and may represent a random selection of heterochromatic origins of replication on a cell by cell basis.

### 2.2.6 Cyclin A-CDK Activity Restricts pre-RC Formation to the Heterochromatin

Prior studies in human cells indicated that cyclin A-CDK activity stimulates repllication in the presence of excess Cdt1 or Cdc6 (Vaziri et al. 2003). Knockdown experiments in Drosophila cells showed cyclin A silencing suppressed the partial repllication induced by geminin depletion (Mihaylov et al. 2002). I sought to investigate whether cyclin A-CDK activity plays a role in restricting pre-RC assembly to the Drosophila heterochromatin. As reported previously (Mihaylov et al. 2002), I also observed that depletion of cyclin A by RNAi arrests cells at G2/M, and after a delay of approximately 24 hours, the cells initiate a complete endoreduplication of their genome (Figure 20A, compare panel 2 and 4). Importantly, in the absence of both geminin and cyclin A, there is only limited re-replication at 24 hours (panel 3) and, similar to the cyclin A depleted cells, I observe a complete reduplication of the genome by 48 hours (panel 5). The suppression of partial re-replication by cyclin A depletion is not due to inefficient depletion of geminin in the cyclin A co-knockdown experiment (Figure 20D). Presumably, the decreased CDK activity associated with the cyclin A depletion allows for the genome-wide re-assembly of the pre-RC which would facilitate the complete reduplication of the genome. I examined the distribution of chromatin-associated Mcm2-
7 in cells depleted for cyclin A, geminin or cyclin A and geminin. In the absence of cyclin A, the majority of cells (~ 80%) exhibited Mcm2-7 loading on the chromatin 24 hours prior to the endoreduplication (Figure 20B and 20C). An equal number of cells with type I (throughout the nucleus) and type II (restricted to the heterochromatin) Mcm2-7 localization patterns were observed. Similar results were obtained for cells depleted of both geminin and cyclin A (Figure 20B and 20C). This is in sharp contrast to the almost complete absence of chromatin associated Mcm2-7 observed in cells depleted only for geminin (Figure 19B and Figure 20C). Together, these results suggest that pre-RC re-assembly may not be specifically targeted to the heterochromatin, but rather the re-assembly of the pre-RC in euchromatin is inhibited by persistent cyclin A-CDK activity.
Figure 20: Cyclin A - CDK activity restricts pre-RC formation to the heterochromatin.

(A) Histogram of DNA content for cells treated with pUC dsRNA, cyclin A dsRNA or cyclin A and geminin dsRNAs for 24 hours and 48 hours.

(B) Immunostaining of chromatin-associated MCMs at 24 hours post cyclin A, cyclin A and geminin, or geminin RNAi treatment.

(C) Quantification of the different MCM localization patterns as described in Figure 5, with error bars indicating standard error. At least 200 cells were counted from three independent experiments. The distribution of MCM localization patterns in geminin
Figure 20 (Continued)

depleted cells was significantly different from cyclin A depleted cells (p<1x10-16) and cells simultaneously depleted of geminin and cyclin A (p<1x10-16). (D) Immunoblot analysis of cyclin A and geminin levels following the individual and co-RNAi depletion experiments.
2.3 Discussion

Maintenance of constant genome ploidy is critical for eukaryotic organisms. If unchecked, disruption of the mechanisms that tightly couple DNA replication with the cell cycle may result in re-replication, aneuploidy and genomic instability. I have found that perturbation of Dup activity by geminin depletion results in the preferential re-replication of heterochromatic sequences in the *Drosophila* genome. Re-replication was limited to pericentromeric heterochromatic sequences which are marked by HP1 and H3K9 methylation. Euchromatin, including gene-poor late replicating sequences and polycomb repressed sequences, was resistant to re-replication. In the absence of geminin, a minimal complement of the helicase Mcm2-7 assembled on the chromatin was sufficient for re-replication. These findings suggest that the 2-3 fold increase in ploidy I observed was regulated by the specific re-assembly and activation of the heterochromatic pre-RCs.

2.3.1 Re-replication of Heterochromatic Sequences

The preferential re-replication of heterochromatic sequences in the absence of licensing controls was particularly striking given the established view that repressive chromatin environments are inhibitory to efficient origin activation (Gilbert 2002). Classic experiments have clearly demonstrated that the heterochromatin in *Drosophila* and other organisms is the last region of the genome to be duplicated during S-phase (Goldman et al. 1984). The complex nature of the heterochromatic sequences has hampered detailed
analysis of the replication program in this part of the genome and it remains possible that the heterochromatin may be populated by a very limited number of ultra-efficient origins of replication. These may be required to ensure that the heterochromatin is duplicated in a timely manner at the end of S-phase and that in the absence of licensing control these origins are preferentially activated. My genomic data did not identify any robust origins of replication in the heterochromatin that were consistently used across the cell population. Similarly, I found that the bulk of heterochromatin was re-replicated to similar ploidy levels suggesting that origin re-activation in the absence of geminin is a stochastic process.

Analysis of total DNA ploidy by FACS revealed that the majority of cells exhibited a DNA content greater than 8N following geminin depletion, suggesting geminin depleted cells had increased their total DNA content by at least 2-fold over that of G2 cells. The heterochromatin constitutes a minimum of 30% of the Drosophila genome (Smith et al. 2007). Assuming that re-replication is specific to the heterochromatin, a 4.5 fold increase in heterochromatic DNA content would be sufficient to account for the increase in DNA ploidy I observe. However, I consistently observed, by multiple methods, only a 2-2.5 fold increase in heterochromatic DNA content (Figure 11, Figure 12 and Figure 15). I speculated that the highly repetitive non-unique heterochromatic sequences might be preferentially re-replicated to higher ploidy levels. I tested this hypothesis by examining the genomic abundance of the 1.688 satellite DNA which
accounts for 4% of the *Drosophila* genome (Hsieh and Brutlag 1979). Again, I only observed a 2-2.5 fold increase in the bulk levels of the 1.688 satellite DNA (Figure 21). It is clear that the heterochromatin is preferentially re-replicated in the absence of geminin; however, I am unable to rule out the possibility that a limited amount of stochastic re-replication is also occurring in the euchromatin.

**Figure 21: Copy number analysis of the 1.688 satellite DNA during geminin depletion induced re-replication.**

Genomic DNA was isolated from cells treated with non-specific (pUC) or geminin dsRNA for 48 hours. The bulk level of the 359bp 1.688 satellite DNA repeat, which comprises 4% of the *Drosophila* genome, was analyzed by digestion with the restriction enzyme *SacI* which linearizes the 359bp repeat unit. The first four lanes are DNA digested with the *SacI* enzyme from independent control and geminin RNAi experiments. The lower band is the linearized 359bp repeat of the 1.688 satellite DNA. The right four lanes are genomic DNA without digestion and serve as a loading control. Quantification of the intensity of the 359bp repeat band was performed with Image J. The first four lanes were normalized to the 359bp band of control cells treated with non-specific pUC dsRNA (pUC1), and the last four lanes were normalized to the genomic DNA of cells treated with pUC dsRNA (pUC1). The copy number of the 1.688 satellite DNA was determined by Image J.
2.3.2 The Re-assembly of pre-RC during Re-replication

In higher eukaryotes, there are many more Mcm2-7 complexes loaded onto the chromatin in G1 than are required to complete an unperturbed S-phase (Ritzi et al. 1998; Edwards et al. 2002; Forsburg 2004; Blow and Dutta 2005). Although these excess MCMs are not required for completion of a normal S-phase, they are critical for protecting the cell from genomic instability during replication stress (Ibarra et al. 2008). However, when geminin is depleted, I only observe a minimal complement of Mcm2-7 being re-loaded onto heterochromatic DNA. These results suggest that there is not a global re-assembly of the pre-RC throughout the genome as occurs in G1 and that limiting amounts of Mcm2-7 are sufficient for the greater than two-fold increase in ploidy that I observe. The Mcm2-7 complex appears to be transiently associated with the heterochromatin as inhibition of DNA re-replication with aphidicolin results in an increase in detectable chromatin-associated Mcm2-7. I propose that in the absence of geminin, the Mcm2-7 complex is loaded onto heterochromatic sequences and that these pre-RCs are immediately activated for initiation of DNA replication.
2.3.3 Geminin Depletion May Not Be Sufficient to Disrupt Dup Regulation

In *Drosophila*, Dup activity is downregulated after origin firing through multiple mechanisms including Cul4-Ddb1 mediated proteolysis in S phase and inhibition by geminin during S, G2 and mitosis. Furthermore, I (Figure 10) and others (Mihaylov et al. 2002; Hall et al. 2008) have reported that Dup/Cdt1 is degraded in the absence of geminin. Thus, geminin is only one factor that negatively regulates Dup/Cdt1 and its depletion may be insufficient to induce genome-wide re-replication. Therefore, the deregulation of geminin and Dup/Cdt1 may have distinct effects on replication control. This may, in part, explain the differences in sequences and chromatin environment which are preferentially targeted for re-replication in human and *Drosophila* cell lines. In human cell lines, Cdt1 overexpression led to the preferential re-replication of early replicating sequences (Vaziri et al. 2003), while in *Drosophila* cell lines, geminin depletion leads to the preferential activation of heterochromatic origins of replication. Future experiments will test whether Dup levels are critical for maintaining ploidy and selecting which origins are activated.

2.3.4 Replication Timing, Chromatin Structure and Re-replication

The observation that the re-replication at pericentromeric heterochromatin was not coupled with late replication timing suggests that origin selection during re-replication and the temporal control of DNA replication in S-phase are regulated by
distinct mechanisms. These data suggest that a key determinant of which sequences will re-initiate DNA replication is the local chromatin environment. *Drosophila* pericentromeric heterochromatin is marked by H3K9 methylation which is maintained by Su(var)3-9 and HP1 (Schotta et al. 2002; Ebert et al. 2004). ORC has been shown to interact with HP1 and localizes to heterochromatin by immunofluorescence in both interphase and mitotic nuclei (Pak et al. 1997; Huang et al. 1998). It is therefore possible that the increased density of ORC in the heterochromatin may stimulate the preferential re-assembly of the pre-RC at those sequences. However, recent studies using GFP tagged ORC and live imaging did not observe an increased density of ORC at the heterochromatic regions of the genome (Baldinger and Gossen 2009).

### 2.3.5 Possible Role of Cyclin A-CDK in Re-replication Control

I found that cyclin A-CDK activity regulates the re-activation of replication origins at two levels. First, cyclin A-CDK activity is required for the large increase in ploidy observed, consistent with the known role of CDK activity in activating the pre-RC for initiation (Diffley 2004). Second, cyclin A-CDK activity appears to differentially inhibit pre-RC re-assembly in the euchromatin and heterochromatin. The simultaneous depletion of both cylin A and geminin results in the global re-assembly of the pre-RC in both euchromatin and heterochromatin (Figure 20). In contrast, depletion of only geminin results in pre-RC re-assembly specific to the heterochromatin, suggesting that cyclin A-CDK activity specifically inhibits pre-RC assembly in the euchromatin. In
humans, the N-terminal domain of ORC1 contains consensus CDK phosphorylation sites which can be phosphorylated \textit{in vitro} by cyclin A-CDK activity and may regulate the SCF/Skp2 mediated turnover of ORC1 during S-phase (Méndez. et al. 2002). In \textit{Drosophila}, the ORC1 N-terminus also contains potential CDK phosphorylation sites, an additional O-box for APC mediated destruction (Araki et al. 2005), and is essential for the binding of ORC1 with HP1 (Huang et al. 1998). I propose that the interaction between ORC1 and HP1 may protect ORC1 from inhibitory cyclin A-CDK signals or destruction by the APC, thereby differentially sensitizing heterochromatic and euchromatic origins of replication to unlicensed pre-RC assembly.

\textbf{2.4 Materials and Methods}

\textit{Cell growth and drug treatment}

\textit{Drosophila} Kc167 cells were cultured at 25°C in Schneider’s Insect Cell Medium (Invitrogen) supplemented with 10% fetal bovine serum and 1% penicillin / streptomycin / glutamine (Invitrogen). DNA synthesis was inhibited by treatment with 1 mM hydroxyurea (Sigma) or 7 µM aphidicolin (Sigma).

\textit{RNA interference}

All double-stranded RNAs were generated using gene-specific PCR products (~700bp) flanked by T7 polymerase-binding sites as templates for \textit{in vitro} transcription reactions with the T7 RiboMax express large-scale RNA production system (Promega).
Primers are available upon request. For each RNAi experiment, cells were washed with and diluted in serum-free medium to a final concentration of $2 \times 10^6$ cells/ml. 15µg dsRNA was added per $1 \times 10^6$ cells, gently mixed, and incubated for one hour. After the incubation, 2× medium was added resulting in a final concentration of $1 \times 10^6$ cells/ml. The cells were incubated for 1-2 days before harvesting.

**Array hybridization and data analysis**

DNA was isolated as described (MacAlpine et al. 1997). Isolated DNA was sheared and labeled with either fluorescent Cy5- or Cy3-conjugated dUTP (GE Healthcare Bio-Sci Corp.) using sequenase (US Biochemicals) and a random nonamer oligo (IDT). The labeled DNA was purified using Microcon YM-30 filters (Millipore), then hybridized to custom Agilent tiling microarrays overnight at 65°C. The slides were then washed and scanned as per Agilent recommendations. The Agilent generated tif files from each genomic microarray were processed and analyzed in R (Team 2010), a free software environment for statistical computing and graphics. The LIMMA package (Smyth and Speed 2003) from the bioconductor project (Gentleman et al. 2004) was used to normalize the ratio of the Cy5 and Cy3 channels across each genomic tiling array by loess normalization. Quantile normalization was used to normalize between replicate slides. The relative copy number between control and geminin depleted cells was determined by the log ratio of Cy5 and Cy3. To assess the significance of the copy number enrichment for different genome features (chromosome, heterochromatin,
euchromatin, etc) a Student’s t-test was utilized and p values are indicated in the figure legends. All genomic coordinates are based on the 5.0 assembly of the *Drosophila* genome. The analysis of the replication timing data is described in MacAlpine et al., 2010.

*Data submission and genomic data*

All genomic data with accompanying metadata (protocols and analysis parameters) are publicly available at the NCBI GEO data repository. The accession numbers are: GSE17279 Kc167 replication timing, GSE20781 S2 H3K27me3, GSE20794 S2 H3K9me3, and GSE20932 Kc167 re-replication CGH data.

*Western blotting and chromatin fractionation*

Antibodies used in western blotting include: rat anti-geminin antibody (a gift from Helena Richardson), anti-Dup guinea pig antibody (a gift from Terry L. Orr-Weaver), anti-acetylated H4 antibody (Upstate), and mouse anti-cyclin A antibody (Developmental Studies Hybridoma Bank). All antibodies were used at a 1:3000 dilution.

Chromatin fractionation was carried out as described (Wysocka et al. 2001). Samples were analyzed by PAGE and western blotting. Primary antibodies used include anti-Orc2 at 1:3000 and anti-MCMs (AS1.1) at 1:100. Secondary antibodies used include Alexa Fluor 680 goat anti-rabbit IgG (Invitrogen), IRDye 800 conjugated anti-mouse IgG
(Rockland Immunochemicals), both at a 1:10000 dilution. Immunoblots were scanned using a LICOR Imaging system.

**Immunofluorescence**

For the BrdU incorporation experiments, cells were fixed with methanol: acetic acid 3:1(v/v) after a 4-hour incubation with 5µg/ml BrdU (Roche). A rat anti-BrdU antibody (Abcam Inc.) was used at 1:200 and Alexa Fluor 594 goat anti-rat secondary antibody (Invitrogen) was used at 1:500. For the double labeling of EdU and HP1, cells were incubated in medium with 10 µM EdU for 30 minutes, treated with 0.5% Triton X-100 in PBS for one minute, fixed with 4% paraformaldehyde in PBS, then run through the EdU Click-iT reaction cocktail (Invitrogen). Cells were then stained with rabbit anti-HP1 antibody (#191, 1:1000, a gift from Sarah Elgin) and Alexa Fluor 568 goat anti-rabbit secondary antibody (1:500, Invitrogen). For double labeling of MCMs and HP1, cells were treated with Triton/PBS and fixed as for EdU and HP1 staining, stained with monoclonal MCM antibody (AS1.1, 1:100) and anti-HP1 antibody (#191, 1:1000), followed by secondary detection with Alexa Fluor 488 goat anti-mouse and Alexa Fluor 568 goat anti-rabbit antibodies (1:500). Chi-square tests were performed for quantifications of immunofluorescence localization patterns and the p values are shown in corresponding figure legends.

**Quantitative PCR**
The relative copy number for seven loci spanning both euchromatin and heterochromatin on chromosome 3R was examined. Specifically, three loci are located in the pericentric heterochromatin, two in the euchromatin proximal to pericentric heterochromatin, and the remaining two in the euchromatin distant from pericentric heterochromatin. DNA isolated from control pUC dsRNA treated cells was used to generate the qPCR standard curve. Primers are available upon request. Quantitative PCR was performed using iQ SYBR Green Supermix (Bio-Rad) on Bio-Rad iQ5 Real-Time PCR Detection System.
3. Re-replication Induced by Dup Over-expression

3.1 Introduction

Cdt1, as a component of the pre-RC, is an essential protein for DNA replication and it is conserved from yeast to humans. The *Drosophila* homolog of Cdt1, Double-parked (Dup), was initially identified in a genetic screen for mutations that affect the G1/S transcriptional program during embryogenesis. Mutation of the double-parked gene causes defects in both genomic replication during the post blastoderm mitotic cycles and chorion gene amplification in follicle cells of the *Drosophila* ovary (Whittaker et al. 2000), suggesting Dup is essential for DNA replication at multiple stages of *Drosophila* development.

In the following description and discussion, Cdt1 is used when referring to other organisms while Dup is used when referring to the *Drosophila* Cdt1 homolog.

3.1.1 Dup Regulation in *Drosophila*

Dup is a critical player in replication licensing in *Drosophila* and its activity has to be tightly regulated to ensure faithful DNA replication. Inhibition of Cdt1 activity outside of G1 is critical to prevent re-replication in metazoans. In *Drosophila*, the level of Dup protein is rapidly down regulated after origin firing through multiple mechanisms. These mechanisms include Cul4^{Ddb1} mediated proteolysis in S-phase and inhibition by
geminin throughout S, G2 and mitosis (Arias and Walter 2007; Hall et al. 2008) (Figure 22). Dup is also rapidly destructed upon DNA damage (Higa et al. 2003; Hall et al. 2008). Cul4, a Cullin-RING family of E3 ubiquitin ligase, ubiquitylates Dup and directs it to the proteasome for degradation both during S-phase and upon DNA damage (Hall et al. 2008). A unique feature of Cul4-Ddb1 mediated ubiquitylation of Dup is the requirement of a conserved motif called the PIP box (PCNA-interacting polypeptide). The PIP box is located at the N-terminus of Dup and binds PCNA (proliferating cell nuclear antigen) directly (Arias and Walter 2006; Hu and Xiong 2006; Senga et al. 2006). It has been suggested that the interaction between Cdt1 and PCNA is necessary to recruit Cul4-Ddb1 to chromatin (Jin et al. 2006). Besides proteolysis through Cul4, Dup is also inhibited from chromatin after origin firing by geminin (Quinn et al. 2001). Geminin binds to Dup in S-phase until its subsequent APC mediated degradation in late mitosis (McGarry and Kirschner 1998; Quinn et al. 2001). These two mechanisms prevent Dup from re-associating with origin chromatin outside of G1 phase.
Regulation of Cdt1 during the mitotic cell cycle.

Cdt1 is ubiquitynated by the E3 ubiquitin ligases Cul4\textsuperscript{Ddb1} and is subsequently targeted to destruction by peoteosome during S-phase. The replication inhibitor geminin binds to Cdt1 during S, G2 and M phase, preventing the re-association of Cdt1 with origins. Geminin is degraded in late M phase and Cdt1 is then free to form pre-RC in the following G1.

3.1.2 Re-replication Induced by Cdt1 Over-expression

Despite multiple and redundant mechanisms preventing re-replication (see section 1.5), recent studies in a number of systems have demonstrated that deregulation of Cdt1 alone is sufficient to induce partial but not complete re-replication of the genome. For example, re-replication induced by Cdt1 over-expression was observed in p53 mutant human cells (Vaziri et al. 2003) and in Drosophila embryos (Thomer et al. 2004). Accumulation of Cdt1 protein after the silencing of Cul4 in C. elegans also led to re-replication (Zhong et al. 2003).

Although excessive Cdt1 leads to re-replication, I and others did not observe an increase but rather a decrease of Dup/Cdt1 in re-replicating cells which were depleted of geminin(Figure 1) (Hall et al., 2008, Mihaylov et al., 2002; Ballabeni et al., 2004). This is
likely because the depletion of geminin exposes the subset of Dup/Cdt1 which is protected from degradation by geminin binding. Given that geminin is only one factor that negatively regulates Dup/Cdt1 and that Dup/Cdt1 level is low when geminin is absent, geminin depletion may be insufficient to induce genome-wide re-replication. In contrast, by manipulating the level of Dup protein, more extensive re-replication of the genome may be observed.

In this chapter, I use an inducible Dup over-expression cell line to investigate whether Dup over-expression is sufficient to induce genome reduplication. I found that in contrast to the specific re-replication of heterochromatin resulting from geminin depletion, re-replication induced by Dup over-expression is not restricted to heterochromatin, but rather includes re-initiation of replication throughout the genome. However, there is a slight preference for heterochromatic re-replication when Dup is initially over-expressed. Studies on re-replication in G2 cells revealed that G2 chromatin loses replication timing control determinants and that re-assembly of pre-RC in G2 is a rapid and continuous process.

3.2 Results

3.2.1 Generation of Inducible Dup Stable Cell Lines

The degree of redundancy between Cul4Ddb1- and Geminin-mediated downregulation of Dup activity during cell cycle is not fully understood. To examine the
effect of bypassing both destruction and inhibitory binding of Dup on cell cycle progression, stable cell lines carrying inducible wide-type (pMt/Dup) Dup were generated and studied.

To determine the responsiveness of pMt/Dup to copper induction, a titration of copper concentrations was tested. After the cells were incubated for 24 hours in the absence or presence of a range of CuSO4 concentrations (from 0.05mM to 0.75mM), immunoblot analysis indicated a 2- to 3-fold increase of Dup protein level relative to the control cell line, whereas there was no significant change in the level of Orc2 which was used as a loading control (Figure 23). There was a subtle increase in Dup level when the concentration of CuSO4 was increased from 0.25mM to 0.5mM; however, the induction levels in 0.5mM and 0.75mM CuSO4 were similar. Higher concentrations of copper have also been tested and the level of Dup did not seem to increase further (data not shown). Therefore, 0.5mM CuSO4 was chosen as the working solution to induce Dup over-expression for studies described in this entire chapter.
The over-expression of Dup is dependent on copper concentration.

A range of copper concentrations (0, 0.05, 0.1, 0.25, 0.5, 0.75 mM) were used to induce Dup over-expression. The relative Dup level was determined by using the analysis tool in the Odyssey® 3.0 application software. A 2- to 3-fold increase of Dup protein was seen for all copper concentrations and the induction exhibited a concentration-dependent manner.

To further characterize the induction of Dup, a time course was carried out to check Dup levels at various time points after incubation in 0.5 mM CuSO4. Immunoblot analysis indicated that the induction of Dup was rapid and the level remained constant for at least 24 hours (Figure 24). An increase in Dup level was clearly detectable by immunoblot as early as one hour after copper induction. Surprisingly, despite the continuous presence of copper and nonstop induction of Dup expression, there was no further accumulation of Dup once it reached a 2- to 3-fold increase as compared to the level in control cells (Figure 24). The modest induction of Dup is presumably due to its destruction upon DNA damage and re-replication caused by Dup over-expression (Hall et al. 2008). Another reason is that DNA replication itself triggers rapid Dup degradation.
(Thomer et al. 2004), therefore Dup cannot accumulate during S-phase even when it is over-expressed.

Figure 24: The induction of Dup over-expression is rapid and modest.

Cells were incubated in 0.5mM CuSO4 for various lengths of time (0, 1, 2.5, 5, 7 and 24 hours) and harvested for immunoblotting. An increase of Dup protein level was detected as early as one hour after copper induction. There was no further accumulation of Dup protein as the induction time lengthened.

3.2.2 Dup Over-expression Induces Re-replication

To examine whether the over-expression of Dup can induce re-replication, DNA content of cells that have been treated with copper under various conditions were assayed by flow cytometry. Dup over-expression induced re-replication in both a dose-dependent and time-dependent manner. In particular, when the cells were induced with copper for 24 hours, low concentrations of copper resulted in less re-replication as compared to high copper concentrations (Figure 25A). When the cells were treated with 0.5mM copper, re-replication was clearly detectable by flow cytometry at 2 hours of treatment, accompanied by a decrease of G2 cells (Figure 25B). There was no significant
change for G2 cell population between 2.5 to 7.5 hours, but re-replicating cells started to
differentiate themselves from G2 and progress toward higher DNA content (Figure 25B).
By 24 hours of copper treatment, there was a large cell population with a heterogeneous
DNA content between 4N and 8N.
Figure 25: The extent of re-replication is dependent on Dup protein level and induction time.

(A) The amount of re-replication by 24 hours of copper treatment exhibits a correlation with the concentration of copper. The extent of re-replication increased as the concentration of copper went up from 0.05mM to 0.75mM.

(B) Flow cytometry profiles reveal that the subpopulation of cells in G2 starts to decrease soon after over-expression of Dup. Re-replication (DNA content beyond 4N) was clearly detectable at 2.5 hours upon copper induction and by 24 hours there was a spectrum of DNA between 4N and 8N.
When Dup was over-expressed, there was no corresponding increase of geminin protein level (Figure 26A). To test whether geminin depletion combined with Dup over-expression may trigger even more extensive re-replication than either alone, cells were treated with geminin dsRNA, copper, and geminin dsRNA together with copper respectively. Flow cytometry analysis revealed that at both 24 and 48 hours of treatment, geminin depletion induced less re-replication than Dup over-expression (Figure 26B). Cells under both treatments for 48 hours showed a similar re-replication profile as copper treatment alone did. When copper was added at 24 hours post geminin RNAi, by 48 hours cells demonstrated a similar profile as cells that were treated with both geminin dsRNA and copper for 48 hours. Immunoblot analysis revealed that Dup over-expression did not significantly rescue the decrease of Dup protein levels caused by geminin depletion (Figure 26C). These observations suggest that geminin levels are not limiting for the increased re-replication during Dup over-expression and that the extent of re-replication is dependent on free Dup which is not bound by geminin and not degraded, instead of the total level of Dup.
Figure 26: Dup over-expression is dominant over geminin depletion in inducing re-replication.

(A) Immunoblot showed that when Dup protein level was elevated 24 hours after copper induction, there was no increase of the level of geminin.

(B) Both geminin RNAi and Dup over-expression induces re-replication. Geminin RNAi in combination with Dup over-expression exhibited a similar profile as Dup over-expression alone, whereas geminin RNAi alone induced re-replication to a less extent.

(C) Dup protein levels were increased both at 24 and 48 hours after copper induction (lane 2 and 3). Geminin RNAi led to a decrease of Dup (lane 4 and 5). The decrease was only slightly rescued by Dup over-expression (lane 6).
3.2.3 Preferential Re-replication of Heterochromatic Sequences Upon Dup Over-expression

To identify sequences that were re-replicated when Dup was over-expressed, comparative genomic hybridization was performed. Briefly, DNA from independent biological replicates was harvested from cells that were treated with copper and from cells with no treatment, differentially labeled with Cy5 and Cy3 conjugated dUTP, and hybridized to custom genomic tiling microarrays containing unique probes for the sequenced Drosophila euchromatin and pericentric heterochromatin. The copy number for each probe on the array was analyzed and plotted as a function of chromosome position. The corresponding copy number for each probe from a 48-hour geminin RNAi microarray experiment was also plotted.

Similar to geminin depletion, Dup over-expression caused an elevation for the copy number of chromosome four but to a much less extent than geminin depletion did (Figure 27C). The relative copy number for the large majority of euchromatic sequences along the chromosome arms of Dup over-expressing cells were constant and equal to one. However, as the sequences approach the pericentromeric heterochromatin on chromosome 2, 3 and X, the copy number showed an increase of approximately 1.5 fold, indicating that DNA sequences in these regions were re-replicated (Figure 28, black and red). In support for the re-replication of heterochromatic sequences, the satellite DNA
1.688 of 24-hour Dup over-expression cells showed a 2-fold increase over that of control cells (Figure 27B).

The relative milder increase of copy number for heterochromatic sequences in 24-hour Dup over-expression cells than in 48-hour geminin RNAi cells is surprising, because both cell populations included a large subpopulation of cells with DNA content spanning from 4N to beyond 8N. However, there are differences between the flow cytometry analyses for these two cell populations. 48-hour geminin RNAi cells showed a broad spectrum of DNA content from 4N to beyond 8N without any peak in between (Figure11). In contrast, 24-hour Dup over-expression cells had a peak between 4N and 8N and there were fewer cells with DNA content greater than 8N as compared to 48-hour geminin RNAi cells (Figure 27A). The differences revealed by flow cytometry analysis suggest that Dup over-expression may lead to genome-wide re-replication with a preference toward pericentric heterochromatin. If the majority of the genome was re-replicated it may appear (after normalization) as no enrichment in the microarray study. It is the difference between a few sequences re-replicating versus most of the genome.
Figure 27: Modest re-replication of heterochromatic sequences when Dup was over-expressed.

(A) Flow cytometry analysis revealed that 24-hour Dup over-expression (green) led to re-replication. The re-replication appeared to be somewhat synchronous, showing a peak near 8N by 24 hours of Dup over-expression.

(B) Copy number analysis of the 1.688 satellite DNA during Dup over-expression induced re-replication. The bulk level of the 359bp 1.688 satellite DNA repeat, which comprises 4% of the Drosophila genome, was analyzed by digestion with the restriction enzyme SacI which linearizes the 359bp repeat unit. The copy number of the 1.688 satellite DNA repeat was calculated as the result of the 1.688 band intensity relative to the band intensity of corresponding genomic DNA.

(C) The relative copy number for array probes following either 24 hours of Dup over-expression (gray) or 48 hours of geminin depletion (black) as a function of chromosomal position for Drosophila chromosome 4.
Figure 28: Heterochromatin is preferentially re-replicated when Dup is over-expressed.

The relative copy number for array probes following either 24 hours of Dup over-expression (black, heterochromatin in red) or 48 hours of geminin depletion (gray, heterochromatin in blue) as a function of chromosomal position for the left and right arms of Drosophila chromosome 2, 3 and the entire chromosome X.
To test whether Dup over-expression induced genome-wide re-replication, I assayed the cytological location of active replication by immunofluorescence. Actively replicating sequences were labeled by the incorporation of 5-bromo-2-deoxyuridine (BrdU). BrdU was added to the medium for a 4-hour window at 20 hours post copper treatment, at which time re-replication was clearly detectable by flow cytometry in cells under copper induction (data not shown). Asynchronous cells exhibited multiple distinct patterns of BrdU incorporation, including: no BrdU incorporation, BrdU incorporation all over the nucleus, and BrdU incorporation localized to a small region at the nuclear periphery (Figure 29A). These distinct patterns of BrdU incorporation were classified as either 'none', 'global', or 'local', respectively. Cells that were over-expressing Dup also exhibited the same three BrdU incorporation patterns (Figure 29A). In sharp contrast to BrdU patterns in geminin depleted cells, BrdU incorporation in Dup over-expressing cells were not predominantly localized to one region of the nuclei but exhibited an equal distribution of both global and local patterns (Figure 29B). Together, these results suggest re-replication induced by Dup is not restricted to pericentric heterochromatin.
Figure 29: Re-replication induced by Dup over-expression is not restricted to pericentric heterochromatin.

(A) Fluorescence microscopy of cells after a 4-hour BrdU pulse at 20 hours post copper treatment (CuSO₄ 24h) or non treatment (Async). BrdU labeled sequences were detected with a rat anti-BrdU antibody (red) and DNA was counterstained with DAPI (blue).

(B) Distribution of the different BrdU incorporation patterns. The distribution of BrdU incorporation patterns was similar between control cells and Dup over-expressing cells. BrdU incorporation patterns in 24-hour geminin-depleted cells were also shown.
3.2.4 Dynamic pre-RC Assembly during Dup Over-expression Induced Re-replication

The assembly of pre-RC onto origins of replication is a prerequisite for DNA replication initiation. During G1 phase of the cell cycle, low CDK levels and the absence of geminin allow for genome-wide assembly of the pre-RC (Arias and Walter 2007). Based on the observation that heterochromatin was preferentially re-replicated over euchromatin and the entire genome of a subpopulation of cells was re-replicated, I hypothesized that re-replication induced by Dup over-expression may favor heterochromatin by initiating re-replication at heterochromatin earlier and more frequently than at euchromatin. For example, when there is abundant Dup available, the re-assembly of pre-RC may first occur in the heterochromatin and initiate DNA replication immediately following pre-RC formation. Re-assembly of the pre-RC at euchromatin, in contrast, may only start when the abundance of Dup reaches a certain threshold. Because it takes time to reach the threshold, any re-replication occurring before the available Dup level reaches the threshold may be restricted to heterochromatin. Alternatively, there may be a global re-assembly of the pre-RC throughout the Drosophila genome once Dup is over-expressed, but initiation of DNA replication occurs earlier at the heterochromatin than at the euchromatin. To differentiate these two models, I assessed the re-loading of the Mcm2-7 complexes onto the DNA by chromatin fractionation and immunofluorescence.
I first examined the global levels of chromatin-associated Mcm2-7 by chromatin fractionation. Chromatin was prepared from control asynchronous cells (As), cells treated with 3% DMSO (G2), and cells treated with copper (Dup) for 24 hours (Figure 30A). Orc2 remained associated with the chromatin fraction in all samples. In contrast, high level of chromatin-associated Mcm2-7 was only observed in asynchronous cells. A marked reduction in Mcm2-7 levels was observed on the chromatin prepared from cells that were arrested in G2 phase of the cell cycle by DMSO (Figure 30A). This result was not surprising because the MCMs are displaced from chromatin during S-phase and pre-RC assembly is prevented in G2. However, the level of chromatin-associated Mcm2-7 in Dup over-expression cells was only slightly higher than that in G2 cells (Figure 30A), suggesting there is not a genome-wide association of Mcm2-7 at 24 hours after Dup over-expression. Despite the reduction in chromatin-associated Mcm2-7 levels in the Dup over-expression cells, a significant number of cells (around 60%) exhibited active DNA synthesis as measured by a 15-minute EdU (5-ethynyl-2´-deoxyuridine) pulse at 24 hours (Figure 31A). Active DNA synthesis with low levels of chromatin-associated Mcm2-7 suggests that re-replication only requires trace amounts of pre-RC assembly to initiate and maintain, or alternatively, that there is a genome-wide re-assembly of pre-RC before the initiation of re-replication and pre-RCs are displaced from the chromatin immediately after the re-firing to origins.
To test whether there is a genome-wide assembly of pre-RC at early stages of re-replication, I took advantage of the observation that Dup over-expression induced re-replication in G2 released cells without passage through mitosis or entry into the next cell cycle (Figure 30C). Because G2 cells have very low levels of chromatin-associated Mcm2-7 (Figure 30A), re-replication in cells released from G2 provides a way to analyze re-assembled pre-RC without the contamination of G1 and S-phase cells. The global levels of chromatin-associated Mcm2-7 was examined in cells treated with hydroxyurea (G1/S), cells treated with 3% DMSO (G2), and cells released from DMSO with either no treatment (G2 R) or copper treatment (G2 R + copper) for 6 hours (Figure 30B). Although the DNA synthesis rate was much higher in cells that over-expressed Dup than in cells that did not at 6 hours after release from G2 arrest (Figure 31A), immunoblotting showed that there were comparable amounts of Mcm2-7 loaded onto chromatin in G2 released cells which were re-entering the cell cycle and G2 released cells which were over-expressing Dup protein (Figure 30B and 30C). Together, these results suggest that, similar to geminin depleted cells, cells undergoing re-replication induced by Dup over-expression are able to synthesize DNA with a reduced complement of chromatin-associated Mcm2-7 proteins.
Figure 30: Limited pre-RC re-assembly occurs when Dup is over-expressed.

(A) Chromatin from control asynchronous (As), DMSO arrested (G2), and Dup over-expression re-replicating cells (Dup) was fractionated biochemically and immunoblotted for Mcm2-7 and Orc2. Levels of Orc2 and Mcm2-7 are also shown for whole cell extracts (WCE).

(B) Chromatin from 24-hour HU arrested (G1/S), 24-hour DMSO arrested (G2), 6-hour DMSO arrest released (G2 R) and 6-hour DMSO arrest released with Dup over-expression re-replicating cells (G2 R + copper) was fractionated biochemically and immunoblotted for Mcm2-7 and Orc2. Levels of Orc2 and Mcm2-7 are also shown for whole cell extracts (WCE).

(C) Flow cytometry analysis of DNA content for cells released from G2 arrest with either no further treatment or copper treatment. Cells with no further treatment entered back the cell cycle, while cells under copper treatment had much fewer cells in G1 but exhibited re-replication instead.
The association of Mcm2-7 with chromatin was also assessed by immunofluorescence of cells treated with a mild detergent and salt prior to fixation (Claycomb et al. 2002). I found that the fraction of Mcm2-7 positive staining cells was small at both earlier (2 and 6 hours) and later (24 hours) time points after the induction of Dup over-expression in G2 released cells (data not shown). To gain a better picture of pre-RC re-assembly when Dup is over-expressed, replication forks were inhibited by the polymerase inhibitor aphidicolin. Aphidicolin treatment of G2 released cells caused cells coming out of mitosis to arrest in G1 (Figure 31C). In contrast, treatment of Dup over-expressing cells with aphidicolin inhibited the initiation of re-replication, and these cells remained in G2 and were unable to have DNA content greater than 4N (Figure 31C). Strikingly, while the number of Mcm2-7 positive cells in G2 released cells with aphidicolin treatment reflected the subpopulation of cells that were arrested in G1, the number of cells stained positive for chromatin-associated Mcm2-7 in Dup over-expression cells was close to 90% of the entire cell population by a 6-hour aphidicolin treatment (Figure 31D). The accumulation of chromatin-associated Mcm2-7 in Dup over-expression cells when replication forks were inhibited suggests that pre-RC re-assembly is a quick event when Dup is over-expressed, and that re-replication itself actively leads to disassociation of Mcm2-7 from the chromatin.

To gain insights into how re-replication and pre-RC re-assembly are correlated, active DNA synthesis was assayed by EdU incorporation and the nuclear location of
EdU was determined by comparing to that of the heterochromatin protein HP1. This study revealed that re-replication at 2 hours post Dup over-expression in G2 released cells exhibited DNA synthesis mostly at heterochromatin (Figure 31B). A longer period of Dup over-expression led to a more distributed EdU pattern as the percentage of heterochromatic EdU incorporation decreased but genome-wide incorporation increased (Figure 31A and 31B). The genome-wide EdU staining pattern is different from early S-phase EdU staining which was described in chapter 2, because it does not exclude the HP1 positive region of the nuclei but rather includes the entire nuclei. Together, these results suggest that pre-RC formation during re-replication is a highly dynamic process and that pre-RC formation and activation of replication origins occur simultaneously.
Figure 31: Re-replication of the genome and continuous re-assembly of pre-RC when Dup is over-expressed.

(A) Re-replication occurs rapidly upon copper induction in G2 released cells. Fraction of G2 released or Dup over-expressing G2 released cells exhibiting active DNA synthesis (EdU incorporation) is shown. DNA synthesis was determined by pulsing the cells for 15 minutes with EdU at the given time points.

(B) Quantification of heterochromatic EdU incorporation. EdU staining was classified into 2 types: EdU localization throughout the nucleus and EdU localization to HP1 region of the nucleus (heterochromatin). The distribution of these two EdU incorporation patterns remained relative constant in G2 released cells once they re-entered the cell cycle. The percentage of heterochromatic EdU incorporation in Dup over-expressing cells decreased as copper induction lengthened.
Figure 31 (Continued)

(C) The inhibition of both replication in S-phase and re-replication in G2 released cells by aphidicolin. The peak at 8N could be due to leaky expression of Dup.

(D) Quantification of cells stained positively for Mcm2-7 after a 6-hour aphidicolin treatment. The rate of Mcm2-7 positive cells corresponds to DNA synthesis rate shown in A.

(E) Examples of Mcm2-7 staining in both non-aphidicolin and aphidicolin treated Dup over-expressing cells. Mcm2-7, HP1 and DAPI are shown in green, red and blue, respectively.

3.2.5 Replication in G2 Does Not Have A Defined Timing Program

DNA replication during S-phase is subjected to a tightly regulated temporal program. However, the mechanisms regulating this temporal program still remain unknown. To examine whether determinants of replication timing are lost during S-phase and whether replication timing program is re-established in G2, I took advantage of re-replication in G2 arrested cells. Specifically, I over-expressed the Dup protein in cells that were arrested in G2 by 3% DMSO, which induced re-replication. The re-replication of G2 chromatin was acute, with occasional EdU detection at one hour post Dup over-expression and flow cytometry detection of DNA content beyond 4N by 2 hours post Dup over-expression (Figure 32A). By 6 hours of Dup over-expression, a subpopulation of cells already possessed DNA content that is close to 8N, meaning these cells almost doubled their genome in a period of 6 hours. A clean peak at 8N by 24 hours post Dup over-expression was observed (Figure 32A). A peak at 16N with a tail spanning from 16N to 32N was observed by 96 hours after Dup over-expression (data
not shown). These observations are surprising because they are different from re-
replication seen in asynchronous cells and G2 released cell. Instead of heterogeneous re-
replication, G2 cells over-expressing Dup undergo re-replication in a way that is similar
to a cell population enters back to the cell cycle from G1/S arrest. The integral
duplication of the genome as evidenced by the appearance of the 8N and 16N peaks
suggests Dup over-expression leads to a complete endoreduplication instead of
incomplete or partial re-replication of the genome.

Dup over-expression induced essentially even re-replication of the genome in G2
cells, as would be expected if each round of endoreduplication corresponded to a
normal S-phase. I therefore investigated whether re-replication of G2 chromatin follows
the same temporal program as S phase replication does. I monitored the replication
timing of heterochromatin which can be labeled by immunostaining with an anti-HP1
antibody in both asynchronous cells and G2 cells over-expressing Dup. Cells were pulse
labeled with EdU at various times after Dup induction by copper treatment and stained
for EdU and HP1. In asynchronous control cells, EdU was either specifically co-localized
with HP1 (actively replicating heterochromatin) or excluded from the HP1 staining
regions of the genome (actively replicating euchromatin) (Figure 32B), which represents
late and early S-phase respectively. In contrast, re-replicating G2 cells displayed neither
of these two EdU localization patterns observed in control cells, but rather exhibited
EdU incorporation across the entire nuclei at both early and late time points during re-
replication (Figure 32B). These results suggest that re-replication in G2 does not follow the normal S-phase temporal program.

Figure 32: Dup over-expression rapidly induces re-replication in G2 arrested cells.

(A) Cells were arrest in G2 phase by treatment with 3% DMSO for 20 hours. 0.5mM CuSO4 was added to G2 arrested cells and cell samples were collected at 2, 6 and 24 hours after copper induction. Flow cytometry analysis indicated that re-replication was detectable after 2-hour copper induction as evidenced by DNA
content beyond 4N (400 in the X-axis). By 6 hours, there was a continuous spectrum of DNA content between 4N and 8N. And by 24 hours, the entire genome had been re-replicated.

(B) Cells were pulse labeled with EdU for 15 minutes at various times after Dup induction by copper treatment and stained for EdU and HP1. In asynchronous control cells, EdU was either specifically co-localized with HP1 or excluded from the HP1 staining regions of the genome, representing late and early S-phase respectively. Re-replicating G2 cells exhibited EdU incorporation across the entire nuclei at all time points checked during re-replication.

3.2.6 The Correlation between Re-assembly of pre-RC and the Level of Dup Protein

There is a major difference between re-replication observed in cells arrested in G2 and those released from G2, which is demonstrated by the different EdU incorporation patterns. Re-replication in G2 released cells showed a preference toward heterochromatin when Dup was initially over-expressed (Figure 31B). In contrast, re-replication in G2 cells seemed to initiate from origins throughout the genome at any given time after Dup over-expression (Figure 32B). To examine possible reasons for this difference, I first studied the re-association of Mcm2-7 with G2 chromatin by immunofluorescence. There was already Mcm2-7 complex bound to chromatin 30 minutes after the induction of Dup over-expression, suggesting that as soon as Dup levels are above normal, the pre-RC will start to form in G2 cells. The Mcm2-7 complex remained associated with chromatin and was not displaced from the chromatin even when re-replication was largely detectable (Figure 33A). This is in sharp contrast to the
low level of chromatin-associated Mcm2-7 in re-replicating G2 released cells. Although the detection of Mcm2-7 at both heterochromatin (HP1) and euchromatin was observed at all times checked except at 30 minutes, the strongest Mcm2-7 staining was at 8-hour Dup over-expression.
Figure 33: High level of Dup leads to persistent association of Mcm2-7 with chromatin during endoreduplication.

(A) Chromatin-associated Mcm2-7 was detected after a 30-minute copper induction in G2 arrested cell. The chromatin association of Mcm2-7 persisted for a long period of time and displayed robust staining across the genome by 8 hours copper induction.

(B) The dynamics of the level of Dup protein after copper induction. Dup level increased 30 minutes after copper induction and peaked 2 hours after induction, from which time the level started to decrease.

(C) Higher levels of Dup were only observed in G2 arrested cells but not in G2 released cells.
To test whether the level of Dup protein could be a reason behind the persistent pre-RC formation in G2 arrested cells, I examined the protein levels of Dup at different times after copper induction. The induction of Dup was modest at 0.5 and one hour. The level of Dup peaked at 2-hour copper treatment and decreased at 6 and 8 hours (Figure 33B). In contrast, the level of Dup protein in G2 released cells which were under copper treatment did not exhibit any increase over G2 released cells with no further treatment (Figure 33C). The higher Dup protein levels in G2 cells and the persistence of pre-RC on the chromatin suggest that the amount of pre-RC assembly is determined by the abundance of Dup.

It is possible that Dup leads to pre-RC formation immediately following its over-expression outside of G2, and these pre-RCs are activated immediately, which results in the rapid destruction of Dup protein. If the over-expression of Dup is just enough to compensate its degradation, a robust pre-RC formation will not be observed as supported by the low levels of chromatin-associated Mcm2-7 in Dup over-expressing G2 released cells. When the over-expression of Dup protein over compensates its degradation, likely the case in Dup over-expressing G2 cells, pre-RC formation is robust and lasting.
3.2.7 Over-expression of Dup Does Not Induce Re-replication in the Presence of DNA Damage

To examine whether Dup over-expression can induce re-replication during S-phase, cells were synchronized at G1/S transition by treatment with hydroxyurea (HU) for 24 hours (Figure 34A). Cells were then released from G1/S to enter S-phase with no further treatment or with copper treatment to induce the over-expression of Dup at either 0 or 24 hours after release. Surprisingly, induction of Dup over-expression right after release from G1/S transition caused cells to arrest in G2 with little re-replication (Figure 34A). When Dup was induced at 24 hours after release from HU, there was more re-replication but the majority of cells were still arrested in G2 (Figure 34A). The G2 arrest phenotype is not simply because cells treated with HU cannot re-enter the cell cycle, as evidenced by the asynchronous flow cytometry profile of cells that were released from HU for 24 hours. Furthermore, cells released from HU do not show a defect in cell division as the number of cells keeps increasing after release.

HU causes replication forks to stall by limiting the nucleotide pool. Extensive DNA damage was observed in HU treated cell (Figure 34B). It is therefore possible that Dup over-expression during S-phase can prevent damage repair cause by HU. Indeed, I observed that when cells were release from HU, the number of rH2Av positive cells decreased to a very low level by 24 hours. However, when Dup was over-expressed at the same time when cells were released from HU, the number of rH2Av positive cells persisted. Noticeably, Dup over-expression in cells that were not pre-treated with HU
also caused DNA damage as indicated by positive rH2Av staining (Figure 34B).

Together, these results suggest that Dup over-expression cannot induce re-replication during S-phase, but rather induces a DNA damage checkpoint.

The apparent G2 arrest caused by Dup over-expression in cells released from HU suggests they might not be able to finish replication from late origins. To test this hypothesis, chromatin-associated Mcm2-7 was studied by immunofluorescence. If late origins were unable to activate in the presence of Dup, Mcm2-7 should remain associated with heterochromatin which harbors a large number of late origins. However, while intensive Mcm2-7 staining was observed at heterochromatin which corresponds to DAPI-bright regions, euchromatin-associated Mcm2-7 was also detected (Figure 35). These results suggest that Dup over-expression leads to the re-assembly of pre-RC during S-phase even though there is pre-existing DNA damage, but there are mechanisms to prevent origin from re-firing. In the presence of excessive Dup, cells released from HU were able to progress through S-phase but arrested in G2 with extensive DNA damage, suggesting either Dup over-expression intervenes DNA damage repair pathway or previous exposure to DNA damage agent sensitizes cells to DNA damage caused by re-replication.
Figure 34: Dup over-expression does not induce re-replication during S-phase.

(A) Flow cytometry analysis of DNA content. HU arrested cells in G1/S transition. 24 hours after release from HU, cells were back in cell cycle, showing asynchronous profile. Dup over-expression immediately following
Figure 34 (Continued)
release from HU did not cause significant re-replication. Dup over-expression at 24 hours after release from HU induced modest re-replication.
(B) rH2Av was highly detectable in cells that were treated with HU, copper after HU release, or copper alone. DNA damage was mostly repaired after release from HU.

Figure 35: pre-RCs were formed despite the lack of re-replication and presence of DNA damage.
At 24 hours after release from HU with copper induction, Mcm2-7 localized to both euchromatin and heterochromatin, with strong rH2Av staining in most of the cells. Cells release from HU without copper treatment exhibited similar Mcm2-7 staining pattern but with much fewer cells stained positive for rH2Av.
3.3 Discussion

I have investigated the effects on DNA replication of Dup, an essential component of the pre-RC that is required for loading of the helicase Mcm2-7 complex to replication origins. I have found that copper induction only resulted in modest over-expression of Dup protein. It is likely that the mechanisms down regulating Dup are very efficient in degrading excessive Dup. The results of copper titration experiments indicate that Dup over-expression induced re-replication in a dose-dependent manner. Together with the results of time course of copper induction, these results suggest that the extent of re-replication is dependent on the abundance of Dup although trace amounts of Dup are sufficient to trigger re-replication as suggested by the low levels of Dup in geminin depleted cells. I have shown that Dup over-expression in both G2 released cells and G2 arrested cells caused re-replication. Re-replication in G2 released and arrested cells exhibits some differences, however, suggesting the possible differences in cell cycle stages between these two cell populations may influence re-replication induced by Dup over-expression. I have also demonstrated that replication of G2 chromatin does not follow a confined temporal program seen during a normal S-phase, suggesting determinants of replication timing program are lost in postreplicative chromatin and are not re-established at G2 arrest caused by DMSO treatment.
3.3.1 Regulation of Dup in Re-replication Control

The results show that Dup over-expression is sufficient to induce re-replication in *Drosophila* tissue culture cells. Re-replication induced by Dup is more profound than that resulting from geminin depletion. This suggests that Dup over-expression bypasses both degradation and inhibition by geminin. I have found, however, that Dup protein only has a modest accumulation when over-expressed, even though the induction from the promoter is persistent. While the precise molecular mechanism for how excess Dup promotes pre-RC formation and re-replication remains unknown, the results indicate that even a slight increase in Dup protein is sufficient to cause re-assembly of the pre-RC and re-activation of origins.

The observation that Dup protein peaks 2 hours after copper induction in G2 arrested cells but not in G2 released or asynchronous cells suggests that there may be additional mechanisms to downregulate Dup as soon as it is over-expressed when the cells are not in G2. Cul4-Ddb1 mediated ubiquitilation and proteolysis of Dup is one mechanism to decrease Dup during S-phase (Jin et al. 2006; Nishitani et al. 2006). It is therefore possible that Cul4-Ddb1 pathway is efficiently able to remove over-expressed Dup in S-phase. Another ubiquitin ligase, the SCFSkp2 complex, exists in mammalian cells to degrade Cdt1 during G2 and M phase but has not been reported in *Drosophila* (Nishitani et al. 2006). The degradation of Dup during G2 and M phase when over-expressed is likely mediated by an ubiquitin ligase which resemble the SCFSkp2 complex.
This speculation has to be tested by further investigation, however. The decrease of Dup protein levels in re-replicating G2 cells after 2 hours suggest that re-replication itself promotes the degradation of Dup protein.

### 3.3.2 Re-replication and Endoreduplication

Dup over-expression in either asynchronous or G2 released cells only induces partial re-replication. When Dup is over-expressed in G2 arrested cells, however, a complete re-replication of the entire genome is seen by 24 hours and a doubling of this re-replicated genome is seen by 96 hours. These results indicate that Dup over-expression in G2 arrested cells induces endoreduplication. The high Dup levels seen after copper induction in G2 arrested cells but not in G2 released cells (Figure 33C) suggest that endoreduplication requires a much higher abundance of Dup than re-replication does.

The acute loading and persistence of the Mcm2-7 complex at both euchromatin and heterochromatin in G2 arrested cells when Dup is over-expressed suggests that high levels of Dup in the cells promote constant pre-RC re-assembly. In contrast, the existence of Mcm2-7 complex on the chromatin of G2 released cells upon Dup over-expression is only observed when the replication fork is inhibited by aphidicolin treatment. These observations suggest that re-activation of origins leads to rapid displacement of Mcm2-7 from the chromatin and that the availability of Dup is the determinant of how many
Mcm2-7 complexes can be reloaded onto the chromatin. The amount of re-licensed origins can determine the subsequent DNA content after re-replication. The observed differences in chromatin-associated Mcm2-7 complex can help to explain the diversity in the effects of geminin depletion and Dup/Cdt1 over-expression on the final DNA content that has been seen in various cell lines (Mihaylov et al. 2002; Vaziri et al. 2003; Thomer et al. 2004; Zhu et al. 2004). Efficient degradation of Dup/Cdt1 leads to limited licensing events and therefore restricts re-replication. Taken together, these results suggest that the abundance of Dup is critical for the extent of re-replication.

3.3.3 S-phase Replication Timing Program Is Not Preserved in Endoreduplication

Replication in S-phase follows a defined timing program. Very broadly, euchromatin replicates early and heterochromatin replicates late during S-phase. The results on replication timing of G2 re-replication indicate that replication of G2 chromatin is independent of determinants that specify the timing of origins during S-phase. Instead of initiating replication from defined timing zones, Dup over-expression induced origin activation in G2 exhibits robust and random replication throughout the genome (Figure 32B). Many of recent genome-wide replication timing studies suggest links between replication timing, chromatin structure and epigenetic marks (Karnani et al. 2010; Eaton et al. 2011). It is likely that replication timing program is disrupted after
replication in S-phase and re-established before next S-phase. The loss of timing
determinants in G2 suggests that the timing program is re-established after G2.

3.3.4 Re-replication and DNA Damage

My results indicate that Dup over-expression in S-phase cells which possess DNA damage leads to G2 arrest instead of re-replication. An important caveat is that in my experiments cells were arrested at G1/S by HU, which causes DNA damage. Dup over-expression in normal S-phase cells does not induce the same phenotype as evidenced by no arrest in Dup over-expressing asynchronous cells (Figure 25A and 25B). Nonetheless, Dup over-expression is sufficient for the reloading of pre-RC, demonstrated by the observed pre-RC in both euchromatin and heterochromatin in these arrested cells (Figure 35). These re-assembled pre-RCs are not able to initiate re-replication, probably due to the high level of DNA damage at the time when the pre-RC is re-assembled. HU treatment leads to DNA damage but this damage is mostly repaired by 24 hours post release from HU. The DNA damage persists if Dup is over-expressed at the time when cells are release from HU. There are two possibilities for the persistent DNA damage. It is possible that Dup over-expression impairs DNA damage repair in cells which are released from HU. The observed DNA damage therefore is caused by HU. Alternatively, as cells pass through S-phase, DNA damage caused by HU is repaired but DNA is sensitized to damage. Under this condition, re-initiation of a subset
of origins resulting from Dup over-expression was able to induce massive amount of DNA damage and trigger a checkpoint activation which blocks activation of origins.

### 3.4 Materials and Methods

**Preparation of inducible plasmids expressing Dup variants**

The plasmid pMt/Dup, in which wide-type full length Dup cDNA is regulated by the metallothionein promoter, was constructed as follows: Pfu PCR amplification of a full-length Dup cDNA from the start codon to the termination codon, subcloning into pMt/Hy vector through SpeI site (kindly provided by…). The primer pair has SpeI at both 5’ and 3’: 5’-GGGTTTACTAGTACACTTACTATGGCCCAGCCA and 3’-GGGTTTACTAGTGGTCTAATTGCTCTTGGCGTTAGC. The plasmid pMt/DupΔPIP (lacking the first 20 amino acids) was constructed in a similar way using a different 5’ primer: 5’-GGGTTTACTAGTATGATCAGTATCAAGAACAGGCGT.

**Generation and induction of stable cell line**

*Drosophila* Kc167 and S2 cells were cultured at 25 °C in *Drosophila* Schneider Medium (Invitrogen) supplemented with 10% fetal bovine serum and 1% antibiotics (…). Cells were transfected using Effecten (Qiagen). Hygromycin-resistant cells were selected with 125 µg/ml hygromycin and then cultured in standard medium supplemented with 125 µg/ml hygromycin. High-level expression from the metallothionein promoter was induced by treating cells with 0.5 mM CuSO4.
Immunoblotting and immunofluorescence

Total cellular protein was fractionated by SDS-PAGE in 12% gels. An affinity-purified guinea-pig anti-Dup antibody provided by Terry Orr-Weaver, Whitehead Institute, Cambridge, MA (Whittaker et al., 2000). Quantitation was performed using Odyssey Application Software version 3.0.

DNA isolation and microarray

See Materials and Methods in Chapter 2.
4. Conclusion

4.1 Conclusions

Eukaryotic cells orchestrate the initiation of DNA replication from hundreds of origins in an efficient, timely and error-free manner. To accomplish this complex task, eukaryotic cells employ an origin licensing system: origins cannot activate without being licensed by the pre-replicative complex which consists of ORC, Cdc6, Cdt1 and the helicase Mcm2-7, and they cannot be re-licensed without passage through mitosis. As discussed in section 1.5, there are multiple mechanisms to inhibit origin re-licensing outside of G1. When I began my thesis research, reports on re-replication in a variety of systems suggested that not all sequences are subjected to re-replication when replication licensing control mechanisms are compromised (Mihaylov et al. 2002; Vaziri et al. 2003; Tanny et al. 2006). It was unclear at that time, however, which sequences are more prone to re-replication when licensing control mechanisms are disrupted. In addition, it was not understood whether origin re-initiation is a stochastic event or a coordinated process. During my thesis research, I have studied the sequences that were re-replicated when geminin was absent or when Dup was over-represented. My studies have also described the dynamic formation of the pre-RC during re-replication and the characteristics of re-replication in G2.
In chapter 2, I studied re-replication induced by geminin depletion. I found that pericentric heterochromatin was preferentially re-replicated when geminin was depleted. The pericentric heterochromatin specific re-replication was due to the restricted re-assembly of pre-RC at heterochromatin, which was, in part, due to the high levels of cyclin A-CDK during S and G2.

In chapter 3, I investigated the genomic consequences of Dup over-expression. I found that Dup over-expression induced genome-wide re-replication with a preference toward heterochromatin. A complete re-replication of the genome (endoreduplication) was only seen when Dup was over-expressed in G2 arrested cells. The coordinated temporal initiation of origins during S-phase was lost in G2 re-replication. Interestingly, whether Dup over-expression led to re-replication or endoreduplication seemed to be dependent on the abundance of Dup.

Taken together, the data presented in this thesis suggests that a subset of origins is more prone to re-replication than others when licensing control mechanisms are disrupted, and that both CDK activities and the abundance of Dup are determinants of the extent of re-replication.
4.2 Discussion and Future Directions

4.2.1 The Significance of the Inhibition of Dup by Geminin

As an important mechanism to regulate the activity of Dup/Cdt1, geminin inhibition of Dup/Cdt1 plays a crucial role in preventing re-replication (Quinn et al. 2001; Mihaylov et al. 2002; Melixetian and Helin 2004; Zhu et al. 2004). Geminin binds to and sequesters Dup/Cdt1 during S, G2 and mitosis (Wohlschlegel et al. 2000; Quinn et al. 2001). Its degradation during late mitosis leads to the recruitment of Cdt1 to ORC bound origins and the loading of the Mcm2-7 complex.

The observation that geminin depletion alone is sufficient to induce re-replication in this thesis work and by others (Quinn et al. 2001; Mihaylov et al. 2002; Melixetian and Helin 2004; Zhu et al. 2004) suggests that geminin is an essential factor in licensing control. No complete reduplication of the genome was detected in geminin-depleted cells, suggesting that there are additional mechanisms to inhibit re-replication after the start of re-replication. Checkpoint activation may be one of these mechanisms. Re-replication induced CHK1 activation has been reported in mammalian (Melixetian et al. 2004), Xenopus (McGarry 2002) and Drosophila cells (Mihaylov et al. 2002). However, checkpoint activation is not enough to cease re-replication but rather provides a means to accumulate re-replication. Indeed, in agreement with reported results in Xenopus and Drosophila, abrogation of the CHK1 checkpoint impairs re-replication induced by
geminin depletion (data not shown). It is therefore possible that checkpoint activation
inhibits the re-activation of certain origins but not others.

Another possible mechanism to prevent further re-replication after initial re-
replication is by degradation of Dup/Cdt1. When geminin was depleted, the level of
Dup/Cdt1 was also decreased, suggesting geminin protects Dup from degradation
under normal conditions. It has been reported that geminin positively promotes Cdt1-
mediated Mcm2-7 chromatin loading in G1 (Ballabeni et al. 2004; De Marco et al. 2009),
suggesting that geminin bound Dup/Cdt1 could function as a reserve of Cdt1 to
promote pre-RC formation. Artificially depleting geminin leads to an abnormal release
of Dup/Cdt1, which could potentially activate the degradation pathways. The fact that
geminin depletion induces re-replication suggests that released Dup binds to origins to
facilitate pre-RC assembly. The decreased Dup could be the result of both direct
degradation upon its release from geminin and replication-coupled degradation. Future
experiments abolishing both geminin and components of the degradation pathways are
needed to determine whether the rapid degradation of Dup in geminin-depleted cells
contributes to the pericentric heterochromatin specific re-replication.

4.2.2 Regulation of Dup during Cell Cycle and Re-replication

In humans, Cdt1 is degraded during S-phase via two different E3 ubiquitin
ligases, the SCF<sup>Skp2</sup> complex which requires prior phosphorylation of Cdt1 by CDKs, and
the Cul4\textsuperscript{Ddb1} complex which requires the interaction between Cdt1 and PCNA (proliferating cell nuclear antigen) (Arias and Walter, 2006; Havens and Walter, 2009) (Figure 37). The SCF\textsuperscript{Skp2} complex not only functions during S-phase but also acts in G2 (Nishitani et al., 2006). Therefore, human Cdt1 is subjected to degradation in both S and G2. However, there is no known Drosophila homolog of Skp2 even though there are 10 consensus CycE/Cdk2 phosphorylation sites at the N-terminus of Dup (Figure 36A). Mutation of these 10 sites partially stabilizes Dup (Thomer et al. 2004), suggesting there is CDK-dependent degradation of Dup in Drosophila. Dup lacking the N-terminal PIP box which interacts with PCNA for its degradation through the Cul4\textsuperscript{Ddb1} complex is resistant to degradation (Figure 36B) (Thomer et al. 2004; Lee et al. 2010). These studies did not assess the protein levels of PIP-depleted Dup by immunoblot, however. It is therefore unclear how efficient is the Cul4\textsuperscript{Ddb1} complex in degrading Dup.
Figure 36: Diagram of Drosophila Dup.

(A) Schematic of the *Drosophila* Dup protein identifying the PIP box, phosphorylation sites, geminin binding domain and the replication licensing domain.

(B) Alignment of the Cdt1 Cul4*Ddb1* 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.


In addition to degradation after origin firing, Dup/Cdt1 is also degraded by proteolysis in response to DNA damage in a number of organisms (Figure 37). In mammalian cells, Cdt1 degradation upon DNA damage caused by both UV and IR is predominantly mediated by the Cul4*Ddb1* pathway (Higa et al., 2003; Hall et al., 2008; Jin et al., 2006). I observed only modest elevation of Dup protein level when it was over-expressed, suggesting degradation pathways in *Drosophila* work very efficiently to remove excessive Dup. Whether this degradation of Dup is dependent on DNA damage or re-activation of origins is unknown. It is also unclear which pathways are involved in
the degradation of Dup when it is over-expressed. Further experiments studying the accumulation of Dup when replication fork is inhibited could provide information on whether replication-coupled degradation is the predominant driving force of Dup degradation upon its over-expression. Depletion of components of the Cul4\textsuperscript{Ddb1} pathway in G2 cells and subsequent assessment on Dup protein levels would help to elucidate the role of Cul4\textsuperscript{Ddb1} in mediating Dup degradation outside of S-phase.

**Figure 37: Degradation of Cdt1 during the cell cycle and after DNA damage.**

In S phase, Cdt1 degradation occurs by the SCF\textsuperscript{Skp2} and by the Cul4\textsuperscript{Ddb1} pathways. Phosphorylation of Cdt1 by cyclin A/Cdk2 leads to the recruitment of the SCF\textsuperscript{Skp2} complex. PCNA binds to Cdt1 on its PIP motif, leading to recruitment of Cul4\textsuperscript{Ddb} complex. Following DNA damage by UV/IR irradiation, Cul4\textsuperscript{Ddb1} mediates Cdt1 degradation on chromatin in a similar manner as S-phase-induced degradation. Adapted from Truong and Wu, 2011, *Journal of molecular cell biology* 3:13-22
4.2.3 All Origins Are Not Equally Sensitive to Re-replication

The preferential re-replication at heterochromatin was observed both during
geminin depletion and early hours of Dup over-expression. The unequal sensitivity of
origins to re-replication has also been reported in yeast and human. Re-replication in
yeast tends to enrich for the subtelomeric chromosomal regions (Tanny et al. 2006;
Mickle et al. 2007; Kiang et al. 2010). In human, re-replicated sequences are enriched in
segments of chromosomes that are replicated early in S phase (Vaziri et al. 2003). The
reason for the high susceptibility of certain portions of the genome to re-replication will
be an interesting question to explore in the future.

To explain the preferential re-replication of heterochromatin in Drosophila, I
suggest three possible mechanisms. The first possibility is that heterochromatin might be
more accessible to replication factors and the replication machinery due to its structure
and subnuclear organization after being replicated in S-phase. For example, it is possible
that geminin binds Dup in close proximity to heterochromatin during normal cell cycle.
When either geminin is absent or Dup is over-supplied, excessive Dup will become
associated with origins at heterochromatin. In the case of geminin depletion, due to the
rapid degradation of Dup, re-licensing of origins is limited to heterochromatin because
of the low availability of Dup. In the case of Dup over-expression, the situation is similar
to geminin depletion when Dup is initially over-supplied. The abundance of Dup
becomes greater when Dup has been over-expressed for a period of time; therefore the
re-licensing of origins at euchromatin becomes possible when the abundance of Dup reaches a certain threshold. By this time, re-replication initiates at both euchromatin and heterochromatin.

Second, cyclin A-CDK activities might specifically inhibit re-replication of euchromatin. Eudoreduplication was observed when cyclin A was depleted for 48 hours independent of geminin levels (Figure 20). CDK inhibition induced endoreduplication has also been reported in mouse cells (Lu et al. 2010). These results suggest that the absence of cyclin A-CDK activities in G2 creates a G1-like environment which promotes genome-wide assembly of the pre-RC. It is possible that CDK blocks euchromatic re-replication via regulation of Dup. When the levels of Dup is high enough to overcome the regulation by CDK, genome-wide assembly of the pre-RC may occur. My observation that high levels of Dup in G2 cells induce endoreduplication is in support of this idea.

Third, the preferential re-assembly of the pre-RC and activation of replication origins at heterochromatin may be an additional control mechanism to prevent catastrophic genomic instability in the absence of replication licensing controls. The heterochromatin may act as a sink, capable of absorbing certain amount of re-replication before cells undergo apoptosis. The repetitive nature of the heterochromatic sequences may facilitate recombination driven repair mechanisms. Future experiments fine tuning the level of over-expressed Dup through a titration of copper concentrations can help to
validate the hypothesis that when Dup is not overwhelmingly abundant, re-replication is restricted to heterochromatin.

4.2.4 The Loss of Replication Timing Program during Re-replication

The re-replication I observe is not specific to either early or late replicating origins. Despite re-replication being restricted to heterochromatin when geminin is absent, it is not limited to late origins which initiate DNA replication late during S-phase. The genome-wide re-replication induced by Dup over-expression further suggests that re-replication is not subjected the mechanisms which regulate replication timing during S-phase.

Endoreduplication in G2 arrested cells provides a great system to examine whether timing program is re-established in G2. Although the assembly of pre-RC is genome-wide during endoreduplication (Figure 33B), the initiation does not follow a defined timing program as replication during S-phase does. The loss of replication timing program in G2 is indicative for two importance features of the cellular environment during this stage of the cell cycle. First, G2 is permissive for pre-RC formation given enough Dup protein. It is therefore critical for cells to maintain proper amount of Dup in G2. The observation that cyclin A silencing also resulted in endoreduplication following a G2 arrest (Figure 20A) suggests that cyclin A-CDK may be the safe-guard during G2 to prevent re-replication. Second, there is no mechanism to
separate pre-RC formation and the activation of the helicase. In other words, Mcm2-7 complex is activated as soon as it is loaded onto chromatin. Interestingly, despite the immediate activation of Mcm2-7 following its loading, re-replication in G2 arrested cells seems to coordinate in a way that ensures the duplication of the entire genome before another round of origin activation. These results suggest that despite the lack of determinants of timing program, there are unidentified mechanisms to regulate origin firing during G2.
References


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Biography

Queying Ding was born on September 25, 1983 in Hunan, China. For her undergraduate degree she attended Tsinghua University in Beijing, China, where she received a B.S. in Biology. She came to Duke University in 2006, pursuing a doctoral degree in Molecular Cancer Biology. Her publications include the following:

