REGULATION OF DNA REPLICATION DURING CONVENTIONAL AND UNCONVENTIONAL CELL CYCLES IN TETRAHYMENA

A Dissertation

by

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ABSTRACT

As the nucleating protein for pre-replicative complex (pre-RC) assembly, the conserved Origin Recognition Complex (ORC) specifies where replication initiates in eukaryotic chromosomes. During the vegetative cell cycle of *Tetrahymena thermophila*, previously published work has shown that DNA replication initiates from defined chromosomal sites in an ORC-dependent manner. *Tetrahymena* exhibits nuclear dimorphism, a polyploid somatic macronucleus (MAC), which is transcriptionally active and maintains vegetative growth, and a diploid germline micronucleus (MIC) responsible for the transmission of genetic information during conjugation. In order to provide more information about the fundamental mechanisms of micro- and macro-nuclear replication programs, I study the impacts of changing in ORC protein contents on the fate of micro- and macro- nuclear chromosomes during the vegetative cell cycle and development in *Tetrahymena*.

I examined the effect of down-regulation of ORC1 on genome stability and intra-S phase checkpoint activation by disrupting ORC1 gene in the macronucleus. Partial depletion of Orc1p leads to genome instability in the diploid mitotic micronucleus, abnormal division of the polyploid amitotic macronucleus, and failure to mount a robust intra-S phase checkpoint response. In addition, the ORC1 knockdown strain fails to execute two developmentally- regulated DNA replication programs, endoreplication and ribosomal DNA (rDNA) gene amplification. I also examined the regulation of ORC and MCM during development. Remarkably, the result suggests that the demand on the

ORC-dependent replication machinery differs during development and the vegetative S phase.

To further gain new insights into fundamental mechanisms that protect chromosomes from replication stress, I examined the impact of replication stress on the regulation of ORC and MCM. This study led to the discovery of a novel DNA replication program that is activated under HU treatment. While Orc1p and Mcm6p were selectively degraded in response to HU, cells were competent to complete S phase in the absence of Orc1p and Mcm6p after HU was removed. In addition, the rDNA origin used exclusively during the S phase of vegetative cell cycle and developmentally programmed gene amplification is suppressed when these replication proteins are selectively degraded under HU treatment. Instead, an alternative program was used to resume the cell cycle progression. These data provide compelling evidence for an ORC-independent DNA replication program in cells recovering from replication stress.

DEDICATION

This dissertation is lovingly dedicated to my grandfather Xsin-Lai Lee and grandmother Yin-Chen Lin who filled my childhood with many precious memories.

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CHAPTER I

INTRODUCTION

Overview

The focus of my dissertation research is to gain insight into the regulatory mechanisms that control eukaryotic DNA replication programs. Our model organism, *Tetrahymena thermophila*, harbors a 21 kb ribosomal DNA (rDNA) minichromosome that undergoes extensive gene amplification (5,000 fold) during development but is subsequently replicated once per cell cycle. In addition, the non-rRNA chromosomes are endoreplicated during development to achieve a copy number of 45 C. Recent work in our lab determined that *Tetrahymena* ORC (Origin Recognition Complex) contains an integral RNA subunit, 26T RNA, which specifically targets ORC to rDNA origins that are used for both rDNA gene amplification and subsequent vegetative DNA replication {Mohammad:2007kf}. Whereas 26T RNA is present in all ORC complexes, ORC is recruited to non-rDNA replication origins by another mechanism since the recognition target for 26T RNA is absent in non-rDNA chromosomes {Donti:2009kh}. Therefore, *Tetrahymena* provides unique opportunities to study the contribution of the ORC to these differential DNA replication programs.

For my research, I focused my attention on replication origin utilization and activation of the intra-S phase DNA damage checkpoint response, a signaling pathway that arrests DNA replication in response to damage or replication stress. The goal of my work is to understand how these two processes are integrated to promote genome

stability during vegetative cell cycles and in response to replication stress.

In the introduction chapter, I first describe properties of eukaryotic replicons (a single replication unit that comprises the three elements: replication origin, replicator and initiator) and mechanisms that regulate replication initiation. I then discuss pathways that are activated by stalled replication forks during DNA replication, such as checkpoint signaling and homologous recombination. Finally, I will introduce the model organism *Tetrahymena thermophila* and illustrate its relevance for the study of DNA replication.

Eukaryotic DNA replication

Eukaryotic replicons and initiation control

According to the replicon model proposed by Jacob and Brenner (Jacob and Brenner 1963), initiation of DNA replication occurs at specific DNA sites, called replicators, that bind a trans-acting regulatory factor, called the initiator. The actual sites where DNA replication begins are called origins of replication. The replicon model was corroborated in bacterial and viral systems. In eukaryotes, however, the situation is more complex as there are many origins to be coordinately regulated on a chromosomal DNA, and there is very little sequence specificity for the origins except in the budding yeast. Many studies suggest that replicators are not determined only by DNA sequence. Furthermore, only a fraction of initiator-bound replicators actually initiate replication in a given cell cycle.

A consensus sequence for replicators has only been found in the budding yeast *Saccharomyces cerevisiae*. These yeast replicators were found to confer the ability to

support extrachromosomal DNA replication, and these DNA elements were termed autonomously replicating sequences, or ARSs (Stinchcomb et al. 1979). Dissection of the most well studied ARS1 replicon revealed that it is about 150 bp in length and contains a single essential segment - 17-bp A element with an 11-bp ACS (ARS consensus sequence) and auxiliary elements (Marahrens and Stillman 1992). The A-T rich ACS is the essential feature for all ARSs (Newlon and Theis 1993) and constitutes half of the binding site for the origin recognition complex (ORC). ORC plays an essential role in the initiation of DNA replication by binding to the origin, and contains six subunits named Orc1 through Orc6. Additional B1, B2, and B3 elements were also identified. The B1 element comprises the second half of the ORC binding site. B2 contains a sequence element important for the assembly of pre-RC (pre-replicative complex) (Wilmes and Bell 2002). B3 is a binding site for the transcription factor Abf1p which is present in ARS1 (Marahrens and Stillman 1992). Other transcription factor binding sites can functionally substitute for the B3 element, and positively or negatively regulate the ARS1 replication activity (Marahrens and Stillman 1992), suggesting a link between transcriptional factors and DNA replication. The effect of transcription factors on the activity of the origin is independent of RNA synthesis, and instead, it reflects chromatin access or nucleosome organization (Cheng et al. 1992). These B elements are not individually essential, but they are required for functional origins. Combinations of these B elements are also found in other ARSs (Newlon and Theis 1993).

In the fission yeast *Schizosaccharomyces pombe,* replicons were identified using a plasmid transformation assay (Clyne and Kelly 1995). *S. pombe* replicons are much

larger than *S. cerevisiae* (0.5–1 kb versus 100–200 bp), and no consensus sequences analogous to the ACS have been identified. Instead, *S. pombe* ARSs contain multiple functional domains for ARS activity rather than one found in *S. cerevisiae* origins, and these essential elements often contain stretches of asymmetrical adenine or thymine residues based on genetic deletion studies in origins (Okuno et al. 1999). The length and number of such stretches are not conserved between different ARSs (Okuno et al. 1999). Further genome-wide analysis revealed that about 90% of these AT-rich stretches (also called AT-islands) co-localize with active *S. pombe* origins (Segurado et al. 2003). In addition, DNA combing studies using DNA fibers labeled by DNA analogue precursors revealed that firing of *S. pombe* origins is stochastic, leading to a random distribution of replication initiation sites. The activity of one origin is not inherited from one cell division to the next. Hence, different origins are used in each generation (Patel et al. 2006).

The unique structure of the *S. pombe* Orc4 subunit accounts for its high preference for AT-rich DNA (Chuang and Kelly 1999). Unlike other eukaryotic ORCs, the N-terminal domain of *S. pombe* Orc4 contains nine copies of the AT-hook motif, found in a number of DNA-binding proteins, and can mediate binding to the minor groove of AT-tracts in DNA with no specificity. Thus, *S. pombe* Orc4 has the potential to bind more than one degenerate element in a given ARS. The interaction between *S. pombe* Orc4 and AT-rich DNA stretches provides the basis that multiple elements contribute to the origin activity.

Metazoan origins are quite similar to the fission yeast DNA replication origins in

that they lack an apparent consensus sequence. In addition, their replicators are large (one to several kilobases) and have a higher AT-rich sequence content. At some metazoan replicons, replication initiates from a single specific location in the genome. Examples include the *Drosophila* chorion locus and human lamin B2 origin. In other metazoan loci, numerous initiation sites are distributed over broad initiation zones, like the Chinese hamster dihydrofolate reductase (*DHFR*) locus (see below; reviewed in Aladjem 2007). Studies on metazoan origins indicate that they are not exclusively determined by DNA sequences; other factors such as chromatin structure and transcription activity may also have significant influence on the selection of DNA replication origins (Bailis et al. 2008; Aggarwal and Calvi 2004; reviewed in Kohzaki and Murakami 2005). I will discuss this subject in more detail later in this chapter.

During *Drosophila* oogenesis, follicle cells surrounding the oocyte undergo a process known as chorion gene amplification. The replicator for chorion gene amplification has been delineated by a P-element transposon transformation experiment testing for amplification of chorion amplicon sequences at ectopic genomic sites (de Cicco and Spradling 1984) and two essential elements on the third chromosome, ACE and Ori-β, were mapped by two-dimensional gel analyses (Lu et al. 2001). ACE3 acts as a replication enhancer element for the initiation of replication from the Ori-β in the amplified chorion locus. However, a study of *Drosophila* ORC DNA binding activity to *in vitro* substrates showed that *Drosophila* ORC bound to origin and non-origin sequences with similar affinities, suggesting that *Drosophila* ORC is not a sequence-specific DNA binding protein (Remus et al. 2004). In fact, a biochemical study reported

that the transcription factor Myb functions in tight complex with four other proteins that interact with ORC and promote ORC binding to ACE3 and Ori-β (Beall et al. 2002). Another study using Chromatin immunoprecipitation (ChIP) analysis reported that *Drosophila* ORC can be tethered to the ACE3 by E2F/Rb complex (Bosco et al. 2001). Thus, these studies on a *Drosophila* amplicon provide evidence that distal sequences that do not function as replicators can also affect the location of initiation events. In addition, they demonstrate a correlation between transcriptional factors and initiation of DNA replication. Moreover, non-ORC DNA binding factors were shown to play an important role in the recruitment of ORC to origins in metazoans.

Another metazoan replicon where origin firing is restricted to a confined site is the human lamin B2 locus. The 1.2-kb lamin B2 replicon comprises the 3' end of the lamin B2 gene and the promoter of the downstream gene. The initiation site was mapped to a □500 bp region 3′ of the lamin B2 gene (Giacca et al. 1994). *In vitro* footprinting analysis displayed a cell cycle dependent footprint that is present at G1 and shrinks in S phase. No footprint is detectable in mitosis (Abdurashidova et al. 1998). These results are reminiscent of the assembly of pre- and post-replicative complexes on the budding yeast ARS sequences (Diffley et al. 1994). A ChIP analysis further demonstrates that at G1 phase the initiation site is surrounded by human Orc1, Orc2, Cdc6 and Mcm3, the pre-RC components that have been seen in yeast and in other metazoan. Only Orc2 was detected in S phase and M phase chromatin contains none of these pre-RC components. (Abdurashidova et al. 2003). These data suggest that the same cell cycle-regulated replication machinery is used in mammals as in other eukaryotes. Importantly, this 1.2

kb fragment supports replication initiation when integrated at ectopic positions of the human genome (Lidonnici et al. 2004), strengthening the argument that lamin B2 is a true mammalian replicator.

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). Deletion of the ori-β locus, which is the preferred initiation site in this intergenic zone, does not reduce origin activity in the remainder of the intergenic zone. However, when the 3′ end of the *DHFR* gene was deleted, the initiation is confined to the far end of the intergenic region, suggesting that an element in the 3' end of *DHFR* constitutes not only for the gene but also acts as a distal element for the neighboring replication origin (Kalejta et al. 1998; Mesner 2005). Further analysis by deletions of the *DHFR* promoter, which abrogates transcription of the gene, showed that the initiation within the intergenic region was reduced but the initiation in the body of the *DHFR* gene was activated. This result suggests that transcription also plays a role in determining the efficiency and location of origin firing from the *DHFR* locus (Saha 2004). The eukaryotic replicons described above are summarized in Fig. 1.1.

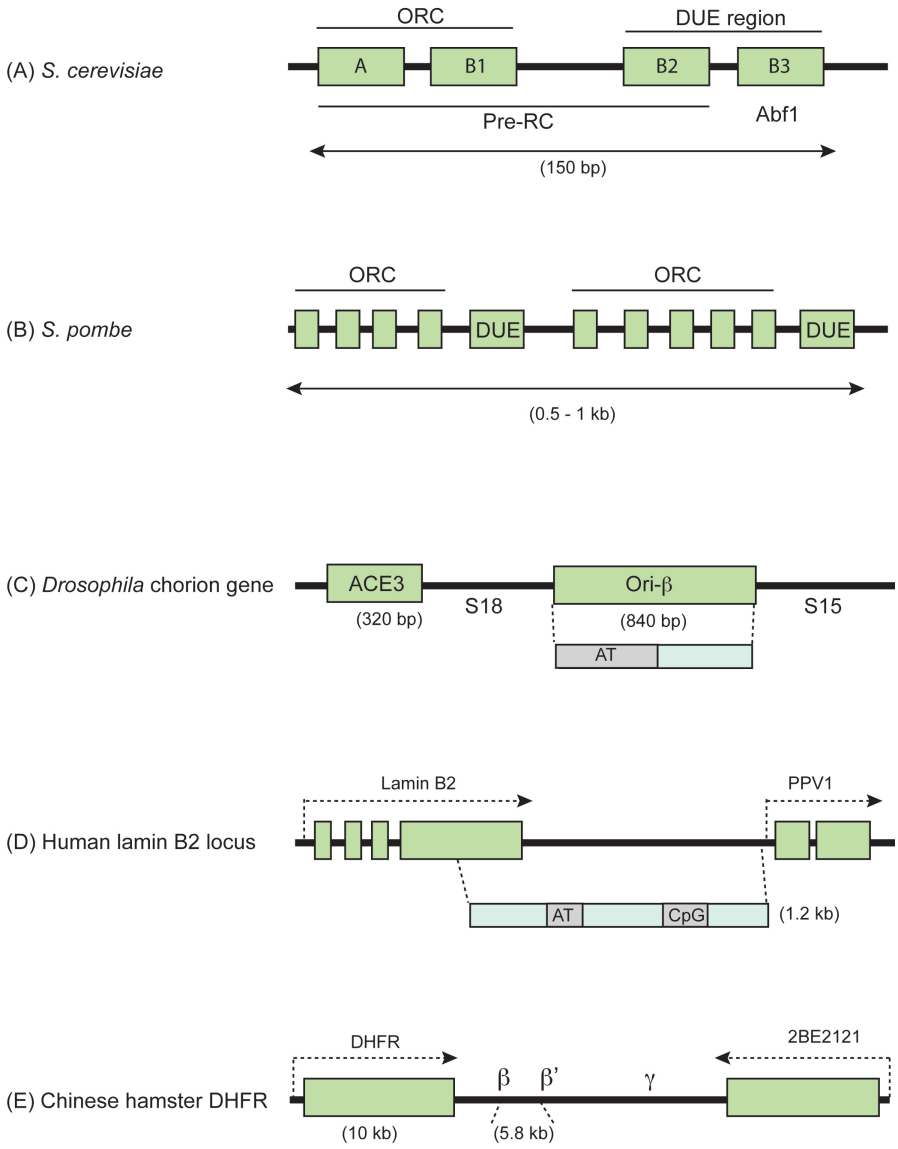

Figure 1.1. Functional elements in eukaryotic replicons.
(A) ARS1 replicon in budding yeast. Showing different *cis*-acting elements important for replication that are described in the text (ACS, B1, B2 and B3); (DUE) DNA unwinding element; (ORC) origin recognition complex and showing its binding region; (Abf1) Transcription factor. The region protected by ORC and pre-RC is indicated. (B) ARS1 replicon in fission yeast. ORC binding seqiences are indicated by small box. (C) *Drosophila melanogaster* chorion locus. (AT) indicates AT-rich stretches. (D) Human lamin B2 locus. (CpG) CpG island. Arrows indicate the direction of transcription. (E) Chinese hamster DHFR locus. Diagram is modified from (Aladjem 2007).

Epigenetic regulation of replication initiation

Chromatin structures and histone modification are important factors not only for transcription but also contribute to origin activation and pre-RC loading. An early nuclease protection analysis revealed that the core sequences (or A element) of the budding yeast ARS1 origin is free of nucleosomes but flanked by well positioned nucleosomes, presumably to facilitate ORC binding (Thoma et al. 1984). Introducing nucleosomes inappropriately into the core sequences of ARS1 origin disrupts origin activity on an episome; however, moving these nucleosomes back to their normal position by insertion of an α2 operator restores normal origin activity (Simpson 1990), suggesting nucleosomes overlapping the origin interfere with the ability of an origin to initiate replication. In the chromosomal context, the alteration of budding yeast ARS1 nucleosomal configuration also affects chromosomal initiation. Notably, the association of ORC with the mutant origins is not altered but formation of the pre-RCs is defective (Lipford and Bell 2001). In addition, ORC and Abf1 are both required to prevent nucleosomes from invading the ARS1 replicon thereby generating a suitable arrangement for origin activation (Lipford and Bell 2001). These results suggest that ORC may contribute to the distribution of nucleosomes for loading of additional pre-RC proteins. Recent advances in genome technology have allowed researchers to use high-throughput sequencing to determine the genome-wide occupancy of nucleosomes in native yeast chromosomes. The data suggest that intrinsic DNA sequence determines the organization of nucleosomes (Kaplan et al. 2009). This result is confirmed by another sequencing experiment mapping ORC binding and nucleosome positioning in budding

yeast and further addresses that ORC is required for the precise positioning of nucleosomes flanking the origin (Eaton et al. 2010).

In *S. cerevisiae*, mutations in the histone deacetylase SIR2 facilitate initiation events (Pappas et al. 2004). At the ACE3 and Ori-β origins of *Drosophila*, the tethering of acetyltransferase to origins at the chorion gene locus promotes the recruitment of the ORC complex, suggesting that histone acetylation modulates the binding or activation of ORC in follicle cells (Aggarwal and Calvi 2004). In addition, *Drosophila* ORC complex localizes to sequences enriched in histone variant H3.3, a marker for active chromatin remodeling (MacAlpine et al. 2010). In human cells, the histone acetyltransferase Hbo1 interacts with Orc1 and Mcm2 and plays a positive regulator for replication initiation (Burke et al. 2001). Histone H4 acetylation at origins by Hbo1 is also critical for replication licensing by Cdt1 (Miotto and Struhl 2010). Taken together, in higher eukaryotes, chromatin structures and epigenetic regulation strongly influences the loading of pre-RC and origin activation. Primary DNA sequence may play a minor role in determining where the DNA replication occurs.

No sequence specification of origin initiation has been found in the *Xenopus* and *Drosophila* embryonic systems where any sequence can be used as an origin of DNA replication (Hyrien and Méchali 1992). However, as development continues, the initiation of replication starts to be localized to specified regions in both *Xenopus* and *Drosophila* cells. In early *Xenopus* embryos, the rDNA replication initiates randomly at 9 – 12 kb intervals. After the mid-blastula, with the resumption of rRNA transcription, the rDNA replication begins to initiate limitedly from the intergenic spacer regions

between the rDNA transcription units (Hyrien et al. 1995). Similarly, during *Drosophila* embryogenesis, DNA replication initiates at unspecified chromosomal sites in pre-blastoderm embryos, but initiation at the DNA polα-dE2F region is specified by 5 h after fertilization (Sasaki et al. 1999). Both cases provide strong evidence for a role of transcriptional activity in origin specification.

Pre-RC formation and regulation

Eukaryotic chromosomal DNA replication is initiated by the formation of the pre-replicative complex (pre-RC) at origins of replication. The pre-RC was originally identified in budding yeast by DNase I protection assays, as it generates a characteristic DNA footprint prior to replication initiation (Diffley et al. 1994). The assembly of pre-RC is a conserved process in eukaryotes and takes place at late M to G1-phase transition when the S-phase cyclin-dependent kinase (CDK) activity is low. This mechanism ensures the genome replicates only once per cell cycle. During this period, ORC acts as a loading pad to recruit Cdc6 to origins, allowing Cdt1 to load two copies of the hexameric MCM ring to complete a pre-RC (Evrin et al. 2009). Loading of the MCM helicases onto DNA is referred to as DNA replication licensing (Fig. 1.2). Once licensed, the activation of pre-RCs requires further ordered recruitment of Sld3, GINS and Cdc45, dependent on CDK and DDK (Dbf4 dependent kinase) activity (Yabuuchi et al. 2006). This process is considered to activate the MCM2-7 helicase, resulting in origin DNA unwinding (Ilves et al. 2010), and assembly of RPA (Replication protein A) and DNA polymerases for initiation of DNA synthesis (reviewed in Masai et al. 2010) (Fig. 1.3).

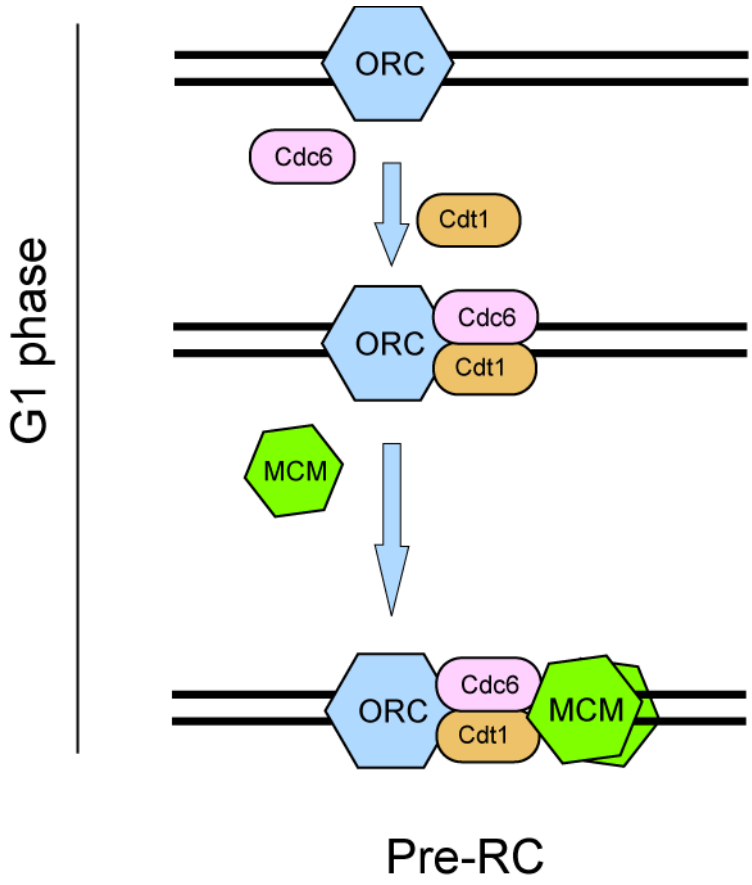

Figure 1.2. A model for pre-replicative complex formation.
Licensing of origins constitutes a conserved process in all eukaryotes that occurs in late mitosis and during G1 phase. It involves the ordered assembly of pre-replicative complexes (pre-RCs) comprising the ORC, Cdt1, Cdc6 and MCM2–7 helicases. Diagram is modified from (Symeonidou et al. 2012).

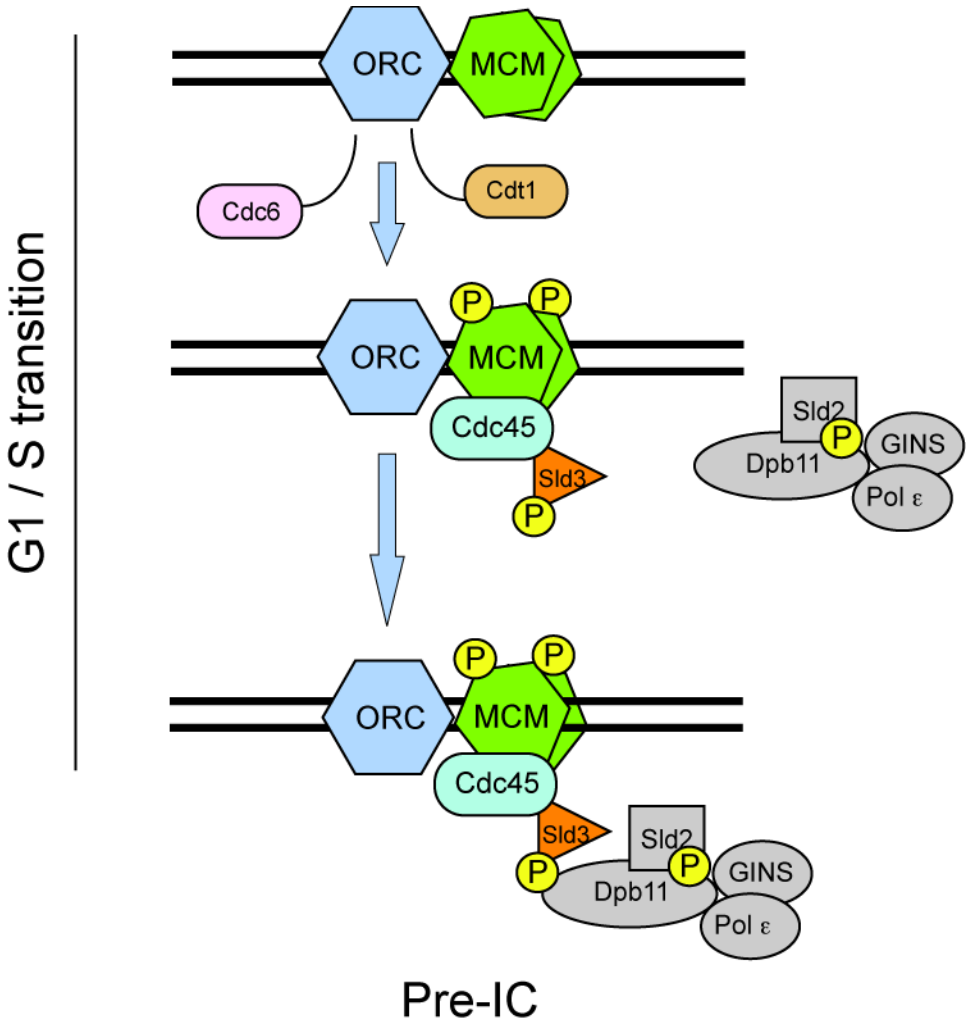

Figure 1.3. A model for pre-initiation complex formation.
At the G1 to S phase transition, the concerted actions of cyclin-dependent kinases (CDK) and Dbf4-dependent kinase (DDK) activate the pre-RCs converting them into pre-initiation complexes (pre-ICs), through the recruitment of additional initiation factors. Diagram is modified from (Symeonidou et al. 2012).

During the transition to S phase, multiple mechanisms are applied in order to prevent pre-RC from re-assembling on DNA that has already been replicated. In *S. cerevisiae*, S-phase CDK reduces Cdc6 levels through phosphorylation of Cdc6, which targets Cdc6 for ubiquitin-mediated degradation (Drury et al. 1997). CDK also promotes the nuclear export of MCM proteins in *S. cerevisiae*, leading to their exclusion from the nucleus during G2/M phase (Nguyen et al. 2000). However, the remaining unphosphorylated Cdc6 (Drury et al. 1997) and constitutively nuclear localized MCM proteins (Nguyen et al. 2000) are not capable of inducing re-replication within a cell cycle, suggesting that there are additional mechanisms to block the pre-RC formation. One inhibitory pathway is through the degradation of Cdt1 during S phase by ubiquitination-dependent proteolysis in a CDK-dependent manner (Arias and Walter 2005), or through the interaction with proliferating cell nuclear antigen (PCNA) (Arias and Walter 2006). In vertebrates, the remaining Cdt1 is inhibited by its specific inhibitor, geminin (Wohlschlegel et al. 2000). These strategies ensure that Cdt1 is not present during S phase and prevent relicensing of origins. In addition, human Orc1p, the largest subunit of the human origin recognition complex, is targeted for ubiquitination in S phase to prevent re-assembly of the pre-RC (Méndez et al. 2002).

The strategies to prevent re-replication may differ in different organisms. However, by using multiple inhibitory mechanisms to target more than one replication protein, the entire genome of an organism can be faithfully replicated once and only once per cell cycle.

The origin recognition complex proteins (ORC)

Origin recognition complex (ORC) proteins were first discovered as a heterohexameric complex in *S. cerevisiae* that specifically bound to origin sequences in vitro in the presence of ATP (Bell and Stillman 1992). The subunits were named Orc1 through Orc6 in a descending order of molecular mass. Orthologs of *S. cerevisiae* ORC1-ORC5 were subsequently identified in organisms such as *Drosophila melanogaster*, *Xenopus laevis*, *Arabidopsis thaliana*, and *Homo sapiens*, suggesting that these genes are likely to be conserved in all eukaryotes (reviewed in Duncker et al. 2009). Orc6 is the least conserved of all ORC subunits, and amino acid alignments between budding yeast and other eukaryotes do not show significant homologies. In *S. cerevisiae*, Orc1-Orc5 are required for origin recognition and binding, whereas Orc6 is dispensable for this process (Lee and Bell 1997). Mutations in ATP-binding motifs of Orc1 have a lethal effect (Klemm et al. 1997). In contrast, *Drosophila* Orc6 is essential for ORC-dependent binding to DNA *in vitro* (Balasov et al. 2007). The conservation of ORC subunits throughout eukaryotic evolution suggests that there must be common mechanisms for the initiation of DNA replication in all eukaryotes.

ORC function is tightly controlled by ATP binding and hydrolysis. Orc1-5 have conserved AAA+ folds (standing for ATPases associated with a variety of cellular activities) (Speck et al. 2005), which are also seen on Cdc6 and Mcm2-7p (reviewed in Duncker et al. 2009). The interaction between the Orc1 subunit and ATP is required for DNA binding and is essential in yeast. When ORC is bound to origin DNA, the ATPase activity of Orc1p is inhibited and ATP remains stably bound to Orc1p (Klemm and Bell

2001). The binding of Cdc6 to ORC on DNA induces ATP hydrolysis and a conformational change that allows ORC to encircle duplex DNA and directs the loading of Mcm2-7 onto DNA in an ATP-dependent process (Sun et al. 2012).

The cellular number of ORCs in budding and fission yeasts is around six hundred, which is about two ORCs per origin. In mammals, the density of ORCs is limited to around one ORC per origin (Natale et al. 2000), suggesting that most ORCs are bound to chromatin. To ensure faithful and timely DNA replication, the cell cycle dependent regulation of ORC, or "the ORC cycle" (reviewed in DePamphilis 2005), provides a mechanism to prevent re-initiation of DNA replication. In budding and fission yeasts, ORC remains intact and bound to chromatin throughout the cell cycle (Fujita et al. 1998; Kong and DePamphilis 2001). However, in human cells, Orc1p is polyubiquitinated and degraded during S phase. It then reappears during the M to G1 transition (Tatsumi et al. 2003). In Chinese hamster ovary cells, Orc1p is selectively released from chromatin under conditions in which other ORC subunits remain stably bound, but it is not degraded (Li and DePamphilis 2002). Restoration of stable Orc1p binding to chromatin occurs during M to G1 transition. *Drosophila* Orc1p is selectively ubiquitinated by the APC/Fzr system during mitosis, degraded as cells exit mitosis, and then synthesized and bound to chromatin during late G1 (Araki et al. 2003). Overall, the purpose of modification (phosphorylation or ubiquitination) on Orc1p is to inhibit the association between the ORC and other components of the pre-RC, or to alter the activities of the ORC, such as ATP-dependent DNA binding. Consequently, initiation of DNA replication is limited to once per cell cycle by preventing re-assembly of pre-RCs

during S, G2 and M phases.

The function of "ORC cycle" not only accounts for restricting the initiation events, but may also contribute to the decision of which initiation sites are used (reviewed in DePamphilis 2005). In metazoana, in which the replication events are randomly initiated, this strategy contributes to the selection of potential initiation sites along the genome. Partial ORC complexes can be assembled at many potential initiation sites awaiting the binding of either Orc1 or an Orc1/Cdc6 complex to dictate which of these sites will be activated. This strategy helps to solve the problem that DNA replication interferes with transcription as chromatin structure changes during development.

Genome-wide analysis of eukaryotic DNA replication

Different genome-scale approaches have been developed in recent years and contributed to look at DNA replication in a comprehensive manner in eukaryotes. The comprehensive nature of these approaches facilitated the research of eukaryotic DNA replication on origin mapping and replication timing, and revealed clear connections between chromosome organization and the pattern of replication (reviewed in Gilbert 2010).

The characterization of early replication intermediates is the straightest way to identify active origins of DNA replication. In this strategy, cells are synchronized before S phase and allowed to initiate replication in the presence of a replication fork inhibitor, such as hydroxyurea (HU), to accumulate nascent strands within a few kilobases of the

origins that fire at the onset of S phase. Replicated sequences are then detected by their copy number increase (~ two fold) on duplication (reviewed in Gilbert 2010) or by BrdU incorporation (reviewed in Schepers and Papior 2010). This genome-scale oligonucleotide microarray approach was initially applied on the budding yeast *S. cerevisiae* and confirmed that most of the known ARS-elements correlate with active initiation sites (Wyrick et al. 2001). However, it has also be shown that not all ARS elements are active origins and that only some of them are used in >50% of cell cycles (Newlon and Theis 2002). Studies in fission yeast *S. pombe* showed that ~ 400 potential origins have an efficiency of 10–60% and additional putative 500 origins are used in <10% of cell cycles, suggesting that these origins in fission yeast fire with low efficiency (Heichinger et al. 2006). In metazoans, the nascent strand DNA microarray-based methods have been used to identify large numbers of replication origins (MacAlpine et al. 2004; Cadoret et al. 2008). These studies in different species all showed a correlation between gene expression and early replication timing, in which origins are preferentially found in GC-rich regions. The advantage of mapping replication initiation sites by microarray-based approach is the high resolution of the data at a genome-wide scale, whereas the disadvantage is that repetitive elements cannot be analyzed by this approach because it generates a bias towards unique regions of genomes (reviewed in Schepers and Papior 2010).

Chromatin immunoprecipitation (ChIP) of pre-RC components, ORC and the Mcm2-7 complex, followed by microarray (ChIP–chip) or sequencing (ChIP–seq) has also been widely used to map potential replication origins (reviewed in MacAlpine and

Bell 2005). In budding yeast *S. cerevisiae*, studies using ChIP–chip (Wyrick et al. 2001) or ChIP–seq (Eaton et al. 2010) identified ~300 ORC-bound sites, and most were in intergenic regions. In addition, ORC and MCM2-7 complexes are co-localized for the most part. In fission yeast *S. pombe*, ChIP–chip showed colocalized ORC and MCM at ~ 460 sites in intergenic AT-rich sequences. In both yeasts, not all sites of ORC and MCM binding are active origins, which can be explained by the presence of dormant origins that are not used under normal growth conditions.

In budding yeast, although a consensus DNA sequence (ARS consensus sequence; ACS) for ORC binding has been identified, ChIP–seq for ORC has revealed that many consensus sequences are not bound by ORC (Eaton et al. 2010). In fact, ORC-bound consensus sites are shown to be located in nucleosome-free regions flanked by phased nucleosomes (positioned at precise intervals from nucleosome-free regions) (Eaton et al. 2010). While *S. pombe* ORC can initiate replication within any sufficiently extensive stretch of AT-rich DNA via the special AT-hook motif in Orc4, a genome-wide nucleosome mapping data showed that origins still seem to align with nucleosome-free regions (Lantermann et al. 2010).

In most metazoa, replication does initiate preferentially at specific sites, and metazoan ORC has no intrinsic affinity for any DNA sequence motifs, despite a 30-fold increase of DNA binding affinity to negative supercoils (Vashee et al. 2003). ORC and MCM2-7 data of *Drosophila* have recently been published on the basis of the modENCODE (Model Encyclopaedia Of DNA Elements) microarray and show the first ChIP data of a metazoan species (Celniker et al. 2009). MacAlpine and co-workers

conducted a genome-scale mapping of *Drosophila* ORC localization. They detected approximately 5000 pre-RC binding sites, and found ORC localized to nucleosome-free regions (MacAlpine et al. 2010). Therefore, these data make a compelling case that ORC targets to nucleosome-free regions in both yeast and *Drosophila*.

Currently, there is no published example of ChIP–chip or ChIP–seq for any pre-RC proteins in mammalian cells due to a lack of significant enrichment over background (reviewed in Schepers and Papior 2010). This revealed one of the disadvantages of this ChIP-based approach that the antibody specificity, expression level of ORC, and DNA sequence specificity will affect the measured enrichment. Another disadvantage of this approach is that the sites of pre-RC assembly only mark potential origins of replication but not directly detecting DNA synthesis products. However, this type of approach does not need to synchronize cells by treating drugs and can map potential origins at higher resolution.

DNA damage responses during DNA replication

DNA damage checkpoints

DNA damage checkpoints are the surveillance and response systems that monitor the cell cycle events involving DNA replication and mitosis. If DNA is subjected to damage, DNA damage checkpoints will be activated to trigger cellular responses, including the arrest of cell cycle progression, inhibition of replication initiation and elongation of replication forks, activation of DNA repair and DNA damage tolerance pathways, and apoptosis if the damage is too extensive (reviewed in Sancar et al. 2004).

Many cell cycle transitions (G1/S, intra-S, and G2/M) are under the control of the DNA damage checkpoints. For the sake of simplicity, I will focus on the intra-S checkpoint that contributes to the stability of replication forks and new origin firing in cells that have already entered S phase.

DNA damage checkpoints contain three components in their signaling pathway: sensors, signal transducers, and effectors (reviewed in Abraham 2001). Sensor proteins recognize DNA damage or replication stress, and initiate the checkpoint cascade. Transducer proteins relay and amplify the damage signal. Effector proteins control cell cycle progression, chromatin remodeling and DNA repair. Two groups of proteins have been identified as sensor proteins: the PIKK (phosphoinositide-3-like kinase kinase) family members, ATM, ATR, and DNA-PK (reviewed in Durocher and Jackson 2001), and the PCNA-related 9-1-1 complex (Melo and Toczyski 2002). While ATM (ataxia telangiectasia mutated) and DNA-PK (DNA-dependent protein kinase) appear to be the primary players in response to DNA double-strand breaks (DSBs), ATR is activated by single-stranded DNA and stalled replication forks (Mordes and Cortez 2008).

ATM function is modulated by a two-step mechanism. The first step involves autophosphorylation of Ser1981, which dissociates an inactive ATM homodimer into active monomers. The second step involves the recruitment of activated ATM to sites of DNA damage. Interaction with its partner, Mre11–Rad50–Nbs1 (MRN) complex (Falck et al. 2005) results in the phosphorylation of downstream substrates, including Chk2 (Matsuoka et al. 2000), p53 (Banin et al. 1998), NBS1 (Lim et al. 2000), and BRCA1 (Cortez et al. 1999).

Similar to ATM, activation of ATR occurs via the following steps. The first step involves recruitment of ATR via its partner ATRIP (ATR-interacting protein) to RPA-coated ssDNA. The second step involves RPA-dependent recruitment of a checkpoint clamp, containing 9-1-1 complex, and the ATR activator TOPBP1 (topoisomerase-binding protein 1) to RPA-coated ssDNA (Ueda et al. 2012). Finally ATR–ATRIP is activated by TOPBP1 and then phosphorylates various downstream substrates, including Chk1. The 9-1-1 complex is a proliferating cell nuclear antigen (PCNA)-like ring structure comprised of Rad9, Hus1 and Rad1. Once assembled, 9-1-1 binds to TOPBP1 through the phosphorylation on Rad9 Ser-387 and Ser-341, and then activates the ATR pathway (Ueda et al. 2012).

DNA-PKcs is also a member of PIKK family, and by far is the most abundant PIKK in human but absent from nematodes, flies and yeast (Yang et al. 2003). The DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and Ku70–Ku80 heterodimer together form DNA-PK complex that plays key roles in the repair of ionizing radiation-induced DSBs through the non-homologous end-joining (NHEJ) pathway. In mammalian cells, the majority of IR-induced DSBs are repaired by the NHEJ pathway (Dobbs et al. 2010). See Fig. 1.4 for the summary of ATR- and ATM-mediated signaling pathways.

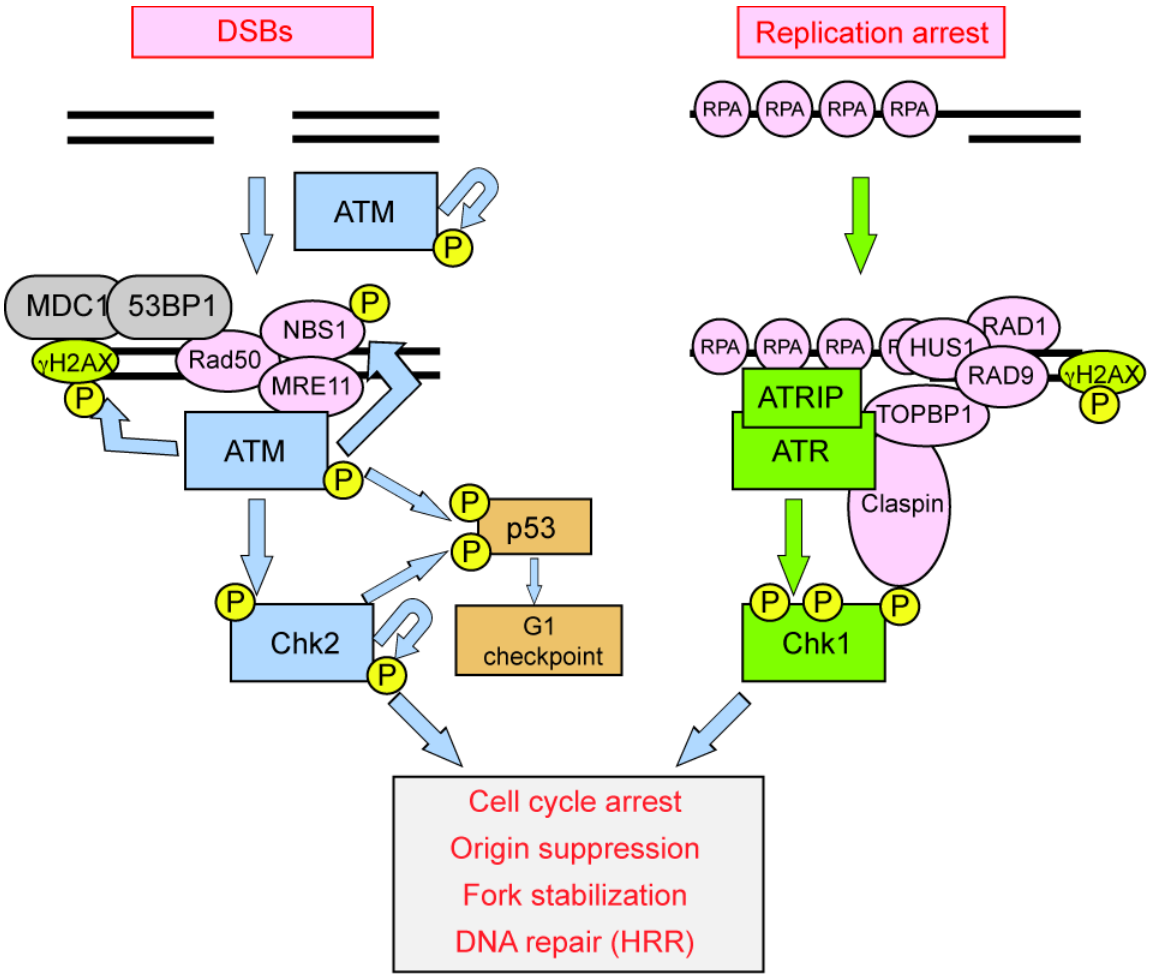

Figure 1.4. Activation of the ATM–Chk2 and ATR–Chk1 pathways.
The ATM–Chk2 and ATR– Chk1 pathways are activated selectively by DSBs and tracts of ssDNA complexed with RPA, respectively. Phosphorylation events are indicated by (P). Diagram is modified from (Smith et al. 2010).

Intra-S phase checkpoint signaling

During DNA replication, the intra-S phase checkpoint is activated when DNA synthesis is disturbed. It will be triggered to stabilize stalled replication forks, suppress the firing of late origins of replication, and delay the onset of mitosis until DNA replication is complete. ATR and ATM are the key sensor proteins for the coordination of checkpoint signaling and DNA repair processes in response to DNA damage. Replication block resulted from the exposure to UV light, DNA methylation by methyl methane-sulphonate (MMS) and replication inhibitors such as HU and aphidicolin can cause uncoupling between DNA polymerases and helicase at the fork. In turn, this uncoupling generates ssDNA tracts that will rapidly be coated by significant amounts RPA. As described above, RPA-coated ssDNA can recruit ATR and its interaction partner ATRIP, and in turn activate ATR-ATRIP by 9-1-1 complexes and TOPBP1.

One important role of ATR in the intra-S phase checkpoint pathway is the phosphorylation and activation of the effector kinase Chk1 (checkpoint kinase 1). Chk1 phosphorylates Cdc25 cell cycle phosphatase family members, causing their inactivation by nuclear exclusion and ubiquitin-mediated degradation. In turn, the degradation of Cdc25 phosphatases prevents activation of cyclin E/A-Cdk2 and cyclin B-Cdk1 (Sanchez et al. 1997; Zhao et al. 2002). These reactions result in slow progression through S-phase and prevent mitotic entry, allowing cells to repair DNA damage of stalled replication forks, and ultimately resume cell cycle progression. In addition, the inactive form of Cdk2 prevents the loading of Cdc45 on the replication origin. This event prevents late origin firing from being activated (Ilves et al. 2010). Other substrates

of Chk1, including p53 and DNA repair proteins, such as Rad51 and BRCA2, participate in DNA repair pathways and the activation of apoptosis.

Several mediators are necessary for the activation of effector kinases. Human Claspin is both a central component of normal DNA replication forks and a mediator of the intra-S phase checkpoint. In unperturbed cells, Claspin interacts with the catalytic subunit of DNA polymerase ε, the primary leading-strand replicative polymerase, to maintain a high rate of replication fork progression (Lou et al. 2008). Within the intra-S phase checkpoint signaling pathway, Claspin is required for ATR-dependent phosphorylation and activation of Chk1 (Chini and Chen 2003). The phosphorylation of Claspin, presumably by ATR, facilitates its interaction with Chk1 (Lee et al. 2005).

ATM checkpoint kinase is activated in response to DSBs, which can be caused by irradiation or replication of a nicked or gapped DNA. As described above, the full activation of ATM relies on the interaction with the MRN complex at the DSBs sites and in turn activates two signaling pathways. The ATM-Chk2-Cdc25-Cdk2 pathway exhibits crosstalk with the ATR-Chk1 pathway on the regulation of Cdk2 activity to slow down S phase progression and inhibit origin firing (reviewed in Smith et al. 2010). In response to DSBs, ATM also initiates a second pathway that leads to strand resection and activation of the downstream ATR–Chk1 pathway. Although the details of the mechanism have not been fully investigated, current thinking is that MRN complex recognizes DSBs, and in turn recruits and activates ATM. The active ATM and MRN complex promotes the translocation of CtIP to DSBs, and CtIP stimulates the nuclease activity of MRE11 subunit of MRN to initiate the strand resection and generates short tracts of ssDNA (You

et al. 2009). The resected ssDNA will be coated by RPA. This becomes the initiating substrate for homologous recombination repair (HRR) and triggers the ATR–Chk1 signaling pathway in a cell cycle dependent manner (Jazayeri et al. 2005) (Fig. 1.5).

Homologous recombination-dependent fork restart

In addition to cell cycle regulation, prevention of the firing of late origins, and induction of homologous recombination repair (HRR), the intra-S phase checkpoint also contributes to the stabilization of stalled forks by preventing replisome disassembly. However, the molecular mechanism by which checkpoints ensure replication fork integrity is not fully understood. Studies in budding yeast have indicated that Mec1 (ATR in mammals) and Rad53 (Chk2 in mammals) mutants are defective in replisome stabilization (Cobb et al. 2003; Lucca et al. 2004). Also, the mediator Claspin that interacts with Rad9 of the 9-1-1 complex to encircle DNA may directly promote fork integrity (Scorah and McGowan 2009). Furthermore, the checkpoint can stabilize stalled forks by targeting the MCM helicases. DNA damage or the depletion of DNA precursors results in the uncoupling of the replication helicase and polymerase. This leads to the accumulation of ssDNA. The MCM2-7 complex is involved in checkpoint activation by unwinding of dsDNA at the fork after replication is blocked to extend ssDNA. Also, MCM subunits are phosphorylated by ATM and ATR as a target of the intra-S phase checkpoint in metazoans and fission yeast (Bailis et al. 2008; Cortez et al. 2004; Ishimi et al. 2003). The phosphorylation on MCM subunits maintains their association with the replisome and exerts a protective effect on the fork. In human cells it has been reported

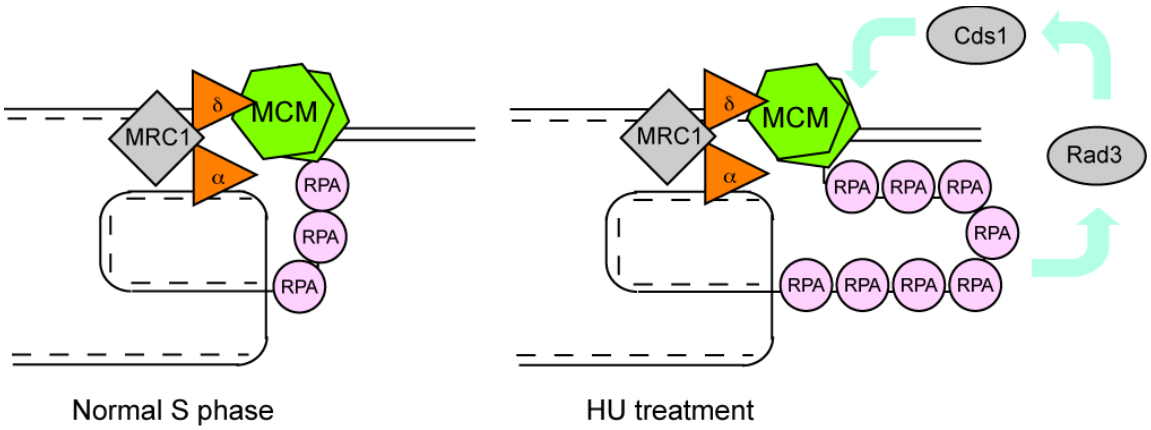

Figure 1.5. Model for replication fork maintenance.
(A) Normal S phase. Leading (δ) and lagging (α) strand polymerases are indicated. M, Mrc1. (B) Under HU treatment. MCM unwinding is limited by Cds1/Rad53 kinase activity to allow sufficient unwinding for a checkpoint signal. Diagram is modified from (Forsburg 2008).

that MCM7 interacts with Rad51 homologous recombinase when the checkpoint is activated (Bailis et al. 2008), suggesting that MCM not only functions to ensure fork stability, but also facilitates DNA repair at stalled forks.

The Intra-S phase checkpoint also functions to promote the resumption of DNA replication after the replication fork has collapsed. HRR plays an important role in fork restart at collapsed forks in situations where there are no converging forks from neighboring origin to complete DNA replication, or when stalled replication forks are converted into DSBs. HRR initiates with resection of the DSB ends by MRN complex and yields a 3′-ended ssDNA tail. This step has been described in the above ATM-dependent checkpoint response. Then the recruitment of 5′–3′ exonuclease Exo1 and Dna2 coupled with Sgs1–Top3–Rmi1 complex extends the resection (Zhu et al. 2008). The resulting 3′ ssDNA tails will be bound by RPA to stabilize ssDNA, and in turn RPA will be displaced by Rad51 with the mediation through BRCA2. BRCA2 protein binds both DNA and Rad51 and loads Rad51 to DNA break sites to modulate Rad51 filament formation (Carreira et al. 2009). Once the Rad51 filaments form, they can invade homologous templates, and the displaced strand anneals with the ssDNA on the other end of the break. Finally, the resulting two Holliday junctions will be resolved by nucleolytic cleavages and generate crossover and noncrossover products (reviewed in Moynahan and Jasin 2010). As noted, HR occurs during the S and G2/M phases while Cdk1 activity is high. Although high Cdk activity does not favor the interaction between BRCA2 and Rad51, the checkpoint signaling induce cell cycle arrest by reducing Cdk activity allowing BRCA2 to interact with Rad51 and engage in homologous

recombination (Jazayeri et al. 2005). In summary, the DSB-induced ATM signaling resects the DSBs to generate RPA-coated ssDNA and in turn activates the RPA-dependent ATR-Chk1 pathway. Chk1 phosphorylates the key HR protein Rad51 (Sleeth et al. 2007) and initiates the downstream HR process.

Biology of *Tetrahymena thermophila*

Tetrahymena thermophila has played a historically significant role as a unique experimental system. The pioneering discoveries on the molecular biology of *Tetrahymena* include the Nobel prize-awarding self-splicing activity of the group I intron within the ribosomal RNA precursor (Cech 2005) and the discovery of the structure of chromosome ends (telomeres) and the enzyme that synthesizes this DNA sequence (telomerase) (Blackburn 2010). Like other ciliated protozoa, *Tetrahymena* contains two nuclei within the same cytoplasm that are genetically related, but differ in structure and function. The germline genome resides in the micronucleus (MIC) while the somatic genome is housed in the macronucleus (MAC). The MIC genome, which contains five transcriptionally silent chromosomes, is the reservoir of genetic material that is transmitted during conjugation. The MAC genome contains ~180 transcriptionally active chromosomes during vegetative growth (reviewed in Karrer 2012). It is responsible for expressing all genes necessary for vegetative growth and determines the phenotype of cell. Consistent with observations correlating histone acetylation with transcriptional activity in other systems, the transcriptionally-active chromatin within the somatic macronucleus contains hyper-acetylated histones relative to that of the

transcriptionally inert micronucleus, which appears to be devoid of this specific histone modification (Vavra et al. 1982). The earliest link between histone variants and transcription activity was established in *Tetrahymena*. For example, the observations that H2A.Z and H3.3 are exclusively localized in the macronucleus suggested these variants may function in transcriptionally active chromatin (Allis et al. 1980). Indeed, H3.3 was confirmed later to serve as a replacement histone deposited to transcriptionally active loci by a replication-independent mechanism (Ahmad and Henikoff 2002).

Tetrahymena's life cycle contains two major phases: vegetative cell division (asexual phase) and conjugation (sexual phase). During vegetative cell division, DNA synthesis and nuclear division of MIC and MAC occur at different points in the cell cycle. MIC S phase occurred in MIC late anaphase (Woodard et al. 1972). With no apparent G1 phase, MIC has a G2 DNA content of 4C for essentially all of the cell cycle. MIC mitosis occurs during MAC G2, and the chromosomes separate on a nuclear spindle that assembles within the nuclear membrane (LaFountain and Davidson 1979). MAC chromosomes have no functional centromeres and divide amitotically. The ~45 copies of each macronuclear chromosome in the polyploid MAC are randomly partitioned between the two daughter cells and do not use any visible mitotic spindle. Therefore, unlike other eukaryotic cells, which undergo conventional cell cycle (G1-S-G2-Mitosis), the *Tetrahymena* cell cycle coincides with the MAC cycle (G1-S-G2-Amitosis). The MIC cycle is offset with the MAC cycle, giving a pattern as G2-Mitosis/S, with no apparent G1 (reviewed in Cole and Sugai 2012) (Fig. 1.6). The unequal division of the MAC generates two heterozygous daughter cells. Continued

vegetative propagations will generate subclones that irreversibly express phenotypes associated with either homozygote. This phenomenon is called phenotypic assortment (Merriam and Bruns 1988; Sonneborn 1974).

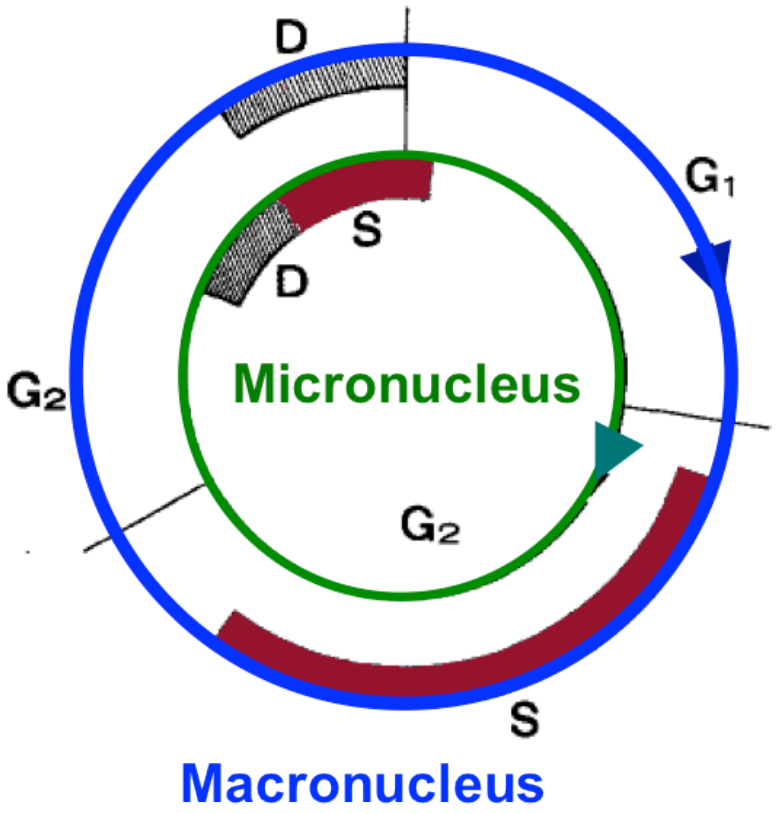

Figure 1.6. Schematic of the *Tetrahymena* vegetative cell cycle.
The MIC and MAC S phases are indicated as (S). The MIC mitosis and MAC amitosis are indicated as (D).

The sexual phase in *Tetrahymena* life cycle is called conjugation. To initiate conjugation, sexually mature cells of complementary mating types are first starved and then mixed together (Allewell et al. 1976). The *Tetrahymena* germline *mat* locus determines a spectrum of seven mating types (I–VII) (Nanney 1959). During development, the *mat* locus, which contains an array of incomplete mating type gene pairs, undergoes rearrangement to assemble one complete and fully functional gene pair. Other mating type genes are deleted from the *mat* locus in the somatic chromosome (Cervantes et al. 2013). When two cells of different mating types have a physical contact for about 1-2 hours, a mating pair will form and initiate a series of conjugation events. First, the parental MIC elongates dramatically to form a crescent structure and undergoes meiosis. The elongated shape of MIC chromosomes promotes the following chromosome pairing and recombination process during meiosis (Loidl and Scherthan 2004). Following the crescent stage, chromosomes condense and undergo two rounds of meiosis. Four meiosis products will be generated and only one will be selected as the nucleus that will be inherited; the three unselected nuclei undergo programmed nuclear degeneration (Akematsu and Endoh 2010; reviewed in Cole and Sugai 2012). The selected meiotic product undergoes one round of mitosis to generate two pronuclei; one will remain in the cell, and the other will be transferred to the mating partner. The pronuclei in each cell will fuse to produce one diploid zygotic MIC. The MIC undergoes two rounds of mitosis to generates four nuclei, two of which will remain as MICs, and two, called anlagen MACs, will develop into new MACs. From this step, the conjugation process enters the stage called “macronuclear anlagen development”. (Fig. 1.7)

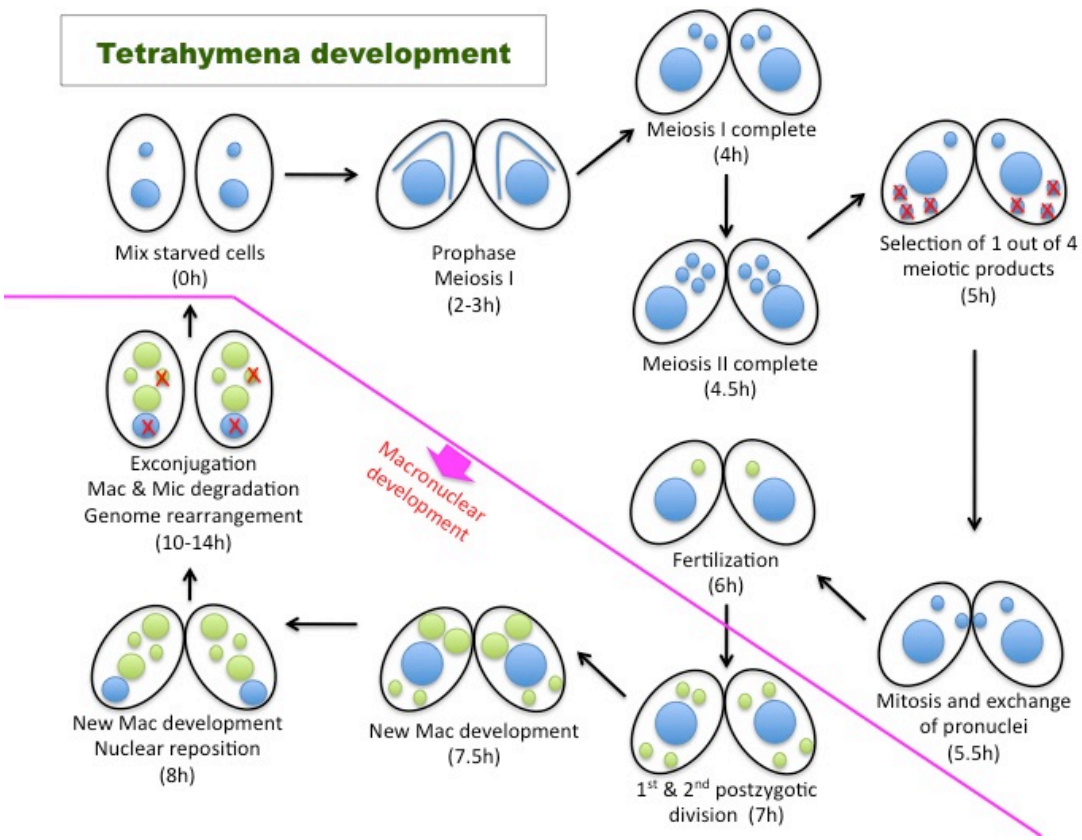

Figure 1.7. Schematic of *Tetrahymena* development.

During the MAC anlagen development, two DNA rearrangement events occur to restructure the macronuclear genome: DNA elimination and chromosome breakage. DNA elimination involves the removal of specific DNA segment (IES; Internal Eliminated Sequences) from internal regions of the chromosomes and followed by ligation of the flanking sequences (Yao and Chao 2005). There are an estimated 6000 distinct IESs in the MIC genome, which make up about 15% of the genome and is thought to be the size difference between MIC and MAC genome (Yao and Gorovsky 1974). IESs contain transposable elements and other repeated MIC sequences often categorized as junk DNA (Fillingham et al. 2004). Mochizuki and Gorovsky proposed a scnRNA model to illustrate how IESs are eliminated from the genome of anlagen MAC (Mochizuki and Gorovsky 2004). In this model, the ~28-29 nt scnRNAs originated from the MIC transcripts are processed by the Dicer-like protein Dcl1 (Malone et al. 2005) and then scnRNAs move to the cytoplasm to assemble with the Piwi family Argonaute protein Twi1p (Mochizuki et al. 2002). Then the Twi1p-associated scnRNAs translocate to the parental macronucleus to "scan" the parental macronuclear genome, and the sorted scnRNAs specific for IES sequences will be translocated to the anlagen MAC to interact with IESs. At this point, the loci for elimination will be marked by histone H3 methylation on lysine 9 (K9) or on lysine 27 (K27) (Taverna et al. 2002). This chromatin modification recruits Pdd1p and associated factors to induce elimination of the target sequences (Taverna et al. 2002).

Another DNA rearrangement event is site-specific chromosome breakage. The five MIC chromosomes will be processed into ~180 MAC chromosomes at the site that

share a conserved 15-bp sequence (CBS; Chromosome Breakage Sequence) (Yao et al. 1990). There are ~200 CBS sites in the genome and this rearrangement can reduce the chromosome size from 25,000 kb to about 800 kb (Preer and Preer 1979). Chromosome breakage in *Tetrahymena* is followed by de novo telomere addition, and the newly formed MAC chromosomes are stably maintained during the vegetative growth (Yao et al. 1990) (Fig. 1.8).

The parental MAC will be degraded into small fragments through PND (Programmed Nuclear Death) (Akematsu and Endoh 2010). Mating pairs separate and one of the two new MICs is degraded. At the end of conjugation, if switched into nutrient medium, the remaining new MIC will undergo mitosis, and the cell divides to segregate the two MACs into daughter cells. Finally, daughter cells enter the vegetative cell cycle with one MIC and one MAC. Transcription in the parental MAC is required for cells to complete the events during the early stages of conjugation, like meiosis, haploid nuclear exchange, and zygotic fusion (Marsh et al. 2000; 2001). After the macronuclear anlagen becomes transcriptionally active, it produces transcripts that drive the process during late stages of macronuclear development and the transition into vegetative growth (Yin et al. 2010).

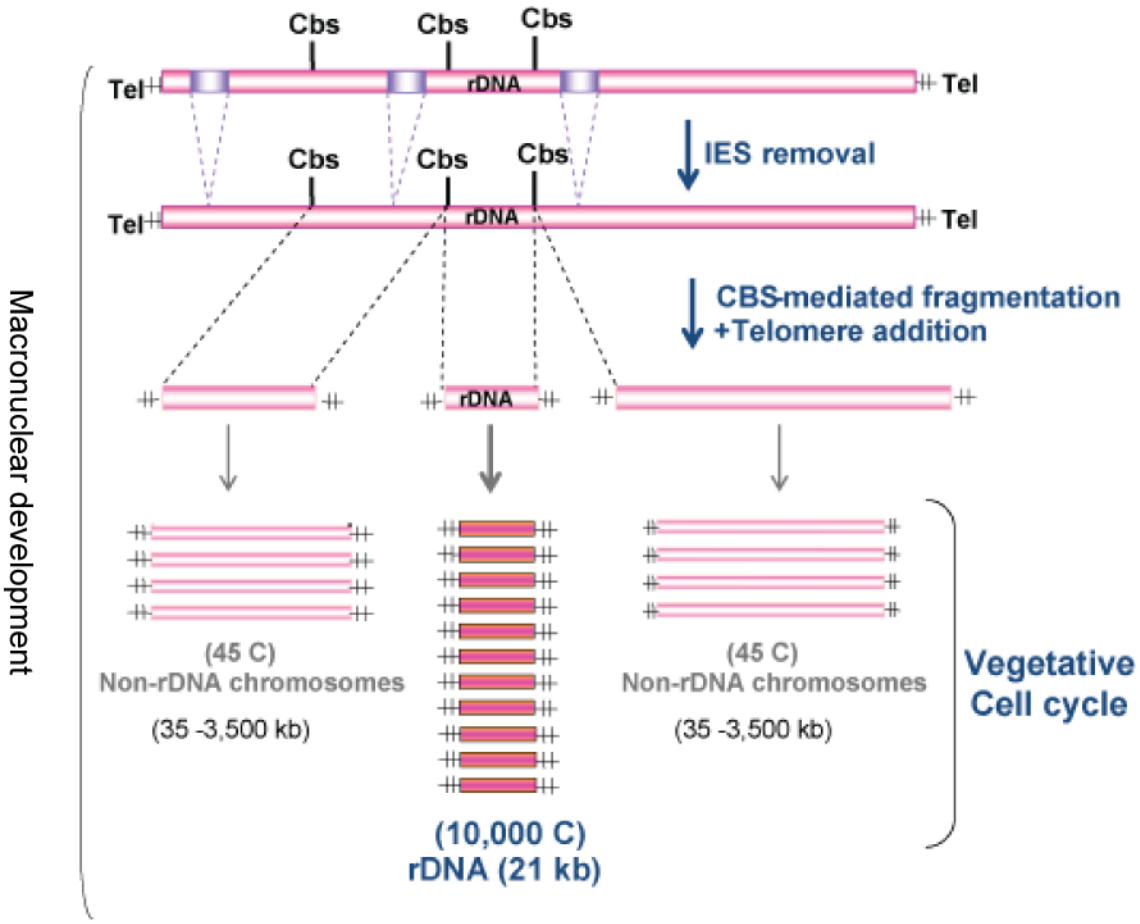

Figure 1.8. Differential DNA replication programs and developmentally programmed DNA rearrangement during macronuclear development.
(Cbs), chromosome breakage, (IES), internally eliminated sequences. Endoreplication of the non-rDNA minichromosomes generates 45 copies. Locus specific amplification of the rDNA increases the copy number in the developing macronucleus up to 10,000 copies. The number of copies is maintained in every vegetative cell cycle.

Cis-acting determinants of rDNA replicon

The advantage of *Tetrahymena* rDNA minichromosome as a tool to study the mechanisms of DNA replication is that both cell cycle-regulated DNA replication and gene amplification are carried out in the 21-kb rDNA minichromosome. During macronuclear anlagen development, the 10.3 kb rDNA locus on the micronuclear chromosome is excised and undergoes DNA rearrangement to generate a 21-kb palindromic rDNA minichromosome (Yasuda and Yao 1991). This rDNA chromosome is amplified from 2 to 9,000 copies during development, but is replicated once per cell cycle during the subsequent vegetative cell divisions.

The rDNA minichromosome encodes 26S, 5.8S and 17S rRNAs. The 1.9-kb 5′ NTS (nontranscribed spacer) contains three nucleosome-free regions. Two of them reside separately in the imperfectly tandem duplicated 430-bp segments, called domain 1 and domain 2 (Palen and Cech 1984). Domains 1 and 2 contain several cis-acting elements, including dispersed type I elements and pause site elements. Dispersed type I elements are required for both rDNA amplification and vegetative replication (Larson et al. 1986; Gallagher and Blackburn 1998; Reischmann et al. 1999; Zhang et al. 1997), and mediate replication fork pausing at pause site elements .

There are four type I elements (IA to ID) in the 5' NTS of the rDNA minichromosome. The type IA and IB elements colocalize with the origins in domain 1 and 2, respectively (Reischmann et al. 1999). Type IC and ID elements map to the rRNA gene promoter, and have been found to regulate rRNA transcription (Pan et al. 1995) and DNA replication (Gallagher and Blackburn 1998). Previously, the genetic screen on the

identification of *Tetrahymena* mutants with defects in rDNA maturation and maintenance (rmm) has reported that the cis-acting element mutations reside within or downstream of the type I elements. For example, the rmm1 mutation is mapped within the type IB element and this mutant has defects in rDNA maintenance so that amplified rDNA chromosomes are lost during the vegetative growth, suggesting this mutation produces a defect in DNA replication, chromosome segregation or copy number control. This rmm phenotype only occurs when the mutant allele is in competition with a wild-type rDNA allele (Larson et al. 1986). Other rmm mutations, rmm3 and rmm8, are found to localize in type IC and type ID elements in the promoter region (Gallagher and Blackburn 1998), suggesting that the type IC and ID elements in the rRNA gene promoter region regulate replication distantly as well. Since mutations in the promoter region may only affect replication or transcription, these data suggest the regulation of these two processes are mediated by different DNA binding proteins. See Fig. 1.9 for the schematic of the *Tetrahymena* rDNA minichromosome.

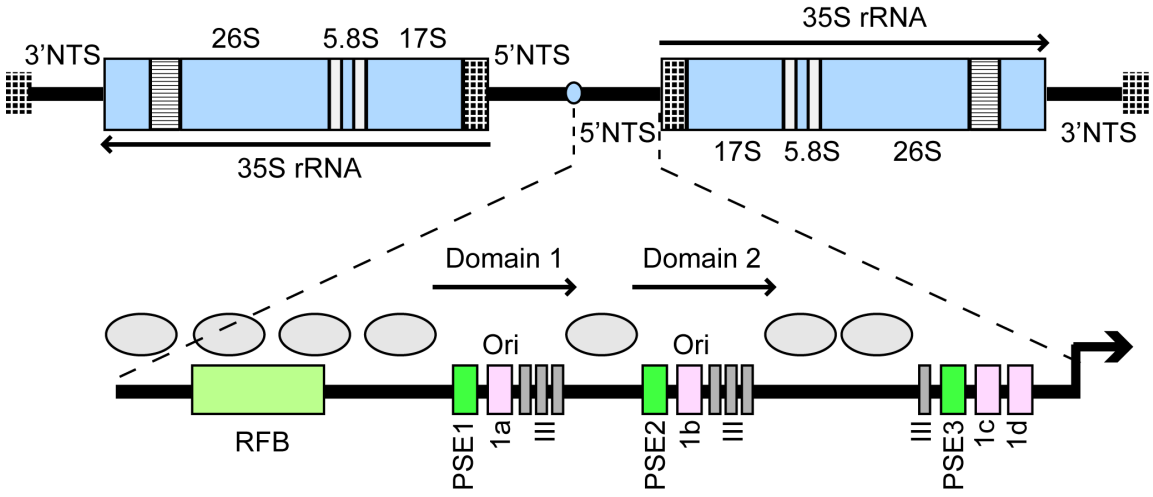

Figure 1.9. Schematic of the *Tetrahymena* rDNA minichromosome.
The 21 Kb macronuclear rDNA minichromosome consists of a palindromic arrangement of two copies of the rDNA gene (in blue) that encode for 17S, 5.8S and 26S ribosomal rDNA. The region encompassing the 5'NTS is expanded and includes *cis*-acting determinants for DNA replication. Type I, II, III and pause site elements are phylogenetically conserved sequences. Type I elements are shown in pink, Type III in grey, replication fork barrier (RFB) in light green and pause site elements (PSE) in green. The locations of the seven positioned nucleosomes are depicted by grey ovals.

Trans-acting factors that regulate rDNA replication

Type I elements, which are required for rDNA amplification and vegetative replication, interact *in vitro* with four different type I element binding factors (TIF1–4), identified by mobility gel shift assays (Mohammad et al. 2000; Saha and Kapler 2000). These TIFs bind to ssDNA in a sequence-specific manner, and are differentially regulated during the cell cycle and development (Mohammad et al. 2003).

TIF1 is a homotetramer with a subunit molecular mass of 21 kDa (Saha and Kapler 2000). In vivo footprinting analysis showed that TIF1 binds to the type I elements A-rich strand at the origin and T-rich strand at the promoter region (Saha et al. 2001), but not to duplex DNA in the gel shift assay (Saha and Kapler 2000). These results suggest that *Tetrahymena* rDNA origin and promoter regions are naturally unwound in native chromosomes. In addition, TIF1 also binds to PSEs, which are shown to be required for the regulation of rDNA replication (MacAlpine et al. 1997).

During the cell cycle, TIF1 origin DNA binding activity and mRNA levels peak in S phase (Morrison et al. 2005). The localization of TIF1 is also dynamic during the cell cycle. It exclusively localizes to the cytoplasm of G1 phase cells, and then relocates to macro- and micronucleus during the vegetative S phases (Yakisich et al. 2006), suggesting its function occurs with the ongoing DNA synthesis. Before cytokinesis, TIF1 is excluded from macronucleus but is transiently retained in the postmitotic micronucleus. In addition, deletion of TIF1 causes a prolonged S phase; however the rDNA origin firing begins earlier than wild type. This result suggests that TIF1 acts as a negative regulator of rDNA replication, and that its absence that accelerates the timing

of origin initiation (Morrison et al. 2005).

TIF1 also functions in the ATR-mediated pathway to regulate the intra-S phase checkpoint response. In response to HU treatment, TIF mutants fail to arrest at S phase or induce caffeine-sensitive HRR Rad51 gene expression. This checkpoint defect may link to the outcome of genome instability in the mitotically divided MIC (Yakisich et al. 2006).

TIF4 has been identified as *Tetrahymena* ORC, which contains multiple subunits that binds specifically to the T-rich strand of type I element in an ATP-dependent manner (Mohammad et al. 2000; 2003; 2007). The ORC DNA binding activity in the gel shift assay was eliminated by RNase A, suggesting *Tetrahymena* ORC is a ribonucleoprotein (Mohammad et al. 2007). The cDNA copy of this RNA was cloned and the sequence revealed this ORC-associated RNA subunit is derived from the 3' end of 26S ribosomal RNA. Therefore, it is designated "26T RNA" (Mohammad et al. 2007).

Chromatin immunoprecipitation (ChIP) analysis with TAP-tagged Orc1p and chromatin pull down assay with aptamer-tagged 26T RNA revealed that 26T RNA is assembled into the ORC and targeted to the rDNA origin. However, neither enrichment was found in the rDNA coding region 3' end which is complementary to the 26T RNA, nor in the promoter region that contain the type I element (Mohammad et al. 2007). This suggests that *Tetrahymena* ORC RNP complex selectively associates with the rDNA origin. When the base pairing between 26T RNA and complementary DNA sequences was disrupted by changing the sequence on the RNA, the in vitro binding specificity of

ORC was completely altered. All these results provide evidence that a non-coding RNA is involved in targeting ORC to rDNA replication initiation sites.

ChIP analysis of synchronized cycling cells showed that the ORC binding to the rDNA is restricted to the origin region and that this interaction changes across the cell cycle. Origin activation during S phase results in the turnover of Orc1p. Therefore, degradation of Orc1p generates a sub-complex that remains bound to the origin (Donti et al. 2009). In contrast, ORC binding to non-rDNA chromosomes are cell cycle-regulated and does not depend on base pairing between the 26T RNA and origin DNA. ORC binds to the origin site in G1 phase and then the entire ORC complex dissociates from the origin upon Orc1p turnover. In G2 phase ORC binds randomly on the newly synthesized daughter chromosomes, and re-localizes to the initiation site in the next G1 phase (Donti et al. 2009).

In the following chapters of this dissertation I will describe the impacts of changing in ORC protein contents on the fate of micro- and macronuclear chromosomes during the vegetative cell cycle and development in *Tetrahymena.* In Chapter 2, I will examine the effect of down-regulation of ORC1 on genome stability and checkpoint activation during the cell cycle, and study the regulation of ORC and MCM during development to gain insights into genome-wide endoreplication and rDNA amplification. In Chapter 3, I will study the impact of replication stress on ORC and MCM and discuss a novel DNA replication program that is activated when cells are recovering from DNA stress.

CHAPTER II

PARTIAL DEPLETION OF TETRAHYMENA ORC1 FAILS TO TRIGGER A CHECKPOINT RESPONSE AND COMPROMISE FOUR DISTINCT DNA REPLICATION PROGRAMS

Overview

During the vegetative cell cycle of *Tetrahymena thermophila*, DNA replication initiates from defined chromosomal sites in an ORC-dependent manner. We show here that sustained partial depletion of Orc1p leads to genome instability in the diploid mitotic micronucleus, abnormal division of the polyploid amitotic macronucleus, failure to mount a robust intra-S phase checkpoint response, and aberrant execution of two developmentally-regulated DNA replication programs, endoreplication and ribosomal DNA (rDNA) gene amplification. Remarkably, the correlation between ORC protein levels and chromosomal DNA replication disappears in wild type cells during macronuclear development. The initial rounds of micronuclear DNA replication, endoreplication and rDNA amplification occur when ORC levels increase by 2.5-fold, but the amount of DNA that has to be replicated decreases by a factor of 2.4 (the ratio of Orc1p to DNA increases by 6-fold). Whereas ORC protein levels dramatically decline by a factor of 30 later in the endoreplication program, the DNA content increases by 6-fold (the ratio of Orc1p to DNA drops by 200-fold). These data provide evidence that the demand on the amount of Orc1p varies in different replication programs of

Tetrahymena, and suggests that replication initiation is differentially regulated at different stages of development.

Introduction

The ciliated protozoan, *Tetrahymena thermophila*, has served as an important model system for assessing the molecular organization of eukaryotic replicons and studying the trans-acting factors that control replication initiation (reviewed in Tower 2004). A distinguishing feature of ciliates is the cohabitation of two functionally distinct nuclei within the same cytoplasm. The 'germline' micronucleus serves as the reservoir of genetic material that is transmitted from parent to progeny during conjugation, and is transcriptionally silent throughout most of the life cycle. The 'somatic' macronucleus controls cellular metabolism: its chromosomes serve as templates for the synthesis of ribosomal, transfer and messenger RNAs during vegetative cell cycles and early development; however, macronuclear chromosomes are not transmitted from parents to progeny during conjugation (reviewed in Karrer 2012). Instead, a new macronucleus is created in exconjugants by the conversion of a diploid post-zygotic micronucleus into a polyploid macronucleus.

The partitioning of chromosomes into two operationally distinct nuclei places unusual demands on the DNA replication program, including replication origin licensing and the coordination of DNA damage checkpoint responses that maintain genome integrity. For example, the diploid micronucleus and polyploid macronucleus replicate their chromosomes at different stages of the vegetative cell cycle (reviewed in Cole and

Sugai 2012). Macronuclear DNA replication precedes micronuclear S phase. Cytokinesis is coupled to amitotic macronuclear division, a process in which sister chromatids are randomly partitioned to daughter nuclei, yet DNA copy number is somehow maintained (Doerder and DeBault 1978). To complicate matters, the order of micronuclear S phase and mitosis is reversed in the cell cycle, with M phase preceding micronuclear S. The temporal regulation of DNA replication in *Tetrahymena* parallels that of higher order chromatin domains in metazoan chromosomes, in that euchromatin (macronuclear chromosomes) replicate early in the cell cycle and heterochromatin (micronuclear chromosomes) replicate late (Woodard et al. 1972).

In contrast to the vegetative cell cycle, micro- and macronuclear S phases are not interdigitated in conjugating cells. Instead, an ordered series of temporally distinct S phases, programmed nuclear death (Davis et al. 1992) and two atypical DNA replication programs- endoreplication and gene amplification occur, leading to the creation of new germline micro- and macronuclei in progeny cells. Through conventional meiosis four haploid pronuclei are produced in each mating partner, three of which are subsequently degraded (reviewed in Cole and Sugai 2012). The sole survivor undergoes one round of DNA replication to produce genetically identical migratory and stationary pronuclei, which are reciprocally exchanged between mating partners. The diploid zygotic micronucleus undergoes two rounds of replication and division. Two micronuclei exit the DNA replication program, while the remaining two differentiate into macronuclei.

Macronuclear development involves the massive, physical reorganization of the five micronuclear chromosomes and re-programming of the entire epigenome. Half of

the micronuclear genome (including centromeres and retrotransposons) is eliminated by two mechanisms: (1) site-specific DNA fragmentation and *de novo* telomere addition, and (2) removal of internal DNA sequences. Non-coding 'guide' RNAs, which are transiently transcribed from the newly formed micronucleus, dictate which internal DNA sequences are eliminated from the developing macronucleus (Yao et al. 2003). Epigenetic reprogramming of histones converts heterochromatic micronuclear chromosomes into pure macronuclear euchromatin. The ~180 macronuclear chromosomes undergo endo-reduplication to achieve a final copy number of ~45C, and the 21 kb ribosomal DNA minichromosome is amplified to ~9000C.

Once development is completed, macronuclear chromosomes replicate on average once per vegetative cell cycle. However, amitotic chromosome segregation and asymmetric division of macronuclei can lead to genic imbalances (Smith et al. 2004). Remarkably, the copy number of macronuclear chromosomes is maintained in a narrow range through the elimination of 'excess DNA' in the form of chromatin extrusion bodies, or partial re-replication of macronuclear chromosomes (Preer and Preer 1979; Bodenbender et al. 1992).

Genotoxic agents (chemical mutagens, ultraviolet and ionizing radiation) and stress (depletion of DNA precursors, physical barriers to replication fork progression) have the capacity to irreparably harm chromosomes. While most deleterious situations are resolved without the need to arrest the cell cycle, eukaryotes elicit a DNA damage/replication stress checkpoint response when the amount of DNA damage or stress exceeds a threshold. Specifically, the mammalian ATM (Ataxia telangectasia-

mediated) checkpoint kinase (*S. cerevisiae* Rad53) prevents cells from transiting the G1/S border in response to an excess of double strand breaks in the genome. ATR (Ataxia telangectasia-related; Mec1 in budding yeast) activates an intra-S phase checkpoint response and induces cell cycle arrest when excess single strand DNA accumulates due to the uncoupling of the replicative helicase and polymerase (reviewed in Nam and Cortez 2011; Cortez et al. 2004). S phase checkpoint activation leads to the phosphorylation of MCM2-7 helicase subunits. This reversible modification inhibits DNA unwinding at replication forks, and prevent late origins from firing (reviewed in Forsburg 2008). *Tetrahymena* encodes a single ATR gene that induces cell cycle arrest and prevents micro- and macronuclear genome instability during the vegetative cell cycle (Yakisich et al. 2006). ATR is also required for the reorganization of chromosomes during meiosis (Loidl and Mochizuki 2009).

In the work presented here, we describe the fate of chromosomes in strains harboring diminished levels of Orc1p under two conditions- chronic, but moderate (~5-fold) down regulation Orc1p in a macronuclear 'knockdown' mutant strain, and transient (~12-fold) down regulation of Orc1p during wild type development. ORC1 knockdown mutants failed to mount a robust intra-S phase checkpoint response, underwent aberrant macronuclear division and mitotic micronuclear chromosome loss, and exhibited defects in endo-replication and rDNA gene amplification. Hence, a modest reduction in ORC abundance can have profound effects on the integrity of micro- and macronuclear chromosomes. To our surprise, the abundance of ORC and MCM proteins plummets in

wild type cells during the second wave of endoreplication in developing macronuclei, when the demands on the DNA replication machinery are the greatest.

Material and methods

Tetrahymena cell culture method and strains

Tetrahymena cells were grown vegetatively in 2% PPYS media (2% proteose peptone, 0.2% yeast extract, 0.003% sequestrine) supplemented with 250 μg/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin B (Invitrogen) at 30°C on a platform shaker (100 r.p.m.). To prepare cells for mating, cells were starved at 30°C in 10 mM Tris-HCl (pH 7.4) for 18-20 h. Mating was carried out between strains of different mating types by mixing in equal numbers at a density of 2 X 10e5 cells/ml. Mating cultures were kept at 30°C without shaking.

The ORC1 knockdown (ORC1-KD) strain was generated by replacing the coding region with an MTT1-neo cassette using flanking ORC1 sequences for targeted homologous recombination. The expression of the neo-cassette (neomycin phosphotransferase provides paromomycin (pm) resistance to the transformed cells) was driven by the Metallothionein 1 (MTT1) promoter which was induced by the addition of cadmium in form of cadmium chloride ($CdCl_2$) (1.2 μg/ml) to the media. Biolistic macronuclear anlagen transformation was performed to produce ORC1/ORC1::MTT1-neo F1 progeny. Progeny cells were propagated in increasing concentrations of pm (100–1000 μg/ml) to obtain phenotypic assortants resulting from replacement of

endogenous ORC1 with the disrupted transgene in the transcribed polyploid macronucleus.

Cell cycle synchronization

Cell cycle synchronization was achieved by starvation or elutriation. To synchronize a log phase cell culture in G1 phase by starvation, cells were harvested by centrifugation and washed twice with 10 mM Tris-HCl (pH 7.4). Cells were then resuspended in 10 mM Tris at a density of 5 X 10e5 cells/ml and incubated for 18 hours at 30°C on a platform shaker (100 r.p.m.). This cell culture was synchronized in G1 phase and then the cell cycle resumed by refeeding with equal volume of 4% PPYS media to reach a final cell density of 2.5 X 10e5 cells/ml. To isolate a *Tetrahymena* cell culture at G1 phase of the cell cycle by elutriation, a 1.5-liter log phase culture at a density of 1 X 10e5 cells/ml was pumped into a centrifugal elutriator rotor (Beckman J6M/E) at a flow rate of 50 ml/min (rotor speed 850 rpm). Once all of the 1.5-liter cell culture was filled into the rotor, two hundred milliliters of cell culture will be collected by increasing the flow rate of the pump to 65 ml/min. This fraction of cell culture corresponds to the population in G1 phase.

Flow cytometry

Flow cytometric analysis was performed to monitor the cell cycle progression in vegetatively growing cells, and to determine the relative DNA content in the isolated micro- and macronuclei in mating cells. For the cell cycle analysis, at least 2.5 X 10e5

cells were collected for each sample, fixed with 70% ethanol, and stored at 4°C. Fixed samples were washed twice with PBS and resuspended in 0.4 ml of propidium iodide staining solution (PBS containing 0.1% Triton X100, 50 µg/ml propidium iodide and 0.2 mg/ml RNase A).

To examine the DNA replication during development, nuclei were extracted by lysing cells in the ice-cold nucleus extraction buffer (10 mM Tris at pH 7.4, 10 mM $MgCl_2$, 3 mM $CaCl_2$, 0.25 M sucrose, 0.2% NP-40, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride), and then staining procedure was performed as described above. Flow cytometry analysis was performed on Becton Dickinson FACSAria II flow cytometer (BD Biosciences). Twenty thousand events were analyzed for each sample on the cytometer. Data were analyzed using BD FACSDiva™ software. Histograms were plotted, in which the y-axis represented the number of events and the x-axis represented the relative DNA content.

Cell fractionation and western blot analysis

To prepare whole cell extracts, 1 ml of cell culture was washed with 10 mM Tris buffer and resuspended in 50 µl of 1% SDS lysis buffer (50 mM Tris at pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1% sodium deoxycholate, 1% SDS) and incubated for 15 min on ice. To prepare the soluble and chromatin-bound fractions, harvested cells were incubated in 50 µl of NP-40 lysis buffer (0.1% NP-40, 20 mM Tris-HCl at pH 8.0, 137 mM NaCl, 10% glycerol, 2 mM EDTA) for 10 min on ice. After centrifugation at 2,000 rpm for 10 min at 4°C, the supernatant was collected as the ‘soluble fraction’. The

pellet was resuspended in 50 µl of 1% SDS lysis buffer and designated as 'chromatin-bound fraction'.

Protein concentrations were determined using the modified Lowry protein assay reagents from Bio-Rad (DC™ Protein Assay, cat# 500-0111). Twenty µg protein of each sample was boiled and subjected to SDS-PAGE. For the preparation of separating gels, 30% acrylamide/0.8% bisacrylamide solution, 1.5 M Tris-HCl (pH 8.8) containing 0.4% SDS, 10% ammonium persulfate, TEMED and H_2O were mixed and poured into the glass-plate sandwich. The percentage of acrylamide in the separating gel depends on the molecular size of the protein being separated. Generally, 7% gels were used for SDS-denatured proteins of 60 to 200 kDa, 10% gels were used for SDS-denatured proteins of 16 to 70 kDa, and 12% gels were used for SDS-denatured proteins of 12 to 45 kDa. After the separating gel was cured, the stacking gel was prepared by mixing the following solutions: 0.65 ml of 30% acrylamide/0.8% bisacrylamide, 1.25 ml of 0.5 M Tris-HCl (pH 6.8) containing 0.4% SDS, 3.05 ml H_2O, 25 µl of 10% ammonium persulfate and 5 µl TEMED. The separating gel solution was poured into the sandwich and 15-well comb was inserted. The gel was run at 80 V at RT for ~3 h in the SDS electrophoresis buffer (0.125 M Tris-HCl, 0.96 M glycine, and 0.5% SDS). Protein samples were then transferred for 2 h to a nitrocellulose membrane (Whatman Protran BA85, GE Healthcare) in transfer buffer (0.025 M Tris-HCl, 0.19 M glycine, and 20% methanol). Membrane was stained with Ponceau S (Sigma-Aldrich) (5 mg/ml of Ponceau S and 1% acetic acid in H_2O) for 1 min at RT for visual analysis of the loading control. Later, the membrane was blocked for 1 h at RT with 10% skim milk in PBS

solution containing 0.1% Tween 20. Immunodetection of Orc1p (1:5,000), Orc2 (1:5,000) and Mcm6p (1:10,000) was carried out using antibodies raised in rabbits (Convance). Rad51 (1:5,000) and Acetyl-histone H3 antibodies (1:10,000) were obtained from Neomarkers (51RAD01, Thermo Scientific) and Upstate (Invitrogen). Blots were incubated with the primary antibodies at 4°C overnight, and then washed 4 times with TBST buffer at RT, 10 min each time. A horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG was used as the secondary antibody (Jackson ImmunoResearch). Blots were incubated with the secondary antibodies at 4°C for 3 h, then the washing steps were repeated as described above. Blots were visualized using ECL reagents (PerkinElmer) according to the manufacturer's instructions. About 1 ml of ECL reagents were applied to cover one membrane and incubated at RT for 3 min. The chemiluminescent signals were detected by x-ray films.

Cytological staining and fluorescence microscopy

For mating experiments, wild type strain CU427 was distinguished from the other wild type CU428 and ORC1 knockdown strains by incorporation of Mitotracker Red-CMXRos (Molecular Probes, Invitrogen) at a concentration of 500 nM during overnight starvation at 30°C. Mitotracker Red-CMXRos dye will be incorporated into mitochondria of cells during the incubation. On the next day, starved cell cultures were washed once by 10 mM Tris and resuspended in 10 mM Tris at the density of 2.5 X 10e5 cells/ml. Mating was induced by mixing CU427 with CU428 or CU427 with ORC1 knockdown cells in equal volume. One-milliliter of mating cultures were harvested each

hour during development. Cells were fixed in 70% ethanol for at least 1 hr before the following process. Fixed cells were washed once with PBS and resuspended in 100 µl of 0.1 µg/ml 4',6'-diamidino-2- phenylidole (DAPI, Sigma Chemical) to stain nuclei. To visualize nuclear division, 1 ml of log phase CU427 and ORC1 knockdown cell cultures were harvested and fixed by 2% paraformaldehyde. Acridine orange was added at a concentration of 0.001% to stain nuclei. Cells were examined with conventional fluorescence microscope.

DNA isolation and enrichment for replication intermediates (RIs)

Total genomic DNA was isolated from *Tetrahymena* culture by a previously described protocol (Zhang et al. 1997). Cells were washed by 10 mM Tris buffer (pH 7.4) and resuspended in NDS lysis buffer (10 mM Tris, 0.5 M EDTA, 2% SDS). Proteinase K (Sigma-Aldrich) was added at the final concentration of 0.1 mg/ml and incubated overnight at 37°C. Samples were then diluted with equal volume of TE buffer (pH 8.0) and extracted with phenol/chloroform/isoamyl alcohol (25:24:1) twice. After additional extraction with chloroform, 2.5 volumes of ethanol were added to precipitated DNA at RT. DNA was resuspended in distilled water containing 10 µg/ml RNase A and incubated at 37°C for 1 h. Finally, DNA was reprecipitated with ethanol and resuspended in 1ml of TE buffer. For RI enrichment, 200 µg of genomic DNA were digested with restriction enzymes (HindIII for the rDNA 5' NTS fragments; ClaI for the rDNA coding region) for 4 h at 37°C, precipitated by ethanol, and resuspended in 400 µl of TNE buffer (0.1 M NaCI, 10 mM Tris and 1 mM EDTA). RIs were applied to the

200-µl packed volume benzoylated naphthoylated DEAE (BND)-cellulose columns (Sigma-Aldrich), washed with 350 µl of TNE buffer for 5 times, and eluted with 1.8% caffeine in 200 µl of TNE for 5 times. Centrifugation at 10,000 rpm for 10 min pelleted the BND-cellulose particles. The supernatant was collected and DNA was precipitated by addition of an equal volume of isopropanol with 20 µg glycogen as the carrier. After centrifugation at 13,000 rpm for 20 min, the DNA pellet was washed once by ice-cold 70% ethanol and finally resuspended in 50 µl of TE buffer. Total DNA recovery was estimated to be ~5% of the input.

Two-dimensional (2D) gel electrophoresis of DNA replication intermediates

Neutral-neutral 2D agarose gel electrophoresis was performed as previously described (Zhang et al. 1997). Approximately 10 µg of BND cellulose-enriched DNA was loaded for each 2D gel experiment. The first dimension agarose gel (0.4% for restriction fragment larger than 3 kb; 0.6% for fragment smaller then 3 kb) was run in 1X TAE buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA) at 1.5 volt/cm for 20 h at RT. The second dimension gel (1% for restriction fragment larger than 3 kb; 1.8% for fragment smaller then 3 kb) was run in 1X TBE buffer (90 mM Tris, 90 mM Boric acid, 2 mM EDTA) containing 0.5 mg/ml ethidium bromide at 3 volt/cm for 18 h at 4°C. For neutral-alkaline 2D gel electrophoresis, the first dimension gel was prepared and run at the same condition as neutral-neutral 2D gel. The second dimension gel was run in alkaline electrophoresis buffer (40 mM NaOH, 2 mM EDTA) at 1.5 volt/cm for 20 h at 4°C using a peristaltic pump to recirculate the electrophoresis buffer. After

electrophoresis, the gel was sequentially soaked in 0.25 N HCl for 20 min and alkaline transfer buffer (0.5 M NaOH, 1.5M NaCl) for another 20 min. The DNA was transferred overnight to a charged nylon membrane (Hybond-XL, Amersham) in alkaline buffer by capillary blotting and DNA was fixed to the membrane using a UV crosslinking apparatus (Stratagene, Agilent Technology). The blot was prehybridized at 37°C for 4 h in the hybridization solution (1M NaCl, 1% SDS, 10% dextran sulfate, 5 mM Tris [pH 7.5], 100 μg/ml of denatured salmon spermon DNA and 25% formamide). Probes labeled by the random primer method are boiled for 10 min, chilled on ice, and added directly to the prehybridization solution. After 18 h the blot was washed 3 times in 2X SSC/ 1% SDS solution for 15 min each at 42°C, and once in 0.4X SSC/ 0.1% SDS solution for 15 min at 42°C. The blot was exposed to x-ray films with an intensifying screen at -70°C.

Alkaline gel electrophoresis

Alkaline gel electrophoresis was performed to examine the replication initiation and elongation under HU treatment. Wild type CU428 and ORC1 knockdown cells were starved overnight to synchronize in G1 phase, and then released into medium containing 20 mM HU. Genomic DNA was isolated by phenol-chloroform extraction as described above from cells harvested at indicated time points under HU treatment. Sixty μg of genomic DNA was loaded in a 1% alkaline gel (40 mM NaOH, 2 mM EDTA at pH 8.0) and separated by electrophoresis in the alkaline buffer (40 mM NaOH, 2 mM EDTA) at 1.5 volt/cm for 20 h at 4°C with buffer recirculation to resolve nascent-strand replication

intermediates. After electrophoresis, DNA was transferred to a charged nylon membrane (Hybond-XL, Amersham) by capillary blotting and hybridized to the rDNA 5'NTS fragment that was radiolabeled with [α-^{32}P]dATP as described above.

Results

Down-regulation of DNA replication components in ORC1 mutants

The conserved six protein subunit Origin Recognition Complex (ORC) specifies where replication initiates in eukaryotic chromosomes by recruiting the MCM2-7 helicase and other replication proteins to sequentially assemble re-replicative and pre-initiation complexes (pre-RC and pre-ICs) (Masai et al. 2010). *Tetrahymena* ORC is unusual in that it contains an integral RNA subunit. This RNA species, designated 26T RNA, selectively targets ORC to the highly amplified rDNA replication origin through conventional Watson-Crick base pairing, but exhibits no complementarity to cis-acting regulatory sequences at non-rDNA origins (Mohammad et al. 2007; Donti et al. 2009). The temporal loading of ORC onto rDNA and non-rDNA origins reflects these fundamental differences: ORC is selectively recruited to the rDNA origin during G2 phase, whereas it is randomly distributed onto other chromosomes. Non-rDNA origin specificity and pre-RC assembly are achieved during G1 phase of the cell cycle. Hence, macronuclear chromosomes are not equivalent.

To evaluate the requirements for ORC1 during vegetative replication of the micro- and macronuclear genomes, ORC1 knockdown mutants were generated by targeted gene disruption in the germline micronucleus. As expected, the null mutant is

homozygous lethal. Consequently, heterozygous mutants were studied, and phenotypic assortment of wild type and ORC1 disruption alleles in the amitotic (45C) macronucleus was used to generate hypomorphic ORC1 strains. Only a 5-fold reduction in the abundance of the ORC1 gene was achieved (TTHERM_00865050) under conditions that selected for retention of the disrupted ORC1 allele. A corresponding reduction in Orc1p was detected by western blotting (Fig. 2.1A, WL); however, the ratio of chromatin-bound to free Orc1p was appreciably altered relative to the wild type control. While the vast majority of Orc1p (~85%) was chromatin-bound in the wild type strain, only 40% of the residual Orc1p was associated with chromatin in the mutant (Fig. 2.1B). Hence the amount of chromosome-associated Orc1p was reduced 6-fold in the mutant. Western blot analyses further revealed that the abundance of Orc2p and Mcm6p were also reduced in the ORC1 knockdown strain (Fig. 2.1B). These findings raised the possibility that the mutant contains fewer active origins and replication forks (see below).

ORC1 knockdown strains grew more slowly than wild type, exhibiting a prolonged S phase (Fig. 2.1C, compare data for 150-210 min time points), consistent with a defect in DNA replication. Flow cytometry analysis of asynchronous log phase cells showed a modest increase in the size of the S phase population in the mutant (Fig. 2.2). Furthermore, ~90% of dividing mutant cells underwent asymmetric macronuclear division with lagging chromosomes at the constricted cytokinetic furrow (wild type frequency: ~2%) (Fig. 2.3). The collective phenotypes are consistent with the failure to fully replicate and partition macronuclear chromosomes.

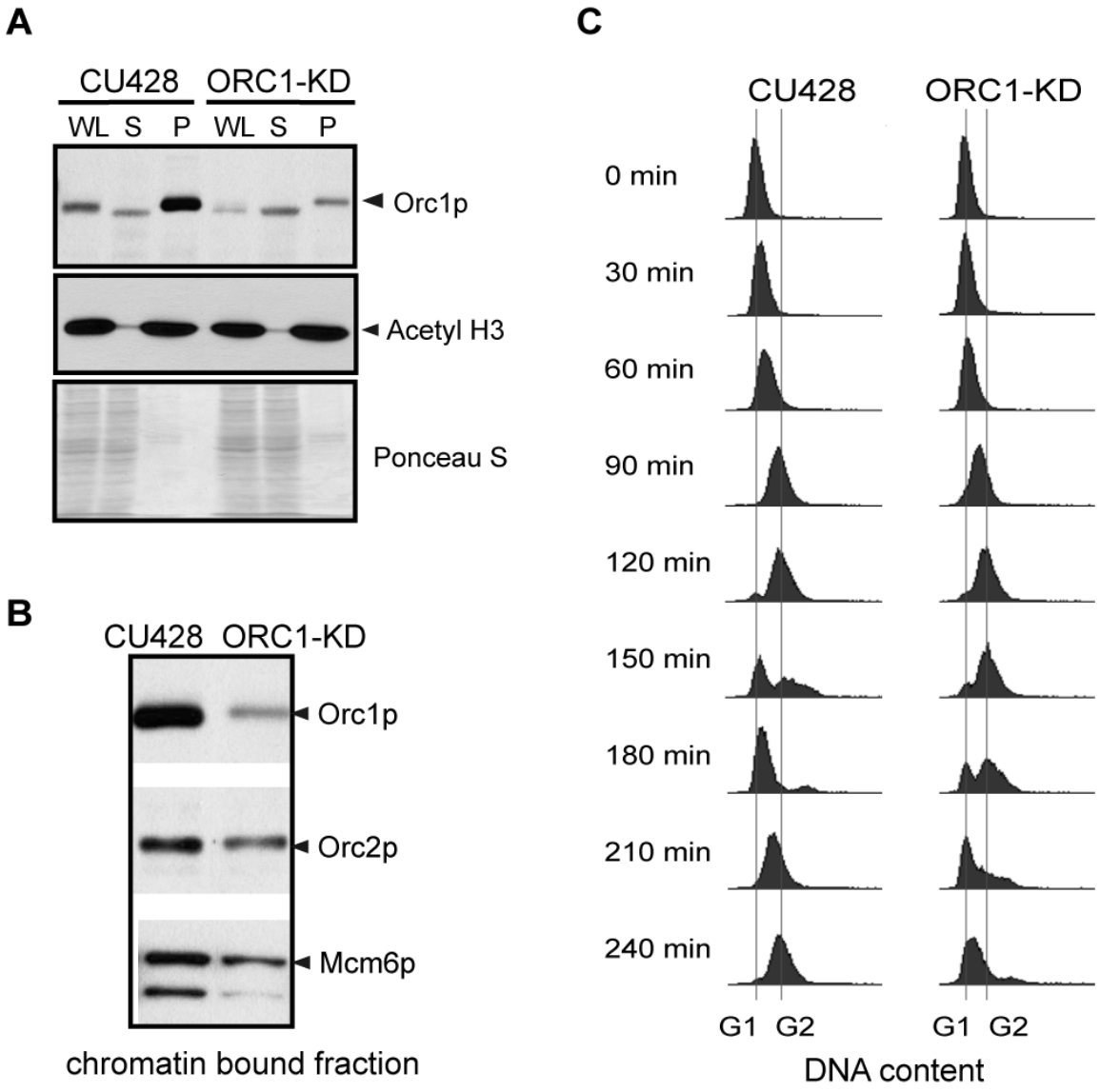

Figure 2.1. ORC1 depletion induces slow cell cycle progression.
(A and B) Western blot analysis of whole cell lysates (WL), NP-40-extractable soluble fractions (S), and nuclear chromatin-bound pellet fractions (P). Samples were prepared from log phase wild type and ORC1-KD cells, and immunoblotted with polyclonal anti-Orc1p, anti-Orc2p, anti-Mcm6p, and anti-acetyl Histone H3 antibodies. (C) Cell cycle progression of wild type CU428 and ORC1 knockdown (ORC1-KD) cells as measured by flow cytometry following staining with propidium iodide. 0 min, point at which G1 phase cells were isolated by elutriated centrifugation.

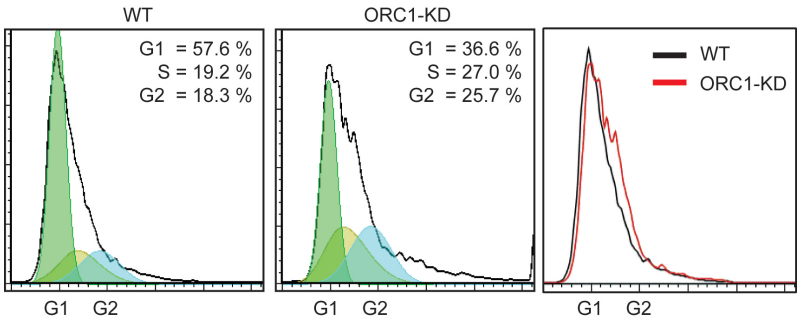

Figure 2.2. Cell cycle distribution analysis of log phase wild type CU428 and ORC1 knockdown (ORC1-KD) cells.
Vegetative growing cell cultures were harvested when the cell density reaches 2.5 X 10e5 cells/ml. Cells were fixed by 70% ethanol and stained with propidium iodide before flow cytometry analysis. Each histogram represents the number of counted cells versus DNA content.

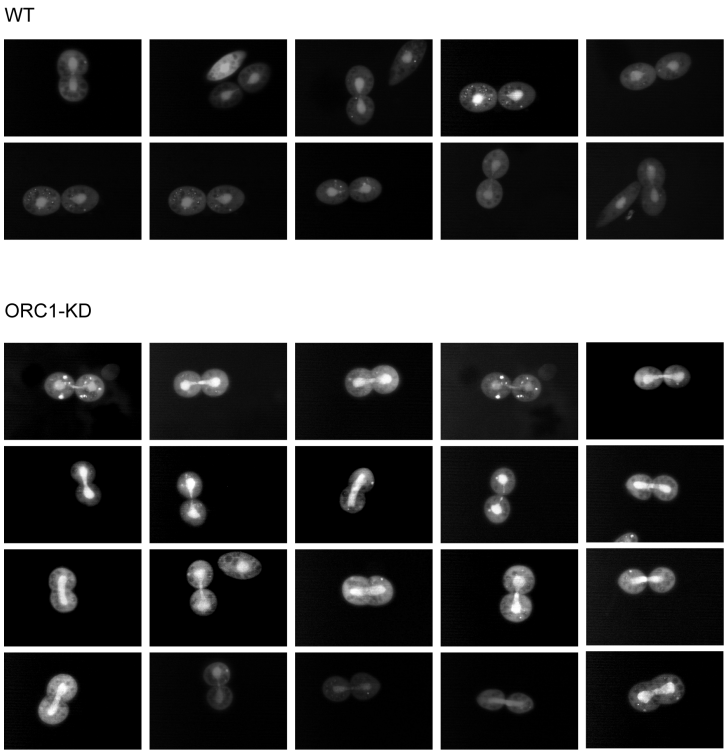

Figure 2.3**.** Nuclear division in wild type (WT) CU428 and ORC1 knockdown (ORC1-KD) cells visualized with acridine orange.

Two dimensional (2D) gel electrophoresis was used to assess replication origin activation and fork progression in the well-characterized rDNA minichromosome. The HindIII-digested genomic DNA isolated from log phase wild type cells generated a bubble-to-Y RI pattern with an rDNA 5' NTS probe, indicating initiation events occurred within the 5' NTS (Fig. 2.4). The same RI pattern was seen in the log phase mutant cells, suggesting rDNA origin site selection and fork progression during vegetative cell cycle were not perturbed in the ORC1 mutant.

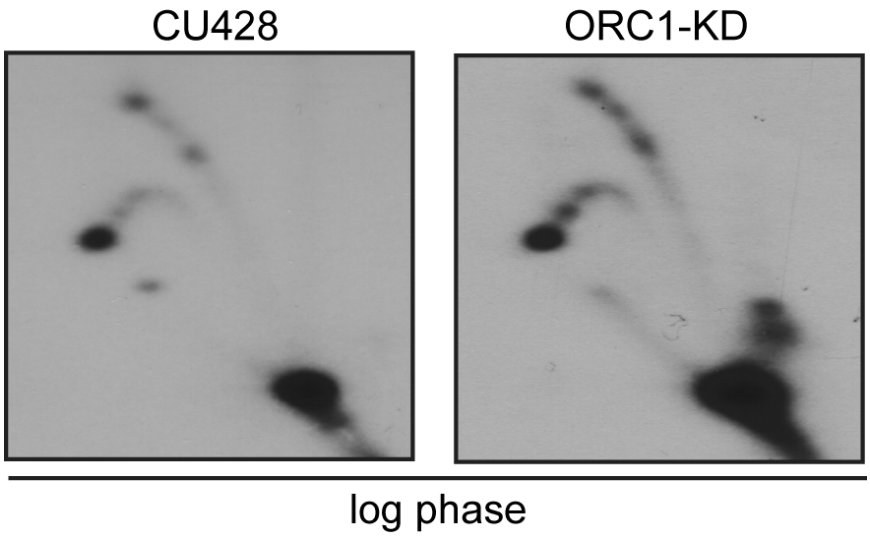

Figure 2.4. Two-dimensional gel analysis of rDNA replication intermediates in wild type CU428 and ORC1 knockdown (ORC1-KD) cells.
DNA samples from log phase cultures were subjected to neutral-neutral 2D gel analysis following digestion with HindIII and enrichment for RIs on BND cellulose. Blots were probed with the rDNA 5' NTS probe.

ORC1 mutants fail to generate a robust intra-S phase checkpoint response

Two key events in the intra-S phase checkpoint response are the suppression of new origin firing and inhibition of replication fork progression. Both processes are arrested by reversible phosphorylation of the heterohexameric replicative helicase, MCM2-7, in all previously examined species (reviewed in Forsburg 2008). To assess the checkpoint response, wild type and ORC1 mutants were treated with hydroxyurea (HU) or methylmethanesulphonate (MMS). Activation of the caffeine-sensitive ATR/MEC1 checkpoint pathway was monitored by flow cytometry and western blot analysis of Rad51p, which is rapidly up-regulated at the mRNA and protein levels in wild type *Tetrahymena* (Yakisich and Kapler 2006; Mochizuki et al. 2008). Synchronized G1 phase cells were obtained by centrifugal elutriation and cultured in media containing 20 mM HU or 0.06% MMS. Whereas HU-treated wild type cells showed no appreciable increase in DNA content, arresting at G1/early S, the ORC1 knockdown mutant entered S phase and continually synthesized DNA (Fig. 2.5A), albeit at a diminished rate compared to mock-treated mutant controls (see Fig. 2.1C for comparison). A similar result was seen in MMS-treated cells (data not shown). Higher concentrations of HU or MMS did not induce cell cycle arrest, suggesting that the threshold for checkpoint activation could not be achieved in the mutant strain (Shimada et al. 2002). Accordingly, Rad51 protein levels increased only modestly in HU-treated mutants, and mutant cells were less sensitive than wild type to the length of exposure or dose of HU (Fig. 2.5B and 2.5C). A diminished Rad51 response was also observed in MMS-treated cells (Fig. 2.5C). HU or MMS induction of Rad51p was inhibited by

caffeine in wild type and mutant cells (Fig. 2.5D), consistent with the involvement of ATR. Hence, while these genotoxic agents are sufficient to arrest DNA replication and activate repair pathways in wild type cells, both responses are compromised in the ORC1 mutant. We conclude that the ORC1 knockdown mutant is defective in checkpoint activation.

Alkaline gel electrophoresis was used to examine the effect of HU on replication initiation and elongation. G1 synchronized wild type and mutant cells were grown in the presence of 20 mM HU and nascent strands from rDNA origin region were visualized by Southern blot analysis with a 5' non-transcribed spacer (NTS) probe (Fig. 2.5E). Wild type *Tetrahymena* generated short nascent strands that gradually chased into high molecular weight species. We interpret this to indicate that fork elongation was slowed down by HU, and that new origin firing was suppressed. In contrast, low molecular weight nascent strands were produced at later time points in HU-treated ORC1 knockdown cultures, suggesting that replication initiation was not repressed. The collective data are consistent with the failure to mount a strong intra-S phase checkpoint response.

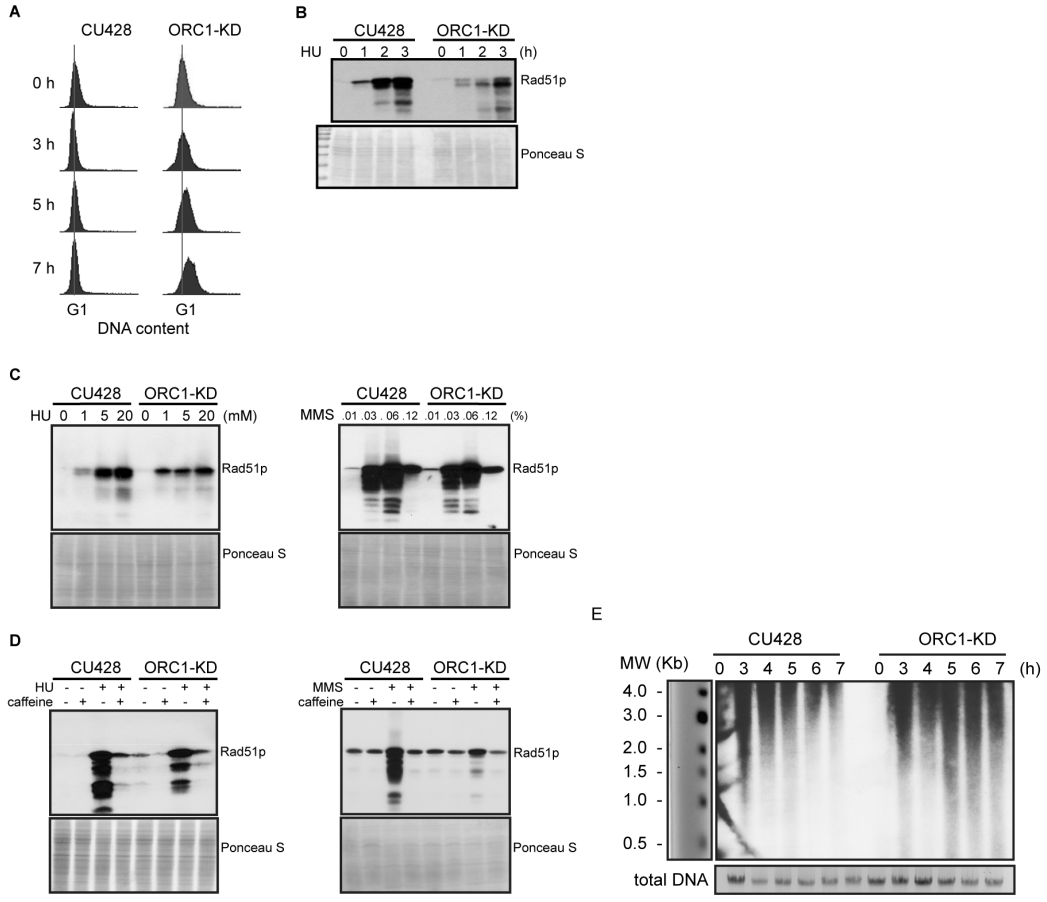

Figure 2.5. Abrogated intra-S phase checkpoint response in ORC1 knockdown cells.
(A) G1 phase wild type CU428 and ORC1 knockdown (ORC1-KD) cells were isolated by elutriation and then treated with 20 mM HU. Samples were collected at the indicated time points for FACS analysis. (B) G1 synchronized wild type CU428 and ORC1-KD cells were released into fresh medium containing 20 mM HU. Samples were collected every hour and whole cell lysates were prepared for western blot analysis with anti-Rad51 antibody. (C) G1 synchronized wild type CU428 and ORC1-KD cells were released into fresh medium in the presence of HU (1-20 mM) or MMS (0.03-0.12%) for 4 hr. (D) G1 synchronized wild type CU428 and ORC1-KD cells were released into fresh medium containing HU (20 mM) / MMS (0.06%), caffeine (1 mM), or HU/MMS+caffeine for 4 hr. Whole cell lysates were prepared and subjected to western blot analysis of Rad51 protein. (E) Alkaline gel electrophoresis of replication intermediates (RIs) accumulated under HU treatment. G1 synchronized wild type CU428 and ORC1-KD cells were released into fresh medium in the presence of 20 mM HU. Genomic DNA was isolated from cells harvested at indicated time point. RIs from the rDNA 5'NTS origin were studied using the alkaline gel electrophoresis method.

Micronuclear genome instability in ORC1 knockdown mutants

A distinguishing feature of ciliated protozoa is the compartmentalization of 'germline' and 'somatic' functions into two distinct nuclei, the micronucleus and the macronucleus. Since micronuclear-encoded genes are not expressed during vegetative cell cycles, a molecular approach was used to evaluate mitotic stability of micronuclear chromosomes. PCR was performed with primer sets that span 10 of the ~180 chromosome fragmentation sites that process the five micronuclear chromosomes into macronuclear chromosomes (Yakisich et al. 2006). Ten clonal ORC1 knockdown lines were generated and propagated for further analysis. PCR primer sets initially yielded products from all ten micronuclear chromosome arms (data not shown). At 120 fissions, all 10 lines failed to produce products with primer sets diagnostic for the left and right arms of chromosome 2 (Fig. 2.6A). Additional micronuclear marker loss was observed in a subset of clonal lines at 250 fissions (micronuclear chromosome arms 1L, 4L, 5L, 5R). While chromosome instability was expected to be stochastic and new events should display a clonal inheritance pattern, the ability to detect chromosome loss did not require repeated sub-cloning. Instead, genome instability was evident in samplings of the entire population. Hence, spontaneous changes in micronuclear genome composition appear to become homogenous within the population. This phenomenon, first reported in a strain bearing a *cis*-acting fragile site in chromosome 2 (Yakisich and Kapler 2006), can now be extended to trans-acting replication initiation factors.

To address the effect of Orc1p dosage on meiotic chromosome transmission and the subsequent rounds of DNA replication associated with development, we mated

knockdown clones that tested positive for all 10 micronuclear chromosome markers to a wild type strain, and used DAPI to follow the fate of micro- and macronuclei. Prior to macronuclear anlagen formation, the abundance of Orc1p is dictated by maternal gene expression in each mating partner. While cytoplasmic exchange could theoretically modulate the level of Orc1p in both mating partners, this does not appear to be the case. The mutant partner exhibited a temporal delay in elongation of the micronucleus into the crescent form, a process in which meiotic chromosomes adopt a widely conserved spatial configuration involving the clustering telomeres and centromeres, respectively (Fig. 2.6B, 3 and 4 h time points) (Mochizuki et al. 2008). Sampling at later time points identified progeny in which post-zygotic micronuclear division was either arrested or developmentally delayed. Differentiation of micronuclei into macronuclear anlagen was similarly perturbed. When post-zygotic defects were detected, both progeny in a mating pair were affected. Hence, these phenotypes were not dictated by the parental cytoplasm of origin (wild type or ORC1 knockdown) (Fig. 2.6B, 7 h and 8 h). Since half of the progeny should inherit two wild type copies of the ORC1 gene, the possibility of zygotic rescue prompted further investigation of the macronuclear development program.

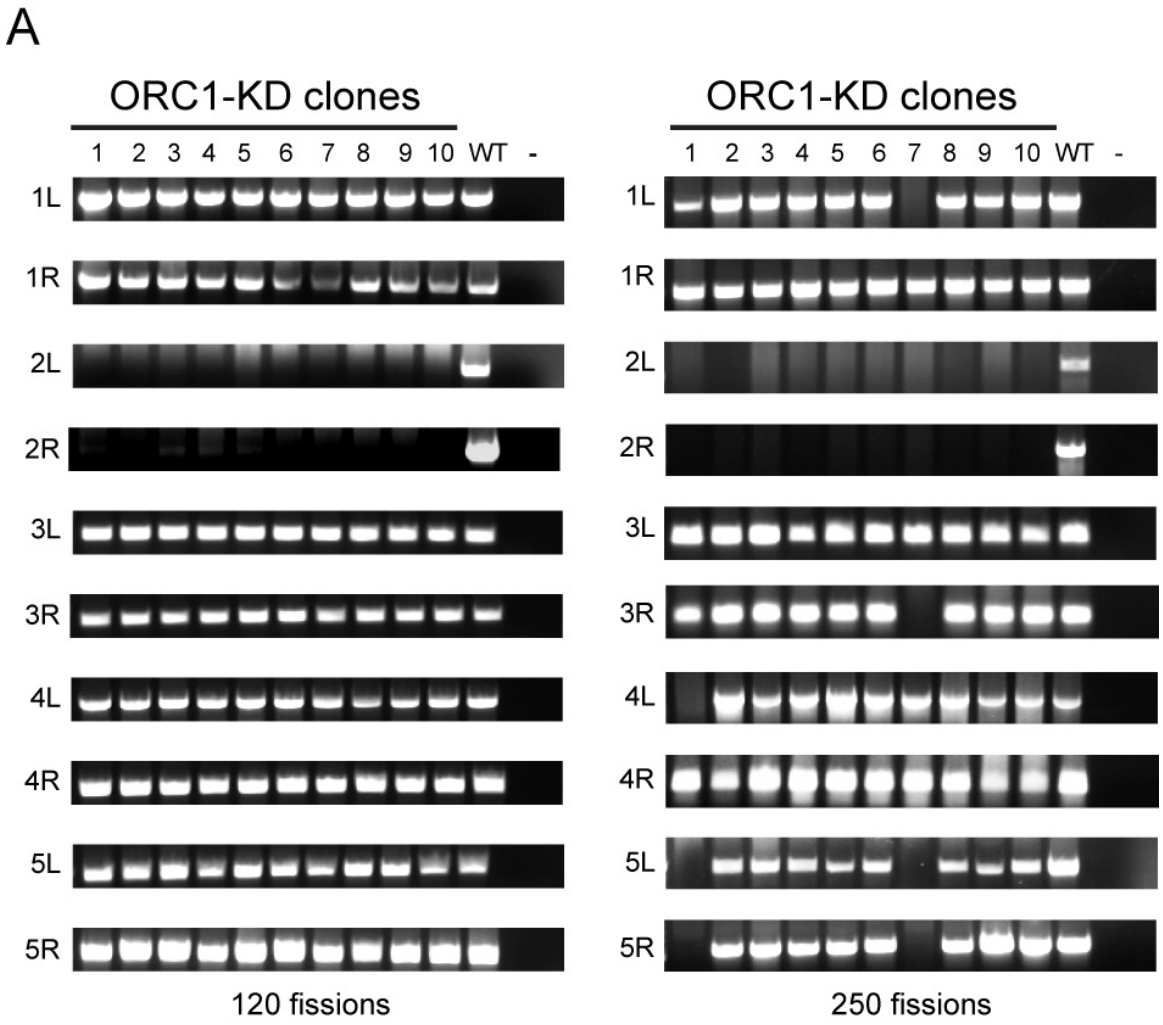

Figure 2.6. Micronuclear genome instability in ORC1 knockdown cells.
(A) ORC1-KD cells were propagated following the establishment of clonal lines. Genomic DNA was isolated at 120 and 250 fissions and subjected to PCR amplification with the primer that span sites for chromosome breakage sequence (CBS)-mediated chromosome fragmentation in the developing macronucleus. PCR primers derived from the right (R) and left (L) arms of all five micronuclear chromosomes were tested. 1–10, clonal ORC1 knockdown lines; WT, CU427 strain; (-), PCR reactions in the absence of DNA templates. (B) Cytological examination of crosses between wild type strain CU427 and CU428, or CU427 and ORC1 knockdown (ORC1-KD) strain with the DNA staining dye DAPI. Cartoon depicts the progression of wild type mating cells during development.

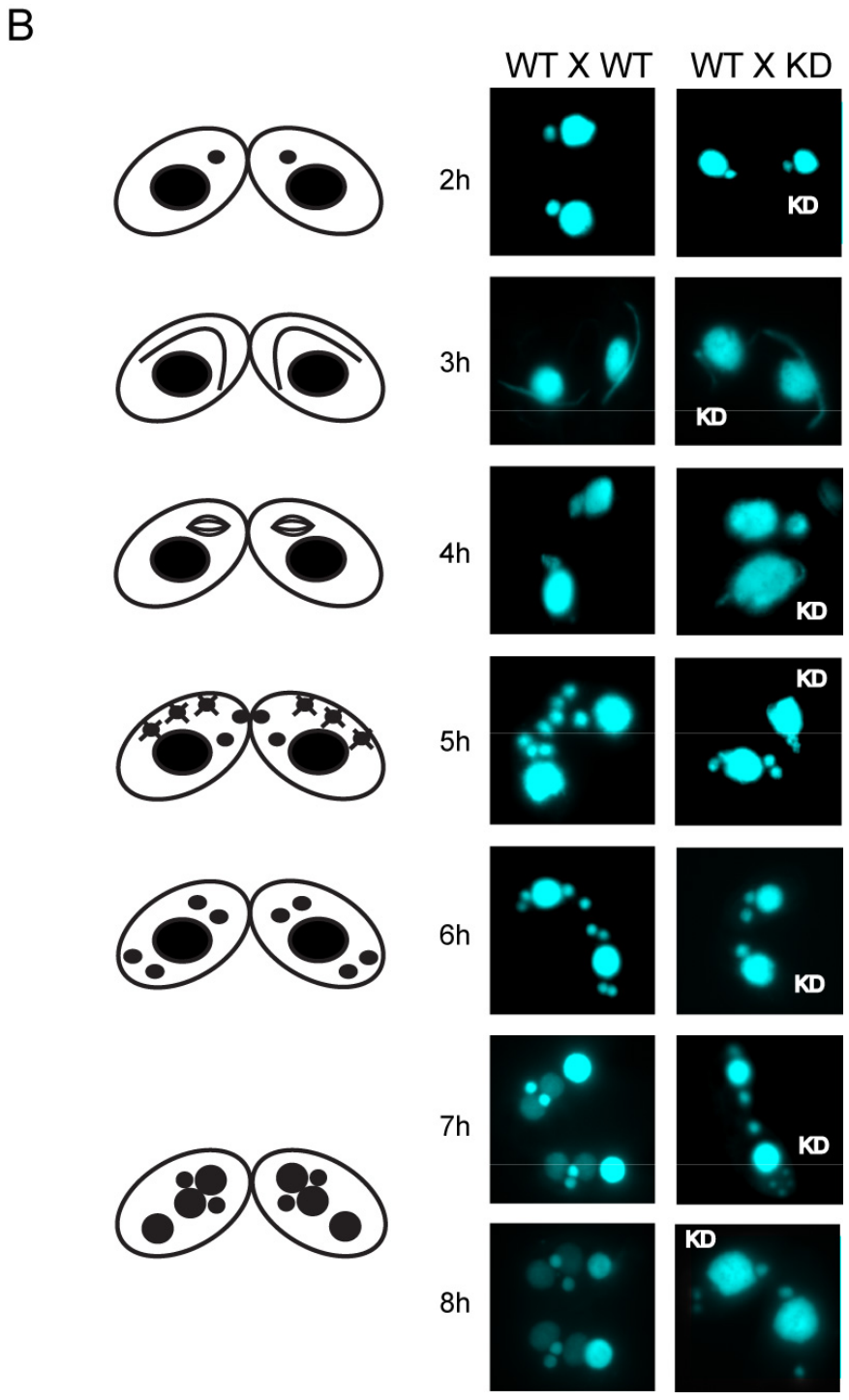

Figure 2.6. Continued.

Developmental regulation of ORC and MCMs subunits

Expression profiling by Miao and colleagues revealed that ORC and MCM gene expression is up-regulated in mating cells relative to asynchronous log phase vegetative cultures (Miao et al. 2009). Two oscillations in mRNA abundance were detected for ORC1, MCM6 and other components of these pre-RC complexes, peaking at 6h and 14h after the initiation of pair formation (Miao et al. 2009). The more prominent 6h peak is derived from 'maternal' gene expression, and correlates with the replication of zygotic micronuclei that generates four genetically equivalent micronuclei, prior to anlagen formation. The second mRNA peak is derived from zygotic gene expression and correlates with endoreplication of the macronuclear anlagen.

To quantify the abundance of pre-RC proteins during development, western blot analysis was performed on a mating between two wild type strains using antibodies directed against Orc1p, Orc2p and Mcm6p. Cytological analysis of fixed cells was used to determine the developmental stage, and flow cytometry assessed DNA content. The 0 h time point corresponds to starved cells arrested at the G1/S border, when Orc1 protein levels are highest in the vegetative cell cycle. A broad peak for Orc1p was detected between 0-12 h in mating cells, encompassing multiple rounds of micronuclear DNA replication and division, and the first round of macronuclear anlagen replication (Fig. 2.7A). Lower signals for Orc1p were detected at 18 and 24 h, correlating with endoreplication phase I in the developing macronucleus (4C to 8C stage). Mating cells were re-fed to complete macronuclear development, a process that involves further

endoreplication cycles, generating 64C macronuclei over a 10 h period (endoreplication phase II) (Yin et al. 2010).

Quantitative analysis of the data indicates that the abundance of Orc1p is increased by ~2.5-fold during the micronuclear replication in mating cells (Fig. 2.7A, 6 – 9 h) relative to the starved G1-phase cells (Fig. 2.7A, 0 h). During endoreplication phase II when anlagen macronucleus undergoes endocycles, Orc1p levels reduced by at least 30-fold relative to the micronuclear replication (Fig. 2.7A, compare 0 h in Endo II with 6 h in starved mating cells). The differential abundance of ORC and MCMs during micronuclear replication and anlagen endoreplication phases suggests that the demands on the DNA replication machinery differ during development.

Endo-replication defects in ORC mutant strains

Flow cytometry was used to determine whether depressed levels of Orc1p inhibit endo-replication altogether or delay onset of this replication program. Under conventional conditions, in which mating cultures are maintained in starvation media for 24h and then refed, wild type macronuclear anlagen exhibit two periods for endo-replication. The first phase (Endo-replication phase I) involves two rounds of DNA replication and generates 8C macronuclei between between 8-16h in starved mating cultures. Upon re-feeding, a second endo-replication phase (Endo-replication phase II) is initiated, generating a DNA content of 16-32C during the next 8h (Fig. 2.7B) (Yin et al. 2010). Both early (starved) and late (re-fed) endocycles were delayed in mating between ORC1-KD and wild type (CU427) strains (Fig. 2.8A). A more exaggerated defect was

observed in crosses between wild type strain SB1934 and ORC1-KD mutant. Macronuclear DNA replication arrested at the 4-8C stage and the old macronucleus was retained.

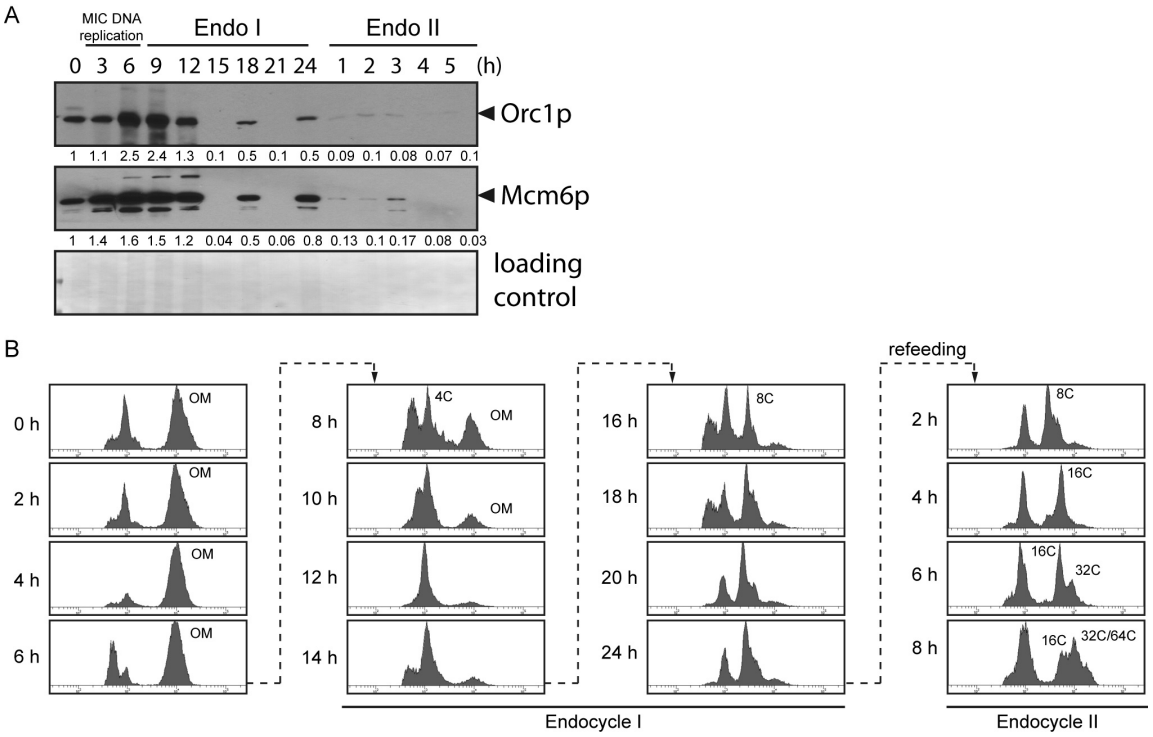

Figure 2.7. Orc1p and Mcm6p expression during *Tetrahymena* development.
(A) Developmental samples were collected from the matings between wild type CU427 and CU428 cells at indicated time points during conjugation (Endo I) and subsequent culture refed with media (Endo II). Whole cell lysates were prepared and subjected to western blot analysis of Orc1p and Mcm6p proteins. Total protein loading control is shown by Ponceau S stained membrane. (B) Flow cytometric analysis of matings between wild type CU427 and CU428 cells. Samples were collected at the indicated time points and nuclei were isolated before stained with propidium iodide. Each histogram represents the number of counted nuclei versus DNA content.

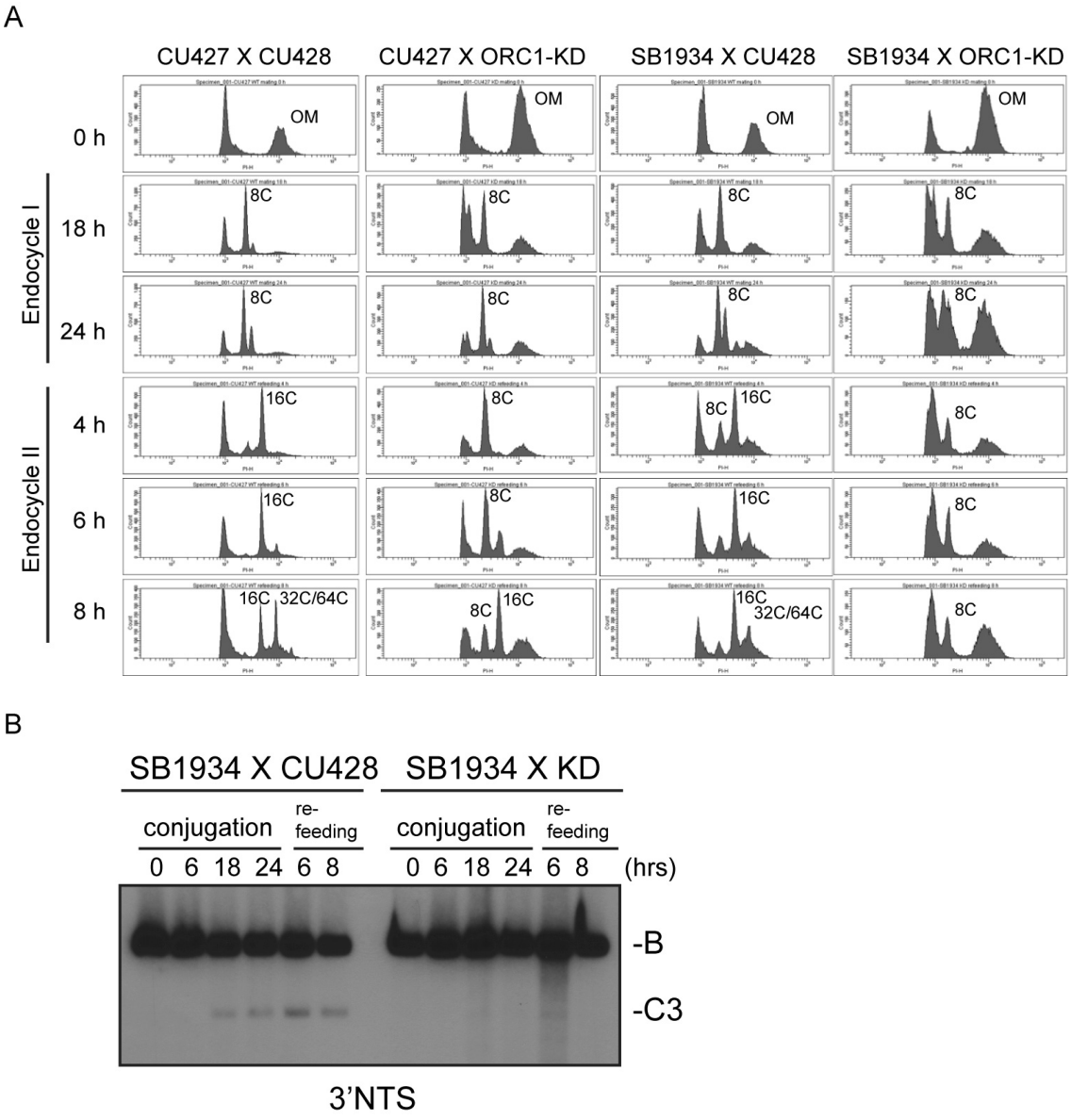

Figure 2.8. DNA endoreplication and rDNA amplification during *Tetrahymena* development.
(A) Flow cytometric analysis of matings between wild type CU427 and CU428 cells, CU427 and ORC1 knockdown (ORC1-KD) cells, wild type SB1934 and CU428 cells, SB1934 and ORC1-KD cells. Nuclei were isolated and stained with propidium iodide. (B) Southern blot analysis of C3 rDNA amplification during development. Mating between wild type SB1934 and CU428, SB1934 and ORC1 knockdown (ORC1-KD) cells were collected at the indicated time points. DNA was digested with BamHI and probed with rDNA 3' NTS probe to identify the macronuclear B and C3 rDNA. (C) Cytological examination of crosses between wild type SB1934 and CU428, SB1934 and ORC1 knockdown (ORC1-KD) with the DNA staining dye DAPI. Samples were collected at 24 h after mating, 4 h and 8 h after refeeding with fresh media. Three photos were shown for each time point.

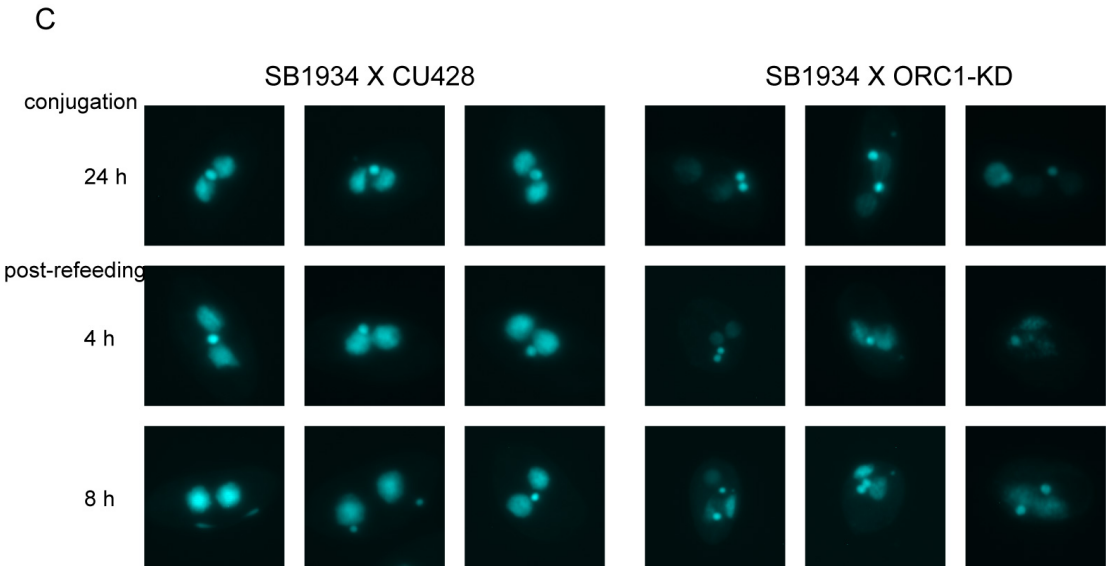

Figure 2.8. Continued.

rDNA gene amplification is compromised in ORC1 knockdown progeny

To determine if depletion of Orc1p affected rDNA amplification, Southern blotting analysis was used to assess C3 rDNA production in matings between ORC1 knockdown and the C3 rDNA strain, SB1934 (Kapler and Blackburn 1994). SB1934 is a heterokaryon strain, with two copies of the integrated C3 rDNA allele in the germline micronucleus and ~9000 copies of the B rDNA minichromosome in its macronucleus. Since the ORC1 knockdown strain contains B rDNA in both the micronucleus and macronucleus, all exconjugants/progeny will have a mixture of B and C3 rDNA in the developing macronucleus. In matings between a wild type C3 (SB1934) and a B (CU428) strain, C3 rDNA was amplified during macronuclear development of progeny cells (Figure 2.8B). In contrast, C3 rDNA was not detected during macronuclear development in the progeny of matings between the B (ORC1-KD) and a wild type C3 (SB1934) strain, indicating that rDNA amplification was also inhibited.

Cytological imaging of mating cells corroborated this finding. When wild type mating cultures were examined during later stages of development (24 h during starved mating, 4 and 8 h after re-feeding), the vast majority of exconjugants/progeny contained two macronuclei and one micronucleus with robust DAPI staining. In contrast, macronuclear DAPI signal was very faint in crosses with an ORC1 mutant (Fig. 2.8C). The collective data demonstrate that modestly diminished levels of ORC1p are not sufficient to support normal macronuclear development.

Discussion

Faithful transmission of genetic information from parents to progeny is critically important for the survival of a species. In all eukaryotes, duplication and segregation of the genome are safeguarded by a conserved set of regulatory processes to assure that a complete copy of the genome is transmitted to each daughter cell (reviewed in Sclafani and Holzen 2007; Harrison and Haber 2006). In *Tetrahymena*, although the macronuclear chromosomes segregate randomly by an amitotic mechanism, the replication of these chromosomes is governed by the same fundamental mechanisms that control DNA replication in normal mitotic cells. These regulatory events include ORC dependent, site-specific initiation of DNA replication (Mohammad et al. 2007; Donti et al. 2009), cell cycle regulated pre-RC assembly (Donti et al. 2009), S phase inactivation of ORC (to prevent re-replication) (Mohammad et al. 2003; Donti et al. 2009), and arrest of the cell cycle in response to DNA damage or replication stress (Yakisich et al. 2006). In this study I explored the effect of modulating the abundance of a critical component of the pre-RC, ORC1, on different DNA replication programs during the vegetative cell cycle and development.

ORC and the vegetative cell cycle

Using gene disruption and phenotypic assortment to drive down the expression of *Tetrahymena* ORC1 gene, we were able to obtain a moderate (~5-fold) reduction in the abundance of Orc1p in vegetative dividing cells. The mutant strains grew slower than wild type and exhibited a slightly elongated macronuclear S phase. In addition, the

majority of dividing mutant cells undergo aberrant macronuclear division with lagging chromosomes. Sustained vegetative propagation correlated with micronuclear genome instability, with the progressive degeneration of micronuclear chromosomes increasing to the point where mutant cells were rendered sterile. The data suggest that Orc1p was a rate-limiting factor for both micro- and macronuclear DNA replication in the mutant. Unexpectedly, the steady state levels of Orc2p and Mcm6p were similarly depressed in the ORC1 knockdown strain. The down-regulation of ORC2 and MCM6 is consistent with the existence of a feedback loop that coordinates the production and/or activity of pre-RC components (Sonneville et al. 2012; Waga and Zembutsu 2006).

Diploid organisms can tolerate a very limited amount of genome instability. Aneuploidy decreases cellular fitness in yeast and higher eukaryotic cells (Tang and Amon 2013) and has been shown to promote further destabilization of the genome (Sheltzer et al. 2011). *Tetrahymena* provides a unique opportunity to study aneuploidy in a diploid mitotic nucleus over time, since the micronuclear genome is not required for vegetative cell viability. Previous work in our lab showed that ATR and a novel *Tetrahymena* protein, Tif1p, protect the micronucleus from genotoxic agents (Morrison et al. 2005; Yakisich et al. 2006). In the work presented here, the mitotic micronuclear genome instability was detected in the partially-depleted ORC1 strain using a PCR assay (Fig. 2.6A).

This result prompted me to examine the S phase checkpoint response by challenging cells with genotoxic reagents, HU and MMS. As I expected, the ORC1-KD strain failed to arrest cell cycle progression, inhibit origin firing, and induce Rad51

expression in response to DNA damage stress (Fig. 2.5). In other eukaryotes, studies have demonstrated that the establishment of replication forks during S phase is required for checkpoint activation (Tercero et al. 2003; Caldwell et al. 2008). Therefore, I hypothesize that defective checkpoint activation in *Tetrahymena* is the consequence of a diminished number of replication forks due to ORC1 depletion, such that the threshold of S phase checkpoint activation cannot be achieved. This checkpoint defect was previously reported in a budding yeast ORC2 mutant (Shimada et al. 2002). The work presented in this chapter extends this model to the amitotic macronuclear and mitotic micronuclear chromosomes of *Tetrahymena*. While amitosis is a normal process in the *Tetrahymena* cell cycle, the integrity of chromosomes that are partitioned by this unorthodox mechanism is carefully monitored. The failure of intra-S phase checkpoint activation allows macronuclear division to occur without complete replication of macronuclear chromosomes (Fig. 2.3)

Previous studies in our lab have defined three modes of ORC binding to DNA in *Tetrahymena*. The first mode is a sequence-specific DNA binding to a single origin site in the rDNA minichromosome, which undergoes selective gene amplification and duplication during vegetative cell cycle. Target site sequence specificity is conferred by an RNA subunit (26T RNA) in *Tetrahymena* ORC through Watson-Crick base pairing between the RNA molecule and rDNA origin sequences, specifically the type I element T-rich strand (Mohammad et al. 2007). The other two modes for ORC binding occur in non-rDNA chromosomes and are temporally regulated across the cell cycle. While G2-phased ORC associates non-specifically with both origin and non-origin sequences,

binding specificity is ultimately achieved in G1 phase when ORC preferentially associates with non-rDNA origins (Donti et al. 2009). Since the specificity of ORC for non-rDNA origins changes across the cell cycle, coordinately with the oscillation of ORC proteins levels, it will be interesting to know if the specificity of ORC binding to rDNA origin is affected by reducing Orc1p levels and presumably the abundance of the ORC holocomplex.

Two dimensional (2D) gel electrophoresis was used to assess replication origin activation and fork progression in the rDNA minichromosome. In the ORC1 mutant strain in which Orc1p was reduced 5-fold, vegetative replication initiated from the 5' NTS, which is the same as the wild type strain (Fig. 2.4). Since the amplified copy number of the rDNA minichromosome is maintained in the ORC1 mutant strain, we speculate that the rDNA origin preference is enriched when ORC becomes rate limiting (see descriptions in the followed sections).

ORC and development

Another remarkable result from chapter 2 is the protein levels of ORC and MCM do not correlate with the DNA content that must be replicated at different stages of development (see flow cytometry profile in Fig. 2.7B for DNA contents and western blot in Fig. 2.7A for the abundance of Orc1 and Mcm6p). The macronuclear genome contains about 104 Mb of DNA while the size of micronuclear genome is around 120 Mb (reviewed in Orias et al. 2011). To make the calculation of DNA content simple, I made an assumption that the DNA content in MAC is comparable to MIC. In addition, I

assume that the Orc1p levels at the initial stage of each replication program are completely involved in the replication of DNA. Therefore, whereas the DNA amount that needs to be replicated during the micronuclear replication is 2.4-fold less than that during the marcronuclear vegetative S phase, the level of Orc1p is 2.5-fold greater (Fig. 2.7A, compare 6 h with 0 h; DNA contents: 20C vs. 47C). During endoreplication phase II (Fig. 2.7A, Endo II 1-5 h) when mating cells were re-fed to complete macronuclear development, the Orc1p levels dramatically diminished (~30-fold reduction, compare 0 h in Endo II with 6 h in starved mating cells), despite a ~ 6-fold increase of DNA content that must be replicated compared to the early stages of development (112C vs. 20C). These results suggest that the demand on the ORC-dependent replication machinery differs during development and the vegetative S phase. The explanation might be that during post-zygotic micronuclear DNA replication, micronuclear chromosomes need to use all potential origins to accelerate DNA synthesis, as they have no transcription constraints. Even though it has been reported that long double-stranded RNAs are produced by bidirectional transcription of the micronuclear genome during development, this nongenic transcription occurs transiently during meiosis (2-3 h after inducing mating) and the transcripts last shortly (5.5-7 h) (Chalker and Yao 2001).

The high demands on replication machinery leads to the increased levels of Orc1p and Mcm6p. After chromatin remodeling and transcriptional activation take place during endoreplication phase I, transcription in the macronucleus would restrain origin use so a relaxed origin usage is established during endoreplication phase II and vegetative cell cycle.

Previous studies in *Xenopus* and *Drosophila* embryonic systems reported that replication initiates randomly at early stages when transcription is off. When transcription resumes replication begins to be restricted to specific sites as is seen in somatic cells of other eukaryotes (Hyrien et al. 1995; Lemaitre et al. 2005). Biochemical studies show that *Xenopus* eggs contain 50-110 nM ORC, or ~10^5 more ORC molecules than in one somatic cell (Carpenter et al. 1996; Tugal et al. 1998). This high concentration of ORC masks the sequence-specific DNA binding preference and this binding preference can be restored by diluting the ORC concentration in vitro (Kong et al. 2003). Therefore, it has been thought that the DNA-binding specificity will be affected by the ratio of ORC to DNA. While the high ratio of ORC/DNA in early *Xenopus* embryos allows DNA replication to initiate randomly throughout the chromosomes, a low ORC/DNA ratio favors initiation at sites with high affinity for ORC (Kong et al. 2003). Another example of dynamic changes in ORC-DNA association has been seen in the *Tetrahymena* ARS1 chromosome. ORC associates non-specifically with newly synthesized chromosomes in G2 phase of the vegetative cell cycle. The ORC binding preference is reset to bona fide replication initiation sites when the levels of ORC proteins peak in G1 phase (Donti et al. 2009). This change in binding activity/specificity does not appear to be associated with a change in the concentration of ORC.

I hypothesize that a similar process might occur during *Tetrahymena* development, in which the origin usage changes dynamically across different stages of development. During micronuclear DNA replication (meiosis & post-zygotic replication)

and macronuclear endoreplication phase I, the high ORC/DNA ratio makes replication initiate randomly. When the ORC/DNA ratio transits from high to low during endoreplication phase II, replication events are restricted to the preferred initiation sites in macronuclear chromosomes.

Another explanation for low levels of ORC1 protein in the endoreplication phase II is that ORC is dispensable for endoreplication of DNA from 8C to 64C, as has been reported in *Drosophila* (Park and Asano 2008). In this recent study, Park and Asano demonstrated that ORC is required for mitotic replication and proliferation of *Drosophila* embryos. ORC is also required for gene amplification in ovarian follicle cells. However, ORC is dispensable for developmentally programmed endocycles in salivary glands, which undergo 10-round endoreplication cycles to reach 1000C DNA content. In contrast to *Tetrahymena*, the *Drosophila* endoreplication cycles still require the MCM complex and the MCM loading factor, Cdt1. The abundance of MCM proteins was not determined in salivary gland tissues so it is not clear whether MCMs are differentially regulated during endoreplication.

The relationship between endoreplication and gene amplification in *Tetrahymena* is still not clear. An allele-specific real-time PCR analysis monitoring the increased DNA content of rDNA and non-rDNA is under development in our lab. If the rDNA minichromosomes undergo endoreplication coordinately with non-rDNA chromosomes during the endo phase II, then the findings in flies can be extrapolated to *Tetrahymena* development. I hypothesize that in endoreplication phase II cells would not require ORC to initiate replication in non-rDNA chromosomes once the rDNA amplification program

has finished. Accordingly, the low level of Orc1p would not affect the endoreplication program, and the little remaining Orc1p could be devoted to DNA replication in the first vegetative cell cycle. Since *Tetrahymena* endoreplication programs are not associated with a terminal differentiation program, the ORC-dependent replication (Donti et al. 2009) must be re-established for vegetative cell cycle in progeny cells.

CHAPTER III

REPLICATION STRESS-INDUCED DEGRADATION OF TETRAHYMENA ORC UNCOVERS AN ALTERNATIVE DNA REPLICATION PROGRAM

Overview

To safeguard the genome, eukaryotes have developed an intra-S phase checkpoint pathway that inhibits the initiation of DNA replication and replication fork elongation when the level of DNA damage exceeds a threshold. Our previous studies revealed that *Tetrahymena* elicits an intra-S phase checkpoint response in the polyploid amitotic macronucleus, and that this pathway shares common characteristics with checkpoint signaling in other eukaryotes. Here, I examined the fate of replication forks during and after treating *Tetrahymena* with DNA damage agents that activate the intra-S phase checkpoint response. I discovered a novel DNA replication program that is activated to regulate progression through S phase when cells are recovering from replicative stress. In this replication program, origin recognition complex (ORC) and replicative helicase (MCM2-7), which are required for site-specific replication initiation and fork elongation, are dramatically diminished in the presence of hydroxyurea (HU). During the recovery from HU-induced cell cycle arrest, cells were competent to complete S phase, yet the abundance of ORC and MCM remained undetectable until the next cell cycle. In addition, the rDNA origin used exclusively during the S phase of vegetative cell cycle and developmentally programmed gene amplification is suppressed when these replication proteins are selectively degraded under replication stress. Instead,

an alternative pathway is used to ensure complete genome replication once the source of replication stress is removed.

Introduction

Cells have evolved complex mechanisms and regulatory networks to faithfully duplicate their genomes. These mechanisms ensure that DNA is replicated exactly once per cell cycle and DNA replication is arrested in response to DNA damage. In many instances the target of these regulatory mechanisms is a key intermediate in the initiation of DNA replication called the pre-replication complex (pre-RC). Pre-RCs are formed on chromatin in late mitosis or early G1 phase. To mark potential sites for replication initiation, ORC acts as the landing pad for other pre-RC proteins. In G1 phase, origin-bound ORC recruits Cdc6p allowing Cdt1p in turn to load the MCM2-7 complex onto origins thereby forming a pre-RC. Subsequently, phosphorylation of Cdc6p and MCM2-7 by a cyclin-dependent kinase (CDK) and Dbf4-dependent kinase (DDK), in G1/S and S phase, results in the degradation of Cdc6p and migration of MCM2-7 with replication forks. The action of CDK and DDK promotes loading of Cdc45, MCM10, GINS, RPA, and DNA polymerases to the ORC binding sites, triggering the initiation of DNA replication (Sclafani and Holzen 2007). These regulated events prevent the pre-RC from reassembling at origins, thereby limiting initiation events to once per cell cycle.

DNA replication fidelity is essential to maintain genome integrity. During DNA synthesis, the progression of replication forks is safeguarded by intra-S phase checkpoint mechanisms in response to exogenous and endogenous genotoxic stress. The intra-S

phase checkpoints are mainly governed by the ATM (ataxia-telangiectasia mutated) and ATR (ATM and Rad3-related) signaling pathways. ATR responds primarily to replication blockage whereas ATM is involved in recognition and repair of DNA double-strand breaks (DSBs). However, there is significant overlap between these pathways (Abraham 2001). In response to DNA replication inhibitors (ex. HU) or DNA damaging agents (ex. methyl-methanesulphonate; MMS), sensor proteins, such as RPA, are recruited to the damage sites and promote the recruitment of protein kinases ATM and ATR. Substrates for ATR and ATM are the protein kinases Chk1 and Chk2, respectively, which act as signal amplifiers in these checkpoint pathways. Chk1 and Chk2 repress initiation of replication from late origins by inducing degradation of Cdc25 and preventing activation of Cdk2, thereby blocking progression through S phase. Additionally, Chk1 coordinates Rad51-mediated homologous recombination (HR), which is involved in DSBs repair and is important for the maintenance of stalled replication forks.

DNA replication and the intra-S phase checkpoint are intimately linked, and replisome proteins have long been proposed to play roles in checkpoint signaling. In most cases, the replisome remains stably associated with the stalled fork (Lucca et al. 2004; Cobb et al. 2003), restraining the activity of recombination enzymes at stalled forks, and then resumption of replication can occur once the block is relieved. In metazoans, for example, MCM subunits are phosphorylated by the ATM/ATR kinases (Cortez et al. 2004; Ishimi et al. 2003). In addition, in both human cells and *S. pombe,* Rad51 protein interacts with MCMs (Sclafani and Holzen 2007; Bailis et al. 2008),

which could provide one way for Rad51 to be recruited to the stalled replication fork, and suggests that MCM function is required for proper recovery from replication stalling. If replisomes dissociate from broken forks or the function of replisome is lost, the resumption of DNA synthesis appears to require replisomes to be rebuilt. In *E. coli*, the restart of stalled replication forks is essential because of the lack of backup origins. The stalled forks are reactivated by recombination-dependent or -independent pathways catalyzed by the RuvABC or PriA and PriC proteins, respectively (Heller and Marians 2006). In eukaryotes, although converging forks from adjacent origins may account for replication of those regions in which the original replication fork has undergone collapse, homologous recombination still plays a major role in restarting stalled and collapsed forks when some lesions are not readily resolved (Branzei and Foiani 2007). However, the mechanism of origin-independent loading of replisomes for replication reactivation is not well characterized.

Like most ciliated protozoa, *Tetrahymena thermophila* contains two genetically related, but functionally distinct nuclei within a common cytoplasm. The diploid germline micronucleus is the reservoir of genetic material and contains 5 transcriptionally inactive heterochromatic chromosomes that divide mitotically during the vegetative cell cycle. Chromosomes in the polyploid somatic macronucleus are euchromatic and transcriptionally active. Without centromeres and spindle formation, they randomly segregate via a poorly understood amitotic mechanism that is temporally coupled with cytokinesis. The differential chromatin organization of micro- and macronuclear chromosomes suggests that replication and repair of DNA in the

micronucleus and macronucleus may be differentially regulated. For example, while the macronucleus is replicated prior to micronuclear S phase during the vegetative cell cycle, micronuclear division (mitosis) precedes amitotic macronuclear division.

Tetrahymena has been an attractive model to study the regulation of alternative DNA replication programs. In the presence of nutrients, *Tetrahymena* reproduces by asexual fission, during which the micronucleus and the macronucleus replicate their genomes at different stages of the vegetative cell cycle. Under conditions of starvation, cells of different mating types conjugate with each other and initiate sexual reproduction. During conjugation, when the parental micronucleus differentiates into the macronucleus, the developing macronucleus rDNA minichromosomes are extensively amplified to ~9,000 copies, while the rest of the macronuclear genome undergo endoreplication to a final copy number of 45 C. Once development is complete, rDNA and non-rDNA chromosomes are replicated once per vegetative cell cycle. The rDNA minichromosome has been extensively studied for its cis-acting elements at a differentially regulated origin and the trans-acting factors that regulate replication initiation. The 1.9 kb rDNA 5' non-transcribed spacer (5' NTS) contains three nucleosome-free regions, two of which function as the initiation sites for rDNA amplification and vegetative rDNA replication (Fig. 1.9). Type I elements found within these nucleosome-free regions, have been shown to regulate replication initiation and transcription of the 35S rRNA precursors (Zhang et al. 1997). *Tetrahymena* ORC contains an integral RNA subunit, 26T RNA, that facilitates rDNA origin recognition in the 5' NTS by Watson-Crick RNA:DNA base pairing (Mohammad et al. 2007).

In previous work, we reported that DNA damaging agents activated a caffeine-sensitive checkpoint response that regulated macronuclear S phase progression and the expression of Rad51 repairing protein (Yakisich et al. 2006). In addition, a caffeine-insensitive checkpoint response was discovered, in which Mcm6p was stabilized in response to MMS to preserve the fork structure while the damage is repaired (Oporto 2011, dissertation). Although the polyploid macronuclear chromosomes segregate randomly during amitosis, the high copy number is stably maintained, suggesting that the DNA damage checkpoint pathway is not the only mechanism for assuring genome balance in the macronucleus. As described in Chapter 2, the abundance of other pre-RC components was also depressed in a *Tetrahymena* strain that was partially depleted for ORC1. This strain failed to activate a robust intra-S checkpoint response due to fewer origins firing to achieve the threshold for checkpoint activation. These results suggest that there is more than one checkpoint pathway responsible for the DNA damage response in *Tetrahymena*. Here, we examined the fate of replication forks during and after treating *Tetrahymena* with DNA damage agents that activate the intra-S phase checkpoint response. Our findings revealed differences in the response to replication stress, and suggest the existence of a novel alternative mechanism to replicate chromosomes during the recovery from replication stress.

It has been widely reported that checkpoint proteins stabilize replication forks and replisomes in response to replication stress (reviewed in Nam and Cortez 2011). However, our findings suggest an alternative mechanism that *Tetrahymena* uses to abrogate replisome functions when the fork encounters obstacles. Therefore, our study

proves new mechanistic insights into the replication stress response. These findings fill the knowledge gap in the regulation of replisome components in the DNA replication program, and present a different fate for pre-RC proteins.

Material and methods

Cell culture synchronization

G1 phase cultures were obtained by centrifugal elutriation or starvation. To isolate G1 phase cells by elutriation, a two liter log phase culture at the density of 1 X 10e5 cells /ml was prepared and pumped into a Beckman J6M/E centrifuge rotor at a flow rate of 50 ml/min, centrifuging at 850 rpm. After all of the 2-liter culture was loaded, 200 ml of G1 phase cell culture was recovered by increasing the flow rate of the pump to 65 ml/min. To synchronize cells at G1 phase by starvation, log phase cells were washed twice with 10 mM Tris (pH 7.5) and resuspended in Tris at the density of 5 X 10e5 cells/ml. Cells were incubated at 30°C for 18 h and shaken at 100 rpm. To resume the cell cycle, cells were refed with equal volume of 4% PPYS to achieve the cell density at 2.5 X 10e5 cells/ml.

DNA isolation and enrichment for replication intermediates (RIs)

Total genomic DNA was isolated from *Tetrahymena* culture by a previously described protocol (Zhang et al. 1997). Cells were washed by 10 mM Tris buffer (pH 7.4) and resuspended in NDS lysis buffer (10 mM Tris, 0.5 M EDTA, 2% SDS). Proteinase K (Sigma-Aldrich) was added at the final concentration of 0.1 mg/ml and

incubated overnight at 37°C. Samples were then diluted with equal volume of TE buffer (pH 8.0) and extracted with phenol/chloroform/isoamyl alcohol (25:24:1) twice. After additional extraction with chloroform, 2.5 volumes of ethanol were added to precipitated DNA at RT. DNA was resuspended in distilled water containing 10 μg/ml RNase A and incubated at 37°C for 1 h. Finally, DNA was reprecipitated with ethanol and resuspended in 1ml of TE buffer. For RI enrichment, 200 μg of genomic DNA were digested with restriction enzymes (*Hind*III for the rDNA 5' NTS fragments; *Cla*I for the rDNA coding region) for 4 h at 37°C, precipitated by ethanol, and resuspended in 400 μl of TNE buffer (0.1 M NaCI, 10 mM Tris and 1 mM EDTA). RIs were applied to the 200-μl packed volume benzoylated naphthoylated DEAE (BND)-cellulose columns (Sigma-Aldrich), washed with 350 μl of TNE buffer for 5 times, and eluted with 1.8% caffeine in 200 μl of TNE for 5 times. Centrifugation at 10,000 rpm for 10 min pelleted the BND-cellulose particles. The supernatant was collected and DNA was precipitated by addition of a equal volume of isopropanol with 20 μg glycogen as the carrier. After centrifugation at 13,000 rpm for 20 min, the DNA pellet was wash once by ice-cold 70% ethanol and finally resuspended in 50 μl of TE buffer. Total DNA recovery was estimated to be ~5% of the input.

Two-dimensional (2D) gel electrophoresis of DNA replication intermediates

Neutral-neutral 2D agarose gel electrophoresis was performed as previously described (Zhang et al. 1997). Approximately 10 μg of BND cellulose-enriched DNA was loaded for each 2D gel experiment. The first dimension agarose gel (0.4% for

restriction fragment larger than 3 kb; 0.6% for fragment smaller then 3 kb) was run in 1X TAE buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA) at 1.5 volt/cm for 20 h at RT. The second dimension gel (1% for restriction fragment larger than 3 kb; 1.8% for fragment smaller then 3 kb) was run in 1X TBE buffer (90 mM Tris, 90 mM Boric acid, 2 mM EDTA) containing 0.5 mg/ml ethidium bromide at 3 volt/cm for 18 h at 4°C. For neutral-alkaline 2D gel electrophoresis, the first dimension gel was prepared and run at the same condition as neutral-neutral 2D gel. The second dimension gel was run in alkaline electrophoresis buffer (40 mM NaOH, 2 mM EDTA) at 1.5 volt/cm for 20 h at 4°C using a peristaltic pump to recirculate the electrophoresis buffer. After electrophoresis, the gel was sequentially soaked in 0.25 N HCl for 20 min and alkaline transfer buffer (0.5 M NaOH, 1.5M NaCl) for another 20 min. The DNA was transferred overnight to a charged nylon membrane (Hybond-XL, Amersham) in alkaline buffer by capillary blotting and DNA was fixed to the membrane using a UV crosslinking apparatus (Stratagene, Agilent Technology). The blot was prehybridized at 37°C for 4 h in the hybridization solution (1M NaCl, 1% SDS, 10% dextran sulfate, 5 mM Tris [pH 7.5], 100 μg/ml of denatured salmon spermon DNA and 25% formamide). Probes labeled by the random primer method are boiled for 10 min, chilled on ice, and added directly to the prehybridization solution. After 18 h the blot was washed 3 times in 2X SSC/ 1% SDS solution for 15 min each at 42°C, and once in 0.4X SSC/ 0.1% SDS solution for 15 min at 42°C. The blot was exposed to x-ray films with an intensifying screen at -70°C.

Flow cytometry

To determine bulk DNA content in the presence of replication stress and during recovery from cell cycle arrest, flow cytometric analysis was performed on a Becton Dickinson FACSAria II flow cytometer (BD Biosciences). Briefly, at least 2 X 10^5 cells were collected for each sample, fixed with 70% ethanol, and stored at 4°C. Fixed samples were washed twice with PBS and resuspended in 0.4 ml of propidium iodide staining solution (PBS containing 0.1% Triton X-100, 50 μg/ml propidium iodide and 0.2 mg/ml RNase A). Stained samples were protected from the light and incubated at RT for at least 1 h. Twenty thousand events were analyzed for each sample on the cytometer. Data were analyzed using BD FACSDiva™ software. Histograms were plotted, in which the y-axis represented the number of events and the x-axis represented the relative DNA content.

Western blot analysis

To prepare whole cell lysates, 1 ml culture washed by 10 mM Tris buffer was resuspended in 50 μl of 1% SDS lysis buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1% sodium deoxycholate, 1% SDS) and incubated for 15 min on ice. The modified Lowry protein assay was performed using reagents from Bio-Rad (DC™ Protein Assay, cat# 500-0111). Twenty μg protein of each sample was boiled and subjected to SDS-PAGE. For the preparation of separating gels, 30% acrylamide/0.8% bisacrylamide solution, 1.5 M Tris-HCl (pH 8.8) containing 0.4% SDS, 10% ammonium persulfate, TEMED and H_2O were mixed and poured into the glass-plate sandwich. The

percentage of acrylamide in the separating gel depends on the molecular size of the protein being separated. Generally, 7% gels were used for SDS-denatured proteins of 60 to 200 kDa, 10% gels were used for SDS-denatured proteins of 16 to 70 kDa, and 12% gels were used for SDS-denatured proteins of 12 to 45 kDa. After the separating gel was cured, the stacking gel was prepared by mixing the following solutions: 0.65 ml of 30% acrylamide/0.8% bisacrylamide, 1.25 ml of 0.5 M Tris-HCl (pH 6.8) containing 0.4% SDS, 3.05 ml H_2O, 25 µl of 10% ammonium persulfate and 5 µl TEMED. The separating gel solution was poured into the sandwich and inserted a 15-well comb. The gel was run at 80 V at RT for ~3 h in the SDS electrophoresis buffer (0.125 M Tris-HCl, 0.96 M glycine, and 0.5% SDS). Protein samples were then transferred for 2 h to a nitrocellulose membrane (Whatman Protran BA85, GE Healthcare) in the transfer buffer (0.025 M Tris-HCl, 0.19 M glycine, and 20% methanol). The membrane was stained with Ponceau S (Sigma-Aldrich) (5 mg/ml of Ponceau S and 1% acetic acid in H_2O) for 1 min at RT for visual analysis of the loading control. Later the membrane was blocked for 1 h at RT with 10% skim milk in PBS solution containing 0.1% Tween 20. Immunodetection of Orc1p (1:5,000), Orc2 (1:5,000) and Mcm6p (1:10,000) were carried out using antibodies raised in rabbits (Convance). Rad51 (1:5,000) and Acetyl-histone H3 antibodies (10,000) were obtained from Neomarkers (51RAD01, Thermo Scientific) and Upstate (Invitrogen). Blots were incubated with the primary antibodies at 4°C overnight, and then washed 4 times with TBST buffer at RT, 10 min each time. A horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG was used as the secondary antibody (Jackson ImmunoResearch). Blots were incubated with the

secondary antibodies at 4°C for 3 h, then the washing steps were repeated as described above. Blots were visualized using ECL reagents (PerkinElmer) according to the manufacturer's instructions. About 1 ml of ECL reagents were applied to cover one membrane and incubated at RT for 3 min. The chemiluminescent signals were detected by x-ray films.

Results

Intra-S phase checkpoint response is regulated through different pathways in response to DNA damage agents in *Tetrahymena*

Previous studies revealed that wild-type *Tetrahymena* elicits an ATR-mediated intra-S phase checkpoint response that can be induced by HU and methylmethanesulfonate (MMS) (Yakisich et al. 2006). To better understand how activation of the intra-S checkpoint affects replication factors in *Tetrahymena*, the level of Orc1p and Mcm6p was monitored following the treatment with HU and MMS. Wild type cells were synchronized at G1 phase by centrifugal elutriation and released into media containing 20 mM HU or 0.06% MMS. Flow cytometry analysis was first performed to examine the cell cycle progression of cells subjected to DNA damage stress (Fig. 3.1A). Whereas mock-treated cells progressed through the cell cycle and completed cell divison within 3 h, as evidenced by the reemergence of cells with 1N DNA content, the HU- and MMS-treated cells arrested early at G1/S border throughout the 4 h treatment. Western blot analysis was performed to examine the expression of Rad51, a biomarker for activation of the ATR-mediated intra-S phase checkpoint

response in *Tetrahymena* (Yakisich et al. 2006). Consistent with the flow cytometry data, Rad51 levels were dramatically elevated in HU- and MMS-treated cells during the 4 h incubation, as compared to mock treated cells (Fig. 3.1B and Fig. 3.1C). These data confirmed that the intra-S phase checkpoint response was activated when cells were subjected to HU and MMS treatment.

In human cells, ATR phosphorylates Mcm2 and Mcm4 in response to multiple forms of DNA damage (Ishimi et al. 2003; Cortez et al. 2004). Also *S. pombe* Mcm4 interacts with the checkpoint protein kinase Cds1 (Chk1 in mammals) and undergoes Cds1-dependent phosphorylation in cells treated with HU (Bailis et al. 2008). These results suggest that the DNA helicase activity of MCM complex is negatively regulated by phosphorylation of MCM subunits to prevent further unwinding during DNA damage. We examined the effect of HU and MMS on the *Tetrahymena* replication factors, Orc1p and Mcm6p. Unexpectedly the abundance of Mcm6p was significantly diminished by HU within 3 h, while MMS-treated cells maintained the Mcm6p level comparable to mock-treated cells. To determine if the different effects of HU and MMS on Mcm6p reflect arresting at different stages of the cell cycle, a similar experiment has been performed by adding drugs in cells entering S phase after elutriation (Oporto 2011). Like the treatments in G1 phase cells, Mcm6p was degraded in HU treated S phase cells; however, its abundance was maintained by MMS. These results suggest that Mcm6p was regulated through different mechanisms under HU and MMS treatments regardless of the stage of cell cycle.

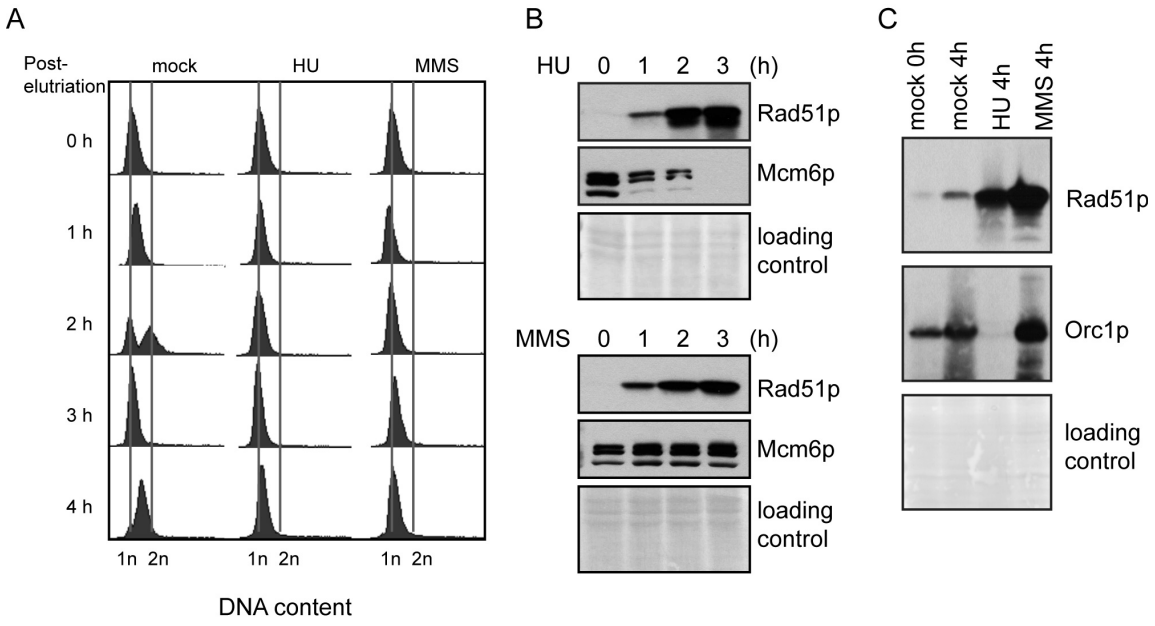

Figure 3.1. Cell cycle arrest and differential regulation of pre-RC proteins under HU and MMS treatment. (A) Flow cytometric analysis of elutriated cultures treated with mock, HU (20 mM) or MMS (0.06%). Each histogram represents the number of counted cells (y axis) versus DNA content (x axis). Cells were synchronized in G1 phase by elutriation and released into fresh media in the presence or absence HU or MMS. Samples were collected every hour after drug addition. (B) Western blot analysis of HU, MMS and mock treated cells showing the effects of DNA damage stress (upper panel: 20 mM HU; lower panel: 0.06% MMS) with Mcm6p and Rad51p. Samples were collected at the same time with samples in (A). (C) Western blot analysis of Orc1p, Rad51p and acetyl-histone H3 after 4 h treatment. G1 synchronized culture were obtained by overnight starvation and then released into the media containing 20 mM HU or 0.06% MMS. Total protein loading control is shown by Ponceau S stained membrane.

Surprisingly, the abundance of Orc1p was also dramatically reduced after treatment with HU for 4 h. Like Mcm6p, Orc1p was stably maintained in MMS treated cells, suggesting Orc1p and Mcm6p are co-regulated in response to replication stress (Fig. 3.1C). The results are consistent with the notion that *Tetrahymena* ORC and MCM2-7 complexes are coordinately regulated during the vegetative cell cycle and development (Chapter 2). Thus, we conclude that HU and MMS induce intra-S phase checkpoint responses in *Tetrahymena*. We postulate that different downstream signaling pathways are involved, and that ORC and MCM complexes, possibly the entire pre-RC, act in concert to respond to replication stress.

Cell cycle progression occurs with diminished level of pre-RC proteins during the recovery from HU arrest

HU promotes the stalling of replication forks by inhibiting the ribonucleoide reductase, thus limiting the pool of deoxynucleotides available for DNA synthesis. Cells respond to stalled forks by activating the intra-S phase checkpoint response and DNA repair mechanism to maintain genome stability (reviewed in Allen et al. 2011). Once the damage is repaired or HU is removed, arrested cells should resume cell cycle progression by firing new origins or elongation of stalled elongation forks. As the first step in exploring these responses in *Tetrahymena*, cells were treated with 20 mM HU for 4 h and released into fresh media lacking the drug. Flow cytometry and western blot analysis were used to monitor cell cycle progression and expression of replication factors. The flow cytometry profile revealed that cells rapidly resumed DNA replication

(T = 60 min) and progressed into the second S phase within 180 min after removal of the HU block, which is reminiscent of the timing of normal cell cycle (Fig. 3.2A). These data suggest that the replication machinery is competent to replicate soon as the HU is removed from the culture. In addition, no clear sub-G1 peak was observed indicating that most cells were viable following HU removal. While DNA replication was efficiently resumed following removal of the HU block, this process was independent of the re-synthesis Orc1p and Mcm6p. Indeed, Orc1p was barely detectable during the first cell cycle (50-fold reduction comparing the 0 min during cell cycle in unperturbed cells and the 0 min time point during the recovery period) and even in cells that reached the second G1 phase (5-fold reduction comparing the 0 min during cell cycle and the 150 min time point during the recovery period)(Fig. 3.2B). Orc2p was coordinately regulated with Orc1p during and after HU treatment, suggesting the entire ORC was diminished in response to HU stress (Fig. 3.2C). Mcm6p re-appeared about 90 min after HU removal, at a time when most cells had already replicated a significant portion of their genome, and remained low throughout the first cell cycle (Fig.3.2B). These data suggest that replenishment of ORC and MCM is not required for DNA replication during the first S phase following the removal of HU.

The rDNA origin is bypassed during recovery from HU induced stress

Previous studies have shown that 21 kb *Tetrahymena* ribosomal DNA (rDNA) is replicated exclusively from the origins in the 5' nontranscribed spacer (5' NTS) during vegetative cell cycle (Donti et al. 2009), and *Tetrahymena* ORC contains an integral

RNA subunit, which confers specificity to the 5' NTS origins by base pairing with the T-rich strand of an essential cis-acting replication determinant- the type I element (Mohammad et al. 2007). Because Orc1p and Mcm6p levels were not restored prior to the first S phase after the removal of HU, we therefore examined replication intermediates from the rDNA 5' NTS origin region by neutral-neutral 2D gel electrophoresis (Brewer and Fangman 1987).

In the mock-treated vegetative S-phase culture (Fig. 3.3B), a bubble-to-Y arc intermediate pattern was observed with an rDNA 5' NTS probe, indicating that all detectable initiation events occurred from origins in the 5' NTS. However, when cells were treated with 20 mM HU for 4 h, a simple Y arc intermediate was detected (Fig. 3.3C, 0 min). The presence of a simple Y arc, indicative of passive replication of the 5' NTS, suggests that alternative initiation sites distal to the Domain 1 and 2 were activated. The simple Y arc intermediates were also detected in cells progressing through the first S phase after the removal of HU (Fig. 3.3C, 90 and 120 min). These results suggest that the 5' NTS origins are inactivated under HU treatment in concert with the degradation of Orc1p and Mcm6p. In the meantime, an alternative mechanism was activated to duplicate the rDNA minichromosome. As noted, X-shaped molecules, which correspond to the homologous recombination–mediated template switch intermediates, accumulated in cells under 4-h HU treatment and during the initial recovery period (Fig. 3.3C, 0 and 90 min). The X-shaped intermediate has shown to be generated under conditions of genotoxic stress by homologous recombination repair in yeasts (Vanoli et al. 2010).

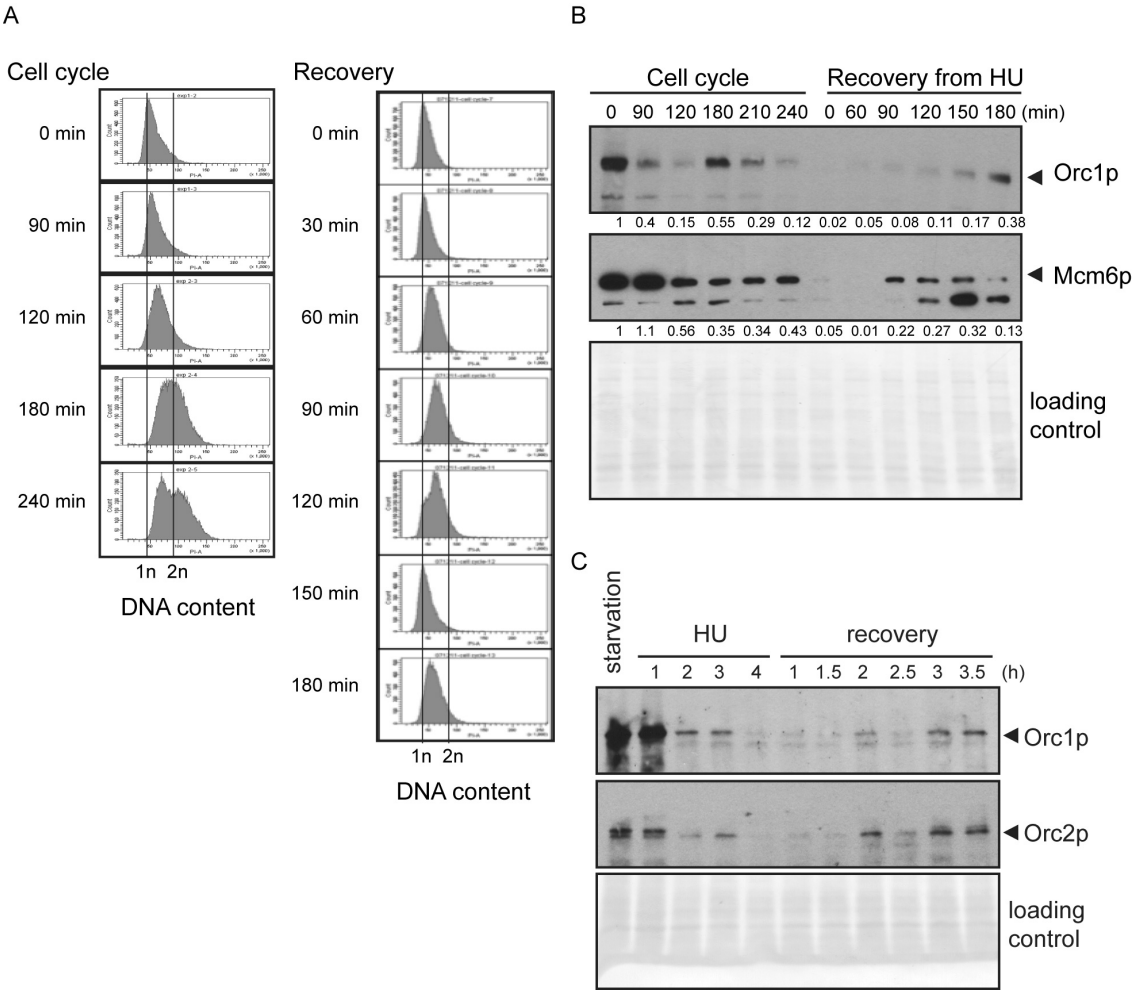

Figure 3.2. Cell cycle analysis following removal of hydroxyurea.
Flow cytometric analysis of cell cycle progression in cells released from HU block. G1 synchronized cultures were generated by starvation in 10 mM Tris. Cells were refed in the absence or presence of 20 mM HU. Following 4h HU treatment, the drug was removed and cells were grown in HU free media. Samples were collected at 30 min intervals (recovery). Each histogram represents the number of counted cells (y axis) versus DNA content (x axis). (B) Western blot analysis of Orc1p and Mcm6p during the recovery from HU block. Total protein loading control is shown by Ponceau S stained membrane. (C) Western blot analysis of Orc1p and Orc2p under HU and during the recovery from HU block. Cells were synchronized in G1 phase by starvation and released into fresh media in the presence HU. Following 4h HU treatment, the drug was removed and cells were grown in HU free media. Total protein loading control is shown by Ponceau S stained membrane.

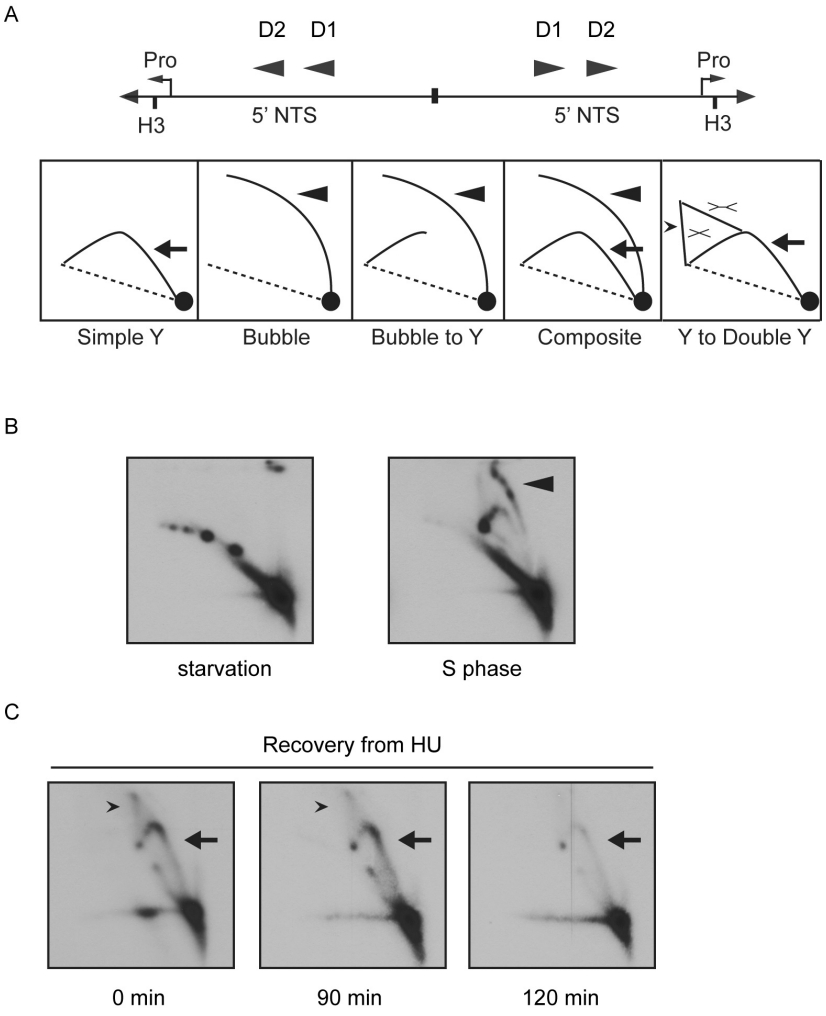

Figure 3.3. Two-dimensional gel analysis of rDNA replication intermediates in cells recovering from HU-induced cell cycle arrest.
(A) Schematic of the *Tetrahymena* palindromic rDNA 5' NTS fragment generated by HindIII digestion, and possible RI patterns following 2D gel electrophoresis. Simple Y arc: passive replication of 5' NTS origins; bubble or bubble-to-Y arcs: initiation within the 5' NTS; composite (simple Y arc and bubbles): active and passive replication of the 5' NTS; x spike and double Ys: converging forks. (B) DNA samples from starvation and S phase cultures were subjected to neutral-neutral 2D gel analysis following digestion with HindIII and enrichment for RIs on BND cellulose. Blots were probed with the rDNA 5' NTS probe. (C) Starved cells were cultured in media containing 20 mM HU for 4 h and then released into media lacking HU. DNA samples were collected at 0, 90 and 120 min after HU removal and subjected to 2D gel analysis as prepared in (B). (See flow cytometry profile and western blot in Fig. 3.1 for cell cycle progression and abundance of Orc1p and Mcm6p).

Activation of an alternative rDNA replication program

Since the rDNA 5' NTS origin was passively replicated in cells exposed to HU, these data indicate that the conventional ORC-dependent origin is inactive. Replication might initiate randomly within the rDNA minichromosome, or from a novel site in the promoter, coding region, or 3' NTS. To address these possibilities, we examined the RI pattern in the adjacent rDNA coding region. If replication initiates within the coding region, a bubble arc should be detected within the 5.5-kb ClaI coding region fragment. This was not the case. Only simple Y arc intermediates were detected in both vegetative S-phase cells and in cells recovery from HU block (Fig. 3.4A), suggesting that the rDNA coding region is passively replicated from origins that reside outside the segment. The passive replication of coding region ruled out the possibility that replication initiated randomly in the rDNA chromosome during the first S phase following HU removal.

To examine whether replication initiated from the 3' NTS, promoter, or both, neutral-alkaline 2D gel electrophoresis was used to determine the direction of fork movement across the rDNA coding region. DNA samples were digested with ClaI, resolved by neutral-alkaline 2D gel, and Southern blotting was performed with probes from opposite ends of ClaI rDNA coding region. Nascent strands were detected with the 5' end probe (Probe 1) and shown as a long diagonal arc from the 5' end ClaI restriction site. In contrast, a short and barely detectable RI was revealed by the 3' end probe (Probe 2). These results suggested that the RIs were initiated proximal to the 5' end of the rDNA coding region, possibly from the promoter region.

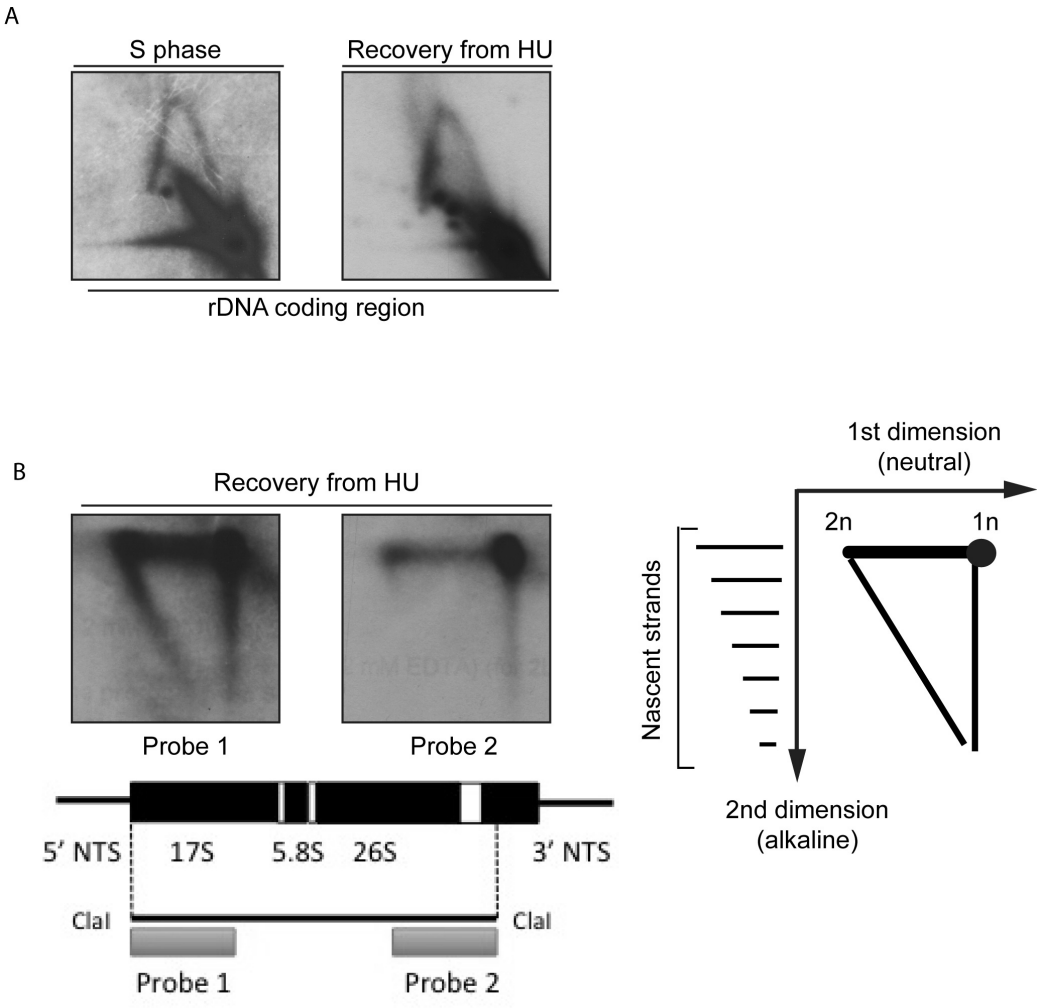

Figure 3.4. rDNA replication following HU removal.
Cells were synchronized in G1 by starvation. The S phase cells were collected after refeeding in the absence of HU for 2 h. The HU recovery cells were obtained by refeeding in the presence of 40 mM HU for 4h and then cultured for 90 min after removal of the drug. (A) Neutral-neutral 2D gel analysis of RIs from vegetative S phase cells and HU recovery cells. Cells were treated with 40 mM HU for 4 h and cultured in drug-free media for 90 min. DNA samples were digested with *Cla*I and enriched for RIs on BND cellulose. A 1.4-kb DNA fragment (nt 5214 to 6676) within the rDNA coding region was used as a probe for hybridization. (B) Neutral-alkaline 2D gel analysis of the direction of fork movement. DNA samples were collected and processed as in (A). BND cellulose enriched RIs were resolved by neutral-alkaline 2D gel electrophoresis {MacAlpine:1997tz} and subjected to Southern blotting with probe 1 (nt 2169 to 3670 of rDNA coding region) or probe 2 (nt 6129 to 7629 of rDNA coding region). Lower panel: schematic of restriction fragments and probes used for neutral-alkaline 2D gel analysis. Right panel: schematic of nascent-strand replication intermediates resolved by neutral-alkaline 2D gel electrophoresis (modified from MacAlpine et al. 1997). The vertical smear is derived from nicked non-replicating DNA, whereas the horizontal smear represents parental strands in RIs of different sizes. The diagonal arc corresponds to nascent-strand replication intermediates from the parental strand by alkali denaturation prior to electrophoresis in the second dimension.

Passive replication of rDNA origin during the recovery from MMS treatment

Unpublished data (Oporto 2011, dissertation) and the above experiments (Fig. 3.1) argued that HU and MMS elicited different checkpoint responses in *Tetrahymena*, since they differentially impact the fate of Mcm6p. Mcm6p was stabilized in cells treated with MMS, presumably allowing for the rapid resumption of DNA replication once DNA damage is repaired. However, Mcm6p is degraded in the presence of HU. To examine the resumption of DNA replication after the removal of MMS, neutral-neutral 2D gel analysis was used to assess RIs originating from the rDNA 5' NTS origin. As shown in Fig. 3.5 and Fig. 3.6, a "composite" RI pattern (a bubble-to-Y arc and a complete Y arc) was seen in cells recovered from 0.03% MMS treatment. Passive replication of 5' NTS origins (complete Y arcs) was more pronounced in cells exposed to 0.06% MMS (Fig. 3.6). Like HU-induced fork arrest, when cells were subjected to higher dose of MMS, the rDNA 5' NTS origin was inactivated and cells utilized an alternative site to initiate replication of the rDNA minichromosome. In addition, X-shaped intermediates were detected in cells under MMS treatment and recovering from MMS, indicating the MMS-treated cells also accumulate recombination intermediates.

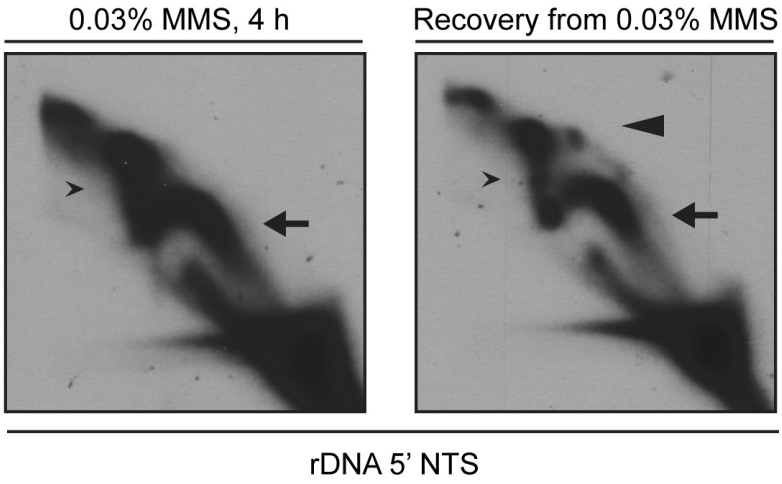

Figure 3.5. Two-dimensional gel analysis of rDNA replication intermediates in cells recovered from MMS-induced intra-S checkpoint response.
Cells were synchronized at G1 by starvation, grown in media containing 0.03% MMS for 4h, and then harvested at 0 and 90 min after MMS removal. DNA were digested with *Hind*III and enriched for RIs on BND cellulose. Blots were probed with the rDNA 5' NTS probe. Left arrow-heads indicate bubble arcs. Arrows indicate simple Y arcs. Right arrow-heads indicate X-spike recombination intermediates.

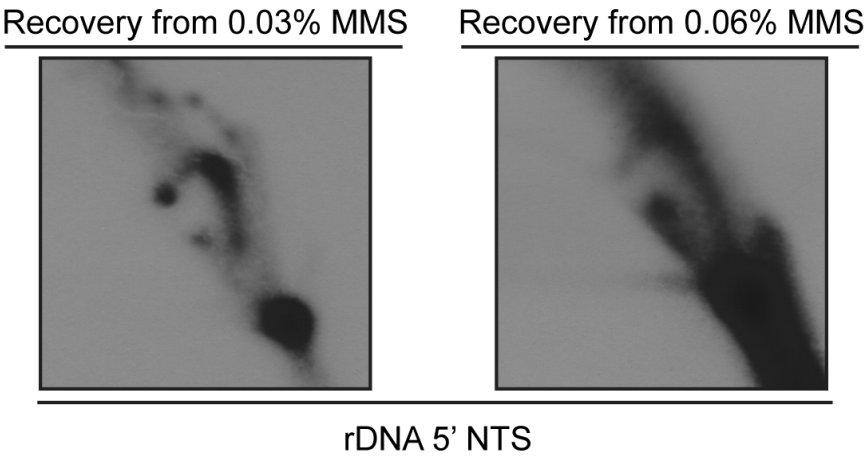

Figure 3.6. Two-dimensional gel analysis of rDNA replication intermediates in cells recovered from MMS-induced intra-S checkpoint response.
Cells were synchronized at G1 by starvation, grown in media containing 0.03% or 0.06 % MMS for 4h, and then harvested at 90 min after MMS removal. DNA were digested with *Hind*III and enriched for RIs on BND cellulose. Blots were probed with the rDNA 5' NTS probe.

Activation of the alternative replication program is ATR and Rad51-independent

Unpublished data (Oporto 2011, dissertation) suggest that genotoxic agents (HU and MMS) activate more than one intra-S phase checkpoint response in *Tetrahymena*. One of these pathways is modulated by caffeine and is responsible for homology-dependent DNA repair mechanisms (as evidenced by the induction of Rad51p). To determine if inactivation of 5' NTS origins occurs through the ATR-mediated pathway, cells were incubated with HU + caffeine or MMS + caffeine, and samples were taken at 90 min after removal of drugs for 2D gel electrophoresis. During the recovery from both treatments, the 5' NTS origins were passive replicated. Furthermore, Rad51p was inhibited by the addition of caffeine (Fig. 3.7). Although the intensity of complete Y arc was less intense in MMS + caffeine treatment, this difference in the amount of RIs may reflect the differential timing of the population entering S phase or the ability to resume DNA replication. In addition, the inhibition of Rad51p by caffeine ruled out the possibility that the passive replication of 5' NTS was induced by Rad51-dependent break-induced replication via homologous recombination (McEachern and Haber 2006). Therefore, inactivation of the rDNA origins is not mediated by ATR.

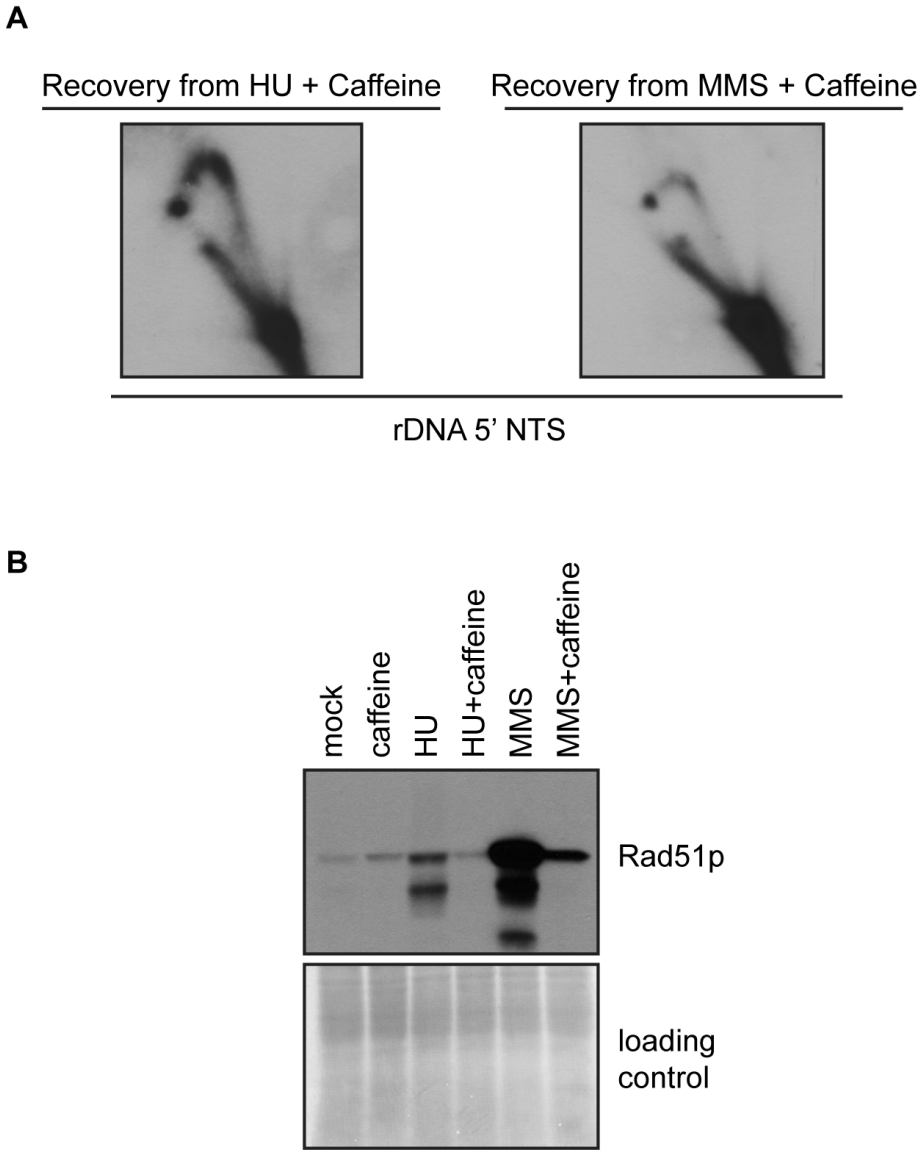

Figure 3.7. The effect of checkpoint inhibition on the initiation of new origin(s) on rDNA chromosome. (A) G1 synchronized cells were grown in the presence of HU (20 mM) + caffeine (1 mM) or MMS (0.06%) + caffeine (1 mM) for 4h, and then harvested at 90 min after removal of treatments. DNA were digested with *Hind*III and enriched for RIs on BND cellulose. Blots were probed with the rDNA 5' NTS probe. (B) Western blot analysis of Rad51p in HU+/- caffeine, MMS+/- caffeine, and mock-treated cells after 4 h treatment.

Discussion

During S phase of the cell division cycle, the genome must be precisely duplicated, with no regions left unreplicated and no regions replicated more than once. To prevent re-replication of DNA, the ability to license new origins of replication by loading MCM2-7 must cease before cells enter S phase. However, if replication forks stall or collapse during S phase, cells cannot respond by licensing new origins but, instead, can only use the origins licensed before entry into S phase. To solve this problem, a growing body of evidence has shown that adjacent dormant origins can be used to facilitate the complete replication of the genome under conditions of replicative stress (Santocanale et al. 1999; Woodward et al. 2006; Ibarra et al. 2008).

The results presented in this chapter reveal a novel DNA replication program that regulates progression through S phase when cells are recovering from replicative stresses. One feature of this DNA replication program is that it occurs under conditions in which ORC and MCM, which are required for site-specific replication initiation and fork elongation, are dramatically diminished under HU treatment. During the recovery from HU-induced cell cycle arrest, cells were competent to complete S phase, yet the abundance of ORC and MCM remained undetectable until the next cell cycle. Another intriguing and unprecedented finding from this study is that the rDNA origin used exclusively during the S phase of vegetative cell cycle and developmentally programmed gene amplification is suppressed when these replication proteins are selectively degraded under replication stresses. Instead, an alternative pathway is used to ensure complete genome replication once the source of replication stress is removed.

The primary function of the intra-S checkpoint response is to coordinate cell cycle arrest with DNA repair to prevent genome instability. When replication forks encounter DNA damage, the replicative helicase will uncouple from DNA polymerase and this action results in the accumulation of single-stranded DNA (ssDNA) bound by RPA, which is a major signal for downstream events, including fork repair and checkpoint activation. The MCM complex is an essential replicative helicase required for the initiation and elongation phases of DNA synthesis. It has been reported that MCM proteins contribute to fork stability and repair during replication arrest induced by HU (Bailis 2004; Labib et al. 2000). In human cells, Mcm4p is phosphorylated in the presence of HU or after exposure to UV irradiation, and ATR-CHK1 as well as CDK2 kinases are involved in this phosphorylation (Ishimi et al. 2003), which leads to the inactivation of MCM4-6-7 helicase activity. Therefore, phosphorylation of Mcm4p in the checkpoint control inhibits DNA replication, which includes blockage of DNA fork progression, through inactivation of the MCM complex. In this study, I found that degradation of MCM is favored over phosphorylation in *Tetrahymena* cells in response to HU treatment (Fig. 3.3A). This result suggests that the decrease in the abundance of Mcm6p during HU treatment may be used as an alternative strategy different than other eukaryotes to inactivate MCM helicase to prevent further unwinding the DNA under replication stress.

Another remarkable finding in this study is that, not only the MCM complex, but also ORC was degraded under HU treatment. Since the primary function of ORC is to nucleate an initiation competent pre-RC at origins, it is possible that the degradation of

ORC is used to suppress late origin firing and prevent further encounters of forks with DNA damage. Evidence from several model systems suggests that HU treatment slows polymerase activity before the replisome starves for nucleotides. In budding and fission yeasts, HU-treated replication forks synthesize 5 to 10 kb of DNA before they stall completely at early S phase (Aparicio et al. 1997; Sabatinos et al. 2012). In Fig. 3.1, FACS analysis shows the DNA content of *Tetrahymena* cells under HU treatment is maintained at G1 phase value, which is different from other eukaryotes that are arresting at early S phase. In addition, an ongoing DNA fiber analysis in our lab found that there is no BrdU incorporation in the presence of HU. Thus, in addition to the degradation of MCM helicase to abrogate elongation of existing forks when cells are exposed to HU, ORC also undergoes rapid degradation to restrict further replication initiation.

Since the two essential factors for replication initiation and elongation are dramatically reduced in response to HU treatment, we were compelled to ask whether the amount of these two factors would be restored when HU is removed. Remarkably, the amitotic macronucleus resumes DNA replication soon after HU removal and the timing and length of S phase appears normal with no indication of replication stress. However, both Orc1p and Mcm6p levels are not restored prior to the first S phase and cell division during the recovery. The absence of Orc1p implies that alternative DNA binding proteins or incomplete ORC complexes are sufficient to initiate and support DNA replication during the recovery period. However, Orc2p was also found to be coordinately regulated with Orc1p under HU treatment (Fig. 3.2), which argues against the above assumption. Interestingly, Park and Asano reported that the endoreplication in

Drosophila ovarian follicle cells can occur independently of ORC. In contrast to our study, the MCM helicase is still required in flies (Park and Asano 2008).

In eukaryotes, a striking feature of the MCM complexes is that they are loaded onto DNA in a 10 to 100 fold excess over the number of DNA-bound ORC and replication origins, and are distributed along the chromatin (Hyrien et al. 2003). However, normal replication rates are maintained when the number of MCM molecules is reduced (Lei et al. 1996; Crevel et al. 2007). This observation is termed the "MCM paradox", which was originally found in *Xenopus* egg extracts. A recent study tested the cellular effects of systematic depletion of MCM2-7 in *Drosophila* S2 cells, and the data showed that reducing MCM2-6 levels had no significant effect on cell cycle distribution or viability. However, depletion of MCM7 caused an S phase arrest (Crevel et al. 2007; 2011). In our study, although currently we don't know if the entire MCM complex is diminished under HU treatment, the results from the *Drosophila* study suggest two explanations for the role of the MCM complex in *Tetrahymena*: (1) Some of the cellular MCM subunits (including MCM6) may be redundant for DNA replication during the recovery from HU treatment. (2) There may be an unknown substitute for Mcm6p to maintain the MCM helicase function during the recovery period. For example, a new new human Mcm gene (MCM8) has been identified (Gozuacik et al. 2003) and shown to form a homo-hexameric helicase. MCM8 has been shown to function in the elongation step of DNA replication (Maiorano et al. 2005).

Coincident with the degradation of ORC, the rDNA origin is not active during the exposure to HU and the S phase following HU removal. Instead, DNA replication

initiates elsewhere in the rDNA minichromosome. A simple explanation for this behaviour is that upon ORC degradation in response to HU, the ORC-dependent rDNA origin initiation is inactivated. Instead, an ORC-independent initiation mechanism is activated to ensure that all of the DNA in the region is eventually replicated.

The *Tetrahymena* rDNA minichromosome contains repeated type I elements in the 5' NTS, which are required for replication and amplification. The type IA and IB elements colocalize with the origins in domain 1 and 2, while type IC and ID elements are proximal to the promoter. We previously discovered that *Tetrahymena* ORC contains an integral 26T RNA subunit, which forms Watson–Crick base pairs with the type I element at the rDNA origin (Mohammad et al. 2007). ChIP analysis with epitope-tagged Orc1p and chromatin pull downs with aptamer-tagged 26T RNA revealed that ORC selectively associates with origin sequences but not with the promoter-proximal type I elements or the 26S RNA coding region, which is fully complementary to 26T RNA (Donti et al. 2009). Remarkably, 2D gel electrophoresis analysis showed that mutations in 26T RNA disrupt the base pairing with the origin sequences and diminish the rDNA origin utilization. Instead, initiation events occur distal to the domains 1 and 2 origins, as 5' NTS origins are passively replicated, suggesting the choice of initiation sites is altered during the normal cell cycle when ORC can not utilize RNA–DNA base pairing to associate with origins. This behavior is consistent with our present findings that the site-specific initiation from the rDNA origin is abolished when ORC is degraded in response to HU.

Break-induced replication (BIR), or recombination-dependent DNA replication, appears to play a key role in the repair of stalled and broken replication forks to continue the elongation of DNA strands (McEachern and Haber 2006). In BIR, the invading Rad51 filament serves to denature the duplex DNA, which might allow the MCM helicase to load independently of ORC (Lydeard et al. 2010). It is not clear how MCM2-7 is recruited to the donor template since ORC and Cdc6 are not required for BIR. In *Saccharomyces cerevisiae* checkpoint-deficient mec1-1 cells, replication forks are incapable of resuming DNA synthesis after the removal of HU, and cells suffer extensive chromosome breakage (Feng et al. 2009). To examine if the DNA replication during the recovery period is recombination-dependent, we treated cells with HU +/- caffeine and then monitored replication initiation by 2D gel electrophoresis. In contrast to the result in the budding yeast, the 2D gel analysis in Fig. 3.7 showed that *Tetrahymena* cells were capable to restart the DNA synthesis when the ATR-mediated checkpoint was inhibited by caffeine, in all likelihood via a recently discovered caffeine-insensitive signaling pathway (Oporto 2011, dissertation). In addition, a previous study from our lab demonstrated that cell viability was comparable between HU and HU + caffeine treatments (Yakisich and Kapler 2006), which also supported the existence of the novel intra-S phase checkpoint pathway. Moreover, the 2D gel analysis detected that the 5' NTS origin was passively replicated during the recovery period, suggesting that the resumption of HU-arrested forks was not regulated by the recombinase Rad51-dependent mechanism.

CHAPTER IV

SUMMARY AND DISCUSSION

DNA replication in eukaryotic cells is a highly regulated process to ensure that the genome is replicated only once per cell cycle. This process is regulated by both cis-acting replication determinants within the DNA, as well as trans-acting factors that interact with these DNA sequences. The primary regulation of DNA replication acts at the initiation of DNA synthesis, when replication origins are licensed by assembly of the pre-replicative complex (pre-RC) in early G1. Pre-RC formation is initiated by recognition of DNA sequences at the origin by the heterohexameric origin recognition complex (ORC), and ORC then recruits Cdc6, Cdt1 and the DNA replicative helicase MCM2-7 complex to form a pre-RC at the origins.

However, eukaryotic chromosomes are still constantly subjected to DNA damage by endogenous and exogenous sources, to which eukaryotes have developed an intra-S phase checkpoint that safeguards the genome by suppressing late origin firing and stabilizing stalled replication forks (reviewed in Harrison and Haber 2006). This checkpoint response is activated by the apical kinase, ATR, which binds to exposed stretches of single strand DNA generated by uncoupling of the replicative helicase MCM2-7 and polymerase. On activation, the ATR checkpoint pathway immediately inhibits replication initiation and elongation across the entire genome through phosphorylation of the MCM complex (reviewed in Nam and Cortez 2011).

Because DNA replication and the intra-S phase checkpoint are intimately linked,

pre-RC proteins have long been proposed to play roles in checkpoint signaling (reviewed in Lau and Jiang 2006). However, the exact mechanistic relationship between pre-RC and checkpoint signaling remains unclear. In my dissertation research, I set out to study the effects of depletion of ORC1 on checkpoint activation and genome stability in the mitotic micronucleus and amitotic macronucleus of vegetatively growing *Tetrahymena* (Chapter 2). Reciprocally, the effects of replication stress on the regulation of ORC1 and initiation of DNA replication were also examined (Chapter 3).

The work presented in Chapter 2 showed that down regulation of ORC1 by targeted gene disruption leads to the coordinate down regulation of ORC2 and components of the MCM2-7 complex (Fig. 2.1), suggesting that the abundance of different pre-RC components is tightly coordinated. In addition, this ORC1 knockdown strain failed to activate an intra-S phase checkpoint in response to DNA damaging agents (Fig. 2.5), resulting in global genome instability in both macronucleus (Fig. 2.3) and micronucleus (Fig. 2.6). Previous studies in other eukaryotes have documented that the establishment of replication forks during S phase is required for checkpoint activation (Tercero et al. 2003; Caldwell et al. 2008). With this in mind, I hypothesize that defective checkpoint activation in *Tetrahymena* is the consequence of fewer active replication forks, such that the threshold of S phase checkpoint activation cannot be achieved.

To determine the degree to which the partialy depletion of ORC1 reduces fork number, a DNA fiber method (Kaufman et al. 2011) is being developed in our lab to address this question. In this method, cells will be sequentially labeled with the

halogenated nucleoside precursors, 5-chloro-2-deoxyuridine (CldU) and 5-iodo-2-deoxyuridine (IdU), which incorporate into actively replicating DNA. DNA fibers will be generated by lysing cells on a glass slide and tilting the slide to generate a moving fluid meniscus that straightens the fibers. The pattern of halogenated nucleoside incorporation can be visualized with fluorescence-labeled secondary antibodies under an immunofluorescent microscope. The global origin firing frequency in wild type and ORC1 knockdown cells will be determined by measuring the inter-origin distance in individual fibers (Fig. 4.1). According to my hypothesis, in which the depletion of ORC1 leads to a decrease in steady state levels of other pre-RC components and fewer established pre-RCs for replication initiation, the expected result from the DNA fiber analysis should display that the average inter-origin distance in the ORC1 mutant strain is longer relative to wild type to reflect the activation of fewer origins.

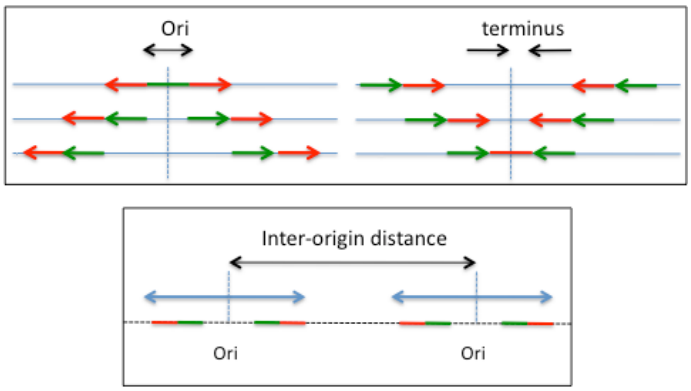

Figure 4.1. Schematic representation of DNA fiber analysis.
Molecules with newly fired origins (top left) or terminating forks (top right). Green: CldU pulse, red: IdU chase.

To expand the lines of this study and further characterize the role of ORC during *Tetrahymena* development, I examined the regulation of ORC and MCM proteins throughout development and the effects of depleting ORC1 on the alternative DNA replication programs, endoreplication and rDNA gene amplification. Consequently, I found that ORC and MCM proteins are coordinately regulated during development, in which Orc1p and Mcm6p levels rise and peak during the micronuclear DNA replication cycles in early development and drop during the macronuclear development when genome-wide endoreplication and rDNA gene amplification occur (Fig. 2.7). This result is inconsistent with the scenario that replication initiation and fork elongation are temporally uncoupled, as was reported for the amplified chorion gene locus in *Drosophila* follicle cells. During the amplification of *Drosophila* chorion genes in follicle cells, ORC2 foci are observed at stage 10, and not in stage 11 embryos. However, BrdU incorporation continues until stage 13 embryos, indicating that chorion gene amplification exhibits "onion skin" replication (Calvi and Spradling 1999). A PCR analysis revealed that the chorion gene origin region and 5 kb of flanking DNA are replicated during stage 10B. During stage 11/12 these forks are subsequently elongated to amplify a total of 40 kb of DNA.

In addition, the abundance of ORC and MCM proteins does not parallel the amount of DNA that needs to be replicated. Compared with vegetative G1-phase cells, the Orc1p levels increase by 2.5-fold during micronuclear DNA replication cycles but the DNA mass that needed to be replicated drops by 2.4-fold. In contrast, when macronuclear DNA mass increases from 8C to 64C during endoreplication phase II, the

Orc1p levels drop dramatically by 30-fold compared with micronuclear replication cycles, despite a 6-fold greater DNA mass that needed to be replicated. All these results suggest that the micro- and macronuclear DNA replication programs during development are fundamentally different from one another, and that they further differ from the respective micro- and macronuclear DNA replication programs in the vegetative cell cycle. In supporting this, hemizygous ORC1 progeny exhibited defects in endoreplication and selective amplification of the rDNA minichromosome (Fig. 2.8).

It has been reported that DNA binding specificity will be affected by the ratio of ORC to DNA. In early *Xenopus* embryos, the high ratio of ORC/DNA allows DNA replication to initiate randomly throughout the chromosomes. In contrast, a low ORC/DNA ratio favors initiation at sites with the high affinity for ORC (Kong et al. 2003). I hypothesize that the dynamic change in the ratio of ORC to DNA during *Tetrahymena* development corresponds to the differential origin usages across different stages of development.

To address whether the origin usage are differentially regulated during development, DNA fiber analysis will be used to compare the inter-origin distance in cells collected during the S phase of vegetative cell cycle, micronuclear replication cycles (meiosis and post-zygotic replication), and the endoreplication phase I and II during development. In addition, a modified chromatin fiber analysis (Kaufman et al. 2011) will be used to visually examine the distribution of ORC and MCM proteins in relation to active sites of DNA replication. In this analysis, the DNA precursor (BrdU or CldU) will be incorporated into the replicating DNA at the timing determined by the

DNA fiber analysis and FACS analysis. Then nuclei will be treated under less denaturing conditions (as compared to the above DNA fiber method) to release chromatin proteins and be spread onto a glass slide. ORC1 or MCM6 proteins can be detected by immunostaining with the fluorescein-conjugated antibody. By overlapping the fluorescent signal of these replication proteins with the labeled DNA tracks, I will be able to determine the contribution of ORC and MCM at different DNA replication programs during development and the vegetative cell cycle. I predict that during micronuclear DNA replication (meiosis & post-zygotic replication) and macronuclear endoreplication phase I, the high ORC/DNA ratio makes replication initiate randomly, which will be reflected by a shorter inter-origin distance value from the DNA fiber analysis. While during endoreplication phase II, the replication events restricted to the preferred sites, and a corresponding longer inter-origin distance will be anticipated.

The work presented in Chapter 2 argues that ORC1 is a rate-limiting factor for both micro- and macronuclear DNA replication. In Chapter 3, I expanded the study to further examine the fate of ORC and MCMs under replication stress. I found that ORC and MCM6 proteins are selectively degraded under HU treatment while the cell cycle is arrested at G1 phase. During the S phase following drug removal, the entire genome is replicated without replenishment of ORC and MCM6 proteins. Degradation of these proteins also impacts where replication initiates. Normal vegetatively growing cells initiate replication from two genetically defined origins in the 21 kb ribosomal DNA (rDNA) minichromosome. However, these origins are inactive in cells that are recovering from genotoxic stress, and replication initiates elsewhere within the rDNA

chromosome. This study led to the discovery of a novel DNA replication program that is activated in cells recovering from genotoxic stress.

I performed 2D gel electrophoresis to examine the replication intermediates from the rDNA 5' NTS origin in cells under and recovering from HU treatment. Surprisingly, passive replication (a simple Y arc pattern) of the 5' NTS origin was detected in cells exposed to HU for 4h. This result suggests that the ORC-dependent 5' NTS origin is inactive and, instead, replication initiates elsewhere within the rDNA chromosome upon cells exposed to HU, in which ORC1 protein is dramatically diminished. When cells were refed with fresh media to recover from HU block, the cell cycle was resumed at normal rate and this passive replication pattern is still detected during the S phase. This result suggests the 5' NTS origin is still inactive and the novel replication program is dominant. Currently we don't know whether this novel replication program utilizes the forks stalled by HU to resume the DNA synthesis, or restarts replication by firing of new origins. Therefore, a detailed time course experiment still needs to be done by 2D gel analysis to examine when the 5' NTS origin becomes inactive and the novel replication program is activated during the course of HU treatment. These data will be correlated with ORC1 protein levels determined by western blot analysis to address the impact of Orc1p degradation on origin utilization.

In addition, the DNA fiber analysis will be applied to examine the fate of HU-stalled fork during recovery. Log phase cells will be pulse-labelled with IdU for 15 min, washed and blocked in HU for 4 h, and then pulse-labeled with CldU for 15 min. Afterward, DNA spreads will be prepared and analyzed by immunofluorescence. To

determine the replication fork restart by elongating the HU-stalled forks, or by firing of new origins, the amount of tracts with continuous IdU and CldU labeling (elongating stalled forks), discontinuous IdU and CldU labeling (firing of new origins), and IdU only (firing of new origins) will be quantified. The mock treated log phase cells will be the control. In yeast, HU-treated replication forks synthesize 5 to 10 kb of DNA before they completely stall at early S phase (Aparicio et al. 1997; Sabatinos et al. 2012). Therefore, a preliminary experiment has been done in our lab to confirm that there is no IdU or CldU incorporation during the 4-h HU incubation period to cause the two labels to become separated. This strategy will allow us to determine whether alternative origins are activated during the course of HU treatment or during the recovery period.

In Chapter 3, western blot analysis was performed to quantify the abundance of ORC1 and MCM6 proteins under HU treatment and after HU removal (Fig. 3.2B). Remarkably, ORC1 protein levels in cells recovering from HU treatment drop 50-fold relative to the mock-treated G1 phase cell. Even in cells that reached the second G1 phase, there is still a 5-fold reduction for the amount of ORC1 protein. This result resembles the DNA replication program in the endoreplication phase II during development, in which both ORC1 and MCM6 protein levels drop ~12-fold, compared with cells in G1 phase (Fig. 2.7). Since the 2D gel analysis revealed that a novel replication program is activated in cells that have 50-fold reduction of ORC1 protein levels by HU treatment, it will be interesting to investigate if a similar replication program is used when ORC1 protein is down-regulated by ~5-fold in the ORC1 knockdown strain, and in the endoreplication phase II during development when ORC1

protein levels transiently drops by ~12-fold. Two dimensional gel electrophoresis was performed to access the origin activation in the ORC1 knockdown strain, and the result is shown in Fig. 2.4. The pattern of replication intermediates in DNA isolated from log phase wild type and ORC1 knockdown strains was indistinguishable, indicating that rDNA origin site selection and fork progression were not perturbed when ORC1 protein was decreased by 5-fold (Fig. 4.2).

However, this is not the case when ORC1 protein level is decreased by ~12-fold during the endoreplication phase II. In the 2D gel analysis examining RIs in the amplifying rDNA minichromosomes during endoreplication phase II (Fig. 4.2; 2, 4 and 8 h after refeeding with medium; the experiment was done by another graduate student in the lab, Xiangzhou Meng), an abnormal RI pattern was detected with a Y-shape structure in which the region close to the fork branch point migrated faster than a normal complete Y arc and ascended from the arc of linear fragment instead of the 1N spot. This RI pattern mimics the fork intermediates that accumulate single-stranded DNA gaps at the branching point due to the annealing of mRNA to the DNA template to generate unreplicated DNA (Alzu et al. 2012). Currently, we are working on dissecting this abnormal RI with nuclease treatment to see if this faster-migrating Y-shape structure can be converted to a normal Y arc, which indicates a passive replication from the 5' NTS origin. If a passive replication from the 5' NTS origin is seen, it suggests that there is a novel DNA replication program in *Tetrahymena* activated under the conditions in which ORC and MCM are limiting, in the likelihood through an ORC-independent mechanism.

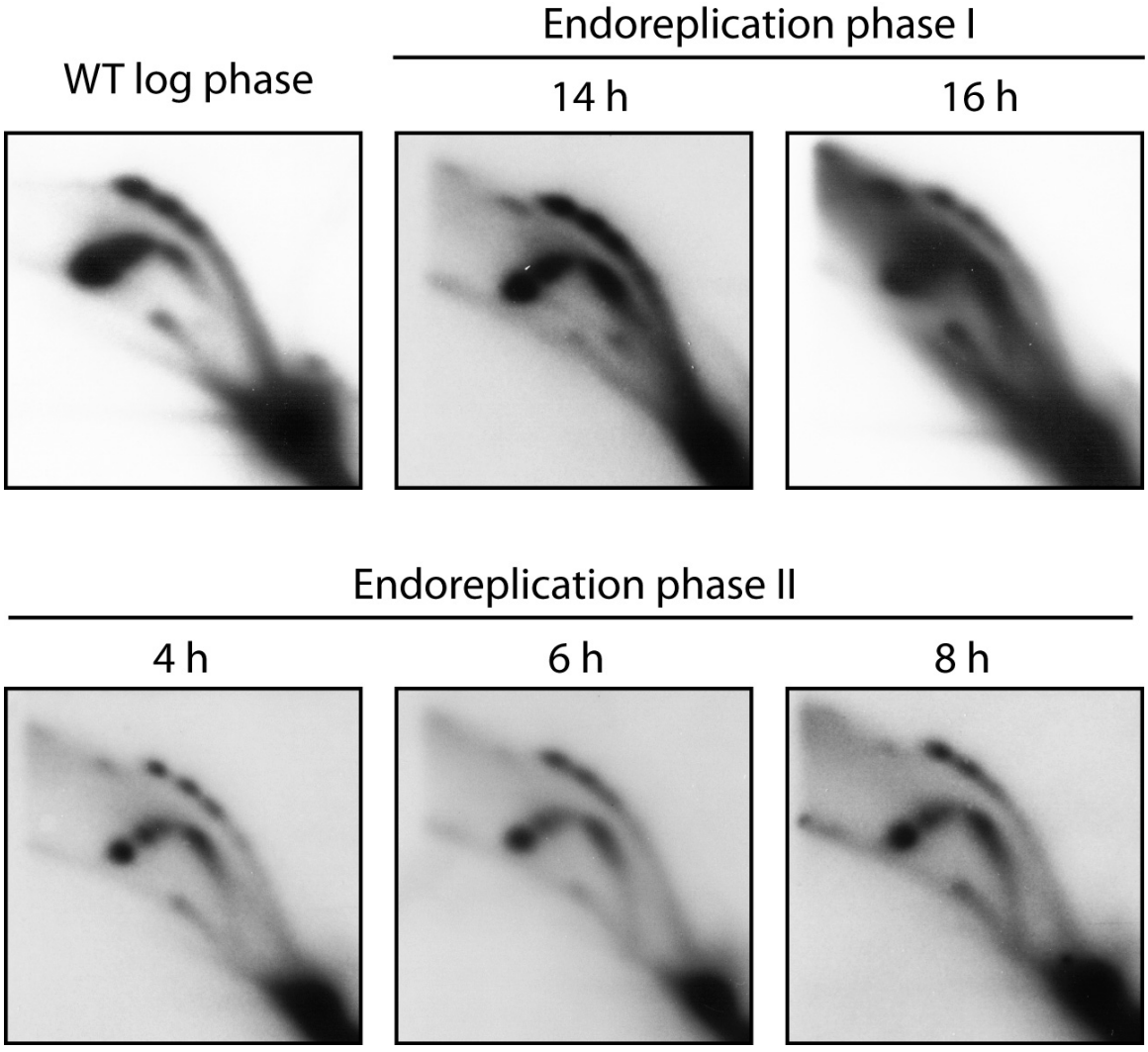

Figure 4.2. Two-dimensional gel analysis of the 5'NTS replication intermediates in rDNA minichromosomes during development.
Strains SB4202 and CU428 were mated, and DNA was isolated at various times during macronuclear development (hours after initiation of mating are indicated). SB4202 is a heterokaryon strain, with two copies of the integrated C3 rDNA allele in the germline micronucleus and B rDNA minichromosome in the macronucleus. In matings between a wild type C3 (SB4202) and a B (CU428) strain, C3 rDNA was amplified during macronuclear development of progeny cells. C3 rDNA amplification intermediates and vegetative C3 rDNA (log phase homokaryon SB4204 as the control for C3 rDNA replication in the macronucleus) were analyzed by 2D gel electrophoresis. DNA samples were digested with *HindIII*, enriched on BND cellulose, and then digested with *SphI*, which cuts within the B rDNA 5'NTS. (This experiment was done by Xiangzhou Meng)

Another way to test whether this novel DNA replication is ORC-dependent and map the localization of the alternative origin would be ChIP analysis. However, since ORC protein levels are dramatically diminished under HU treatment, it is difficult to utilize ChIP analysis to examine how origins are populated by Orc1p during the recovery period. In Fig. 3.5 and Fig. 3.6, 2D gel analysis revealed that the 5' NTS origin is passively replicated in cells recovering from MMS treatment. MMS and HU have been shown to trigger different signaling pathways to regulate ORC and MCM protein levels (Oporto 2011, dissertation). I can take advantage of abundant Orc1p levels in MMS-treated cells to perform ChIP analysis to explore the ORC binding sites in the rDNA minichromosome. The mapping can be narrowed down to the rDNA promoter region according to the data from 2D gel analysis of the direction of fork movement (Fig. 3.4). If no Orc1p enrichment is detected, then we can argue that this is an ORC-independent replication.

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