Regulatory Mechanisms That Define Precise DNA Replication Origin Utilization

ELIZABETH SUZANNE DORN

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Approved by

Dr. Jeanette Cook

Dr. Jason Lieb

Dr. David Kaufman

Dr. Aziz Sancar

Dr. Brian Strahl

ABSTRACT

ELIZABETH SUZANNE DORN: Regulatory Mechanisms That Define Precise DNA Replication Origin Utilization

(Under the direction of Dr. Jeanette Gowen Cook)

Each time a cell divides its DNA must be replicated so that a complete genome is passed on to each daughter cell. To duplicate the entire genome within a single S phase, eukaryotic cells initiate replication at multiple sites, termed origins. All potential origins require recruitment and assembly of a pre-replication complex (preRC). ORC, Cdc6, and Cdt1 are coordinated to facilitate loading of the final preRC component, the MCM complex. Once MCM is loaded, an origin is prepared or "licensed" for replication. There are many mutually reinforcing mechanisms that regulate replication to ensure that an exact copy of DNA is created, and that genome instability is avoided. All origins share three regulatory stages: origin licensing, initiation, and inhibition of preRC assembly. Nevertheless, origins are not utilized identically; they fire asynchronously in S phase, are utilized with varying efficiencies, and are differentially prone to re-firing. This dissertation investigates the mechanisms that define precise replication at individual origins.

In this work, a novel method to detect re-replication at the single molecule level was developed and this method revealed that a portion of origins in untransformed cells undergo re-replication. Furthermore, the baseline level of re-replication is increased in cancer cells. These studies also implicate the chromatin

environment, most extensively H3K4me, as a critical factor in regulating origin activity. These observations provide insight into the replication program and will be valuable in understanding how the cell maintains a stable genome to avoid oncogenesis.

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LIST OF ABBREVIATIONS

ACS – ARS consensus sequence

APC – Anaphase promoting complex

ARS – Autonomously replicating sequence

ATP – Adenosine triphosphate

BrdU – Bromo-deoxyuridine

Cdc6 – Cell division cycle 6

CDK – Cyclin-dependent kinase

Cdt1 – Cdc10 dependent transcript 1

ChIP – Chromatin immunoprecipitation

Chk1 – Checkpoint kinase 1

Chk2 – Checkpoint kinase 2

CldU – Chloro-deoxyuridine

COMPASS – Complex associated with Set1

CTD – C-terminal domain

DDK – Dbf4-dependent kinase

ENCODE – Encyclopedia of DNA elements

FISH – Fluorescent in situ hybridization

GINS – Go, ichi, ni, and san complex

GST – Glutathione-S-Transferase

HAT – Histone acetyltransferase

Hbo1 – Histone acetyltransferase binding to ORC

IdU – Iodo-deoxyuridine

MCM – Minichromosome maintenance complex

MLL1 – Mixed-lineage leukemia complex

NHF – Normal human fibroblast

NURF – Nucleosome remodeling factor

ORC – Origin recognition complex

PHD – Plant homeo domain

preRC – Pre-replication complex

siRNA – Small interfering RNA

UV – Ultraviolet radiation

Chapter 1

Introduction

BIOLOGICAL SIGNIFICANCE

Cancer is a widespread disease that affects people of every age, race, and gender. In the United States, it is estimated that 41% of people will be diagnosed with cancer at some point in their life. Trends indicate that 1.5 million new cases of cancer were reported in 2010 and that approximately 570 thousand people died due to cancer-related causes [1]. Since 1990, mortality rates have consistently declined in part due to insight gained from research focused on the disease. Unfortunately, cancer still remains the second leading cause of death for people in the United States [2]. In order to address this high mortality rate, researchers must continue to investigate the underlying causes of cancer so that we can improve prevention recommendations, detection methods, and treatment options.

Each time a human cell divides, over three billion base-pairs must be replicated. This duplication must be efficient and rapid while also being precise. Replication must also be limited to the appropriate phase in the cell cycle. In cancer cells, exact duplication and maintenance of the genome of is severely perturbed. Mutations that lead to improper expression of replication-associated proteins are often found in cancers and dysregulation of these proteins can lead to uncontrolled cellular proliferation. Furthermore, failure to produce an exact copy of the DNA during replication can lead genome instability, which contributes to tumor formation. To understand how normal cells avoid transformation, this work focuses on understanding the mechanisms that precisely regulate DNA replication at individual origins to maintain stability of the genome.

EUKARYOTIC CELL CYCLE

To ensure that the genetic material is passed from the parent cell to the daughter cells without error, a cell progresses through four defined stages known as the cell cycle (Fig 1.1). These four stages ensure that the genome is duplicated in its entirety, and that the identical copies are segregated exactly between the two daughter cells. The cell begins in gap 1 phase (G1 phase) where it prepares the DNA for duplication of its genome. The cell then enters synthesis stage (S phase) where the DNA is duplicated, or replicated, completely. Following S phase is gap 2 phase (G2 phase). During this time, the cell confirms that replication of the genome is complete and accurate, and it also prepares for mitosis (M phase). In mitosis, the duplicated genetic material is segregated and distinct nuclei are formed. This is followed by cytokinesis in which two genetically identical daughter cells are formed.

DNA REPLICATION ORIGINS

One of the most critical tasks that must be accomplished during G1 phase of the cell cycle is preparation of the DNA for replication. In order to duplicate the entire genome in S phase, eukaryotic DNA replication initiates at hundreds of sites in budding yeast and thousands of sites in mammalian cells, termed origins. Although many aspects controlling DNA replication initiation are highly conserved across eukaryotes, origins in *S. cerevisiae* are more defined than their higher eukaryotic counterparts.

Replication origins in *S. cerevisiae,* termed autonomously replicating sequences (ARS), were identified by their ability to maintain transformed

extrachromosomal plasmids [3, 4]. The ARS elements contain several cis-acting elements including an ARS consensus sequence (ACS), which is a conserved 11 base-pair sequence [5]. Despite the requirement that all potential budding yeast origins contain an ACS, only a small subset of the ACS sequences function as active origins in the context of the chromosome, suggesting that sequence alone cannot define origins [6, 7].

Mammalian origins have proven more challenging to identify, because in contrast to yeast, a sequence-specific origin element does not exist. Nevertheless, mammalian origins do show some preference for localization within certain types of sequences such as AT-rich regions, dinucleotide repeats, and asymmetrical purine-pyrimidine sequences [8]. It has been suggested that within these types of sequences, mammalian origins function as large initiation zones rather than discrete loci. Origins in *X. laevis* egg extracts, though not dependent on a specific sequence, are still spaced at a regular distribution across the genome indicating that there are mechanisms in place to ensure an initiation program that will support complete replication [9].

Despite the variable methods for determining origin location, all eukaryotic origins undergo the same three distinct regulatory phases that are coordinated with the cell cycle. First, origins are “licensed” for replication by assembly of the pre-replication complex (preRC). Second, at the G1-to-S phase transition, licensed origins are fired by protein kinase-mediated recruitment of initiation factors to individual origins. Finally, origins that have fired are prevented from re-firing by inhibiting the reloading a preRC prior to the next cell division (Fig 1.2).

Origin Licensing: Assembly of the preRC

In every eukaryote, all potential origins must prepare for initiation by assembling a preRC in late M/early G1 phase. PreRCs are formed in a step-wise manner beginning with the origin recognition complex (ORC) binding to DNA. The cell division cycle 6 (Cdc6) and the cdc10-dependent transcript 1 (Cdt1) proteins are then recruited and with ORC they coordinate the loading of the minichromosome maintenance complex (MCM) onto chromatin. Once MCMs are loaded, an origin is said to be "licensed" for replication [10-12].

Origin recognition complex (ORC)

ORC is a complex comprised of six members, Orc1-6. ORC was initially identified in *S. cerevisiae* as selector of origins through its interaction with the ACS elements [13]. It identifies potential origins by binding to specific locations on the genome. Although ORC binding to DNA is partially sequence-specific in yeast, this specificity for a particular sequence is lost in other organisms (they lack ACS elements). Nevertheless, ORC and its role in initiation are conserved across all eukaryotes including *X. laevis*, *D. melanogaster*, and human cells [14-16]. In fact, tethering assays in mammalian cells revealed that ORC localization to any location on the DNA is sufficient to confer origin potential for that particular site [16]. However, ORC is commonly found at unused origins and at silenced chromatin indicating that although ORC binding is necessary for origin function, additional factors are needed to induce *utilization* of an origin [17, 18]. In addition to selecting

origins, ORC serves as the platform for loading other preRC factors, Cdc6 and Cdt1. ORC is an ATPase, and its ability to bind ATP is required to facilitate both Cdc6 recruitment and MCM loading onto chromatin [19, 20]

Cell division cycle 6 (Cdc6)

A screen performed in *S. cerevisiae* for mutants with defective progression through the cell cycle first identified Cdc6 [21]. In yeast, cells with mutant Cdc6 arrest at the G1-S phase transition implicating Cdc6 in replication initiation, and footprinting analysis revealed that Cdc6 is required for the formation and maintenance of the preRC [22-24]. Transcription of the human and yeast *cdc6* gene is cell cycle regulated and its protein expression oscillates accordingly [25]. Human Cdc6 expression peaks at the end of G1 phase and mitosis while APC-mediated degradation occurs in early G1 phase [26]. This expression profile helps ensure that preRC assembly is limited to the appropriate time in the cell cycle. Cdc6, like ORC, is an ATPase, and the coordinated ATP hydrolysis activity of the two proteins is required for loading of the final licensing factor, MCM, onto chromatin [27].

Cdc10 dependent transcript 1 (Cdt1)

Originally isolated in *S. pombe*, the expression of Cdt1 is cell cycle regulated and is dependent on the cdc10 transcription factor [28]. Its expression is highest from the end of M phase through the beginning of S phase during which time it is recruited to origins in an ORC-dependent manner [29, 30]. Depletion of fission yeast Cdt1 in early S phase (but not late S phase) prevents the completion of DNA

synthesis and demonstrates the need for Cdt1 in licensing [29]. The requirement for Cdt1 in licensing is conserved across many species including *S. cerevisiae*, *X. laevis*, and in mammalian cells [31-33]. Coordinating with Cdc6, Cdt1 interacts with MCM through its C-terminal domain and acts as a shuttle, bringing multiple MCM complexes to chromatin to complete preRC assembly [30, 33, 34].

Minichromosome maintenance complex (MCM)

MCM is the final component of the preRC to be recruited to origins. It is a six member (mcm2-7) ring-shaped complex that was identified in a screen of mutants that showed defective maintenance of a transformed minichromosome [35]. Like the other preRC components it is conserved across all eukaryotes. The human mcms are E2F-regulated and their expression peaks at the end of mitosis [36, 37]. As stated above, MCM is recruited to the chromatin through the concerted efforts of Cdc6 and Cdt1, and its loading onto chromatin is dependent on the ATPase activities of both ORC and Cdc6 [27, 33]. Once MCM is loaded, the origin is licensed; at this point, ORC, Cdt1 and Cdc6 become dispensable for replication initiation [19, 38, 39]. MCM travels with replication forks and is presumed to be the replicative helicase [40-42]. Interestingly, although only 1-2 MCM complexes per origin are needed to complete S phase, they are typically loaded in excess of the number that is required for normal replication in unperturbed cells [19, 43-45]. It is speculated that the excess MCM complexes are utilized at normally dormant origins during times of replicative stress [46, 47].

Origin initiation: "Firing"

PreRC assembly is completed in G1, and the loading of MCM designates all potential origins. In untransformed cells, a checkpoint ensures that a sufficient level of licensing has occurred before progression through the cell cycle continues [48]. However, individual origins must recruit additional factors for an origin to actually initiate or "fire". To transition from a licensed state to an active one, two types of kinases mediate the recruitment of initiation factors and limit their loading to the proper phase.

Initiation factors & polymerases

After loading of the preRC complex members, additional proteins are recruited to origins that are critical for unwinding the DNA and initiating synthesis. These factors include Cdc45, the Go Ichi Ni San complex (GINS), Mcm10, and the replicative primase pol α. One of the rate-limiting factors for origin firing is Cdc45. Cdc45 is required for both replication initiation and elongation [49]. Unlike MCM which is loaded in excess, only two Cdc45 molecules are loaded for every chromatin-bound ORC [50]. Cdc45 is an excellent marker for origin activation because its binding is coincident with firing [51, 52]. Proper loading of Cdc45 is dependent on the DDK-mediated phosphorylation of MCM, CDK activity, and the GINS complex [50, 53]. Cdc45 interacts with both MCM and pol α and because it binds origins prior to polymerase association, Cdc45 is presumed to play a role in coordinating the replication fork [54, 55].

The GINS complex is also an essential protein that is found at the fork and is important for unwinding the DNA [56]. Loss of any GINS subunit or Cdc45 in *D. melanogaster* leads to an accumulation of G1/S cells demonstrating its role in initiation and elongation [57]. GINS and Cdc45 compose a scaffold for coordinating units of the mcm2-7 motor. The GINS, Cdc45, and MCM proteins form a stable complex that is integral for the recruitment of replication polymerases to origins [56-58]. Finally, MCM10 is thought to coordinate the replication fork [10, 50, 59]. MCM 10 is recruited to the chromatin in two steps. First, it is recruited to chromatin before Cdc45 in order to facilitate Cdc45 binding to chromatin [60]. Mcm10 also forms a soluble complex with pol α to help stabilize and target the polymerase to origins [61].

Kinases

Dbf4-dependent kinase (DDK) and cyclin-dependent kinases (CDKs) play a large role in the formation of the initiation complex and activation of replication origins. Cdc7 and Cdk2 (yeast Cdk1), the catalytic components of the complexes are required for replication initiation [54, 62-64]. The kinase activity of both Cdc7 and Cdk2 is high in S phase when expression of their binding partners, Dbf4 and cyclin E (yeast Clb5/6) peaks [10].

Cdc7 phosphorylates several replication factors, and genetic and biochemical evidence shows that Cdc7 is required for replication initiation [54, 62, 63]. Cdc7 targets include pol α, Cdc45, and MCM [65-67]. The concerted action of Cdc7 and CDK to phosphorylate MCM facilitates Cdc45 loading onto chromatin [68-70]. Evidence suggests that phosphorylation of MCM induces a conformational change

which stimulates the MCM to interact with and subsequently load Cdc45 [71]. Furthermore, the *mcm5-bob1* mutation bypasses the need for yeast Cdc7 likely because the mutation confers a conformational change that mimics the phosphorylation-induced structural change [71].

Similar to DDK, CDK activity is cell cycle regulated through a binding partner, in this case a cyclin. Like DDK, CDK is required for replication initiation [64]. CDKs have been shown to physically interact with a variety of preRC components and these physical interactions likely help recruit the kinase to chromatin [10]. The targets of CDK include several replication factors including DNA primase pol α, MCM and in yeast Sld2 and Sld3 [50, 72]. In yeast, Sld2 and Sld3 are targets of CDK phosphorylation and this is required for replication initiation [73]. The Dpb11, Sld3, Cdc45 and GINS proteins assemble onto origins in a mutually dependent manner to initiate replication [53]. This assembly requires CDK-mediated phosphorylation of Sld2 and Sld3. Dpb11 binds Sld2ph and Sld3ph and stimulates the Sld2-Dpb11-GINS-pol ε factors to bind to Sld3-Cdc45. The Sld3ph also stimulates the Cdc45-MCM interaction to promote initiation [74] Despite extensive work, the order in which these kinases act remains unclear, but it is clear that they are critical for triggering initiation [75].

PreRC inhibition: preventing origin re-firing

Origins are prepared for replication in late M and G1 phase by assembly of the preRC and cells inactivate preRC formation upon entrance into S phase to prevent re-licensing. Re-licensing must be avoided to ensure that re-replication and

genome instability are limited. To maintain the preinitiation and postinitiation state as two distinct phases, the cell employs a variety of tactics including regulation of protein activity, expression, degradation, and cellular localization.

In addition to facilitating recruitment of initiation factors, CDKs are also critical for inactivating preRC components to prevent re-licensing. Depletion of the mitotic CDK in fission yeast leads to re-replication [76]. Likewise, increased Cdk2 in an *X. laevis* cell-free system inhibits replication initiation [77]. These outcomes illustrate the role of CDK in preventing re-initiation.

The role of CDK in preventing aberrant preRC formation is conserved. For instance, Cdc6 is phosphorylated by CDK in late G1/S phase. This phosphorylation signals for the degradation of the protein in budding yeast, and induces nuclear export in mammalian cells to block preRC formation [78-80]. During normal progression through S phase, human Cdt1 is also a target of the cyclin E/Cdk2 complex to regulate preRC assembly. Cdt1 phosphorylation promotes $CRL1^{skp2}$(an E3 ubiquitin ligase) – mediated degradation of Cdt1 in fission yeast and in human cells [81, 82]. In budding yeast CDK-dependent phosphorylation MCM results in the nuclear export of MCM and Cdt1 [31, 83, 84]. The CDK-dependent phosphorylation and nuclear export in yeast also prevents re-licensing by blocking interactions between MCM and ORC, and Cdc6, [84, 85]. There is also evidence that CDK targets ORC to prevent re-licensing [86]. Recent studies show that CDK blocks MCM recruitment through steric and phosphorylation-dependent inhibition of Cdt1 binding [87].

Although budding yeast relies largely on CDK-dependent mechanisms to prevent re-licensing, higher eukaryotes utilize additional methods as well. Metazoan Cdt1 is phosphorylated and targeted for degradation through the $CRL4^{Cdt2}$ E3 ubiquitin ligase pathway [88, 89]. In addition to nuclear export in S phase, metazoan Cdc6 is down regulated in late mitosis by APC-mediated degradation [26]. Cdt1 is also regulated by a cell cycle dependent interaction with geminin. Geminin expression is high in S and G2, and its binding to Cdt1 prevents Cdt1 from binding Cdc6 and MCM. This prevents recruitment of MCM to chromatin during this phase of the cell cycle [33, 90, 91].

Consequences of aberrant preRC regulation

The regulation of the preRC is extensive in order to pass an exact copy of DNA onto each daughter cell. Any aberrations in proper regulation of these factors can have real and dire consequences (Fig 1.3). Insufficient preRC assembly by overexpression of geminin, depletion of MCM, or depletion of Cdc6 & Cdt1 leads to cell cycle arrest, and ultimately cell death [47, 48, 92]. Furthermore, inactivation of Cdc7 prevents cell cycle progression in mammalian cells [93] . Perhaps more serious are the potential consequences of too much origin licensing. In budding yeast, improper activation of Cdc6 and ORC combined with deregulated MCM nuclear export results in origin reinitiation [86]. Human cancer cell lines that overexpress Cdt1 or have limited geminin also show an increase in overall DNA content and induce double-strand breaks and the DNA damage response [94-96].

Certain normal human cells also promote re-replication when ORC and Cdt1 or Cdc6 and Cdt1 are simultaneously overexpressed [97].

A long-term consequence of aberrant replication is genome instability which is a marker of cancer [98]. Several replication factors have been linked to cancer. Elevated levels of Cdc6 and Cdt1 have been observed in tumors and in cancer-derived cell lines indicating that improper regulation of origin-associated proteins may contribute to tumorigenesis [99-101]. Overexpression of mouse or human Cdt1 or human Cdc6 in premalignant cells led to cellular transformation and formation of tumors in mice that had been injected with the cells [100, 102]. Furthermore, there is evidence that improper regulation of replication leads to genome instability. Deregulated licensing by overexpression of Cdt1 in *Drosophila* egg extracts showed evidence of head-to-tail fork collision and the generation of chromosome fragmentation and short re-replications [103]. The yeast CDK inhibitor Sic1 was demonstrated to prevent genome instability by promoting licensing in late G1 [104]. The balance of Cdt1 and geminin has also been demonstrated to be critical in maintaining genome stability; the depletion of geminin led to centrosome overduplication [105, 106]. Together, these studies illustrate that deregulation of replication factors can lead to re-replication and also that cancer cells often have deregulated replication factors.

However, a major challenge to the field as been directly linking the aberrant re-replication mediated by deregulated preRC to tumorigenesis. Under conditions of massive re-replication and genome instability, the cell undergoes apoptosis. In contrast, we presume that moderate levels of aberrant preRC regulation mediates

re-replication that leads to genome instability. This hypothesis is supported by the appearance of tumors in xenograft models that overexpress Cdt1, but show an undetectable increase in overall DNA content by canonical methods [100, 102]. Also, recent work in yeast shows that re-replication can induce the initial steps of gene amplification [107]. Therefore, more precise methods are needed for mammalian cells to directly attribute tumor development to re-replication and also to help discern where in the cell cycle the aberrant replication is occurring.

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Origins act independently

Paradox:

The re-replication induced by improper preRC regulation leads to an increase in the overall DNA content of a cell. Interestingly, the increase in DNA content is variable from cell to cell and rarely is a complete doubling of the content of the unperturbed cell. This argues that only a portion of the DNA has been re-replicated; it indicates that some origins have re-fired while others have not. Up until this point, I have treated origins as though they are uniform. All origins assemble a preRC, they recruit the same initiation factors, and they are prevented from re-firing by the same inhibitory mechanisms. And yet they don't all re-replicate. Therefore we are

presented with a paradox; how do origins act independently when they share the same regulatory steps?

Re-replication, origin identity, origin use

In addition to the variable propensity to re-replicate, there are many additional features of origin activity that illustrate their propensity to be regulated independently. First, not all potential origins initiate at the same time in S phase. While all potential origins assemble a preRC in G1, origins can be characterized as early, mid, or late origins based on the time they fire in S phase. Although defining specific origins is elusive in mammalian cells, nearly 80% of human initiation zones are predicted to display temporally specific firing [108]. In yeast, confirmed ARS elements have been demonstrated to consistently fire at variable points in S phase. For instance, ARS822 consistently fires late in S phase while ARS315 consistently fires in early S phase [109-111]. The characteristic timing of human and yeast origins lends further support to the prediction that mechanisms beyond the preRC exist to regulate origin function.

Moreover, origin use and efficiency varies greatly. Some origins initiate in every cell cycle, while others rarely fire [112]. In yeast, chromatin immunoprecipitation (ChIP) studies have shown that some ORC binding sites never initiate replication [18]. Furthermore, as previously discussed, metazoans have origins that remain dormant and are activated only during times of replicative stress [47, 113]. While it is well documented that Cdc45 and DNA polymerases mark

active replication, the factors that regulate the timing and efficiency of origin firing are unknown.

Furthermore, preRC regulation does not explain how ORC selects the genomic sites that become potential origins. Budding yeast origins contain ACS sequences, but there are 30 times more occurrences of this sequence in the yeast genome than *bona fide* origins; thus functional origins are not defined by an ORC-binding sequence alone [5, 50].

Additional regulation

Together these observations indicate that additional elements beyond preRC regulation and nucleotide sequence are important for defining origin location and regulating origin function. Over the past several years, differences in local chromatin structure have been implicated in defining origins and controlling their activity.

CHROMATIN STRUCTURE AND REPLICATION INITIATION

In order to fit into the nucleus, DNA is highly compact, and is formed into units called nucleosomes. Nucleosomes consist of 147 base-pairs of DNA wrapped around a histone octamer. Each histone octamer contains four core histones H2A, H2B, H3 and H4 (Fig 1.4) [114]. Histones, DNA, and other DNA-associated proteins are collectively referred to as the chromatin. Because the DNA of a cell is so highly compact, chromatin must be extremely organized and dynamically regulated to provide access to particular regions of DNA at specific times. Histones are subject to post-translational modifications including acetylation, methylation, ubiquitination,

and sumoylation, which can alter interactions between chromatin-associated proteins and the DNA [115]. Also, histone remodelers can affect access to DNA by physically altering the DNA-histone interaction. Nucleosome positioning and post-translational modifications of histones have been shown to play a role in regulating a variety of cellular processes including transcription, chromosome silencing, DNA repair, and replication fork progression [116, 117].

In recent years, differences in local chromatin structure have been implicated specifically in defining origins and in controlling their activity. Although it remains largely unclear how the changes work in conjunction, or by what mechanism they affect origins, significant correlations have been identified linking nucleosome positioning, histone acetylation, and histone methylation to origin use (Fig 1.5).

Nucleosome positioning

Nucleosome mapping studies in both yeast and human cells have shown that early replicating regions are most frequently located in open, nucleosome-free regions [118]. In budding yeast and fission yeast, highly efficient origins are correlated with sites of nucleosome depletion, and firing efficiency was severely reduced in fission yeast when nucleosomes were allowed to encroach upon an origin by deletion of a nucleosome-disfavoring element, polyA(20) [118, 119]. A system that shifted nucleosomes even further away from origins demonstrated, however, that initiation of replication requires nearby nucleosomes as well [120]. These observations suggest that nucleosomes must be exactly positioned for accurate origin activity. Corroborating this model, disruption of proper regulation of the histone remodeler

FACT limited the effectiveness of MCM loading (preRC assembly) at early origins [121]. Thus, it is clear that nucleosomes must be precisely positioned near, but not on origins; what determines where nucleosomes are positioned?

Utilizing high throughput ChIP-seq, Eaton *et al.* identified 238 sequences in the budding yeast genome that ORC does *not* bind despite bearing sequences predicted to be highly compatible with ORC binding and therefore origin function [122]. Nucleosome positioning at these non-origin sites differed from that of functional (ORC-binding) origins. Both classes of sequences intrinsically repel nucleosomes; however the ORC-binding site of the functional sequences is located asymmetrically in the nucleosome-free region and further, nucleosomes are positioned with a high degree of periodicity. Also, the *bona fide* ORC-binding sequences include the ORC-binding site followed by an A-rich element, but the non-origin sites did not. Interestingly, the nucleosome-free region is 90 base-pairs larger than the ORC footprint. The additional sequence elements may maintain a larger open region to accommodate MCM loading [122]. Together, these studies indicate a role for DNA sequence in positioning the nucleosomes near origins. Even human replication initiation zones, which lack a consensus ORC-binding sequence, are AT-rich, suggesting that sequence may also function in positioning nucleosomes at human origins.

Nevertheless, strict nucleosome positioning and phasing, which are characteristics of the most highly efficient origins (in yeast), cannot be explained by sequence elements alone. Not only do higher eukaryotes such as human cells lack canonical origin-identifying sequences altogether, but even budding yeast requires

additional factors such as ORC to maintain proper nucleosome localization. In the absence of ORC, nucleosomes shifted inward toward, but did not cover, the ORC binding sequence, and the periodicity of nucleosomes near origins was reduced [123]. ChIP studies in budding yeast indicated that in addition to DNA interactions, ORC interacts with nucleosomes through the N-terminal BAH domain of the Orc1 subunit of ORC for positioning nucleosomes and stable association with chromatin at select origins. Thus, both ORC-DNA and ORC-chromatin interactions may contribute to determining nucleosome positioning and where ORC will stably bind to establish origin location [124].

Histone acetylation

The emerging appreciation for the importance of chromatin structure in replication function prompted experiments to determine which histone modifications can be found at origins. Acetylation has been extensively linked to stimulation of DNA replication initiation. Early-firing origins are often found in hyperacetylated and highly-transcribed regions, while late-firing origins are often localized to heterochromatic regions which are depleted for histone acetylation [125, 126]. In addition, Hbo1, an H4 histone acetyltransferase is required for replication [127]. Furthermore, several groups provide evidence that changing the acetylation status at an origin changes the timing of its firing. Stimulation of replication by histone acetylation is highly conserved and has been observed in follicle cells of *D. melanogaster*, *X. laevis*, and human cells [125, 128, 129]. At specific erythrocyte development stages, artificial recruitment of the histone acetylase or deacetylase to

the human β-globin locus accelerates or delays, respectively, origin-firing [130]. In yeast, upon inhibition of the histone deacetylase Rpd3, or tethering of an acetylase Gcn5 to late-firing origins, global levels of acetylation are increased, and late firing origins are shown to fire earlier in S phase [131, 132]. Specifically, the Rpd3L complex, which gets targeted to specific origins is known to be the dominant Rpd3 at these sites [133].

Together, these observations illustrate a role for acetylation in promoting firing; but by what mechanism does acetylation act? Which replication factors are affected, and how are they affected? Yankulov et al. have shown that limiting global Gcn5-mediated acetylation induces delays in origin firing by inhibiting proper preRC formation [134]. Similarly, deletion of the Sir2 deacetylase promotes assembly of the preRC [135]. Hbo1 facilitates MCM loading and deregulation of the Hbo1:Cdt1 interaction can induce re-replication [136, 137].

Acetylation may promote preRC assembly by recruiting additional proteins. Lysine acetylation is known to recruit several factors including, for example, the RSC remodeling complex. This complex contains bromo domains, which recognize acetylated lysine residues, and mutation of the bromo domains was demonstrated to inhibit proper cell cycle progression [138]. Alternatively, acetylation can function directly to induce an open chromatin state. Acetylation of lysine neutralizes the positive charge. This disrupts the histone:DNA and histone:histone interactions to induce a more open chromatin state. Histone acetylation can regulate DNA-associated processes by multiple mechanisms. There are several examples where acetylation has been implicated in changing the chromatin structure around origins.

For instance, acetylation of H3K56 is a marker for nucleosome deposition and exchange. Early origins of replication have shown high levels of nucleosome exchange during G1 that is decreased in M phase, pointing to H3K56ac-mediated chromatin dynamics in regulating firing [139]. The Sir2-mediated mechanism for inhibiting firing also functions through maintenance of chromatin structure and is not simply an indirect effect of origins localized to heterochromatin. Sir2-sensitive origins share a common structure where an inhibitory element is near to the B2 regulatory sequence. These inhibitory elements lead to nucleosome positioning that is unfavorable for preRC formation. Increased H4K16ac as a result of Sir2 inactivation promotes origin firing by disrupting the unfavorable nucleosome positioning and opening the chromatin for preRC formation [140]. These are just a few of the acetylation-associated effects that function at origins, and new links are continuously being identified. For instance, several new correlations between origins and acetylated histone residues were identified this past year, including H4K79ac H3K23ac, and H4K4ac [141]. It is clear that histone acetylation plays an important role in promoting origin firing.

Histone methylation

Although the positive role of histone acetylation at origins has been well documented, acetylation alone cannot be the only chromatin modification that affects replication. Histone acetylation is enriched at promoters of active genes, however not all promoters contain origins and not all origins are near promoters [142, 143]. Furthermore, if acetylation were the only chromatin element origins required, then

deacetylated regions such as heterochromatin and telomeric regions would exclude origins, but functional origins have been identified in these regions [144].

In addition to histone acetylation, several methylated histone lysine residues have been found near origins. In particular, novel marks such as methylation of H3K37 and H2BK111 have been identified and correlated with origins along with several well-studied marks such as methylation of H3K4, H3K36, and H4K20 (Fig. 1.5) [141, 145]. Unlike acetylation, which is typically associated with a general opening of chromatin and active genes, methylation has been shown to both activate and repress transcription and replication [115, 146]. Additional complexity stems from the fact that the extent of methylation on a particular lysine can have opposite effects [147, 148]. Thus, diverse histone methylation events may function along with acetylation to control the precise sequence of events that are required for efficient but regulated origin firing.

Histone H3K36 methylation

Genome-wide studies of budding yeast chromatin found that trimethylation of histone H3 lysine 36 (H3K36me3) is low at early-firing origins relative to late-firing origins [146]. The abundance of H3K36me3 at origins also decreases throughout S phase at the same time that monomethylation of H3K36 (H3K36me1) increases. These observations correlate H3K36 methylation with early or late replication, but do individual H3K36 methylation states directly affect replication? Studies that disrupt all forms of methylation at H3K36 have suggested opposing answers to this question. For example, deletion of Set2, the H3K36 methyltransferase, suppresses

the replication stress phenotype of a mutant form of FACT, a remodeling factor known to promote replication, at least at replication forks. This genetic interaction is consistent with H3K36 methylation playing a *negative* role in replication [149]. On the other hand, H3K36 methylation was required for the accelerated S phase phenotype caused be deletion of the histone deacetylase, Rpd3 [146]. Deletion of Set2 also resulted in a delay in the recruitment of the replication initiation factor, Cdc45, to origins [146]. Although the change was subtle, this result indicates that H3K36 methylation may play a *positive* role in replication. Subsequent investigation suggested a means to reconcile these seemingly conflicting conclusions.

A variety of replication parameters suggest that H3K36me1 plays a positive role in regulating replication initiation whereas H3K36me3 plays a negative role. For instance, reduction in H3K36me3 by overexpression of the human tridemethylase JMJD2A resulted in earlier replication initiation at select origins [150]. Also, the recruitment of the origin initiation factor, Cdc45, to yeast origins was correlated with high levels of H3K36me1 but low levels of H3K36me3 [146]. Additional work shed light on how H3K36me3 may mediate an inhibitory effect. Eaf3 associates with both H3K36me3 and the Rpd3S deacetylase [151]. H3K36me3 is already known to be linked to Rpd3-mediated deacetylation in actively transcribed genes [151]. Since histone acetylation is positively correlated with origin firing, H3K36me3 may inhibit origin firing through the Eaf3-mediated recruitment of the histone deacetylase, Rpd3, and the resulting reduction in acetylation and chromatin accessibility [151]. In support of this model, S phase progression was accelerated in the absence of Rpd3 or the H3 trimethyl-binders Eaf3 and Nto1 [146]. Furthermore, overexpression of the

human H3K36me3 demethylase results in reduced H3K36me3 and increased chromatin openness [150]. It still remains to be determined if all of the effects of H3K36 methylation are attributable to histone deacetylase recruitment. Interestingly, the S phase accelerating effects of Rpd3 deletion required H3K36 methylation, suggesting a role for H3K36 methylation that is independent or downstream of Rpd3. It is also not clear if all effects on S phase progression are due to events at origins or if H3K36 methylation affects replication fork progression and therefore S phase length.

Histone H4K20 methylation

Studies of cell cycle-dependent changes in global histone modifications in human cells found that histone H4K20me1 is high in late mitosis and throughout G1 but low in S phase [152]. Depletion of Set8 (PR-Set7), the enzyme responsible for H4K20me1 in humans, causes replication stress and cell cycle arrest suggesting that H4K20me1 may be important for replication [153, 154]. Experimental manipulations leading to aberrant persistence of Set8, and therefore H4K20me1, *during* S phase resulted in extensive genome re-replication, a phenomenon characterized by re-firing of origins within a single cell cycle [155, 156]. Furthermore, H4K20me1 can be detected at each of a select group of tested human origins.[157] Thus the question arises: What role does H4K20 methylation normally plays in origin function?

Tethering Set8 to an artificial locus by expression of a Gal4 fusion protein not only induced ectopic H4K20me1, but also promoted preRC formation at that site

indicating a positive role for H4K20me1 in defining origin location or promoting preRC assembly [157]. It is not yet clear how H4K20me1 promotes origin identification, but H4K20 methylation has been detected in conjunction with acetylation on lysines 5, 8, and 12 of histone H4, which themselves are thought to promote preRC assembly by facilitating DNA accessibility. One model for the role of H4K20me1 proposes that a burst of H4K20me1 in mitosis leads to an increase of H4 acetylation in G1 which then facilitates preRC formation [157].

As with H3K36 methylation, the mechanism by which H4K20me1 affects recruitment of replication proteins to origins is still unknown. H4K20me1 may promote a particular chromatin structure at origins that is compatible with replication factor recruitment. It is also possible that the different methylation states, either alone or in combination with other marks, are recognized specifically by a replication protein through a mechanism similar to that used by 53BP1 which binds to H4K20me2 as part of the checkpoint response.[158] Alternatively, these marks may function in a signaling cascade leading to other histone modifications, such as lysine acetylation, that ultimately promote origin licensing.

In contrast to the suggested stimulatory role of H4K20me1, H4K20me2 may function to inhibit re-licensing of origins in S phase. Unlike H4K20me1 which declines in S phase, H4K20me2 (catalyzed in humans by the Suv4-20h1 and Suv4-20h2 enzymes) increases during S phase, thus potentially replacing the permissive monomethylation mark with dimethylation [158]. Interestingly, the simultaneous presence of H4K20me2 and H4K16ac at early replicating domains in S phase persisted until it was removed in the following G1 when preRC formation occurs

[159, 160]. This dual mark of the same histone H4 molecule was also detected in *D. melanogaster*, where H4K16ac was enriched at origins containing H4K20me2 [140, 161-163]. This association could implicate H4K20me2 (like H4K16ac) in maintaining an open, but also inactive or poised chromatin state with respect to origin licensing. The presence of H4K20me2 in cells undergoing a normal S phase (i.e. without re-replication) indicates that H4K20me2 may inhibit re-licensing of origins during S phase either directly or by replacing the monomethylation mark. Additionally, the association with H4K16ac suggests that H4K20me2 (and possibly H4K20me3) may prevent re-licensing of origins in S phase without limiting origin accessibility for replication in subsequent cell cycles. While deletion of Suv4-20h has been shown to have major consequences for proliferation in both mice and *Drosophila, [158]* assays specifically monitoring replication are needed to determine if H4K20me2 or H4K20me3, like H4K20me1, directly impact origin function.

Histone H3K4 methylation

Histone modification localization data gathered by the ENCODE consortium have identified a correlation between high levels of H3K4 trimethylation (H3K4me3) and early replicating regions in human cells [108, 164]. In particular, regions shown to replicate early in the cell cycle were enriched for H3K4me2/3 specifically while late-replicating sites were depleted of H3K4me2/3 [165]. While these correlations may be partially influenced by gene density at the resolution of the ENCODE projects, evidence from other organisms also supports a role for H3K4me at origins. For instance, H3K4me3 has been correlated with distinct sets of ORC-binding sites

in *D. melanogaster* [166]. The H3K4me3 mark was originally identified at the 5' ends of actively transcribed genes and has thus been intensively studied for its role in gene expression [167, 168]. Less is known about the dynamics of H3K4 methylation specifically at origins however, and it remains to be determined if these marks play direct roles in origin activity.

The enrichment of H3K4me3 near early-firing origins compared to late-firing origins suggests a positive role in regulating firing rather than origin licensing, since both early and late-firing origins are licensed together in G1. ChIP studies of budding yeast examined the location of H3K4me3 genome-wide in synchronized cells. As cells progressed through S phase, H3K4me3 was lost at early-firing origins before late-firing ones [169]. ChIP analysis at a subset of the few defined human and monkey origins in synchronized cells found a similar enrichment of H3K4me3 at known early-firing origins compared to late-firing origins [170]. Interestingly, both studies also demonstrated transient increases in H3K4me3 during origin firing. This H3K4me3 spike occurred at origins specifically, not generally across all chromosomal locations [170].

Correlation between H3K4me3 localization to origins and replication initiation has clearly been established; but it is still unclear whether H3K4me3 regulates replication or if replication regulates the placement of H3K4me3. Technical hurdles currently make it difficult to precisely determine if the changes in H3K4 methylation precede or follow origin firing. That is, it remains unknown whether the mark regulates firing or is a result of the firing itself.

MULTIPLE LAYERS OF ORIGIN REGULATION

The significant amount of effort the cell expends to regulation replication initiation is an indicator of how important this process is. Though all origins undergo the same phases of preRC regulation, they still act independently. The importance of the nucleosome environment has been extensively linked to transcription, DNA damage repair, and more recently studies have correlated aspects of the chromatin with replication initiation. It remains largely unclear, however, whether these marks contribute to the regulation of replication initiation, or if they are simply consequences of the replication process itself. In this work, a new tool was developed to examine re-replication of origins at the level of single molecules and established that the propensity of individual origins to re-fire is variable. This work also explored the role of the chromatin environment, and focused most extensively on characterizing the role of H3K4 methylation specifically in origin regulation.

Figure 1.1

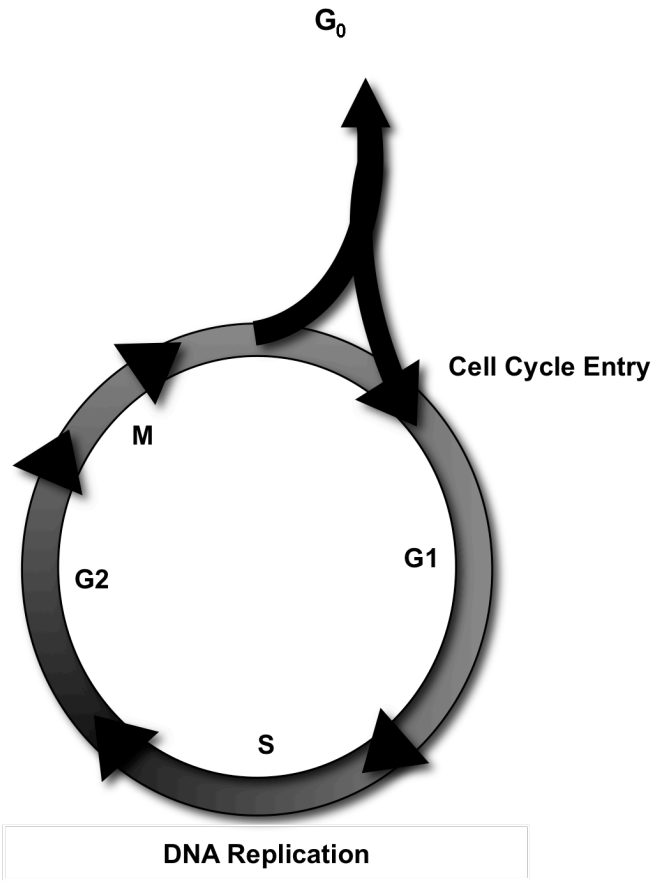


Figure 1.1 The eukaryotic cell cycle. A cell progresses through four phases, termed the cell cycle, when proliferating. The cell begins in gap 1 phase (G1) where the DNA is prepared for duplication. This is followed by synthesis phase (S) where the DNA is replicated. Following S phase is gap 2 phase (G2). During this time the cell confirms that DNA has been duplicated and prepares for cell division. Finally the cell enters mitosis phase (M) where the genetic material is segregated, and this is followed by cell division.

Figure 1.2

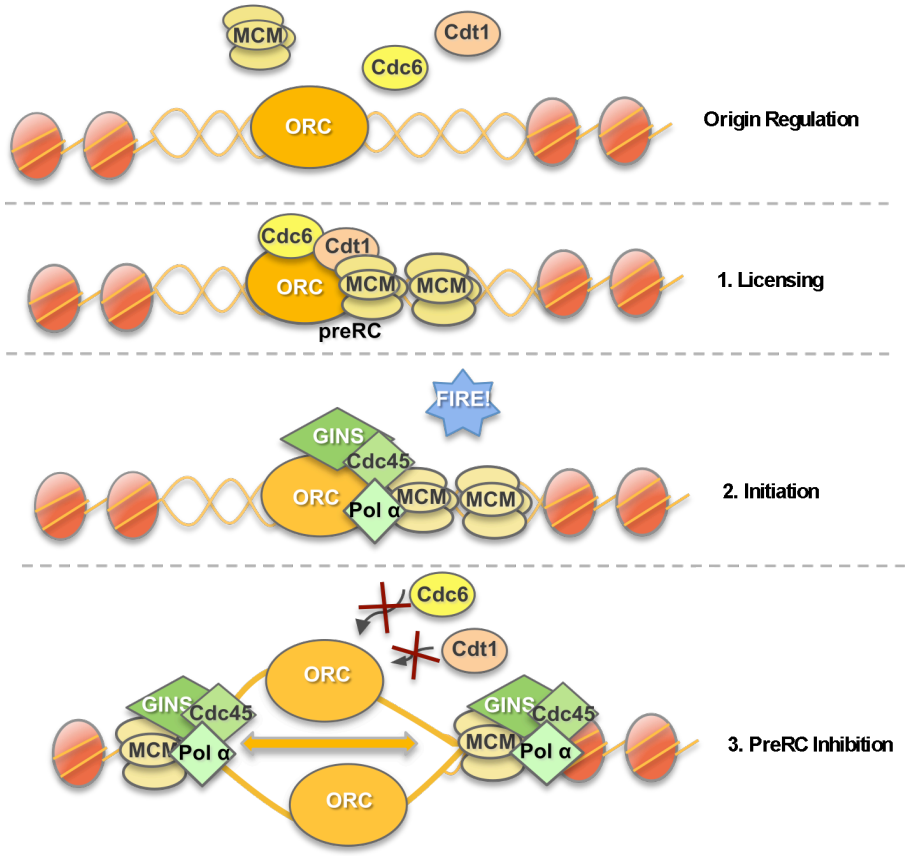

MCM
Cdt1
Cdc6
ORC
Origin Regulation
Cdc6
Cdt1
ORC
MCM
MCM
preRC
1. Licensing
FIRE!
GINS
Cdc45
ORC
Pol α
MCM
MCM
2. Initiation
Cdc6
Cdt1
ORC
GINS
Cdc45
MCM
Pol α
GINS
Cdc45
MCM
Pol α
ORC
3. PreRC Inhibition

Figure 1.2 PreRC regulation at origins. A schematic of the regulatory phases of DNA replication that occur at origins. An origin is licensed for replication when ORC, Cdc6, and Cdt1 have loaded the MCM complex. This four member complex is the pre-replication complex (preRC). DNA replication is then initiated upon recruitment of the GINS complex, Cdc45, and DNA pol α. Finally, preRC formation is inhibited from the end of G1 until the beginning of the subsequent G1.

Figure 1.3

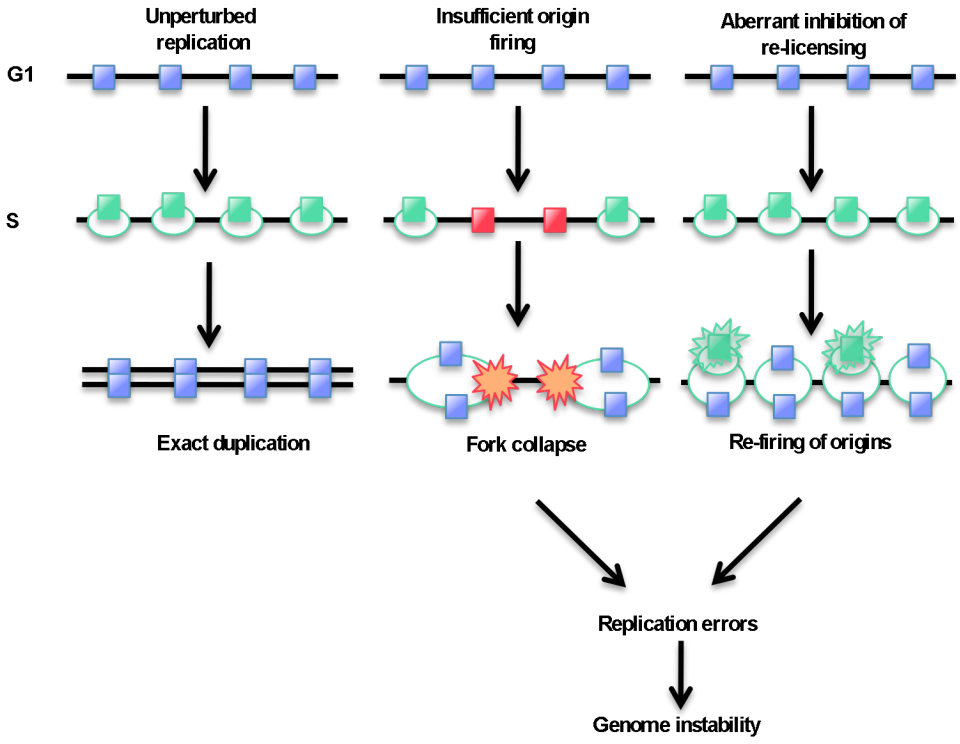

Unperturbed replication
Insufficient origin firing
Aberrant inhibition of re-licensing
G1
S
Exact duplication
Fork collapse
Re-firing of origins
Replication errors
Genome instability

Figure 1.3 Aberrant origin licensing. All potential origins are licensed by the assembly of a preRC in G1, and DNA replication is initiated as cells enter S phase. In an unperturbed system, adequate origin firing results in exact duplication of the DNA (left column). Insufficient origin firing, or re-licensing of previously-fired origins can lead to DNA damage and contribute to genome instability and oncogenesis.

Figure 1.4

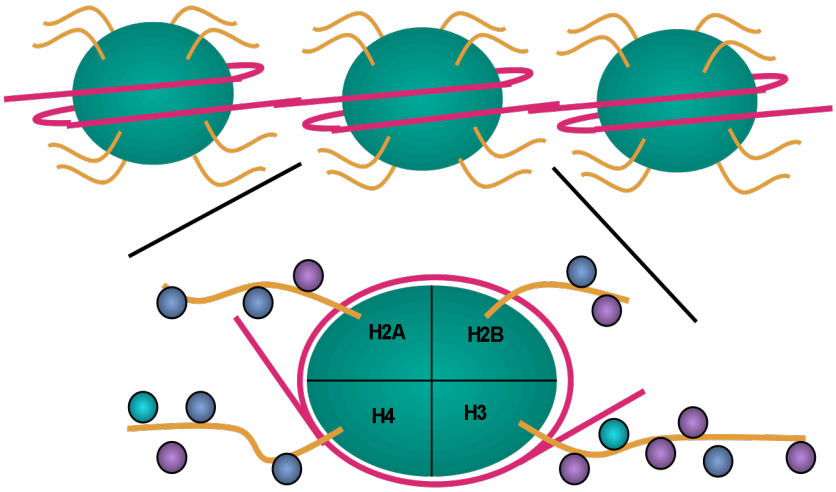
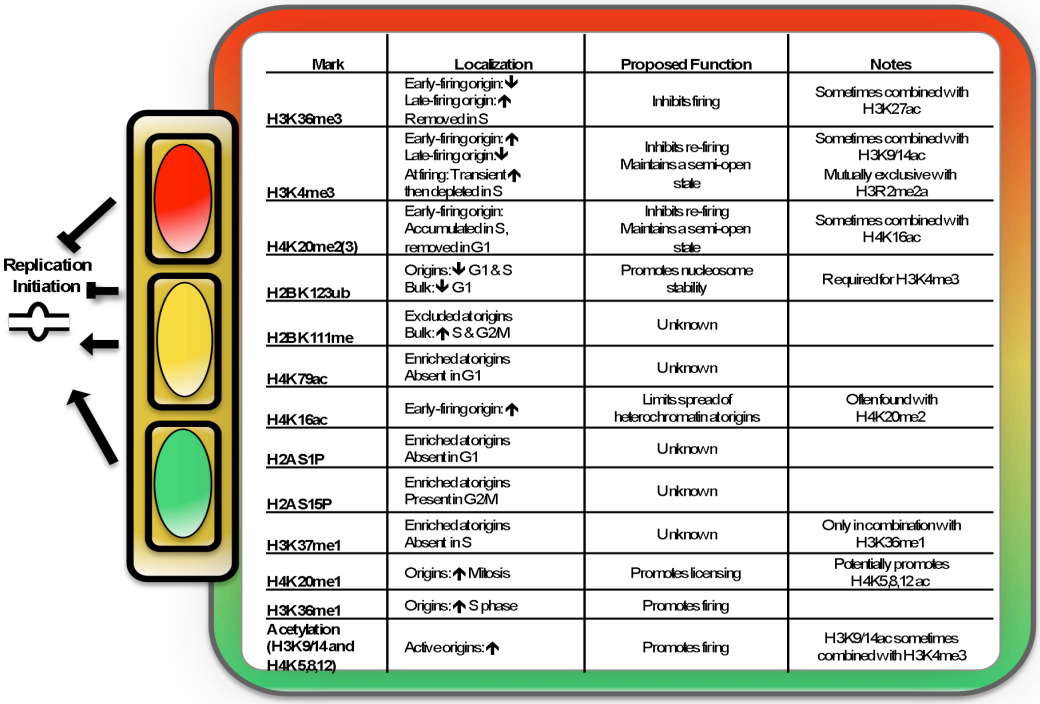


Figure 1.4 Histone post-translational modifications. To fit into the nucleosome DNA is highly compact, and it is formed into units termed nucleosomes. 147 base-pairs of DNA are wrapped around a histone octamer consisting of two subunits each of histone H2A, histone H2B, histone H3 and histone H4. The histone core and protruding N-terminal tails (orange) are subject to a variety of post-translational modifications (blue, teal, purple circles) including acetylation, methylation, ubiquitination, among others.

Figure 1.5



Mark	Localization	Proposed Function	Notes
H3K36me3	Early-firing origin: ↓ Late-firing origin: ↑ Removed in S	Inhibits firing	Sometimes combined with H3K27ac
H3K4me3	Early-firing origin: ↑ Late-firing origin: ↓ At firing: Transient ↑ then depleted in S	Inhibits re-firing Maintains a semi-open state	Sometimes combined with H3K9/14ac Mutually exclusive with H3R2me2a
H4K20me2(3)	Early-firing origin: Accumulated in S, removed in G1	Inhibits re-firing Maintains a semi-open state	Sometimes combined with H4K16ac
H2BK123ub	Origins: ↓ G1 & S Bulk: ↓ G1	Promotes nucleosome stability	Required for H3K4me3
H2BK111me	Excluded at origins Bulk: ↑ S & G2/M	Unknown	
H4K79ac	Enriched at origins Absent in G1	Unknown	
H4K16ac	Early-firing origin: ↑	Limits spread of heterochromatin at origins	Often found with H4K20me2
H2AS1P	Enriched at origins Absent in G1	Unknown	
H2AS15P	Enriched at origins Present in G2/M	Unknown	
H3K37me1	Enriched at origins Absent in S	Unknown	Only in combination with H3K36me1
H4K20me1	Origins: ↑ Mitosis	Promotes licensing	Potentially promotes H4K5,8,12ac
H3K36me1	Origins: ↑ S phase	Promotes firing	
Acetylation (H3K9/14 and H4K5,8,12)	Active origins: ↑	Promotes firing	H3K9/14ac sometimes combined with H3K4me3

Figure 1.5 Histone post-translational modifications at replication origins. Chart of post-translational histone modifications that show localization or regulation distinct from bulk chromatin that have been implicated in replication. The mark, its localization, timing, proposed function in regulating origin firing, and notes on correlation (or anti-correlation) are listed. The marks are arranged from top to bottom depending on whether they are proposed to inhibit origin firing, maintain a semi-accessible origin (or have an unknown function) or promote origin firing.

Chapter 2

Analysis of re-replication from deregulated origin licensing by DNA fiber spreading

Modified from Dorn ES, Chastain PD II, Hall JR, and Cook JG. Nucleic Acids Research. January 2009, Volume 37, pages 60-69.

INTRODUCTION

In each cell-division cycle, a human cell must duplicate over three billion DNA base pairs precisely once.

In order to efficiently copy a large genome in a single cell cycle, eukaryotic cells initiate replication at thousands of chromosomal locations known as origins of DNA replication. Initiation of DNA synthesis, or origin 'firing', takes place in the S phase of the cell cycle with individual origins firing at different times during that period. Each origin that fires must simultaneously be prevented from firing again until the next cell cycle. Even modest re-replication from failure to maintain this 'once and only once' rule results in DNA damage and genome instability which has been linked to oncogenesis [12, 100, 102, 171, 172].

Origins are licensed for DNA replication during the G1 cell-cycle phase by the assembly of an origin-bound pre- replication complex (preRC). PreRCs are assembled by the recruitment of the Mcm2–7 complex through the combined action of the Origin Recognition Complex (ORC) and the Cdc6 and Cdt1 proteins. Once S phase begins, licensed origins containing a preRC are stimulated to fire by the S phase-specific protein kinases, Cdk2 and Cdc7, but no new preRCs can be assembled, thus avoiding relicensing and reinitiation of origins that have already fired [10, 11]. To prevent re-replication a variety of overlap- ping non-redundant mechanisms restrict origin licensing in all cell-cycle phases except G1 by directly affecting the activity or abundance of individual preRC components. These mechanisms include ubiquitin-mediated degradation, Cdk-mediated phosphorylation and the accumulation of the Cdt1 inhibitor, geminin [12, 171-173]

Overexpression of Cdt1 or depletion of the Cdt1 inhibitor geminin can induce substantial re-replication in human cancer cell lines that is detectable as an aberrant increase in the overall amount of DNA per cell [94-96, 174]. It is presumed that re-replication at more physiological (sublethal) levels promotes genomic instability. In support of that assertion, modest overproduction of Cdt1 or Cdc6 did not induce detectable re-replication in cultured cells but markedly increased tumorigenesis in xenograft assays [100, 102]. The increased tumorigenesis may have been the result of limited re-replication, but it is unclear if re-replication actually occurred in those studies or if the tumorigenesis was related to potential other functions of Cdt1 and Cdc6. Conventional cell-based techniques to detect re-replication are restricted to the subpopulation of cells that accumulate a DNA content greater than 4C (more than the normal G2 DNA content) and require lethal extents of re-replication to reach detectable levels. For this reason, detection of re-replication has required extensive origin refiring and fork elongation over periods of time longer than the normal S phase to allow hyper- accumulation of chromosomal DNA. It is thus impossible to determine when in the cell cycle the re-replication actually occurred. In addition, during these long incubations DNA becomes fragmented triggering a secondary cell-cycle DNA damage checkpoint and/or apoptosis [95, 96, 103, 175]. Moreover, most primary and nontransformed cells appear to be resistant to re-replication induction when analyzed for total DNA content, though cell- cycle checkpoints are still activated [96, 175]. Re-replication in these cells can only be inferred from cell-cycle check- point activation, but it has not been demonstrated that these cells re-replicate after geminin depletion or Cdt1 overproduction.

The limits of available re-replication assays prompted us to develop a more sensitive method to directly quantify re-replication. We report here a protocol for detecting re-replication by single molecule DNA fiber analysis, also known as 'fiber spreading'. We have used this technique to demonstrate for the first time that re-replication can occur in very early S phase, in geminin-depleted untransformed cells, and further that HeLa cells may re-replicate at a low level even in unperturbed cell cycles.

MATERIALS AND METHODS

Cell manipulations

Normal human fibroblasts immortalized with human telomerase (NHF1-hTert) and HeLa cells were cultured in Dulbecco's modified Eagle's medium (Gibco) containing 10% fetal bovine serum (Sigma, St Louis, MO) and 2 mM L-glutamine (Sigma). Purified adenovirus producing HA2-tagged Cdt1 was previously described [176], and a derivative truncating Cdt1 after amino acid 321 was constructed by standard methods. siRNA oligonucleotides were previously described [177] and introduced into cells using Dharmafect 1 reagent (Dharmacon). Cells to be analyzed for flow cytometry were trypsinized, fixed with ethanol and treated with propidium iodide/RNAse solution by standard methods. DNA content was analyzed using the CyAn (DakoCytomation) and cell-cycle distributions were determined using Summit v4.3 software (DakoCytomation). Total cell lysates were separated by SDS–PAGE and transferred to PVDF membrane (Millipore) and probed with antibodies to detect

the following proteins: anti-geminin (FL-209) and anti-HA (y-11) purchased from Santa Cruz Biotechnology (Santa Cruz, CA), anti-tubulin (DM1A) purchased from Sigma, phosphospecific antibodies to p53 and Chk1 purchased Nucleic Acids Research, 2009, Vol. 37, No. 1 61 from Cell Signaling Technology (Beverly, MA) and poly-clonal anti-Cdt1 described in Cook et al. [33].

Fiber spreading

Culture medium was supplemented with CldU to 100 mM for 30 min, the medium containing CldU was removed and fresh medium was added. After 30 min, IdU was added to 50 mM for 10 min. Cells were trypsinized and resuspended in PBS to a density of 250 cells per microliter. DNA spreads of approximately 500 cells per slide and the staining of the fibers were as previously described [178, 179] with the following modifications: the amount of antibody that detects IdU was diluted 1:500 instead of 1:250 and the length of the stringency buffer wash increased to 15 min. In addition, IdU-only and CldU-only slides were also stained alongside the slides from the experimental conditions. If more than 5 of 100 tracks stained with both antibodies in the IdU-only or CldU-only slides (i.e. appreciable staining from both antibodies when only one nucleotide was used), then slides from the whole set were not analyzed. IdU and CldU were considered coincident if the IdU and CldU fluorescence were equal to each other (i.e. the red and green signals were similar). To determine whether they were equal in intensities, the red channel and green channels were visually estimated simultaneously using Image J software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA,

http://rsb.info.nih.gov/ij/, 1997–2006)) alongside the composite image.

The length of the yellow tracks were measured using Image J software, the length converted to micrometers using five arbitrary units per micrometer and the micro- meters were converted to kilo base pairs by multiplying the micrometers by 2.5kb as previously described [179]. The tracks were scored as being all green (green-only), all red (red-only), tracks containing red adjoined to green (red-green tracks), or tracks containing more than one micrometer of yellow (tracks that contained red and green signal with substantial overlap). Red-green tracks on occasion contained yellow at the joint, but the length of the yellow track was less than 1mm (2.5kb in length) and were due to a single replication fork being active during both pulses (which is rare due to the gap between signals). True re-replication tracks containing yellow were always much larger and not due to a single replication fork that was active during the first pulse and continued to be active during the second pulse. Any closely spaced tracks that appeared to have come from separate DNA strands due to their angles relative to each other or thickness of signal were ignored.

Statistical analysis

In experiments characterizing re-replication levels and re- replication track length, a minimum of 200 individual fibers was analyzed between at least two independent experiments. Because re-replication level studies compared two categorical values, Fisher's two-tailed exact test was utilized to determine statistical

significance. Re-replication track length studies compared populations of lengths so Student's two-tailed t-test was used to determine statistical significance.

RESULTS

Cdt1 overproduction induces an S phase delay

Re-replication induced by Cdt1 overproduction can be demonstrated in human cells by the appearance of a sub- population of cells with DNA contents greater than 4C [96, 175, 180]. We recapitulated this result by infecting an asynchronous population of HeLa cells with adenovirus expressing HA epitope-tagged Cdt1 and quantifying the amount of DNA per cell using flow cytometric analysis of propidium iodide-stained nuclei. Cdt1-overproducing cells displayed the typical re-replication phenotype characterized by a reduction in the G1 population (2C) and an increase in both the G2 population (4C) as well as a subpopulation with DNA content greater than 4C (Fig 2.1 A). Due to the long incubations required to produce the re-replication phenotype (typically 24h or more) [96, 175, 180], it is not clear if the re-replication only occurred after S phase was completed (i.e. in G2 phase) or if origins continuously reinitiated throughout S phase. If re-replication does occur early in S phase, it is not possible to detect the re-replicated DNA by flow cytometry because cells still have DNA content less than 4C.

To determine if there might be cell-cycle restrictions on the opportunities for re-replication, we evaluated synchronized cells overproducing Cdt1 in S phase by

the protocol outlined in Fig 2.1 B. In early S phase, endogenous Cdt1 levels are kept low by ubiquitin-mediated proteasomal degradation [82, 181, 182] (Fig 2.1 C, lane 1). High-level expression of ectopic Cdt1 presumably overwhelmed the capacity of the cells to properly degrade Cdt1 allowing it to persist into S phase (Fig 2.1 C, lane 2). Control cells completed S phase 8 h after release (Fig 2.1 D), but cells overproducing Cdt1 failed to complete S phase on schedule (Fig 2.1 D). Instead, the majority of Cdt1-overproducing cells still harbored near-G1 DNA content, indicating that Cdt1 overproduction interferes with S phase progression. By 24 h after release (and held in noco- dazole to block mitosis), these cells showed a heterogeneous DNA content with some cells accumulating DNA to levels greater than 4C (Fig 2.1 D). Previous studies in asynchronous cells or using in vitro replication assays had indicated that high levels of Cdt1 can interfere with S phase progression, presumably due to re-replication [103, 183]. One interpretation of these results is that Cdt1 overproduction induced re-replication in early S phase shortly after S phase entry and that this re-replication interfered with S phase progression.

An alternate interpretation hinges on the fact that Cdt1 has multiple binding partners including other components of the preRC, geminin and cyclin A/Cdk2. Early studies suggested that cyclin A/Cdk2 activity is critical for S phase entry and/or progression [184, 185], so it was possible that the S phase progression defect in Cdt1 overproducing cells was due to interference with cyclin A/Cdk2 rather than an immediate consequence of re-replication. To address this question, we constructed a recombinant adenovirus in which Cdt1 was truncated after amino acid 321 ‘Cdt1 ΔC’ (Fig 2.1 E). The corresponding Xenopus laevis truncation is defective for

origin licensing, but retains geminin binding and the cyclin A binding motif [183, 186]. We have also recently demonstrated that this truncation is defective for induction of re-replication in asynchronous cells [187]. We confirmed the binding proper- ties of Cdt1∆C using bacterially expressed GST-Cdt1 fusions incubated with HeLa cell lysate. Both full length ('FL') and the truncation mutant ('∆C') bound cyclin A and geminin as expected (Fig 2.1 F).

A derivative of the Cdt1 adenovirus bearing the '∆C' truncation was constructed and tested for the ability to delay S phase. Overproduction of Cdt1 ∆C to levels simi- lar to that of full-length Cdt1 (Fig 1G, immunoblot) failed to induce a substantial S phase progression defect, whereas full-length Cdt1-producing cells again progressed very slowly through S phase (Fig 2.1 G). Taken together the results described above are consistent with excess Cdt1 in S phase immediately re-licensing previously fired origins followed by a re-replication-induced S phase progression defect. Nevertheless, these indirect assays do not definitively demonstrate that re-replication took place within S phase.

Single fiber analysis quantifies early S phase re-replication in Cdt1-overproducing cells

DNA replication can be directly detected by incubation with halogenated nucleotide analogs followed by spreading DNA fibers on glass slides and staining with fluorescent antibodies to the nucleotide analogs [188, 189]. This procedure is referred to as 'fiber spreading' because the DNA fibers are uniformly stretched on a glass slide so that individual replication tracks can be identified. Cells can be

sequentially incubated with two different halogenated nucleotides detected by two different fluorescent antibodies so that ongoing replication tracks can be distinguished from replication terminations and origin firing events. Ongoing replication forks produce tracks with the first label adjoining the second label, forks that terminated during the first incubation contain only the first label and origins that fired during the second incubation contain only the second label [190]. We hypothesized that re- replication would produce a unique signal where both labels coincide on a single replication track. Since the anti- bodies to detect the nucleotide analogs are labeled with either red or green fluorescent dyes, the coincidence of these signals would produce a yellow track on merged micrographs.

To test this hypothesis, we infected synchronized HeLa cells with control adenovirus or adenovirus overproducing Cdt1 as in Fig 2.1 B. These cells were released from the aphidicolin block immediately into medium containing the thymidine analog CldU. Thirty minutes later, the medium was removed and fresh medium lacking nucleotide analogs was added for an additional 30 min. Cells were then labeled with IdU for 10 min and harvested and subjected to fiber spreading as described in Materials and Methods. The chase period between pulses was introduced to ensure that any residual CldU was exhausted from the intracellular nucleotide pools when the second thymidine analog was added. With this protocol, actively progressing replication forks produce two distinct tracks with a clear unlabelled region between them and no overlap (Fig 2.2 A, left panels). We confirmed the staining specificity by labeling a set of control cells with either CldU only or with IdU only but staining with both antibodies (for an example, see Fig 2.3

D). Small numbers of cells were applied to the slides and only tracks that were clearly separate from neighboring tracks were analyzed. Finally, the fluorescence emissions of the two secondary antibodies were scanned sequentially by confocal microscopy rather than at the same time to minimize spectral overlap.

The analysis of several hundred fibers from each sample clearly revealed a significant number of yellow tracks when Cdt1 was overproduced in very early S phase (Fig 2.2 B). Some tracks containing extensive yellow regions had nearly complete overlap of the red and green signals (an example is shown in Fig 2.2 A, right panels) and some had more complex combinations with flanking single-labeled regions (data not shown). Presumably these differences related to the relative timing of the first and second replication events. Quantification of hundreds of distinct tracks from each sample revealed an approximate four-fold increase in the number of tracks with substantial regions of yellow signal from Cdt1-overproducing cells compared to control cells (Fig 2.2 B). The presence of coincident replication tracks from two distinct labeling pulses is consistent with the immediate refiring of early origins and we suggest that these yellow tracks are the direct visualization of single molecules of re-replicated DNA in early S phase.

HeLa cells produce coincidently labeled tracks in unperturbed cell cycles

In the course of these experiments we were surprised at the high number of yellow tracks that were detectable in the control HeLa cells not overproducing Cdt1. Individual yellow tracks in the control cells were indistinguishable in length or staining pattern from the yellow tracks in Cdt1-overproducing cells (data not shown).

The control cells had not been intentionally manipulated to perturb re-replication control and yet they consistently produced yellow tracks (Fig 2.2 B). Because we have shown that the number of yellow tracks increase when HeLa cells are manipulated to induce re-replication, we were interested to determine if the yellow tracks in these unperturbed cells represented a basal level of re-replication in HeLa cells. First, we considered the possibility that the synchronization procedure that arrested cells in early S phase might have been responsible for this observation, so we repeated the experiment in asynchronously growing HeLa cells. In the absence of cell-synchronizing drugs, the percentage of yellow tracks dropped 2-fold (6%), suggesting that holding cells for a period of time in early S phase could promote origin relicensing and re-firing. Importantly however, these findings may suggest that re-replication is not a rare event even in unperturbed HeLa cells.

HeLa cells are transformed cells and as a result suffer multiple disruptions to normal cell cycle and replication controls. Because of these genetic alterations, the HeLa cell line, like most tumor cell lines, shows relative genomic instability [191, 192]. A higher rate of re-replication in each cell cycle is one potential contribution to genomic instability in cancer cell lines. If so, then HeLa cells would be predicted to re-replicate at a higher rate than non-trans- formed (more normal) cells. To test that idea, we assayed NHF1-hTert fibroblasts (hereafter NHF1) for re-replication tracks. This cell line was established by telomerase expression in primary human diploid fibroblasts and thus has not been subjected to selection for endogenous mutations to escape senescence or promote aberrant growth [193]. Using the same labeling

and staining protocol in Fig 2.2 A, samples of asynchronously growing NHF1 and HeLa cells were processed simultaneously and assayed for yellow replication tracks. Strikingly, compared to NHF1 cells, unperturbed HeLa cells are 2.5 times more likely to produce yellow tracks (Fig 2.2 C).

While these observations point to re-replication as the most likely source of the increased yellow tracks in HeLa cells, it was also possible that they were produced by some unrelated form of DNA synthesis, such as DNA damage. To determine if DNA damage can induce similar yellow tracks we irradiated asynchronous HeLa cells with 1 J/m2 of UV and analyzed the replication tracks both before and after UV. This dose of UV is sufficient to induce significant replication stress, but is sublethal (P.D. Chastain, unpublished observations). Rather than stimulating the generation of yellow tracks however, UV irradiation actually reduced the number of yellow tracks produced by HeLa cells (Fig 2.2 D). These observations still cannot exclude the possibility that some form of unusual replication could lead to yellow tracks. However, the reduced number of yellow tracks in UV-treated cells argues against the interpretation that damage-induced stress contributes to the generation of yellow tracks and further supports the conclusion that these events include re-replicated DNA.

Re-replication in geminin-depleted non-transformed cells

Cdt1 overproduction or geminin depletion induces many tumor cell lines to accumulate DNA content greater than 4C, but some tumor cell lines and virtually all non-transformed cells do not [96, 175]. For this reason, it has been difficult to directly

quantify re-replication in non-transformed cells. Re-replication can be induced by depleting cells of the Cdt1 inhibitor geminin and in tumor cell lines this treatment results in a robust re-replication phenotype measurable by flow cytometric analysis of total DNA content [94, 95]. We attempted to induce re-replication in NHF1 cells by transfecting them with geminin siRNA or a control siRNA targeting GFP and then labeling as in Fig 2.2 A prior to harvesting at 24, 30 or 48 h post-transfection. Samples were analyzed by flow cytometry (Fig 2.3 A), by immunoblotting to confirm geminin depletion (Figs 2.3 B and 2.3 C) and DNA fibers were analyzed for re-replication (Fig 2.3 D). We observed no change in overall DNA content in these cells even 48h after geminin siRNA transfection (Fig 2.3 A). Nevertheless we inferred that some re-replication took place because two checkpoint markers known to be induced during re-replication, phosphorylated Chk1 and p53 [94-96], were induced in the geminin-depleted NHF1 cells (Fig 2.3 C). Strikingly, the proportion of yellow replication tracks steadily increased from 24 to 48 h after geminin depletion (Fig 2.3 D). By 48 h, more than 13% of all replication tracks contained both nucleotide analogs, a difference of more than 5-fold compared to the control transfected cells ($P<0.001$). The accumulation of yellow tracks appeared at the expense of ‘green only’ and ‘red only’ tracks. These singly-labeled tracks include newly fired origins and replication termination events respectively but due to the 30 min chase period, many of these could also represent ongoing replication forks. Geminin depletion also induced an 2-fold increase in tracks that contained both labels (Fig 2.3 D, table), but by our stringent standards these did not score as re-replication that was clearly distinguishable from ongoing replication. We thus conclude that robust origin refiring

occurred in geminin-depleted NHF1 cells despite the fact that the overall cellular DNA content was not detectably increased.

Since the labeling with IdU followed the labeling with CldU by 40–70 min, we assume that some origins fired at least twice in relatively close succession. Immediate refiring of an origin would generate a second set of bi-directional forks that travel on the same stretch of DNA. Some have speculated that replication forks from re-fired origins could travel faster than normal since the chromatin structure behind the first fork may be temporarily more permissive to fork movement [103]. Others have speculated that two replication forks on the same strand would slow fork movement because re-replication triggers DNA damage checkpoints that slow replication [194]. Our ability to directly visualize re-replication on single DNA fibers permitted the estimation of replication fork speed from re-fired origins. We measured the length of yellow tracks in NHF1 cells depleted of geminin for 24, 30 or 48 h. The average yellow track at 24 and 30h was approximately 18kb, (Fig 2.3 E) and since the pulse of the second label was 10min, this corresponds to a fork speed of 1.8kb/min. We note that these tracks are much more than 10 times longer than the 200-bp fragments recently described by Gomez et al. [195], although we cannot rule out a relationship between the released origin fragments observed by that group and re-replication detected here. At 48 h post-transfection, geminin-depleted NHF1 cells show robust checkpoint activation as determined by phosphorylation of Chk1 and p53 (Fig 2.3 C). At this time point, the average yellow track length dropped slightly to 15kb, but this difference was not statistically significant (P = 0.2). Normal replication fork speeds in unperturbed S phase have been measured by us in NHF1 cells and

by other investigators and all are in close agreement of 1.5 kb/min. By this analysis, re-replicating forks travel at close to the same speed as normal forks. We noted that in control cells, the average length of the IdU tracks (second label) was less than half that of the CldU tracks (first label), which is expected given their respective labeling times. Interestingly however, geminin- depleted cells produced shorter CldU tracks (Fig 2.3 E, table) suggesting that these tracks were produced by forks that have slowed in response to the effects of geminin depletion, perhaps due to the effects of Chk1 activation on elongation [196]. More importantly for the purposes of this study however, the fact that the yellow tracks are of a similar length to the singly labeled tracks further supports the conclusion that the yellow tracks are the result of re-replication.

DISCUSSION

In this study we have developed a highly sensitive method for single-molecule detection of re-replication and have applied it to the analysis of re-replication in early S phase in HeLa cells and in a non-transformed cell line. Our approach relies on the detection of two nucleotide analogs incorporated at different times in S phase into the same chromosomal DNA. Staining with red and green fluorescent antibodies specific to the different nucleotides produces yellow replication tracks on merged micrographs that can be readily quantified. It is possible that yellow tracks have been noted by other investigators during fiber spreading experiments, but these signals would likely have been attributed to cross-reactivity from antibodies or chance deposition of tracks from dif- ferent DNA fibers on the slide, and thus might

not have received much attention. Several features of this study rule out such trivial explanations for the yellow tracks in our experiments however. First, we increased the staining stringency for our antibodies to reduce cross-reactivity to less than 5% and we confirmed the specificity of the staining with single-label controls (e.g. Fig 2.3 D). Second, we introduced a chase period between the two labeling periods equivalent to the first pulse to ensure that all of the first label was depleted before the second label was added (e.g. the gap in Fig 2.2 A). Third, we were very conservative in scoring yellow signals as true re-replication tracks. A few small foci of yellow signal can sometimes be seen in standard fiber spreading proto- cols where red signal meets green signal on the same track, but these small signals were not scored as positives in our analysis. Fourth, small numbers of cells were applied to the slides for the combing experiments to ensure that tracks were separated enough to be clearly identified. We have noted extremely rare instances of tracks that lie together on slides, and these pairs are easily identified (and therefore excluded) by the double

thickness of the fluorescent signal and the fact that their ends are offset from one another. Fifth, we showed that neither DNA damage nor its associated replication stress can account for the increased yellow tracks produced by Cdt1-overproducing cells. This observation argues against an interpretation that the yellow tracks are the result of DNA repair synthesis. Moreover, the length of the yellow tracks is consistently much longer than any characterized DNA repair synthetic events in eukaryotic cells and this length is quite close to the normal length of tracks produced from fired origins (Fig 2.3 E). Finally and most importantly, any artifacts that could

have produced yellow tracks cannot account for the marked increase in yellow tracks that was reproducibly observed when Cdt1 was overproduced or geminin was depleted. We induced re-replication by two entirely different techniques, recombinant adenoviral transduction and siRNA transfection, targeting two different genes, Cdt1 and geminin, so any off-target effects to explain this increase can be ruled out.

Using this fiber-spreading procedure we were able to detect re-replication in early S phase long before cells had accumulated supraphysiological DNA levels. The ability to detect re-replication shortly after it begins (within 1h) permits the evaluation of immediate effects that are not influenced by the long-term cellular responses to re-replication-induced DNA damage. For example, we observe slow S phase progression from Cdt1 overproduction, but little to no Chk1 or Chk2 phosphorylation within the first 8–9h of re-replication (E.S.D., unpub- lished observations), whereas long-term overproduction of Cdt1 induces robust Chk1 and Chk2 phosphorylation [96, 175, 187]. The mechanism by which Cdt1 overproduction slows S phase is likely a direct consequence of re-replication in early S phase. Continual re-licensing and re-firing of early origins may act to titrate replication factors away from the rest of the genome or may induce other forms of a replication stress response not detectable as activation of Chk1 and Chk2.

A somewhat unexpected result that stems directly from the sensitivity of this assay is the high number of re-replication tracks produced by HeLa cells compared to the more normal NHF1 cells. NHF1 cells produce a small but quantifiable number of yellow tracks in unperturbed cell cycles, but it is difficult to determine if those tracks are from re-replication or if they are produced by some other process related

to the experiments themselves, telomerase activity, background staining, etc. Importantly however, these cell lines were labeled and processed simultaneously, so the difference between their re-replication rates almost certainly reflects a real biological difference rather than an effect of the experimental technique. This observation implies that HeLa cells already have perturbations in origin licensing control and routinely re-replicate in culture even when there has been no acute experimental manipulation. If so, then HeLa cells must have some means of accommodating the aberrant additional replication forks without triggering either a permanent checkpoint arrest or acquiring lethal amounts of DNA damage. Moderate deregulation of re-replication control in budding yeast causes no overt growth defect but renders those cells highly dependent on DNA repair activities [28]. This observation suggests that cells with moderate re-replication can appear to grow normally, but these cells are constantly subjected to a level of re-replication-associated DNA damage. It may be that HeLa cells exist in a similar state with a constant amount of low-level re-replication. Repeated rounds of re-replication, DNA damage and repair would likely con- tribute to genome instability. It is possible that absolute ‘once and only once’ DNA replication is not actually achieved by HeLa cells and by extension, other tumor cell lines as well.

A number of genetic lesions in the HeLa cell line are likely to account not only for the possible low-level re- replication, but also the ability to re-replicate significant portions of the genome when Cdt1 is overproduced (Fig 2.1 A and 2.1 D). Deregulation of the Rb-E2F transcriptional program by the HPV E7 protein results in high-level expression of the majority of the replication proteins including those that

are directly involved in licensing control such as Cdt1 and Cdc6 [197-200]. Excessive endogenous amounts of replication factors may not be regulated as tightly as they are in normal cells leading to more opportunities for origin re-licensing. Low expression of p53 as a result of the HPV E6 protein has multiple effects on cell-cycle progression and a variety of DNA metabolic events including replication and repair. Insufficient p53 could promote S phase Cdk activity and increase the likelihood that a relicensed origin actually fires, though we note that the absence of p53 is not a strict requirement for re-replication and therefore is not the sole explanation for differences in the propensity to re- replicate [94, 95]. In addition, recent studies have demonstrated correlations between the activity of the ATR-Chk1 pathway and the ability of cells to re-replicate when Cdt1 is overproduced [175, 201]. An exciting implication from our findings is that different cancers may be characterized by different propensities to re-replicate based on individual constellations of genetic abnormalities. If so, then the ability to sensitively quantify endogenous re-replication rates may contribute to predicting differences in overall genome stability.

In assays for increases in DNA content above 4C NHF1 cells did not re-replicate when Cdt1 was overproduced (J.R.H., unpublished observations) or when geminin was depleted (Fig 2.3 A). Similar failure to detect greater than 4C DNA content has also been observed in other untransformed cells [94-96, 175]. In such cases re-replication can usually only be indirectly inferred from the activation of DNA damage checkpoint markers. NHF1 cells have constitutive telomerase expression, but apparently normal Rb, p53 and ATR pathways [187, 193, 202]. Failure to

observe overt re-replication by flow cytometry could have been a consequence of profound resistance to origin relicensing and refiring, or it could have been the result of strong checkpoint effects on replication elongation or other events required to produce cells with greater than 4C DNA content or both. Our finding that NHF1 cells can produce robust re-replication tracks when geminin is depleted suggests that these cells are not extraordinarily resistant to origin relicensing and refiring. The undetectable increase in genomic DNA content from these refired origins could be explained if only a subset of origins is sensitive to origin licensing perturbation. In support of that idea, even when large increases in DNA content were induced by Cdt1 overproduction in tumor cell lines, the re-replication was unevenly distributed across the chromosomes [96]. It may be possible in the future to combine this technique with sequence specific probes (once more human origins have been mapped) to deter- mine if some origins are more likely than others to re- replicate. The ability to directly examine re-replication tracks in a wide variety of cell lines is likely to be a useful addition to the available tools to study genome stability.

Figure 2.1

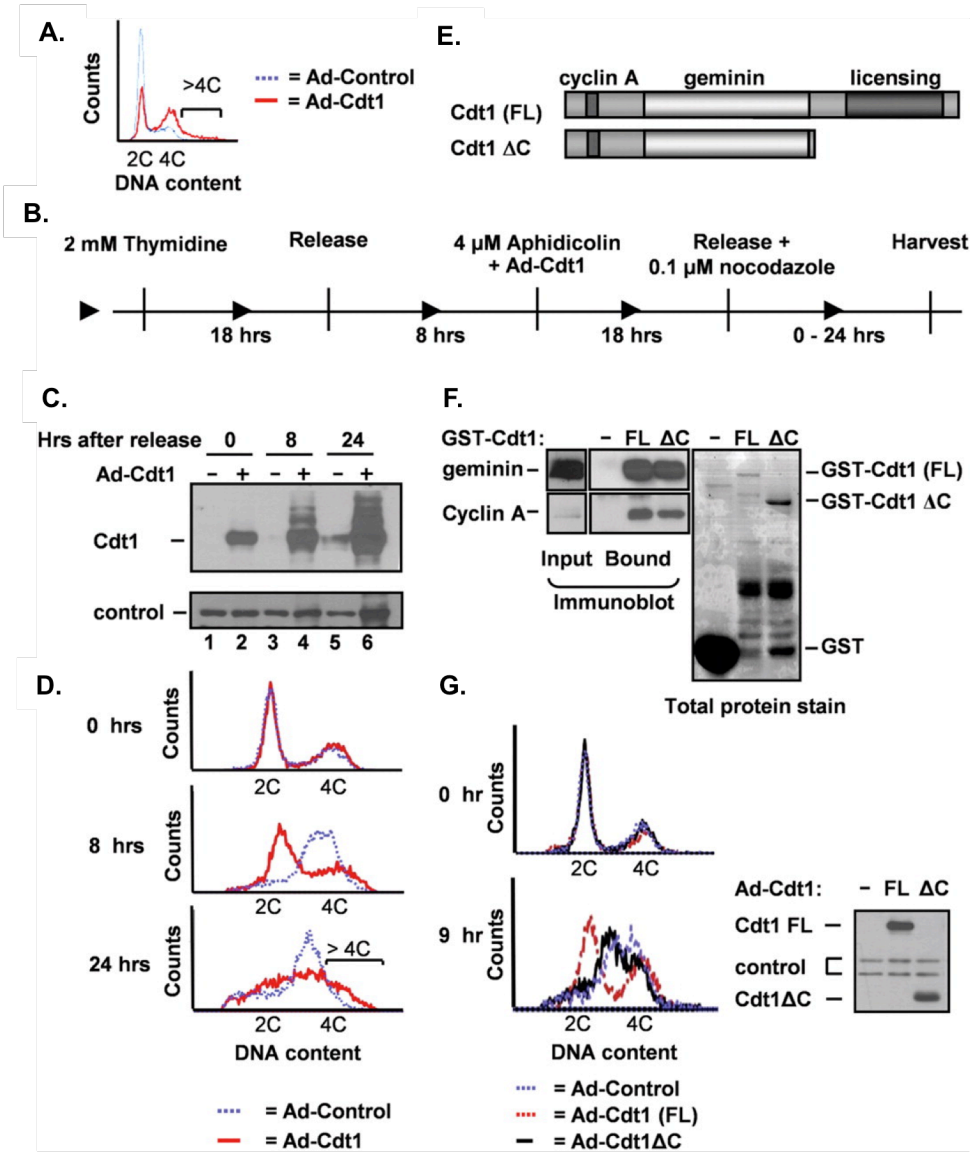

A.
Counts
>4C
= Ad-Control
= Ad-Cdt1
2C 4C
DNA content
E.
cyclin A
geminin
licensing
Cdt1 (FL)
Cdt1 ΔC
B.
2 mM Thymidine
Release
4 μM Aphidicolin
+ Ad-Cdt1
Release +
0.1 μM nocodazole
Harvest
18 hrs
8 hrs
18 hrs
0 - 24 hrs
C.
Hrs after release 0 8 24
Ad-Cdt1 - + - + - +
Cdt1
control
1 2 3 4 5 6
F.
GST-Cdt1: - FL ΔC - FL ΔC
geminin
Cyclin A
Input Bound
Immunoblot
GST-Cdt1 (FL)
GST-Cdt1 ΔC
GST
Total protein stain
D.
0 hrs
8 hrs
24 hrs
Counts
2C 4C
> 4C
DNA content
= Ad-Control
= Ad-Cdt1
G.
0 hr
9 hr
Counts
2C 4C
DNA content
= Ad-Control
= Ad-Cdt1 (FL)
= Ad-Cdt1ΔC
Ad-Cdt1: - FL ΔC
Cdt1 FL
control
Cdt1ΔC

Figure 2.1 Cdt1 overproduction induces slow S phase progression. (A) Asynchronously growing HeLa cells were infected with control adenovirus (Ad-CMV) or adenovirus expressing epitope-tagged Cdt1 (HA2-Cdt1) at a multiplicity of infection of 500. Cells were harvested 48h post-infection and evaluated for DNA content by flow cytometric analysis of propidium iodide-stained nuclei. The percentage of cells with greater than 4C DNA content is 1.2% for Ad-Control and 9.4% for Ad-Cdt1. (B) Workflow of the cell synchronization. HeLa cells were synchronized in early S phase with a thymidine-aphidicolin double synchronization protocol and infected with recombinant adenovirus expressing either HA2-tagged Cdt1 or empty virus (CMV promoter only) as a control at a multiplicity of infection of 500. This viral dose leads to Cdt1 overproduction by 25–30-fold over endogenous Cdt1 in asynchronous cells (data not shown). About 18h post-infection, aphidicolin was removed and cells were collected at various times after release. (C) Immunoblot of Cdt1 in cells collected at 0, 8 and 24 h after release from aphidicolin; 'control' is a non-specific band serving as a loading control. (D) DNA content of synchronized HeLa cells from C determined by flow cytometry. The percentage of cells with greater than 4C DNA content was the following: for Ad-Control, 0h 0.3%, 8h 3.6%, 24h 5.4%; for Ad-Cdt1, 0h 0.6%, 8h 8.7%, 24h 14.9%. (E) Diagram of human Cdt1 identifying the cyclin A binding motif, geminin binding domain and the replication licensing domain. The truncation to remove the licensing domain 'Cdt1 C' is illustrated below. (F) Fusions of full-length Cdt1 ('FL') and Cdt1 C to glutathione-S-transferase (GST) were produced in E. coli, bound to glutathione agarose, then incubated with lysates of asynchronous HeLa cells. Endogenous geminin and cyclin A were detected in the lysate ('input') or bound fractions by immunoblotting and the purified GST fusions were detected by Coomassie staining. (G) HeLa cells were infected with control Ad-CMV (control virus), Ad-HA2-Cdt1, or Ad-HA2-Cdt1 C and synchronized in early S phase as in (B). Cells were collected at the 0 and 9 h timepoints and analyzed for DNA content. Portions of the cells from the 0 h samples were analyzed for ectopic Cdt1 expression by immunoblotting with anti-HA antibody. Non-specific bands serve as a loading control.

Figure 2.2

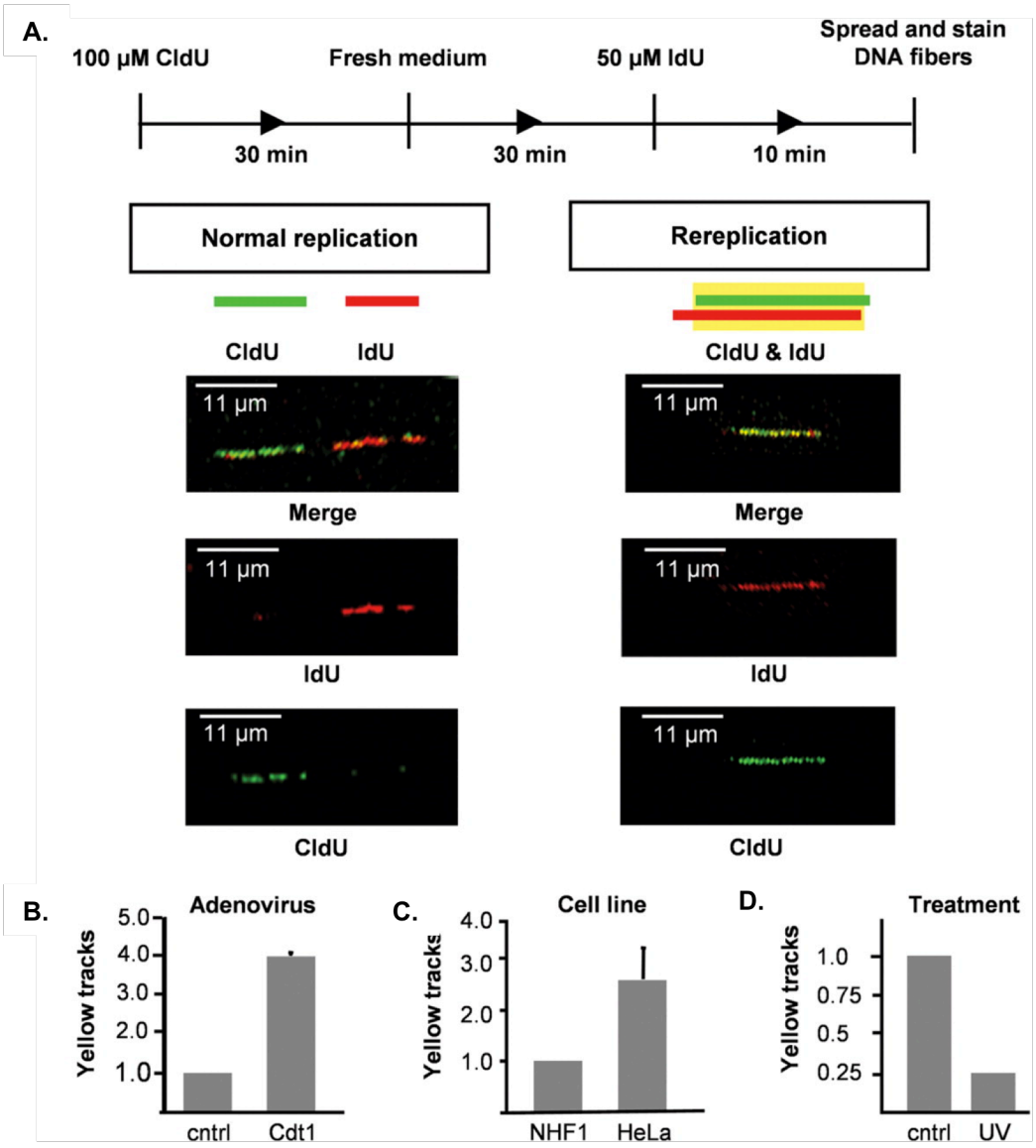
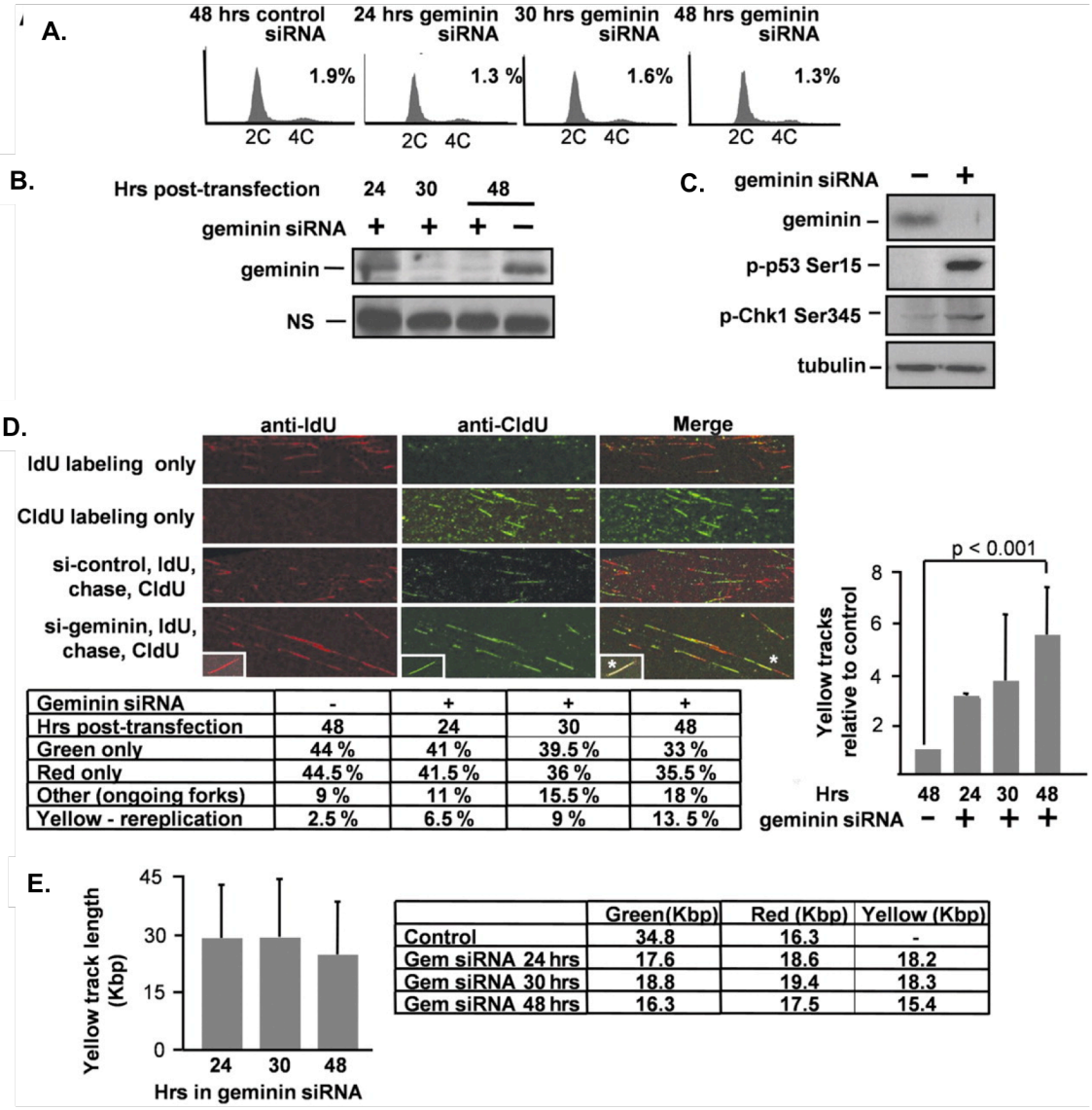

A.
100 µM CldU
Fresh medium
50 µM IdU
Spread and stain DNA fibers
30 min
30 min
10 min
Normal replication
Rereplication
CldU
IdU
CldU & IdU
11 µm
Merge
IdU
CldU
B.
Adenovirus
Yellow tracks
cntrl
Cdt1
C.
Cell line
NHF1
HeLa
D.
Treatment
cntrl
UV

Figure 2.2 Fiber spreading detects re-replication. (A) Workflow of the labeling protocol and representative replication tracks. See Materials and methods section for details. (B) DNA fibers from HeLa cells synchronized and transduced with control adenovirus or Cdt1 adenovirus as in Figure 1A. Labeling was initiated immediately after the release from aphidicolin and cells were harvested approximately 70min after release. Bar graph: a total of at least 200 replication tracks were analyzed from each sample and the relative fraction of yellow tracks (re-replication) in Cdt1-overproducing cells compared to control is plotted; $P<0.001$. (C) Quantification of re-replication in asynchronous unperturbed HeLa cells and NHF1 cells. The number of yellow tracks produced by NHF1 cells was set to 1 and the relative number of yellow tracks in HeLa cells is plotted; $P<0.05$. (D) Asynchronous HeLa cells were treated with 1J/m2 UV immediately before labeling with CldU and IdU by standard protocols; the 30 min chase period was omitted. Yellow tracks were scored as in B and reported as the fold-change in the irradiated cells compared to unirradiated control cells.

Figure 2.3



Geminin siRNA	-	+	+	+
Hrs post-transfection	48	24	30	48
Green only	44 %	41 %	39.5 %	33 %
Red only	44.5 %	41.5 %	36 %	35.5 %
Other (ongoing forks)	9 %	11 %	15.5 %	18 %
Yellow - rereplication	2.5 %	6.5 %	9 %	13. 5 %

	Green(Kbp)	Red (Kbp)	Yellow (Kbp)
Control	34.8	16.3	-
Gem siRNA 24 hrs	17.6	18.6	18.2
Gem siRNA 30 hrs	18.8	19.4	18.3
Gem siRNA 48 hrs	16.3	17.5	15.4

Figure 2.3 Quantification of re-replication in unperturbed and geminin-depleted cells. (A) NHF1 cells were transfected with siRNA targeting geminin or GFP as a control and harvested at 24, 30 and 48 h after transfection. DNA content was determined by flow cytometric analysis of a portion of the harvested cells. The percentage of cells with greater than 4C DNA content is indicated on each histogram. (B) Immunoblot analysis of a portion of cells from (A) to detect endogenous geminin and a non-specific band as a loading control. (C) Immunoblot analysis of phospho-53 (Ser 15) and phospho-Chk1 (Ser 345) from cells in A transfected with siRNA for 48 h. (D) Representative fields of stained DNA fibers. Single labeling with CldU or IdU only (stained with antibodies to both) demonstrates minimal cross-reactivity. Examples of representative yellow tracks are marked with asterisks. Table: Quantification of all re-replication tracks in siRNA transfected NHF1 cells from (A). The category 'Other' includes tracks with green into red, green flanked with red and red or green tracks with yellow ends. Bar graph: Comparison of geminin-depleted cells to control cells: 24 h, $P = 0.02$; 30 h, $P < 0.001$; 48 h, $P < 0.001$. (E) Measurement of re-replication track lengths from the samples in D. Comparison of yellow track lengths to those in geminin-depleted cells: 24h; 30h, $P=0.98$; 48h, $P=0.2$.

Chapter 3

Methylation of histone H3 lysine 4 is linked to replication initiation

INTRODUCTION

Every time a cell divides, its DNA must be duplicated so that a copy can be passed on to each daughter cell. In order to make a complete copy of their DNA within a single S phase, eukaryotic cells initiate replication at hundreds to thousands of sites along the genome termed origins. It is critical that these origins are coordinated and regulated properly to prevent inappropriate initiation. Failure to control origin activity can lead to DNA damage and genome instability, which contributes to oncogenesis.

All origins undergo three phases of regulation: licensing, initiation, and inhibition. In G1 phase all potential origins are prepared, or "licensed", for replication by assembling a preRC complex. Following ORC binding to DNA, Cdc6 and Cdt1 act together with ORC to load MCM complexes onto chromatin. At this point preRC formation is complete and an origin is licensed [10]. As the cells progress into S phase, the initiation factors GINS, Cdc45 and the primase pol α are recruited to the preRC at the time of origin firing. Finally, reassembly of the preRC during S phase is inhibited by a variety of methods to prevent re-firing of origins until the subsequent G1 phase.

Although these regulatory steps are common to all origins, preRC regulation alone is insufficient to explain all aspects of replication initiation. First, origins are utilized with different efficiencies. Some origins fire every cell cycle while others rarely fire [112, 203]. Certain metazoan initiation sites are known to remain dormant except under times of replicative stress [47, 113]. Also, as shown in chapter 2, re-replication is limited to a subset of origins. Furthermore, origin firing is not

synchronous, some origins fire early in S phase and others fire late [108, 164]. Finally it is unclear how origins are identified within the genome. In mammalian cells, there is no sequence-specific element that identifies origins. In yeast, ORC binds to a conserved 11 base-pair DNA sequence termed the ACS. However, there are 12,000 copies of the ACS in the yeast genome and it is unclear why only approximately 350 of these sites have been confirmed as functional origins [50].

Histone modifications and chromatin organization have been extensively linked to regulation of transcription. Therefore, we hypothesized that the chromatin landscape at an origin may be important for regulating the aspects of replication that cannot be explained by preRC assembly alone. Acetylation across the genome has been shown to be critical for stimulation of DNA replication initiation. In yeast, inhibition of the histone deacetylase Rpd3 or tethering of an acetylase Gcn5 to late-firing origins is shown to accelerate the time of origin firing to earlier in S phase [131, 132]. In addition, deletion of the Sir2 deacetylase promotes preRC formation [135]. In the time since initiating this study, various histone methylation marks have also been correlated with origins, but their regulatory roles remain largely unknown [115, 146].

This study utilizes *S. cerevisiae* as a model system for eukaryotic origins. Origins in budding yeast have been clearly defined, these cells are easily manipulated by extensive genetic tools, and the high conservation of both replication machinery and histone modifications from yeast to humans make it an attractive system to work in. To identify and prioritize elements of the chromatin that likely play a role in regulating origin firing, we undertook a data mining approach coincident

with a screen for genetic interactions between chromatin modifiers and replication factors. These initial studies identified a possible link between the regulation of origin initiation and methylation of lysine 4 of histone H3. This interaction was validated by a variety of independent methods. Our results also suggested that the degree to which H3K4 is methylated might be an important distinction, as preliminary results indicate that H3K4me3 has an effect that is different from H3K4me1/2.

MATERIALS AND METHODS

Strains and Plasmids

Genetic Screen:

Deletions in histone modifying enzymes were introduced into the Ruy028 hypermorphic strain by a mating and selection procedure. The gene deletion strains were marked by KanMX and were obtained from the Research Genetics strain collection in the BY4741 background (*MAT **a** geneX::KanMX his3 leu1 met15 ura3*). The Ruy028 strain was a gift from Dr. Fred Cross (*MAT α LEU2::ORC6-rxl URA3::GAL-CDC6ΔNT-HAs mfa::MFA1pr-HIS3 trp1 ade2 can1 leu2 his3 lys2 ura3*). The two strains were mated overnight on YPD. The mated strains were then streaked for individual colonies onto selection medium (SCD-His/Leu/Ura/Arg plus 3-AT and canavinine) to select for *Mat **a** mfa;;MFA1pr-HIS3 ORC6-rxl::LEU2 URA3::GAL-Cdc6ΔNT-HAs* haploid spore progeny. Several individual colonies were patched onto selective medium. These strains were then patched onto sporulation medium and grown overnight at 30 degrees and then five subsequent days at room

temperature. The strains were plated onto SCD-His/Ura/Leu/Arg + 3-AT + canavinine + G418 to select for haploid double mutants.

Plasmid Maintenance:

The strains utilized in the plasmid maintenance assay were obtained from a variety of sources. BY4741 strains were transformed with pcr products to introduce the *set1*::His3 and *bre1*::KanMX alleles. The *swd1*::kanMX strain was obtained from the Research Genetics deletion library. The hypomorphic strain was created by Dr. Candice Carlile in the Cook lab by introducing the *cdc6-1::Hph* cassette into BY4741. The 1xARS (ARS1) plasmid was YCplac33 and the 3xARS (ARS1 + 2xARS209) plasmid was created in by the Cook lab by adding two ARS209 sequences to the YCplac33 plasmid.

Chromosome Loss:

The chromosome loss assay strains recipient strains YKN10 (*Mat **a** HIS4 leu2-Δ1 ade2 kar1Δ15*), *YKN10 rad9::KANMX* and donor strains F510αA1-4 (*MAT α his4-290 LEU2 C26::ADE2 Tel 5oriΔ(305,306,307,309,310)*) and F013αB2C-1C (*Mat α his4-290 LEU2 C26::ADE2 Tel)* were gifts from Dr. Carol Newlon ([204] for complete genotypes). The YKN10 recipient strain was transformed with a pcr product amplified from a *bre1::KANMX* strain to create the YKN10 *bre1::KANMX* strain. To create the partially disomic strains, the strains were mated and plated on chromoductant medium (SCD-Leu-Trp + canavinine and cyclohexamide) to select haploid cells that had received single donor chromosome transfers.

Tethering Assays:

The GAL-LexA-Set1 expression construct was constructed by pcr amplification of LexA-Set1 from pADH URA Set1 (WT)(Strahl lab) or p1170 (CD)(Cairns lab) by JGC. The pcr amplification also introduced XhoI and XbaI restriction sites that were used to clone into pglx2 (which I created for N-terminal tagging by inserting LexA into yEP352). The LexA-operator tagged ARS822 maintenance plasmid was a two-step process. First, the URA3 gene with flanking homology to an 822-adjacent site was amplified off of pRS316 and introduced into BY4741. PCR amplification of LexA-operator sites with homology to URA3 was amplified from pSH18-34 and replaced the URA3 marker. Recombined strains were selected on 5-FOA. A 3kb piece of the tagged-822 genomic region was amplified by pcr and was introduced into Ycplac111 by SpeI restriction digest and ligation. The plasmid was confirmed by diagnostic digest and DNA sequencing.

Spotting Assay

Five-fold serial dilutions starting with an equivalent number of cells (10^5 cells) were made in sterile water. These dilutions were spotted onto YPD (2% dextrose) and YPG (2% galactose). Growth was assayed after two days.

Plasmid Loss Assay

1xARS (ycplac33) or 3xARS (ycplac33 + two ARS209) were transformed into appropriate strains. Three independent transformants were selected for measuring plasmid stability. Transformed strains were grown overnight in SCD-leu. A portion of this culture was plated at a predicted density of 200 cells/plate onto selective and

non-selective plates to determine initial baseline loss rates. The overnight cultures were diluted into YPD at a concentration of 10^5 cells/ml and were grown for approximately 24 hours. The concentration of these cultures was determined to calculate the number of generations underwent. Also, a portion was plated at a predicted density of 200 cells/plate onto selective and non-selective plates to determine final plasmid loss rates. P values were calculated an unpaired t-test.

Artificial tethering of Set1

Appropriate strains were co-transformed with maintenance plasmids (LexA-822 or Untetherable-822) and LexA fusion plasmids (LexA alone, LexA-Set1WT, LexA-Set1CD). Strains were grown overnight –leu-ura 2% dextrose. The second day the cells were grown in SC –Ura + 1% raffinose1%galactose to induce expression of the LexA-fusion constructs. Loss rate of the maintenance plasmid was determined as described in the plasmid loss rate section

Sectoring Assay

All tester and donor strains 5oriΔ (F510alpha4A1-4) and 0oriΔ (F013alphaB2C-1C) were patched from frozen culture onto YPD or CA plates and allowed to grow overnight. The next day, the donor and recipient strains were mixed together and incubated at 30 degrees overnight for mating. To induce the chromoduction, the mated strains spread densely onto chromoductant medium. The strains were incubated at 30 degrees for 5-10 days until individual colonies appear. Individual colonies were re-patched onto chromoductant plates. These isolates are

streaked onto YPD and incubated at 30 degrees for 2 days, followed by incubation at room temperature for 5-7 days to allow color to develop. Photos documented plate sectoring.

Immunoblotting

Cell lysates were prepared according to standard methods (Corbett), separated by SDS-PAGE and transferred to PVDF membrane and probed with antibodies to detect the following proteins: anti-trimethyl Histone H3 (Lys4) Millipore 04-745, anti-dimethyl Histone H3 (Lys4) Active Motif C39142

RESULTS

The chromatin landscape at origins is distinct from other genomic regions

To identify aspects of the chromatin environment that may be important for origin regulation, we took advantage of previously published data. There have been several genome-wide mapping studies of nucleosome positioning in *S. cerevisiae*. Each of these studies reported patterns of relative nucleosome occupancy and the levels of histone modifications at transcription domains such as promoters, transcription start sites, and gene bodies. Because of the success of these studies in identifying significant patterns of histone modifications at transcription motifs, we sought to identify the histone signature at origins of replication by mining the raw data of these published mapping studies.

We utilized the mapping data provided by Pokkholok *et al* and examined genome locations corresponding with the 314 confirmed replication origins as

reported by the DNA Replication Origin Database [144, 205]. In collaboration with the UNC Bioinformatics Core Facility the nucleosome position and the enrichment or depletion of various histone modifications at these origins was established by overlaying the origin sequences with the raw data from the published study. H3K9ac, H3K14ac, H3K4me, and H3K36me localization at origins was examined. The average enrichment of each modification at origins was compared to the average level of that specific modification at all non-origin sequences, promoters, intergenic regions, and open reading frames.

The initial screen yielded interesting and instructive findings. As expected, this approach confirmed previous studies showing that origins tend to be depleted for nucleosomes (Fig 3.1 A). While origins are like promoters with respect to histone occupancy, analysis of acetylation revealed that origins are not simply promoter-like, but have their own unique histone signature. As observed by others, both H3K9ac and H3K14ac are enriched at promoters. However, neither mark is enriched at origins over the global non-origin average (Fig 3.1 B). This observation was somewhat surprising because acetylation has been shown to induce origin firing [131, 132]. An explanation for this observation may be that acetylation at origins is very transient, or that the level of acetylation required to induce origin firing is less than what is required at promoters. An alternative, but not mutually exclusive explanation is that because this study was performed in an asynchronous population, changes that are cell cycle regulated might not be apparent.

Studies linking acetylation to origin firing report that in general, acetylation promotes origin firing [203]. Histone methylation, which has previously been

implicated in regulating gene expression, has more complex effects. Methylation can both promote and inhibit transcription, and furthermore, its effects have been shown to depend on the residue that the modification occurs on, and also the extent of methylation (mono-, di-, or tri-) at that residue [148]. Origins are depleted for H3K4me1 and H3K4me3 compared to non-origin sequences while promoters are enriched for H3K4me3, but depleted for H3K4me1 compared to non-origin sequences (Fig 3.1 C). Interestingly, genome-wide studies executed since our analysis was conducted have found that H3K4me3 is enriched at early-firing origins compared to late firing origins, and that this enrichment is depleted as cells progress through S phase [169]. Again these seemingly contrasting observations may be due to transient changes at origins that are not seen when examining data from an asynchronous population of cells. Alternatively, this may be a function of increased resolution in the more recent studies. Furthermore, the comparison between origins and non-origins may be less valuable than examining how the H3K4me3 status varies across groups of origins with specific characteristics (early-firing *vs.* late-firing, efficient *vs.* inefficient) at different points in the cell cycle.

Finally, H3K36me3 was analyzed, and both origins and promoters were hypomethylated for this mark compared to non-origin sequences (Fig 3.1C). This modification is absent from promoters but enriched in gene bodies where it limits cryptic transcriptional initiation by recruiting Rpd3 to remove acetylation [151]. It is possible that a similar mechanism is utilized to regulate replication. Therefore, the absence of a mark may be just as critical as the presence of one.

While informative, additional methods were needed to confirm the observations gleaned from this data-mining approach.

Histone modifying enzymes display genetic interactions with replication factors

In addition to data mining, we employed a genetic screen to identify interactions between histone modifiers and replication factors. We utilized a hypermorphic replication strain, Ruy028, developed by the laboratory of Dr. Fred Cross that contains mutations that make it susceptible to re-replication under certain conditions. This strain expresses an ORC ^{RXL} mutant, which makes the protein hyperactive and unable to be targeted by the S-phase cyclin Clb5. In addition, the strain contains a GAL-inducible HA-tagged truncated form of Cdc6 (Cdc6-NTΔ) that is unable to be degraded because it lacks the N-terminal cyclin-binding regulatory domain. Under conditions where expression of the stable Cdc6-NTΔ mutant is induced, the likelihood for re-replication to occur in this strain is increased. With these mutations alone, cells are able to recover from any moderate re-replication that is induced, but additional mutations that promote re-replication or impair the ability of the cell to respond to damage results in impaired growth or cell death [28].

Initially, gene deletions for 33 histone-modifying proteins were introduced individually into the hypermorphic replication strain utilizing a mating and selection strategy. Spotting assays were used to compare growth between the hypermorphic replication strain and the hypermorphic strain containing the gene deletion. After two days on galactose medium, differences in growth were observed. Of these 33 gene

deletions, 11 showed consistent growth phenotypes that were different from the replication mutant alone (Table 3.1). We utilized deletion of *clb5*, an S-phase b-type cyclin that prevents re-replication by binding to ORC in S phase and *mre11*, a component of the DNA repair machinery, as controls for our assay. Deletion of *clb5* removes one of the mechanisms the cell uses to prevent re-licensing of origins and the *mre11* deletion prevents the cells from properly responding to and repairing low levels of re-replication induced damage. Therefore, we expected that deletion of these controls would lead to further growth defects in the Ruy028 strain. As expected, deletion of our controls, *mre11* and *clb5,* exacerbated the growth phenotype of the Ruy028 strain alone (data not shown).

Deletion of some histone modifiers helped rescue the growth deficiency while others exacerbated it. Interestingly, preliminary assays demonstrated improved growth upon deletion of two proteins that are linked to H3K4 methylation. This observation led us to focus on the role of H3K4me in replication for the remainder of this work. Swd1 is part of the COMPASS complex, which is the complex that mediates H3K4 methylation through the catalytic activity of the Set1 methyltransferase (Fig 3.2 C). Swd1 is required for COMPASS integrity and for methylation of K4 [206]. Deletion of *swd1* improved growth on galactose (Fig 3.2 A top panels) Deletion of *bre1* in the Ruy028 strain also improved growth (Fig 3.2 A middle panels). Bre1 is required for H2BK123ub which has been shown to contribute to efficient H3K4me2/3 [207]. Western blotting with an antibody recognizing the HA-tag confirms that the mutant Cdc6-NTΔ is still induced and that a compensatory mutation limiting its expression is not responsible for the improved

growth phenotype. One interpretation of the improved growth is that deletions of the histone modifiers in the Ruy028 strain help to limit re-replication. Therefore, we propose that Swd1 and Bre1 normally promote replication. Because Swd1 and Bre1 are important for H3K4me, these results suggest a positive role for H3K4 methylation in replication.

In light of these observations, we subsequently deleted additional members of the COMPASS methyltransferase complex including Spp1 and Bre2 in the Ruy028 strain. Both deletions improved growth (Fig 3.2 A lower panels). The growth rescue was subtle for Bre2, which is only required for trimethylation, and it was more robust for Spp1 [208, 209]. These results further implicate H3K4me in promoting replication. The observation that deletion of several members of the COMPASS complex exhibited improved growth compared to the Ruy028 strain alone increases the likelihood the H3K4me mark itself is important for replication and that these effects are not the result of other functions of the proteins.

Plasmid maintenance assays support a role for H3K4me in replication regulation

Our screen identified genetic interactions between replication factors and histone modifiers. However, spotting assays alone cannot eliminate the possibility that changes in growth resulted from changes in gene expression of cell cycle genes or defects in other processes like DNA repair. Maintenance of yeast plasmids containing an ARS element is a convenient way to quantitatively measure deficiencies that may reflect replication defects.

The maintenance plasmid, 1xARS, (containing a single copy of ARS1) was transformed into BY4741, *set1Δ*, *swd1Δ*, *bre1Δ* (1x data from Ms. Lindsay Faircloth) and *cdc6-1* yeast strains. The *cdc6-1* strain is a hypomorphic replication mutant strain that was used as a control for the assay. The *cdc6-1* hypomorphic strain contains a temperature-sensitive conditional allele that prevents the strain from robustly initiating DNA replication at semi-permissive temperatures. We measured the stability of the 1xARS plasmid by comparing colony numbers on selective and non-selective plates after growth in non-selective medium. Our control strains confirm that the assay performs as expected. At the semi-permissive temperature of 31°, we found that the plasmids were quite stable in wild-type cells (loss rate = $2^{\%}$ / generation) and that the plasmid was less stable in the *cdc6-1* strain (loss rate = $11.2^{\%}$ / generation) (Fig. 3.3 A).

Our experimental strains also showed decreased plasmid stability compared to the wild-type loss rate per generation increasing from $2^{\%}$ to $5.2^{\%}$, $5.9^{\%}$, and $8.0^{\%}$ for *swd1*Δ, *set1*Δ, and *bre1*Δ strains respectively (Fig 3.3 C). Immunoblotting confirms that H3K4me2 and H3K4me3 are absent in these strains (Fig 3.3 B). Because deletion of these proteins led to decreased plasmid maintenance (which results from defective replication of the plasmid) one interpretation is that these factors normally promote replication. Furthermore, because these proteins are all important for H3K4me, the data then also suggests that H3K4me may be important for promoting replication.

Next, we wanted to confirm that the defects in plasmid stability were specifically due to defects in origin initiation, and not the result of other explanations

such as limited fork progression or DNA repair. Plasmid instability in licensing-defective strains has previously been shown to be rescued by the inclusion of additional origins on the maintenance plasmid [23]. We compared the loss rate of the 1xARS to the loss rate of a 3xARS plasmid (ARS1 + two copies of ARS209). Plasmid stability was significantly improved in all strains by the additional ARS elements (Fig. 3.3 B light bars). These results confirmed that the plasmid maintenance defect is replication associated lending further support to a role for H3K4me in origin regulation. Alternatively, had the loss rate not been rescued, one interpretation would be that these proteins are critical for fork progression or chromosome segregation.

Chromosome loss assays further implicate H3K4me in replication

Because plasmid maintenance assays revealed a link between H3K4me and replication initiation, we employed another type of maintenance assay as an independent method to confirm the link between H3K4me and replication. A study by Theis *et al* examined the stability of two chromosome fragments [204]. One fragment, 0oriΔ, was a segment of chromosome III that was lost at a rate less than once per 10,000 cell divisions. This loss rate was compared to the loss rate of 5oriΔ, a DNA fragment of the same segment of chromosome III, but deleted for the five efficient origins known to exist on this segment of DNA. Interestingly the plasmid loss rate was increased about 20-fold, but the chromosome was still replicated and was only lost about once per 700 divisions. They hypothesized that normally inactive/inefficient origins become active in the absence of the efficient origins to

maintain replication of the DNA. Chromosome stability of the 5ori∆ (but not the 0ori∆) became highly dependent on factors that contribute to replication fork progression and stability (like Rad9) [204]. Therefore, we hypothesized that maintenance of the 5ori∆ chromosome may also be reduced when factors important for the initiation step of replication are mutated. In strains with mutant H3K4 histone methyltransferase activity, we would interpret a change in the stability of the 5ori∆, but not the 0ori∆, as evidence for a positive role for H3K4me in replication.

To test this hypothesis, partially disomic strains containing balancer and test chromosomes were created and the stability of the test chromosome was assayed (Fig 3.4 A). Three different recipient strains were utilized. The background of each of these strains was WT, *rad9*∆ or *bre1*∆. Attempts to delete *set1* have not yet been successful. The WT and *rad9*∆ (a positive control for chromosome loss) strains were gifts from Dr. Carol Newlon. The *bre1*∆ was constructed for this study. These recipient strains contain a balancer chromosome and are mutant for *kar1,* which prevents efficient nuclear fusion. Together, these characteristics facilitate induction of partial disomic status when mated with the donor strain and placed under proper selection. The donor strains carry the test chromosome and these were also gifts from Dr. Carol Newlon. Loss of the test chromosome, 5ori∆ (all (5) efficient origins mutated) from a disomic strain was compared to loss of a control test chromosome, 0ori∆, (all efficient origins intact). The test chromosomes also harbor the ADE2 gene providing an easy method to monitor loss of the test chromosome. Red pigment accumulates in strains that have lost the test chromosome, which carries the ADE2

marker. Accumulation of red pigment in strains mated with the 5oriΔ donor, but not the 0oriΔ donor signifies a replication-associated chromosome maintenance defect.

The recipient and donor strains were mated and then plated under conditions that selected for the appropriate partially disomic strains (haploid, one copy of the balancer and one copy of the test chromosome). The resulting strains were streaked onto YPD, grown overnight at 30°, and red pigment was allowed to develop at room temperature for 10 days. Qualitative analysis revealed a consistent pattern. The wild-type cells showed little accumulation of red sectors with either donor chromosome (Fig 3.4 B Row 1). *Rad9Δ,* the positive control for chromosome loss showed significant chromosome loss (as detected by abundant accumulation of red sectors) with the 5oriΔ test chromosome but not the 0oriΔ (Fig 3.4 B Row 3). A similar phenotype was observed with the *bre1Δ* recipient strain (Fig 3.4 B Row 2). Increased chromosome loss over wild-type was observed with the 5oriΔ test chromosome only. These results were consistent, robust, and visually striking, and additional analysis will be required to obtain quantitative rates of chromosome loss. The accumulation of red sectors indicates increased chromosome loss, (possibly as a result of replication defects) suggesting that deletion of *bre1* (and thus reduction of H3K4me2/3) results in replication inhibition. Therefore, one interpretation is that this is an independent method that provides further evidence implicating H3K4me in positively regulating origin initiation.

Targeting Set1 to an extrachromosomal plasmid affects the plasmid stability.

Because changes in post-translational modifications also affect transcription, it is possible that the replication effects we have observed in strains deleted for histone modifying enzymes are indirect responses resulting from changes in expression of replication or cell cycle genes. To address this possibility, Set1 was targeted to a single origin on a plasmid. The effect of this tethering was monitored by the stability of the plasmid over several cell divisions.

To specifically target Set1 to a single origin, a maintenance plasmid (LexOp-822) was constructed that included 3 kb of the genomic region surrounding the ARS 822 element (Fig 3.5). Four LexA operator sites were inserted 300 base-pairs from the ARS element to facilitate recruitment of the histone modifier to the origin while leaving the ARS element unimpeded for recruitment of replication factors. An additional construct that contained a galactose-inducible LexA-Set1WT fusion protein was created. Both the expression and maintenance plasmids were transformed into a *set1*Δ strain. The assay was performed in a *set1*Δ strain to ensure that the level of H3K4me at the targeted plasmid was in fact enriched over the level that might be achieved by endogenous Set1 in a wild-type strain. Additional expression plasmids LexA-alone and LexA-Set1H017K (catalytically dead) were included as controls to ensure that expression of LexA alone did not affect replication and that the catalytic activity of Set1 was required for changes in the stability of the maintenance plasmid. In parallel, assays were performed using a control maintenance plasmid (NoOp-822) that contained the same 3kb surrounding ARS822, but lacked the LexA operator sites to confirm that changes in maintenance were due to specific targeting of the LexA-Set1 fusion.

Surprisingly, induction of LexA-Set1WT *decreased* the stability of the maintenance plasmid. This trend was specific to the LexOp-822 plasmid and the catalytically active LexA-Set1 fusion (Fig. 3.6 A). This result indicates that additional H3K4me may inhibit origin initiation. Immunoblotting confirmed that H3K4me2 and H3K4me3 was induced in the presence of 1% raffinose + 1% galactose in appropriate strains (Fig 3.6 C). Because previous results suggested that H3K4me played a positive role in replication initiation, we considered the possibility that the degree to which H3K4 is methylated is a critical determinant in the regulatory output. We proposed a model where H3K4me1/2 promotes replication initiation and that H3K4me3 (or the absence of H3K4me1/2) inhibits initiation (Fig 3.7). To test this model we utilized an *spp1*Δ strain and again added back LexA-Set1 fusions. Spp1 is needed for efficient Set1-mediated trimethylation of H3K4 through its interaction with the Phe/Tyr switch of Set1 [210]. Without Spp1, H3K4me3 is severely depleted. Our rationale was that expression of LexA-Set1WT would increase the H3K4me1/2 at the targeted origin, but that H3K4me3 would not be efficiently induced as a result of the absence of Spp1. In this strain, expression of Set1 helped *increase* the stability of the plasmid (Fig 3.6 B). Immunoblots of whole cell lysates examined overall H3K4me2 and H3K4me3 levels, and revealed that H3K4me3 is significantly reduced in *spp1*Δ deletion strains globally. If we assume that this reduction in H3K4me3 is also seen at our targeted origin, our still model fits.

Preliminary efforts targeting Set1 to LexOp-ARS822 in a *bre1*Δ background by Ms. Lindsay Faircloth also fit this model. In this strain, plasmid loss rates showed that tethering Set1 in *bre1*Δ strains also decreases the plasmid stability. *In vivo* Bre1

contributes to H3K4me2 and H3K4me3. However, unlike Spp1, which is required for the catalytic activity of Set1, the role for Bre1 in H3K4me is upstream of Spp1, largely mediated through maintaining stability of the nucleosome, and is not critical for the actual catalytic activity of Set1 [211]. Therefore, tethering Set1 to an origin may bypass the need for Bre1 for di- and tri-methylation of H3K4. Taking into account these assumptions, these results suggest that increasing the local enrichment of H3K4me3 at an origin inhibits stability, which can be interpreted to mean that H3K4me3 plays an inhibitory role in replication.

DISCUSSION

Changes in the post-translational modifications and specifically methylation of histones have been extensively linked transcriptional regulation. More recently, histone methylation has been correlated with certain origins at particular times in the cell cycle [146, 152, 164]. However, it has largely remained unclear whether methylation is involved in regulating replication or if the methylation is a result of replication. In this study, we have utilized two screening methods to identify aspects of chromatin structure that may contribute to efficient replication initiation. Initial results led us to focus on methylation of H3K4, and we have provided several pieces of evidence linking H3K4me to replication regulation.

Methylation of H3K4 promotes efficient replication

Deletion of several proteins that have previously been shown to be important for efficient methylation of H3K4 (Swd1, Spp1, Bre1 and Bre2) improved the growth

of a hypermorphic replication mutant strain. One interpretation of the improved growth in these strains is that deletion of these proteins prevents excess re-replication in the hypermorphic strain; these proteins normally promote replication. Therefore, because they are all important for H3K4me, these results could also indicate a positive role for H3K4 in replication. Similarly, deletion of Set1, Swd1, or Bre1 led to increased plasmid and chromosome instability, which can be interpreted as impaired replication efficiency. Because each of these proteins is required for H3K4me and also for plasmid stability, this provides further evidence that H3K4me promotes replication initiation.

The degree to which H3K4 is methylated may have distinct effects on the regulation of replication

To limit the possibility that indirect effects were responsible for the replication phenotypes, we set out to change the methylation status at a single origin by tethering Set1 to a specific ARS element. This type of targeting has been used successfully in the past to change local chromatin structure [132, 157]. Surprisingly, we found that tethering Set1 to ARS822 on a plasmid actually decreased the stability of the plasmid. It has been suggested that methylation on a single residue, such as H3K36me can have distinct effects depending on the extent to which it is methylated. Therefore, we tested the effect of tethering Set1 to an ARS822 maintenance plasmid in an *spp1*Δ strain, which has reduced ability for facilitating trimethylation of H3K4 but not dimethylation of H3K4. We found that in this case, Set1 targeting actually improved the stability of the plasmid. One model is that

H3K4me2 is important to promote replication, and that H3K4me3 limits H3K4me2, thereby inhibiting replication (Fig 3.7). At first, it is hard to reconcile this model with the growth assays showing that *bre2*Δ improved growth of the hypermorphic replication strain. If H3K4me1/2 promotes replication (and these cells are still capable of me1/2), we might expect the impaired growth phenotype to be exacerbated. However, this outcome may reflect the limitations of this assay and could be explained by pleiotropic effects of the *bre1*Δ. Changes in growth may be attributed to replication defects, but could also be the result of changes in transcription or mitotic deficiencies.

The interpretation of these tethering studies, and our proposed model require several assumptions that must be tested. First, it is imperative that ChIP studies be performed to confirm that the H3K4me state is changing specifically at our targeted origin, and that the extent to which it is methylated is also what is expected. Several additional experiments will also be valuable in determining the role of each degree of H3K4me at replication origins. Tethering the H3K4me2/3 demethylase, Jhd2, to the maintenance plasmid in a WT strain would help further characterize the role of each methylation state. Additional LexOp-tagged origins, and monitoring origin firing in a genomic context will also be valuable. Finally, once the role of H3K4me is more defined, it will be interesting to determine the mechanism by which it functions. Does H3K4me act at the licensing step to prevent MCM loading, or does it act later in the pathway to prevent firing by limiting recruitment of the Cdc45 or other initiation factors? In any case, these observations represent the growing body of evidence linking H3K4me to the regulation of replication origins.

Table 3.1

Ruy028 x ___Δ	**Growth**
Asf1	x
Bre1	**+**
Bre2	**+**
Bur2	x
Chd1	**+**
Clb5	**-**
Ctk1	x
Dig2	x
Dot1	x
Eaf3	x
Eaf6	x
Gcn5	x
Hat1	x
Hir1	x
Hos2	x
Hpa2	x
Isa1	x
Jhd1	**+**
Jhd2	**+**
Mre11	**-**
Rad6	**+**
Rph1	x
Rtt109	**-**
Sap30	x
Set2	x
Set3	x
Snf1	**-**
Spp1	**+**
Spt7	x
Spt8	**+**
Swd1	**+**
Tof1	x
Tom1	x

Table 3.1 Results of genetic screen. Several factors associated with modifying chromatin were screened. The factors that were individually deleted in the hypermorphic replication strain, Ruy028, are listed on the left. Under inducing conditions, the growth of these strains was compared to the growth of the Ruy028 strain alone. The observed growth is denoted as better (+) than the Ruy028 strain alone, worse (-) than the Ruy028 strain alone, or no change (x) relative to the Ruy028 strain alone. Strains that demonstrated a change in growth are highlighted in bold.

Figure 3.1

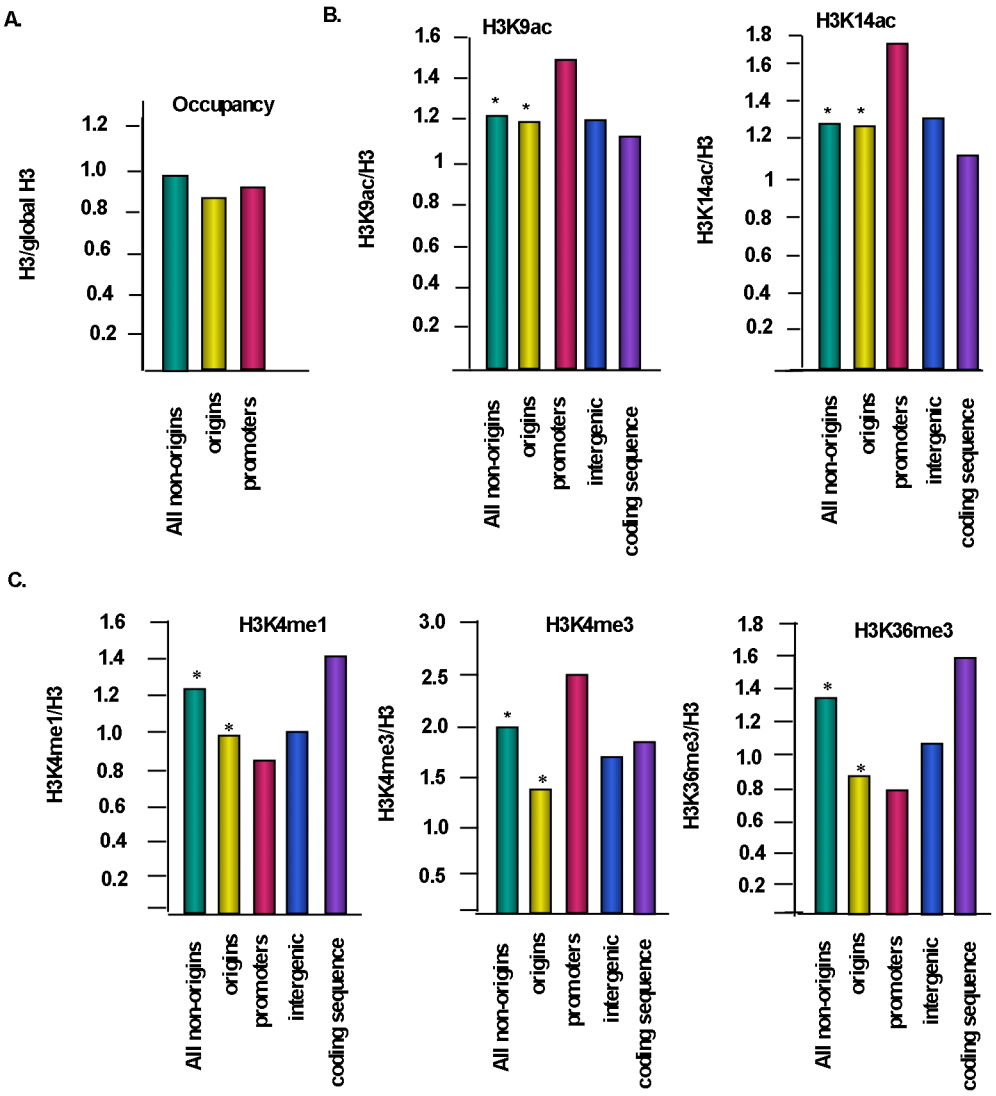

A.
Occupancy
H3/global H3
1.2
1.0
0.8
0.6
0.4
0.2
All non-origins
origins
promoters
B.
H3K9ac
H3K9ac/H3
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
*
*
All non-origins
origins
promoters
intergenic
coding sequence
H3K14ac
H3K14ac/H3
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
*
*
All non-origins
origins
promoters
intergenic
coding sequence
C.
H3K4me1
H3K4me1/H3
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
*
*
All non-origins
origins
promoters
intergenic
coding sequence
H3K4me3
H3K4me3/H3
3.0
2.5
2.0
1.5
1.0
0.5
*
*
All non-origins
origins
promoters
intergenic
coding sequence
H3K36me3
H3K36me3/H3
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
*
*
All non-origins
origins
promoters
intergenic
coding sequence

Figure 3.1 Comparison of nucleosome occupancy and histone modification status between origins and other genomic loci. 314 confirmed origin sequences were overlayed with published nucleosome mapping data. The nucleosome occupancy or enrichment of a specific modification at origins was compared to all non-origin sequences, promoters, intergenic regions, and open reading frames. (A) Nucleosome occupancy is reported as the signal of H3 at a subset of genomic loci / global H3. Both promoters and origins were nucleosome depleted relative to non-origin sequences; *$p < 2 \times 10^{-10}$. (B) The average histone acetylation / total H3 was reported for H3K9ac and H3K14ac. The difference in histone acetylation between origins and non-origin sequences is not statistically significant; *$p > 0.5$. (C) The average histone methylation / total H3 was reported for H3K4me1, H3K4me3, and H3K36me3. Relative to non-origin sequences, origins were depleted for all three modifications; *$p < 4.0 \times 10^{-12}$.

Figure 3.2

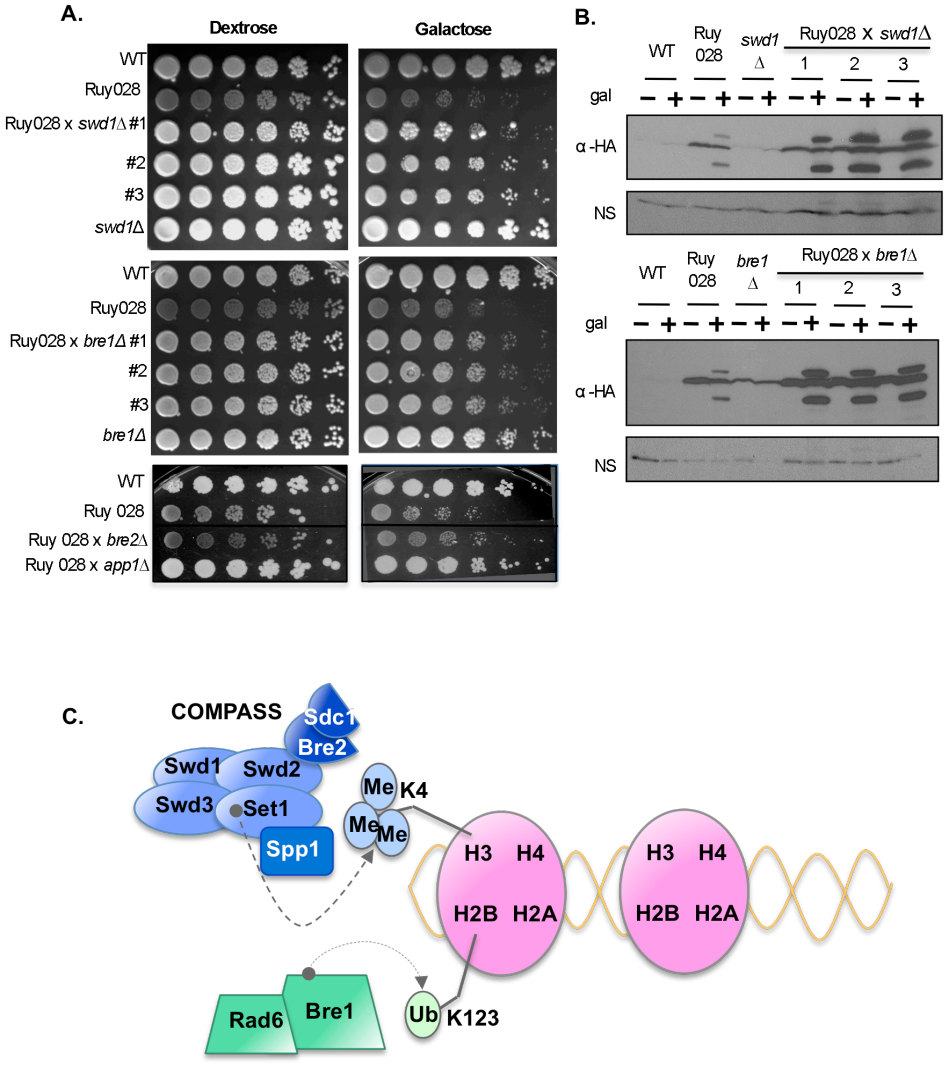

A.
Dextrose
Galactose
WT
Ruy028
Ruy028 x swd1Δ #1
#2
#3
swd1Δ
WT
Ruy028
Ruy028 x bre1Δ #1
#2
#3
bre1Δ
WT
Ruy 028
Ruy 028 x bre2Δ
Ruy 028 x app1Δ
B.
WT
Ruy 028
swd1 Δ
Ruy028 x swd1Δ
1
2
3
gal
α -HA
NS
WT
Ruy 028
bre1 Δ
Ruy028 x bre1Δ
1
2
3
gal
α -HA
NS
C.
COMPASS
Sdc1
Bre2
Swd1
Swd2
Swd3
Set1
Spp1
Me
K4
Me
Me
H3
H4
H2B
H2A
H3
H4
H2B
H2A
Rad6
Bre1
Ub
K123

Figure 3.2 Growth assays detect genetic interactions between histone modifying enzymes and replication factors. (A) Five-fold serial dilutions of WT, Ruy028 (hypermorphic replication strain), *swd1*Δ*, bre1*Δ*,* three independent isolates of both Ruy028 x *swd1*Δ and Ruy028 x *bre1*Δ (constructed by mating), and single isolates of Ruy028 x *bre2*Δ and Ruy028 x *spp1*Δ (constructed by mating) were plated on dextrose (control) and galactose (induce expression of the stable Cdc6-NTΔ mutant). (B) Immunoblot of expression of HA-tagged Cdc6-NTΔ mutant in dextrose and galactose corresponding to Swd1 and Bre1 spotting panels. A non-specific band served as loading control. (C) Model. The Rad6-Bre1 complex catalyzes monoubiquitination of H2BK123. This promotes nucleosome stability to facilitate efficient H3K4me2 and H3K4me3. The COMPASS complex mediates the H3K4 methyltransferase catalytic activity of Set1. Swd1, Swd2, and Swd3 are required for complex integrity. Swd2 is also critical for H3K4me2 and H3K4me3 specifically, and Spp1, Sdc1 and Bre2 are necessary for efficient H3K4me3.

Figure 3.3

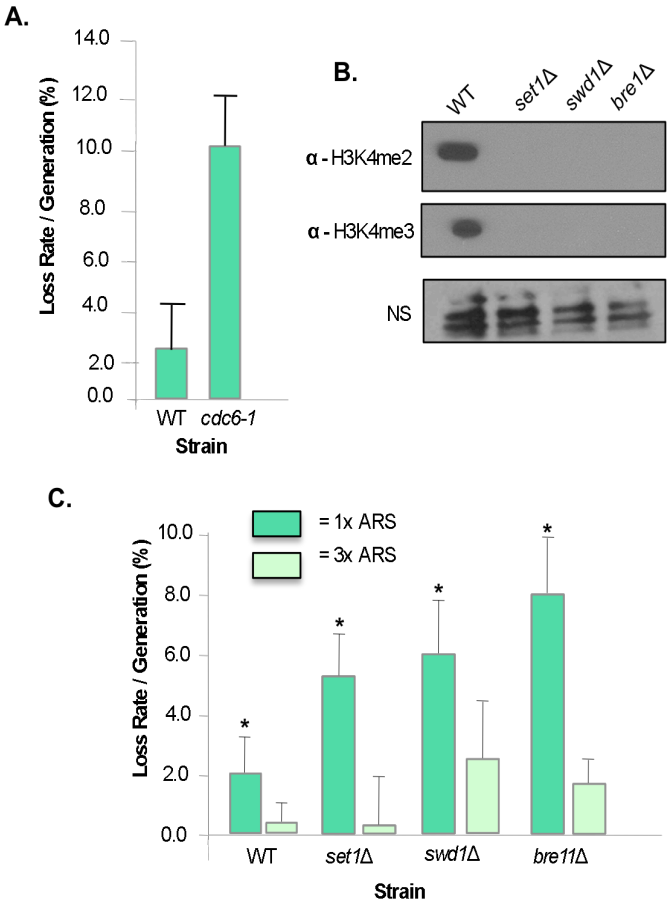
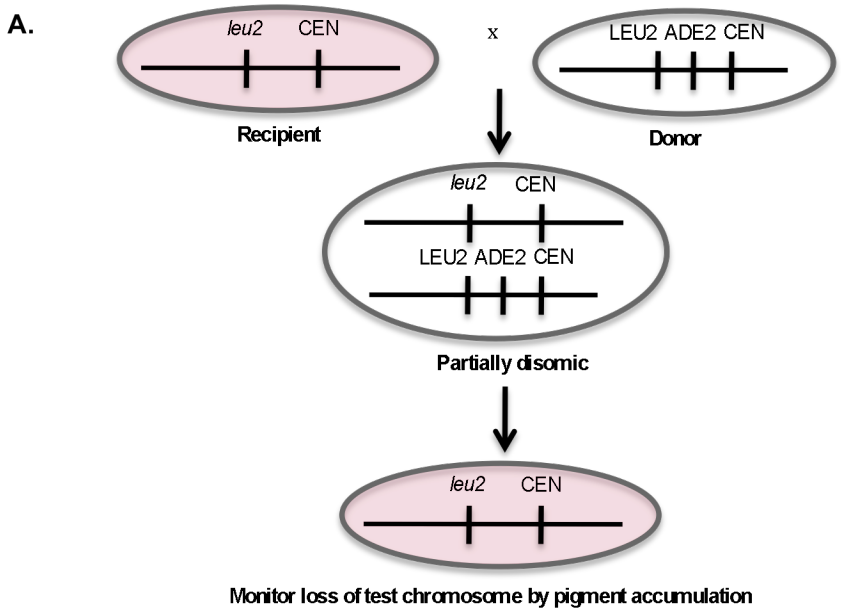

A.
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
Loss Rate / Generation (%)
WT
cdc6-1
Strain
B.
WT
set1Δ
swd1Δ
bre1Δ
α - H3K4me2
α - H3K4me3
NS
C.
= 1x ARS
= 3x ARS
10.0
8.0
6.0
4.0
2.0
0.0
Loss Rate / Generation (%)
*
WT
set1Δ
swd1Δ
bre11Δ
Strain

Figure 3.3 Plasmid maintenance assays are consistent with a role for H3K4me in replication regulation. The plasmid loss rate was compared between several strains that were grown in non-selective medium at 31° for several generations. The stability is reported as a percentage (loss rate / generation). (A) WT and *cdc6-1* (replication hypomorphic strain) were transformed with 1xARS. (B) Iummunoblotting of H3K4me2 and H3K4me3 in WT, *set1*Δ, *swd1*Δ, and *bre1*Δ cells. A non-specific band of the H3K4me2 immunoblot served as loading control. (C) WT, *set1*Δ, *swd1*Δ, and *bre1*Δ strains were transformed with 1xARS (dark green) or 3xARS (light green). P-values comparing the 1xARS loss rate between WT and *set1*Δ, *swd1*Δ, and *bre1*Δ were statistically significant; *p < .04 for all.

Figure 3.4


A.
leu2
CEN
x
LEU2 ADE2 CEN
Recipient
Donor
leu2
CEN
LEU2 ADE2 CEN
Partially disomic
leu2
CEN
Monitor loss of test chromosome by pigment accumulation

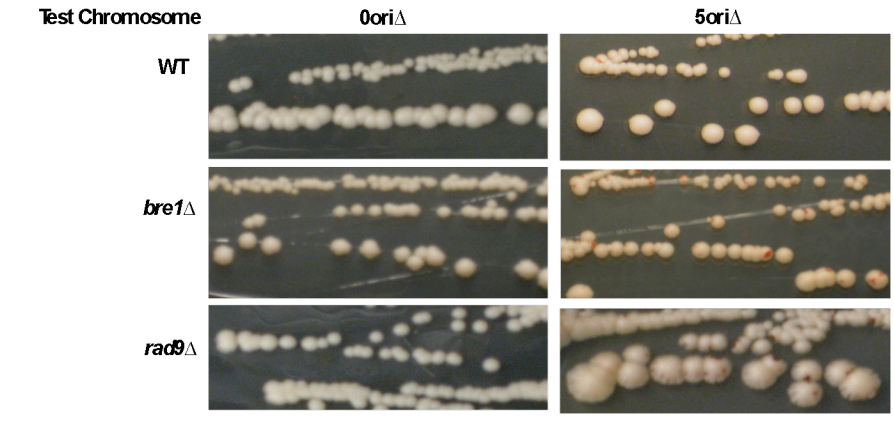

B.
Test Chromosome
0oriΔ
5oriΔ
WT
bre1Δ
rad9Δ

Figure 3.4 Chromosome stability assays are consistent with a role for H3K4me in replication regulation. (A) Schematic of the chromosome stability assay. Strains are mated and undergo selection to create partially disomic strains. Loss of the test chromosome, which contains ADE2, is monitored by pigment accumulation in the *ade*$^-$ background. (B) Representative colonies of the WT, *bre1*Δ, or *rad9*Δ carrying the 0oriΔ test chromosome (control, origins intact) or the 5oriΔ test chromosome (all efficient origins deleted) were photographed after growth overnight at 30° followed by 10 days at room temperature.

Figure 3.5

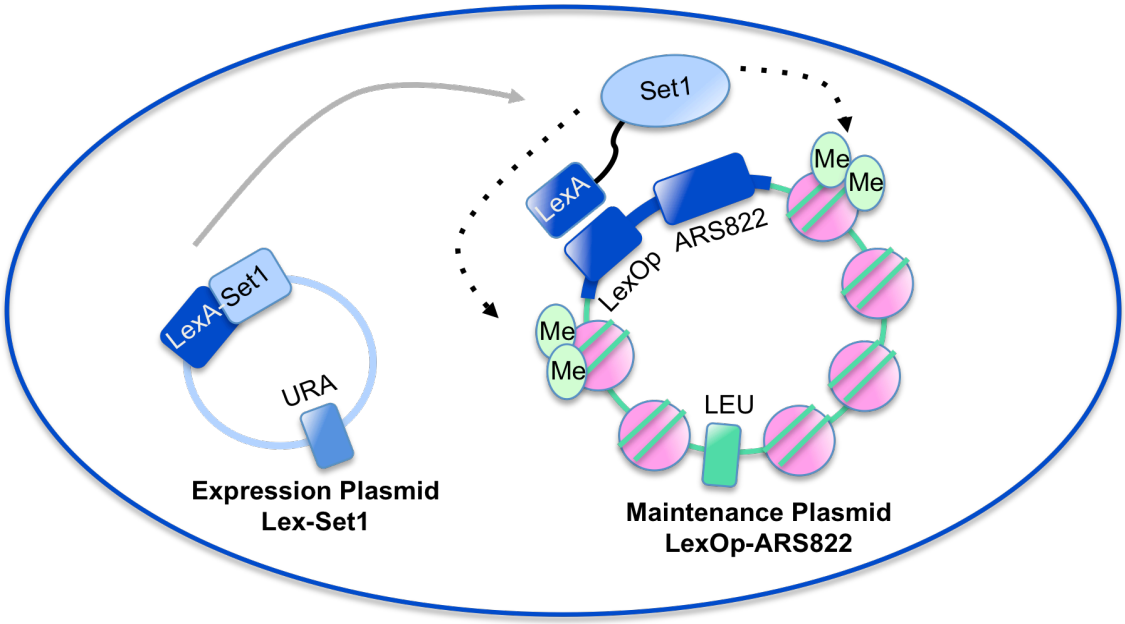

Set1
LexA
Me
Me
LexOp
ARS822
Me
Me
LEU
LexA
Set1
URA
Expression Plasmid
Lex-Set1
Maintenance Plasmid
LexOp-ARS822

Figure 3.5 Artificial targeting of Set1. Schematic of the strategy used to target Set1 to LexOp-ARS822. LexOp-ARS822 cotains 3kb of the genomic region surrounding the ARS822 with four LexA operator sites inserted 300 base-pairs from the ARS region. The LexA-Set1 plasmid was constructed to express a galactose-inducible fusion protein of LexA and Set1. The LexA portion of the fusion protein binds to the LexA operator sites on the plasmid, specifically recruiting Set1 to the plasmid ARS822. The expression plasmid (LexA, LexASet1WT, or LexA Set1CD) are co-transformed with the maintenance plasmid (LexOp-ARS822 or NoOp-ARS822). These cells are then grown in medium that maintains the expression plasmid but is non-selective for the maintenance plasmid (SC-ura), so plasmid stability may be measured.

Figure 3.6

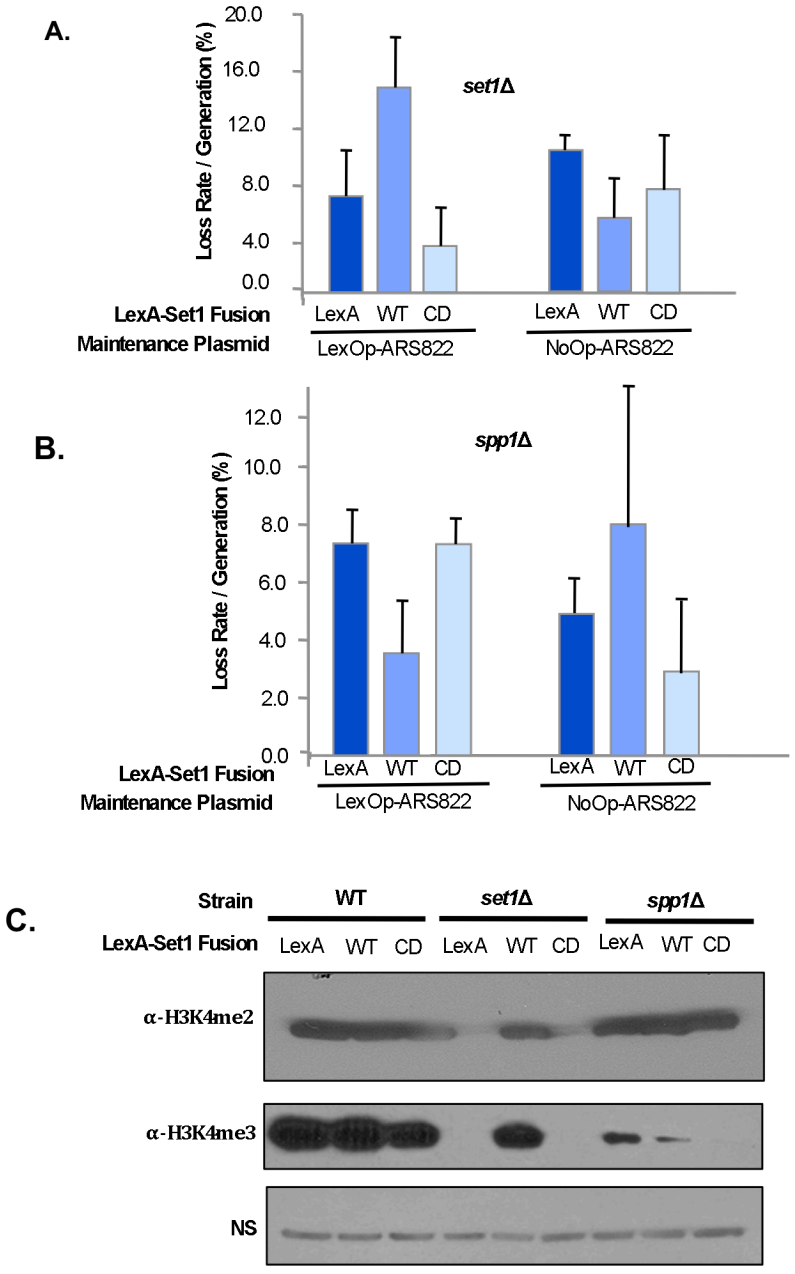

A.
Loss Rate / Generation (%)
20.0
16.0
12.0
8.0
4.0
0.0
set1Δ
LexA-Set1 Fusion
Maintenance Plasmid
LexA WT CD
LexA WT CD
LexOp-ARS822
NoOp-ARS822
B.
Loss Rate / Generation (%)
12.0
10.0
8.0
6.0
4.0
2.0
0.0
spp1Δ
LexA-Set1 Fusion
Maintenance Plasmid
LexA WT CD
LexA WT CD
LexOp-ARS822
NoOp-ARS822
C.
Strain
WT
set1Δ
spp1Δ
LexA-Set1 Fusion
LexA WT CD LexA WT CD LexA WT CD
α-H3K4me2
α-H3K4me3
NS

Figure 3.6 Set1 targeting to an extrachromosomal plasmid affects plasmid stability. Cells were co-transformed with an expression plasmid carrying a LexA-fusion (LexA-Set1WT, LexA-Set1CD, or LexA-alone) and a maintenance plasmid (LexOp-ARS822 or the untargeted NoOp-ARS822). The strains were grown for 24 hours (or at least seven generations) in SC-ura + 1%raffinose + 1% galactose (to induce expression of the LexA-fusion). The plasmid stability is reported a percentage (loss rate / generation). (A) (B) Plasmid maintenance assays were performed in a *set1*Δ or *spp1*Δ background, respectively. The difference in maintenance between the LexA-Set1WT and LexA-alone was statistically significant in both backgrounds for only the LexOp-ARS822 plasmid; *p < .02 and not for the NoOp-ARS822 plasmid. There was no significant difference in maintenance when LexA-alone or LexA-Set1CD was expressed. (C) Immunoblotting of H3K4me2 and H3K4me3 on whole cell lysates of WT, *set1*Δ, and *spp1*Δ cells. A non-specific band from a LexA immunoblot was used as loading control.

Figure 3.7

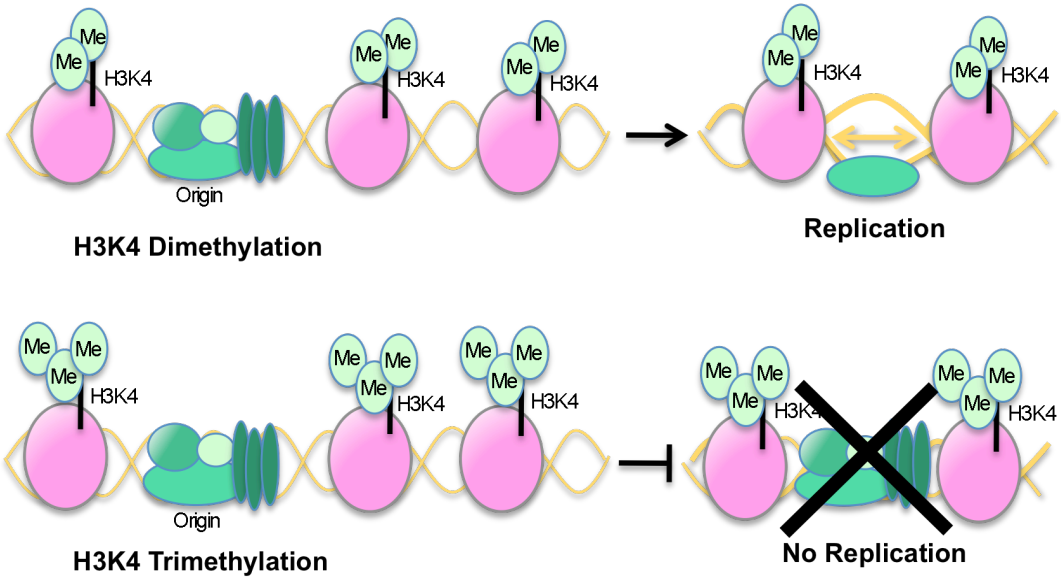

Me
H3K4
Origin
H3K4 Dimethylation
Replication
H3K4 Trimethylation
No Replication

Figure 3.7 Model for the role of H3K4me in origin regulation. Dimethylation (or mono-) of H3K4 contributes to a chromatin environment that is consistent with origin firing. Trimethylation of H3K4 contributes to a chromatin environment that does not promote origin initiation, either through H3K4me3-specific inhibition or by limiting the level of H3K4me2 at the origin.

Chapter 4

Future directions and perspectives

FUTURE DIRECTIONS

Differentiate the effects of mono-, di-, and trimethylated H3K4

Evidence presented in chapter three indicates that the extent to which an individual H3K4 residue is methylated may have variable consequences on the replication program. To separate the role of each methylation state, several additional experiments should be performed. First, plasmid maintenance assays in cells with altered COMPASS function would be valuable. For instance, Spp1 and Sdc1 are required specifically for efficient trimethylation of H3K4 and strains mutant for them would be good candidates for this assay. If trimethylation inhibits replication initiation in wild-type cells, we would expect to see no change or improved plasmid maintenance in strains lacking these COMPASS subunits.

Additional tethering studies could also help to differentiate the replication phenotypes of each H3K4 methylation state. Tethering the H3K4 demethylase, Jhd2, to an origin in a wild-type strain should result in loss of both H3K4me2 and H3K4me3 without affecting the H3K4me1 [212, 213]. A significant decrease in plasmid maintenance when Jhd2 is tethered would indicate a role for H3K4me2 in promoting replication (as previous data indicates that H3K4me3 inhibits replication). If tethering Jhd2 to an origin does not alter the origin's ability to be maintained, the result becomes harder to interpret. One possibility is that H3K4me2 is not involved in replication of the plasmid. Alternatively, over the entire population of cells, the removal of the suggested inhibitory H3K4me3 and removal of the potentially promoting mark, H3K4me2, may balance out any potential effect of the other. This

outcome would depend on whether H3K4me3 is inhibitory itself, or if it only inhibits origin function by preventing the presence of H3K4me2.

Constructs tethering mutant forms of Set1 would also be useful. Schlicter and Cairns characterized the methyltransferase ability of a variety of Set1 mutants [214]. Of particular interest would be the Set1ΔRRM mutants, which show diminished ability to trimethylate but can still effectively mono and dimethylate H3K4. Targeting the Set1ΔRRM mutant to the maintenance plasmid in a *set1*Δ background would direct H3K4me1/2, but not H3K4me3 to the tagged origin. This experiment would be an independent method to confirm the results observed when Set1 was targeted in an *spp1*Δ strain.

Determine if there is a direct effect on origin firing:

The assays that have been employed thus far indirectly link H3K4me with the regulation of origin function. Moving forward it will be critical to show directly that origin firing is affected by changes in H3K4me. BrdU-IP assays can be used to monitor origin function directly and therefore would be a good candidate to monitor changes in replication when the H3K4me status is altered. BrdU-IP has been used successfully to immunoprecipitate newly synthesized DNA and to detect changes in replication origin activity under varying conditions [133]. This method utilizes *in vivo* incorporation of the thymidine analog BrdU into newly replicated DNA. The newly replicated DNA (marked by BrdU incorporation) is immunoprecipitated using an antibody to the BrdU moiety, which is only found in the nascent DNA. Quantitative PCR using origin-specific primers would measure the amount of newly-synthesized

DNA at that origin. Increased signal specific to the origin and not found at nearby genomic regions would indicate the firing of that origin. Additionally, a modified version of the fiber analysis assay developed in chapter two to detect changes in firing and re-firing of an origin could be used to detect replication initiation changes at the single molecule level. Following the labeling and DNA stretching method in our protocol, fluorescently-labeled probes to specific DNA sequences could be hybridized to the DNA to monitor specific origins in varying conditions. DNA combing has been used effectively in many different model systems including yeast, and this two-step method has been optimized and used successfully in mammalian cells [215-218].

Identify the step at which histone modifications regulate replication.

Presuming that H3K4me and other marks identified in our screen are demonstrated to affect replication, we will determine which step of replication is affected. That is, which stage of origin regulation (licensing, initiation, or inhibition) is altered by the presence of the post-translational modification and how are the individual components of the replication machinery affected? For instance, does H3K4 methylation status affect origin identity and modify the ability of ORC to recognize and/or bind to initiation sites? Or, does the modification cause defects in licensing, preventing efficient MCM loading onto chromatin? To determine the step of replication that is affected, ChIP analysis of replication factors in synchronized cells will be conducted in WT, *set1*Δ*, spp1*Δ*,* and *swd2*Δ strains. We plan to ChIP for ORC, MCM, and Cdc45 to determine if changes in the chromatin modifications affect

origin identity, licensing, or firing. In the past, several groups have reported difficulty in successfully immunoprecipitating certain replication factors during specific cell cycle phases (ex/ ORC in G1). To address this, members of the Cook Lab have created strains expressing biotinylated forms of these replication proteins with the hope that the extended tag and the strong interaction between biotin and streptavidin will improve immunoprecipitation efficiency.

Alternatively, or as a follow-up to canonical ChIP, ChIP-Seq may be a logical next step. ChIP-seq has been successfully used to examine the DNA associated with ORC binding sites, and we will examine the binding of other replication factors such as MCM and Cdc45 [122]. The advantage of this method is that all origins may be examined at once eliminating any bias in choosing origins. Because most of the histone-modifying enzymes are not essential, I would expect that not all origins would show the same change in replication factor association. A subset of origins may be more or less susceptible to a change in local chromatin architecture and genomic analysis will determine the characteristics of these origins. For example, early-firing origins may require one specific H3K4me state whereas recruitment of replication factors to late-firing origins may be unaffected by the methylation status of H3K4.

Determine the role of the chromatin environment in regulating human origins

Characterizing the role of the chromatin environment is critical because abundant changes in the post-translational modifications of histones have been shown to be common among tumor cells [219]. H4K20me3 and H4K16ac are two

examples of modifications that have been correlated both with transformed cells and with origins of replication [219]. It follows then, that understanding the chromatin environment at origins in human cells may also help our understanding of tumor formation. The work in this project utilizes budding yeast as a model system. Unlike human cells, budding yeast provides a myriad of genetic tools and has well-defined origins . However, the purpose of the model system is to gain insight into the human system. I expect that many findings will be transferable due to the high degree of conservation of both histone modifications and the conservation of catalytic domains of histone modifying enzymes. Nevertheless, there is not absolute conservation in either of these categories and therefore experimental manipulation of human cells will be critical. There have been several genome-wide studies (discussed in the introductory chapter) that correlate histone modifications with human origins [152, 156]. Additionally, it will be important to perform functional studies on human cells. Methods to induce overexpression of proteins, by adenovirus transduction for instance, will be useful. Likewise, down-regulation of histone modifying enzymes alone and in combination by siRNA will be valuable. Although knockdown may be less complete than the full deletions that are constructed easily in yeast strains, our plasmid maintenance data of targeted origins provides evidence that these methods will nevertheless be effective. For instance, when expressing Set1 in an *spp1*Δ strain, we can still detect low levels of H3K4me3, but the change in H3K4me3 relative to the control is large enough to see robust changes in plasmid stability. These and other techniques will be necessary to characterize the effect of the chromatin environment on human replication origins.

UNANSWERED QUESTIONS

What regulates the methylation state of individual histone residues?

Single enzymes often have the ability to methylate a single residue to varying extents. Several studies have made significant progress in explaining *how* a single enzyme can be responsible for various methylation states. These studies have shown that changes in the association of the methyltransferase with other proteins can affect the extent of methylation. For instance, the WDR5 cofactor stimulates MLL1 to trimethylate targets in human cells, and similarly Pdp1 is required for efficient di- and trimethylation of H4K20 in *S. pombe* [220, 221]. In the case of H3K4me in budding yeast, it is proposed that Spp1, which facilitates H3K4me3, helps position the hydroxyl group of the tyrosine that is found in the “phe/tyr” switch region to make space for the addition of the third methyl group [210]. Despite the understanding that binding partners can alter the extent to which a methyltransferase modifies a lysine, it remains unclear how the cell determines the extent of the methylation to place at a specific genomic location or particular phase in the cell cycle.

Future studies that characterize the regulation of these co-factors (Spp1 for example) will be critical. How does their localization, expression, and degradation change throughout the cell cycle? Are they substrates for post-translational modification(s) that affect their interaction with the histone methyltransferase or regulate the catalytic activity of other complex components? Budding yeast Set1

associates with the phosphorylated CTD of pol II and other components of the transcription machinery as it functions in transcription. Perhaps post-translational modifications of other DNA-associated proteins can function in an analogous manner and affect the association of Set1 with origins.

What is the mechanism by which H3K4me status regulates replication?

A few explanations have been put forward to describe how the chromatin environment can affect DNA-associated processes. One of the first hypotheses proposed was that acetylation of histones facilitates an open chromatin conformation thereby making the DNA more accessible to factors for transcription, etc. The basis behind this theory is that acetylation neutralizes the basic charge and disrupts the histone:histone and histone:DNA contacts [222]. The 'loosening' of chromatin as a result of histone acetylation has been confirmed by several studies, and H4K16ac has been shown to inhibit chromatin compaction [223, 224]. It is possible that the chromatin conformation may allow DNA replication factors to access origins more easily.

Alternatively, "readers" of post-translationally modified histones may provide the link to replication. A myriad of domains have been identified that recognize specific histone modifications. The chromo, tudor, PHD, MBT, and Wd40 repeats are just some of the recognition motifs for histone methylation alone [225]. There are a few methods by which these histone readers may affect replication or other DNA processes. First, readers may also modify the openness of the chromatin. Chromo and tudor domains have been found in histone remodelers which change

the chromatin structure by altering the DNA:histone interactions [225]. This conformation could change the accessibility of origins to regulate access for replication factors.

The readers, or mediators, may also interact directly with replication factors to assist in their recruitment. Examples of mediators linking histone modifications to replication initiation factors have not yet been identified, however this strategy has been used for recruiting factors involved in transcription. TAF3 has a PHD finger that binds selectively to H3K4me3 and is required for efficient TFIID binding to DNA [226]. Also, H4K16ac together with H3S10ph act as a platform for Brd4 binding which recruits a positive transcription elongation factor to the chromatin [227].

Finally, instead of recruiting replication factors, the mediators may recruit additional chromatin modifiers. There is some evidence that this mechanism is utilized; specifically, H4K20me2 has been shown to stimulate H4K14ac [157]. It remains a possibility that all marks function with the ultimate goal of recruiting acetylation to induce structural changes of the chromatin and change the accessibility of specific genomic loci. H3K4me2 has been demonstrated to recruit the Set3 complex through its PHD domain, and two subunits of the Set3 complex, Hos2 and Hst1, have histone deacetylase function [228]. In the context of gene expression, recruitment of these factors near the 5' end of genes plays a positive role in transcription [229]. It remains a possibility that Set3 or other histone modifying enzymes may also be recruited by H3K4me at origins.

How do combinations of histone modifications function?

As mentioned previously, most histone modifiers are not essential for cell viability. How then, can histone modifying enzymes be essential for the replication program? One possibility is that modifying enzymes may have redundant functions. On the other hand, there are several examples where a single known histone modifier exists for catalytic transfer of a specific mark (ex/ Set1 is the only known histone methyltransferase for H3K4 in yeast). Taking this fact into account, an alternative possibility is that each single histone modification doesn't change the recruitment of a particular replication factor, but instead is important for contributing to the creation of a general origin chromatin environment that favors a particular outcome (firing, inhibition of licensing, etc). In this model, the chromatin environment is essential, but any single histone modification that contributes to the environment may be dispensable. The prediction is that the elimination of several marks at origins (thus destroying the local chromatin environment) would result in growth phenotypes and heightened sensitivity to treatments that interfere with replication (HU for example). This model could explain how Set1, the only H3K4 methyltransferase in yeast, may play a role in regulating replication, but can be deleted without affecting the cell viability or causing significant changes in cell cycle progression. This model could be tested by changing several aspects of the chromatin landscape at once. To determine which combination of histone modifications should be altered, identification of common combinations of histone modifications will be extremely valuable.

Already, approaches that have attempted to examine multiple modifications at once have been successful in identifying histone modifications that may function

together. Top-down mass spectrometry, which analyzes intact protein samples (as opposed to digested ones) have identified interesting patterns of combinations of modifications [230]. Genome-wide association studies by ChIP-seq find that modifications in combination may in fact favor specific outcomes. For instance, the status of H3K27me3 and H3K4me3 together are correlated with commitment to a defined cellular lineage [231]. Utilization of peptide arrays and genome-wide analyses taken on by the ENCODE consortium and more recently the modENCODE project have also been important in identifying genome-wide patterns of chromatin signature and effector protein interactions [108, 232, 233]. Moving forward, one challenge to these techniques will be determining how unmodified residues affect the DNA templated processes that are regulated by the chromatin environment. For example, modification of H3R2 inhibits the binding of certain factors to methylated H3K4 [234]. These and other studies illustrate the mounting evidence that the combination of chromatin modifications (as opposed to any single mark) is critical for regulating DNA-associated processes.

Why are there so many potential origins?

The eukaryotic genome is large, and to duplicate it in its entirety in a single S phase, replication initiates at multiple origins. In fact, there are checkpoints to ensure that the entire genome will be duplicated. First, a G1-licensing checkpoint prevents progression into S phase unless adequate preRC assembly has taken place [48]. Another checkpoint prevents mitosis unless enough firing has occurred in S phase to replicate the entire genome [235]. Interestingly, although these

checkpoints are in place to guarantee that there is adequate replication, there is no shortage of potential origins. Curiously, there is an abundance of potential origins in eukaryotic cells, and estimates suggest that in any one cell cycle only 50% of yeast origins and less than 20% of metazoan origins actually initiate DNA replication [112, 236]. In *S. pombe* origins exist in clusters and only one origin in the cluster is "chosen" to fire. Together, these observations suggest that although there are mechanisms in place to track the global replication program, origins are individually regulated by a variety of factors including the chromatin environment. However, the question still remains; why are there so many potential origins to begin with? Because disruption in origin regulation can lead to re-replication as demonstrated in chapter two, it seems counterintuitive to have an abundance of potential origins.

One hypothesis is that excess origins provide flexibility in the replication program. Cells have to respond to environmental changes such as stress signals and DNA damage, both of which can inhibit the replication machinery. Higher eukaryotes also undergo development-associated genome reorganization and all eukaryotes show variations in gene expression depending on the cell type. It is known that the genomic loci that get used as origins are not just defined or affected by DNA sequence, but by chromatin modifications as demonstrated by this work and other studies. Other groups have also shown that distal regulatory elements, nearby origins, transcription and chromosome topology are also important for determining origin regulation [237]. Many of these aspects of DNA (topology, active transcription sites, etc) can change depending on cell type and exogenous factors (like temperature). Because the organization, access to, and use of the DNA changes

with the cellular context, the abundance of potential origins may be needed to provide origin locations that are optimal for firing in specific DNA conformations.

What factors affect the functional outcomes of chromatin features?

How influential is the chromatin structure on origin activity; is proper chromatin structure sufficient to create an origin? It is known that improper chromatin environment such as positioning a nucleosome over an ACS sequence can destroy its ability to fire [238]. Artificial targeting of Set8, the human mono-methyltransferase for H4K20 was sufficient to induce preRC formation [157]. Nevertheless, the evidence presented throughout this discussion favors the model that chromatin modifications function combinatorally. Moreover, as touched upon in the previous section, even the overall chromatin state at an origin is not maintained in a vacuum.

There are many additional factors, like transcription and chromosome topology that depend on the cellular context. I hypothesize that many aspects of the cellular context contribute to the formation of a specific DNA conformation, and that this conformation and all of the factors that establish it must be accounted for to accurately predict the functional outcome of the chromatin state on origin regulation. It is apparent from the previously mentioned studies that chromatin landscape can affect origin regulation. It is also clear that other aspects of the cellular environment can affect replication. For instance, in mammals, imprinted genes have been shown to replicate asynchronously while homologous alleles replicate synchronously [239]. This pattern is set early in development, but is erased in the germ-line prior to

meiosis [240]. Importantly, there is mounting evidence that the cellular context affects the functional outcome of histone modifications. For instance, the genes that are transcribed change depending on the cell type; and induction of transcription has been demonstrated to silence previously active origins that are found within coding regions in mammalian cells [241]. Also, recruitment of the chromatin remodeler ISWI to chromatin is dependent on H3K4me3 (as part of NURF complex) and its recruitment is required for replication – but only through heterochromatic regions [242]. This demonstrates that understanding factors beyond the post-translational modification signature are critical for predicting the functional outcome DNA-templated processes. Illustrating this concept further, H3K9me, for instance, can either repress or activate transcription depending on whether it is found at promoters or coding regions, respectively [243]. Also, H3K4me3 is implicated in a two independent DNA-related processes, transcription and VDJ recombination. Without the cellular context it is unclear whether H3K4me3 is present at a specific genomic loci to promote transcription or recombination. In total, these observations demonstrate that chromatin landscape is important for regulating DNA-associated processes, but the functional outcome of the chromatin landscape depends on additional contextual elements of the cell.

CONCLUDING REMARKS

Before these studies began, there were many aspects of the replication program (origin identity) and the individual activity of origins (asynchronous firing, susceptibility to re-firing, etc), which suggested that mechanisms beyond regulation

of preRC components were critical for replication. Preliminary work by other groups had found evidence for histone acetylation promoting origin firing. In this study, we have provided evidence to implicate additional histone post-translational modifications, most extensively H3K4me in regulating replication. During the course of this work, independent laboratories have also correlated H3K4me and other modifications with origins. Additional investigations will be needed to characterize all of the contextual attributes of the cell that contribute to the functional outcome of the chromatin landscape at origins. Understanding these elements will enable us to advance our understanding of the replication program and cellular proliferation overall. Aberrant proliferation has been shown to induce premature cellular aging, affect tissue regeneration after heart attacks, and may play a role in oncogenesis [100, 102, 219, 244, 245]. Therefore, expanding our understanding of DNA replication and cellular proliferation is critical for the development more accurate disease detection methods, and improved treatment options.

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