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Chromatin Unfolding by Cdt1 Regulates MCM Loading via Opposing Functions of HBO1 and HDAC11-Geminin

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
Department of Cancer Biology
College of Arts and Sciences
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Keywords: Cdt1, HDAC11, HBO1, chromatin, DNA replication

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DEDICATION

To my mother, Ming Hsieh
and my father, Quon Wong

And to my wife,
Nancy Parquet

ACKNOWLEDGEMENTS

I would first like to thank Dr. Mark Alexandrow for his mentorship and guidance. I am grateful that he has put forth the effort to teach me what it means to become a scientist and I feel extremely fortunate to have been a member of his lab.

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I thank the Cancer Biology Program for giving me the opportunity to pursue my doctoral training and providing an outstanding educational environment. The members of my lab, past and present, have helped me a great deal and I owe them my gratitude.

Finally, I owe my greatest thanks to my family and friends. I thank my parents for their continual support and love and I am grateful for the laughter BCP and HJW have brought me. I am forever indebted to Nancy Parquet for being a constant source of inspiration and encouragement, without her I would not have been able to complete this dissertation.

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LIST OF ABBREVIATIONS

AAA+	ATPases Associated with a variety of cellular Activities
APC	Anaphase Promoting Complex
ARS	Autonomously Replicating Sequence
ATP	Adenosine Triphosphate
CDC	Cell Division Cycle
CDK	Cyclin Dependent Kinase
Cdt1	Cdc10 Dependent Transcript
DNA	Deoxyribonucleic Acid
FEN1	Flap endonuclease 1
G1-phase	Gap 1 phase
G2-phase	Gap 2 phase
GINS	Go Ich Nii and San replication complex
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylase
HBO1	HAT Binding Orc1
KAT	Lysine Acetyltransferase
MCM	Mini-Chromosome Maintenance complex of proteins

M-phase	Mitosis
Ori	Origin of Replication
ORC	Origin Recognition Complex
PCNA	Proliferating Cell Nuclear Antigen
PHD	Plant Homeo Domain
PIP	PCNA Interaction-Protein Motif
pol	Polymerase
preRC	Pre-Replication Complex
RFC	Replication Factor C
SAHA	suberoyl anilide bishydroxamide
Sir2	Silent Information Regulator 2
S-phase	Synthesis phase
TSA	Trichostatin A

ABSTRACT

The efficiency of metazoan origins of DNA replication is known to be enhanced by histone acetylation near origins. Although this correlates with increased MCM recruitment, the mechanism by which such acetylation regulates MCM loading is unknown. We show here that Cdt1 induces large-scale chromatin decondensation that is required for MCM recruitment. This process occurs in G1, is suppressed by Geminin, and requires HBO1 HAT activity and histone H4 modifications. HDAC11, which binds Cdt1 and replication origins during S-phase, potently inhibits Cdt1-induced chromatin unfolding and re-replication, suppresses MCM loading, and binds Cdt1 more efficiently in the presence of Geminin. We also demonstrate that chromatin at endogenous origins is more accessible in G1 relative to S-phase. These results provide evidence that histone acetylation promotes MCM loading via enhanced chromatin accessibility. This process is regulated positively by Cdt1 and HBO1 in G1 and repressed by Geminin-HDAC11 association with Cdt1 in S-phase, and represents a novel form of replication licensing control.

CHAPTER 1

INTRODUCTION

Cancer

Cancer is not one specific disease, but instead is a grouping of over 100 distinct diseases that share the fundamental characteristics of uncontrolled cellular growth and invasiveness (Stratton et al. 2009). Individual cancer cells escape the biological regulatory mechanisms that control normal cellular proliferation leading to their accumulation and dissemination (Collins et al. 1997; Frank 2007). The spread of cancerous cells ignores anatomical constraints, utilizing physical and chemical means to invade surrounding tissues and metastasize to other areas of the body (Kufe et al. 2003; Folkman 2006). Since tumor size is directly proportionate to nutrient and oxygen demand, angiogenesis, or neovascularization, is required to support the growth of a tumor and its metastatic colonies (Folkman 2006). Tumor expansion, therefore, negatively affects the normal function of organs not only by physical disruption due to increasing mass, but also by robbing neighboring tissues of nutrients and oxygen. Without

intervention, the malignant nature of these neoplasms ultimately leads to death (Kufe et al. 2003).

Currently, cancer ranks behind only heart disease as the leading cause of mortality both in the United States and worldwide. Nearly one in every four deaths in the United States and more than one in every ten deaths worldwide are a direct result of invasive tumors. As an indication of how commonplace cancer is, especially in developed nations, the lifetime probability of developing invasive tumors for American men and women are a daunting 44% and 37%, respectively (American Cancer Society 2009).

The high incidence of occurrence and mortality has propelled cancer to the forefront of medical research, however the complexity and heterogeneity of these diseases have impeded the progress towards efficacious treatments, let alone cures. Tumors can arise from nearly every cell type and organ within the human body, encompassing a broad-spectrum of differing cellular biologies. Although every tumor type encapsulates different causal mechanisms that involve differing hereditary and environmental factors, one shared characteristic underlying the transformation of a normal cell into a tumor cell is the alteration of the genome (Stratton et al. 2009).

Genomic Instability in Cancer

The transformation of a normal cell to a tumor cell is a complex process that involves circumventing the many safeguards inherently in place that prevent such a negative event from occurring (Hanahan and Weinberg 2000). The genetic material of a cell is the fundamental blueprint upon which all cellular processes and controls are based,

therefore maintaining genomic integrity is of the utmost importance. Improper alterations of the genome, ranging from point mutations to chromosomal rearrangements to gains and losses of genetic material, are known collectively as genetic instability and are associated with the vast majority of cancers (Collins et al. 1997; Negrini et al. 2010). In cancer cells, genomic instability commonly emerges as chromosome translocations, gene amplifications, aneuploidy, and polyploidy. The ultimate result of such genetic changes result in phenotypic shifts that are favorable to growth and proliferation, while genes encoding growth inhibitory proteins are often compromised (Lengauer et al. 1998; Negrini et al. 2010). Genomic instability often arises out of defects in mitotic control, DNA repair, telomere control, and DNA replication (Cahill et al. 1998; Blow and Gillespie 2008). The basis of this research project was to investigate one such mechanism of control that is associated with proper assembly of the proteins involved in DNA replication initiation.

Loss of Proliferative Control

The propelling force in cancer development is the loss of control of the underlying mechanisms responsible for regulating cellular proliferation, also known as the cell division cycle (Hanahan and Weinberg 2000; Evan and Vousden 2001). As such, regulatory vigilance of the cell division cycle at the molecular level is necessary to prevent any minute deviations that may contribute to the transformation of a normal cell into a neoplastic one. Extracellular growth signals and intracellular signaling cascades act in a concerted manner to coordinate cellular growth by regulating the downstream cell

cycle machinery. The fundamental steps of the cell cycle include copying parental DNA (S-phase), physical division of the two daughter nuclei (Mitosis), and the periods separating these events (Gap phases, G1 precedes S-phase and G2 precedes M). While each of these phases is tightly regulated, the control of the G1-S transition is of particular importance (Hartwell and Kastan 1994; Hook et al. 2007). During this period, the controlled assembly of the pre-Replication Complex and subsequent loading of the MCM helicase represents an essential regulatory mechanism and proper control of this step is crucial to ensuring the fidelity and timing of genome replication (Vaziri et al. 2003; Hook et al. 2007). Elucidating a novel molecular mechanism by which cells regulate MCM helicase loading, and thereby maintaining proper G1-S control, is the focus of this research project.

Cell Cycle

To proliferate, individual cells must duplicate themselves via a series of coordinated events known collectively as the cell cycle (Norbury and Nurse 1992). In its essence, the cell cycle involves two basic processes: a single round of DNA replication in which the entire genome of a cell is copied and the segregation of the replicated chromosomes into two separate daughter cells (Collins et al. 1997). During the cell division cycle, DNA is replicated during the synthesis phase (S-phase) and the two copies of each chromosome are segregated into daughter nuclei during mitosis (M-phase) (Norbury and Nurse 1992). This is followed by the separation of the cytoplasm into two

daughter cells, a process known as cytokinesis, which represents the final step of the cell cycle.

S-phase and M-phase are separated temporally by what are known as gap phases, G1 occurs between M-phase and S-phase while G2 occurs between S-phase and M-phase. During these gap phases, cells prepare for entry into either S-phase or M-phase and gather information from throughout the cell to determine its readiness to progress to the next phase (Pardee 1989; Johnson and Walker 1999).

Cell cycle progression is controlled by two major mechanisms. The first mechanism of regulation involves a cascade of phosphorylation events modulated by a group of heterodimeric protein kinases, which drives the cell cycle forward. The activation of these kinases requires the physical association of a regulatory subunit, called a cyclin, with a catalytic subunit, or cyclin-dependent kinase (CDK) (Pardee 1989; Norbury and Nurse 1991). The substrate specificity of this activated complex derives from the specific combination of cyclin and CDK. The cellular levels of cyclins increase and decrease in concert with progression through the cell cycle as different cyclin-CDK combinations are required during the different stages of the cell cycle. The second mechanism by which the cell cycle is regulated is known as checkpoint control (Collins et al. 1997). Cell cycle checkpoints integrate sensory signals from within the cell to determine if the processes of each cell cycle phase has been properly completed before progression is permitted (Johnson and Walker 1999). In a sense, checkpoints play a supervisory role and sense imperfections in cell cycle processes that could lead to

imprecise replication of DNA or flawed segregation of chromosomes potentially resulting in unequal daughter cells or other mutations (Collins et al. 1997).

The cell cycles of normal dividing cells are tightly regulated and coordinated to ensure faithful reproduction of the parental cells. Cancer cells must also utilize the cell cycle machinery in order to proliferate and grow, however the regulatory mechanisms of the cell cycle are often altered to permit the uncontrolled growth that is inherent to neoplasia (Cahill et al. 1998; Lengauer et al. 1998).

DNA Replication

Cellular proliferation requires the faithful duplication of the parental genome during the S-phase of the cell division cycle to ensure that each daughter cell receives an exact copy of the mother cell's genetic material (Dutta and Bell 1997; Bell and Dutta 2002; Masai et al. 2010). Imprecision during the replication of parental template DNA can potentially lead to alterations in the genome that favor cancer development (Blow and Gillespie 2008). Due to the size of the genetic template, DNA replication initiates from thousands of separate genomic regions known as origins of replication. The DNA that is replicated from a single origin of replication is known as a replicon (Huberman 1995). Mammalian somatic cells have large replicons that range in size from 50-kb to 500-kb and these replicons appear to replicate in clusters and in a staggered manner such that not all active origins fire simultaneously at the onset of S-phase (Berezney et al. 2000; Ge et al. 2007; Cadoret et al. 2008). Furthermore, in a given S-phase, not all

licensed preRCs are activated, as many are kept in reserve and only fire in response to replicative stresses (Ge et al. 2007).

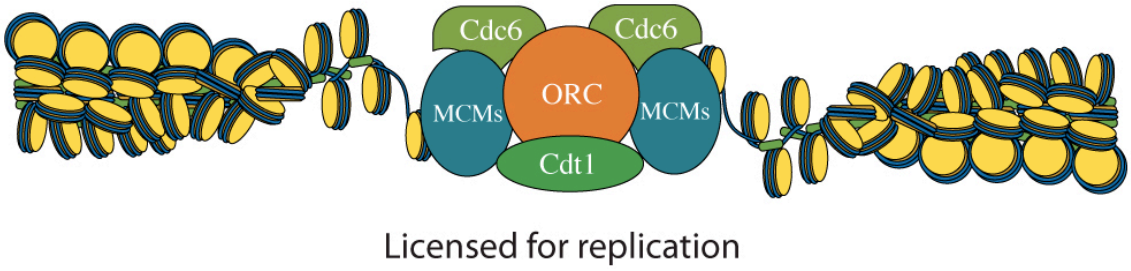


Fig. 1 The Pre-Replication Complex. The preRC is required for the initiation of replication at a given replication origin. The preRC is comprised of an ORC hexamer, which recruits Cdt1 and Cdc6 to the origin of replication. Cdt1 and Cdc6 are required to load the MCM complex, which serves as the putative replicative helicase. Once the MCM helicase is loaded, the preRC is considered to be licensed for replication.

Initiation of DNA replication requires the stepwise assembly of a macromolecular complex of proteins known as the pre-Replication Complex (preRC) during G1-phase of the cell cycle (Fig. 1). The hexameric origin recognition complex (ORC) binds to the origins of replication on DNA, forming the foundation of the preRC. ORC then recruits Cdt1 and Cdc6, which are both required to load the hexameric Mini-Chromosome Maintenance (MCM) helicase complex, comprised of Mcm2-7, onto DNA (Bell and Dutta 2002; Mendez and Stillman 2003). Once loaded, the MCM complex is believed to serve as the replicative helicase or act at the initial DNA unwinding step, or both (Chong et al. 2000; Shechter et al. 2004). At the G1-S transition, Cdk2 and Cdc7 kinases are activated and Mcm10, Cdc45, and DNA polymerase α/primase are recruited, initiating bidirectional nascent strand synthesis from the origin (Fig. 2), or preRC site (Mendez and Stillman 2003; Masai et al. 2010).

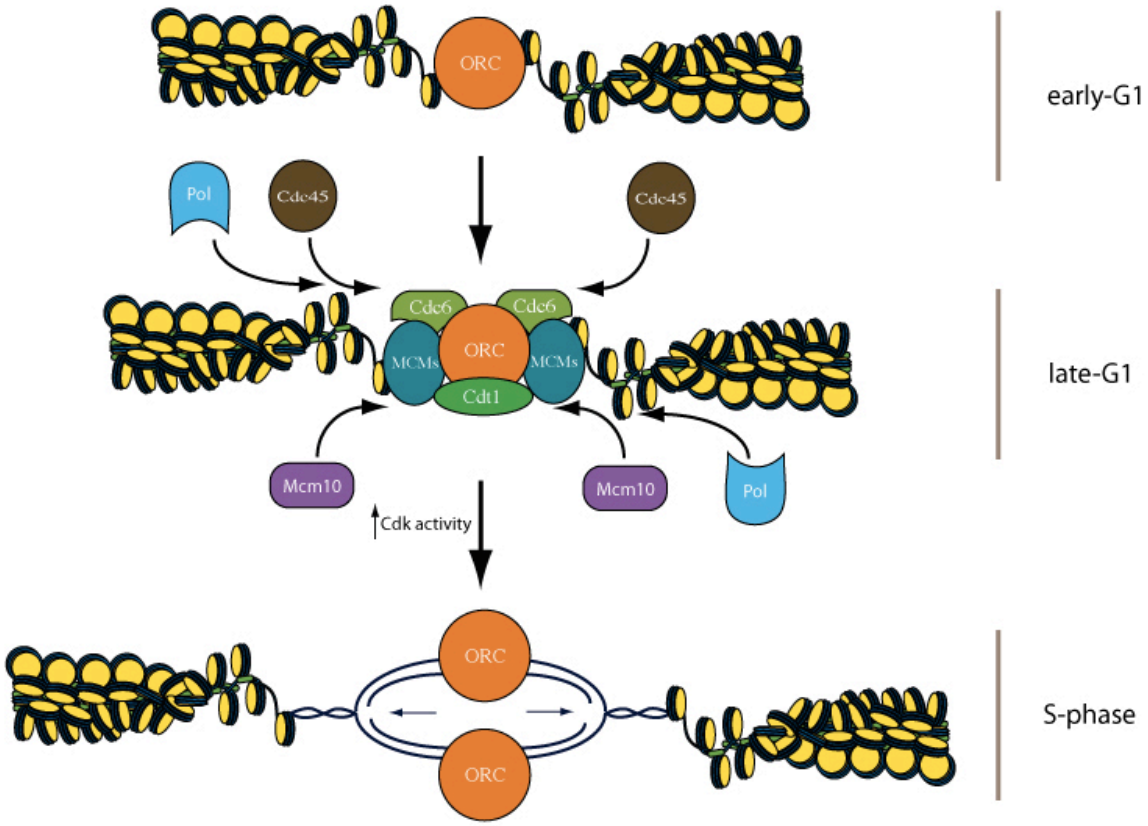


Fig. 2 Origin of Replication during G1-S. PreRCs at origins of replication are licensed during G1-phase. ORC recruits, Cdt1 and Cdc6, which in turn load the MCM helicase onto chromatin. At the G1-S transition, there is an increase in cellular CDK activity, and Cdc45, Mcm10, and DNA polymerases are recruited to the activated origins, which is followed by bidirectional nascent strand synthesis.

After DNA replication initiation, two replication forks originate from a single activated preRC and origin of replication. As the replicative helicase unwinds and separates the DNA substrate, RPA binds the single stranded DNA and DNA polymerase α/primase is loaded in a process termed primosome assembly, as reviewed by Waga and Stillman (Waga and Stillman 1998) and depicted in Figure 3. Mcm10 and Cdc45 are necessary for the loading and retention of pol α/primase onto chromatin (Waga and Stillman 1998; Garg and Burgers 2005). Since DNA polymerases can only extend existing oligonucleotides and not create them *de novo*, pol α/primase serves an important role in fork progression as it is able to synthesize short RNA primers complementary to the parental DNA strand and then extend them by approximately 20 nucleotides. Polymerase switching then occurs and other, more processive polymerases can take over allowing replication to continue (Garg and Burgers 2005).

DNA polymerases only synthesize DNA in the 5' to 3' direction, as such within any given replication fork, one strand will be copying DNA in the same direction of helicase unwinding, termed the leading strand (Fig. 3). The other strand, termed the lagging strand, will copy its template in a more discontinuous fashion. Replication of the leading strand is performed by DNA polymerase ε (Pursell et al. 2007), in a process that is inherently faster than lagging strand synthesis. To rectify this, pol α/primase also acts as a molecular brake for pol ε synthesis as a mechanism to coordinate leading strand and lagging strand synthesis (Lee et al. 2006). Lagging strand replication is performed by DNA polymerase δ and, unlike the leading strand, lagging strand synthesis requires frequent priming by pol α/primase (Fig. 3). Thus, lagging strand synthesis has several

discrete steps: synthesis of an RNA primer by pol α/primase, subsequent extension of this primer by the polymerase subunit of pol α/primase, polymerase switching to allow for pol δ to continue synthesis, excision of the RNA primers by FEN1 or RNase HI, gap filling by pol δ, and then nick sealing by DNA Ligase I (Waga and Stillman 1998; Garg and Burgers 2005). The latter three steps described here are known as Okazaki Fragment Maturation and occurs 20-50 million times in every mammalian cell cycle (Garg and Burgers 2005). PCNA, or Proliferating Cell Nuclear Antigen, plays an important role in DNA replication elongation by acting as a processivity factor for pol δ and pol ε and is loaded onto the DNA by RFC in an ATP-dependent manner (Tsurimoto and Stillman 1990; Waga and Stillman 1998; Garg and Burgers 2005).

The assembly of preRCs is subject to numerous limitations, especially with respect to the timeframe when assembly can occur during the cell cycle in order to prevent replication origins from firing more than once. Collectively, this control over preRC assembly and MCM loading is known as "replication licensing" and is completed once the MCMs are loaded (Blow 1993; Blow 2001; Blow and Hodgson 2002). In fact, once the complex of MCMs has been recruited, ORC and Cdc6 (and likely Cdt1) are no longer required for origin firing and S-phase progression.

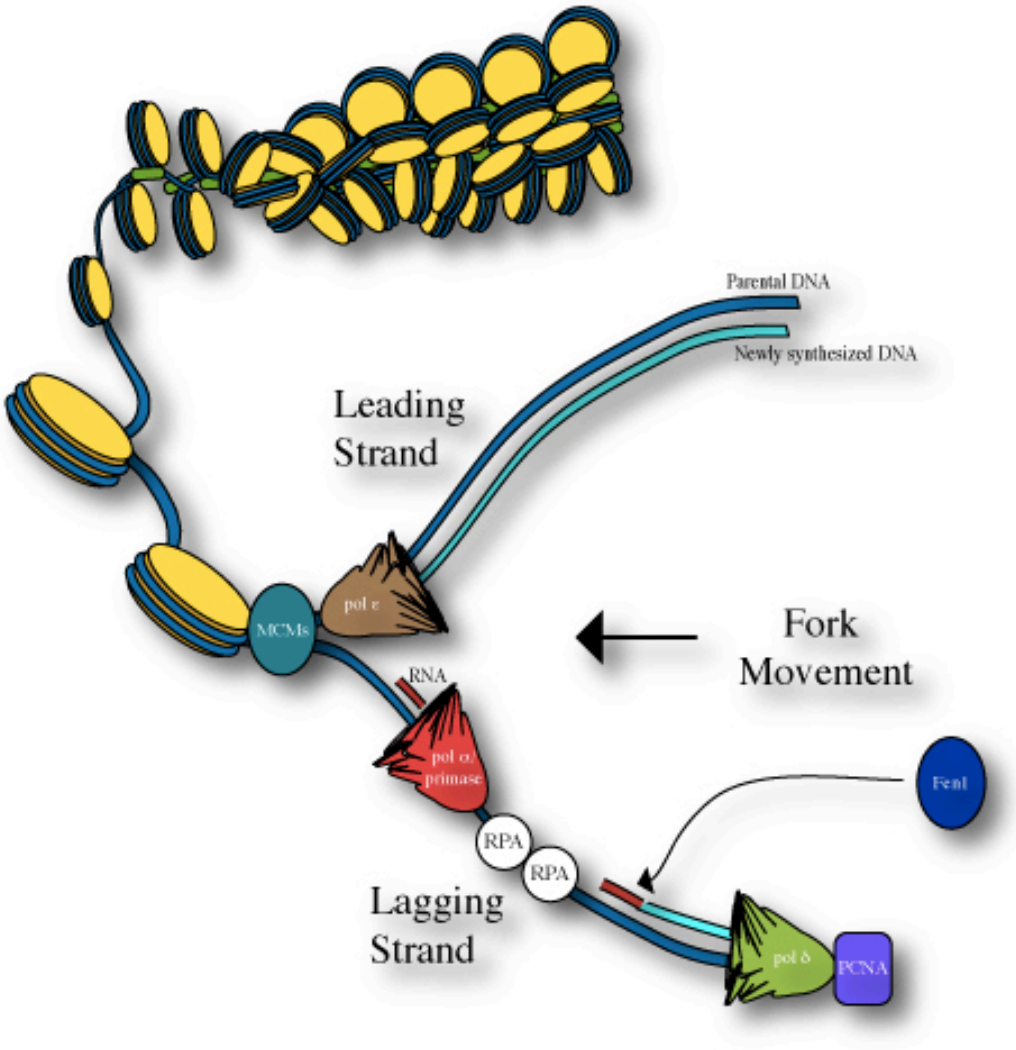


Fig. 3 The Replication Fork. Initiation of DNA replication involves the generation of two replication forks moving in opposite directions from an activated origin of replication. One such replication fork is depicted, which includes a leading strand that synthesizes DNA in the direction of fork progression, and a lagging strand that is forced to replicate DNA in a discontinuous fashion due to the unidirectional nature of polymerases.

Origin Recognition Complex

The evolutionarily conserved, hexameric Origin Recognition Complex (ORC) binds directly to DNA and acts as the initiator of DNA replication. ORC was initially identified in *S. cerevisiae* budding yeast and demonstrated binding specificity to the autonomously replicating sequence (ARS) via the A-element (or ARS consensus sequence), which contains the origins of replication in yeast (Bell and Stillman 1992; Loo et al. 1995). ORC has since been found to be a vital component in all eukaryotic DNA replication, however, unlike in yeast, a specific binding site or sequence does not seem to exist in other organisms (Mendez and Stillman 2003; Vashee et al. 2003; Masai et al. 2010). Instead, ORC binding to origins of replication in higher eukaryotes seems to be more plastic and occurs in zones rather than at discrete sites (Hamlin et al. 2010). Orc2-5 stably bind to chromatin through the cell cycle, while Orc1 seems to be bound only during G1 and becomes degraded during progression through S-phase (Ohta et al. 2003; Tatsumi et al. 2003).

Orc1, Orc4, and Orc5 belong to the AAA+ (ATPases associated with a variety of cellular activities) family of proteins and ATP binding stimulates ORC association with DNA (Giordano-Coltart et al. 2005; Duncker et al. 2009). Once ATP bound ORC has loaded onto chromatin, it serves as an anchoring point for the remainder of the preRC proteins to bind to. ATP hydrolysis by ORC is necessary for reiterative loading of preRCs onto chromatin (Bowers et al. 2004).

Cdc6

Cdc6, or Cell Division Cycle 6, was initially identified in *S. cerevisiae* and is indispensable for the loading of MCMs and preRC licensing (Bell and Dutta 2002; Cook et al. 2002). Cdc6 shows sequence similarity to subunits of clamp-loaders in both eukaryotes and prokaryotes and have functionally been shown to be involved in the loading of Mcms onto chromatin (Perkins and Diffley 1998). Similar to several of the ORC components, Cdc6 is an ATPase that belongs to the AAA+ family of proteins (Randell et al. 2006). Once ORC binds to the origin of replication, Cdc6 complexed to ATP is then recruited to the site of preRC assembly along with Cdt1 to load Mcm2-7. Cdc6 then hydrolizes ATP which results in both Cdc6 and Cdt1 dissociation from the preRC but simultaneously stabilizes the MCM complex to chromatin (Randell et al. 2006).

Cdc6 is regulated by several mechanisms. Phosphorylation of *S. cerevisiae* Cdc6 (and its *S. pombe* fission yeast homolog Cdc18p) by CDKs results in ubiquitin-dependent proteasomal degradation, while Cdc6 phosphorylation in *X. laevis* results in export from the nucleus (Pelizon et al. 2000; Cook et al. 2002). Interestingly, phosphorylation of human Cdc6 has the opposite effect and leads to protein stability by preventing APC/C E3 ubiquitin ligase-dependent proteolysis (Mailand and Diffley 2005).

Cdt1

Cdt1, CDC10 dependent transcript 1, was initially identified in *S. pombe* as an essential gene product of cdc10 (Hofmann and Beach 1994). Cdt1 is highly conserved (Bell and Dutta 2002) and homologs have since been identified in many eukaryotic species including humans (Wohlschlegel et al. 2000), mice (Arentson et al. 2002), *S. cerevisiae* (Tanaka and Diffley 2002), *X. laevis* (Maiorano et al. 2000), and *Drosophila* (Whittaker et al. 2000). CDT1 is an essential gene as its product is required for chromosomal DNA replication (Maiorano et al. 2000) and mutations result in a DNA synthesis block and errors in the S-phase checkpoint (Hofmann and Beach 1994). Cdt1 is loaded onto chromatin prior to DNA replication in an ORC dependent manner as an integral part of the pre-Replication Complex and is required to load the MCM2-7 protein hexamer (Maiorano et al. 2000). In *S. pombe*, Cdt1 has been shown to physically interact with the C-terminus of Cdc6 and together they act to cooperatively promote the association of MCM proteins on chromatin (Nishitani et al. 2000). Furthermore, Cdt1 is known to bind several DNA replication proteins directly, including PCNA (Tsurimoto 1999), Mcm4 (Cook et al. 2004), and Mcm6 (Yanagi et al. 2002) (Fig. 4).

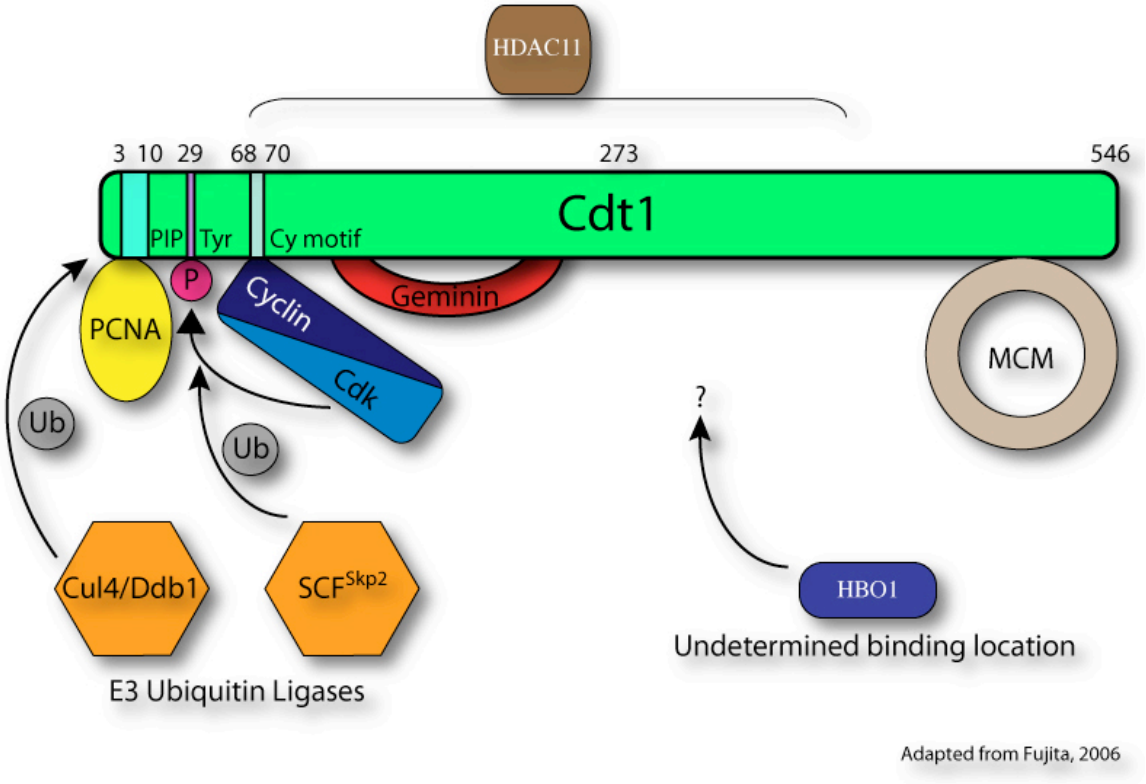


Fig. 4 Schematic of Cdt1. Cdt1 serves as a scaffolding protein that interacts with many different proteins. Shown here is the C-terminal Cdt1 interaction with the MCM helicase. The central portion of Cdt1 is responsible for the bipartite interaction with Geminin and the interaction with HDAC11. The N-terminal region of Cdt1 contains the PIP box for PCNA interaction and Cy motif for Cyclin/Cdk binding, both of which mediate polyubiquitination by distinct E3 ligases. The HBO1 binding region of Cdt1 has yet to be defined.

Cdt1 is regulated by a small protein called Geminin (Wohlschlegel et al. 2000). Initial reports demonstrated that Geminin acts to inhibit Cdt1 function (Wohlschlegel et al. 2000; Yanagi et al. 2002), however, more recent evidence indicates Geminin acts also in a positive manner to facilitate Cdt1 function (Lutzmann et al. 2006; Xouri et al. 2007). This dual role of Cdt1 regulation by Geminin is determined by the stoichiometric ratio of Geminin to Cdt1 within the complex where a high Geminin:Cdt1 ratio acts in an inhibitory manner whereas a low Geminin:Cdt1 ratio facilitates Cdt1 function. The ratio of Geminin binding to Cdt1 increases to become inhibitory after origin firing, presumably to prevent improper MCM loading by Cdt1 (Lutzmann et al. 2006).

It is of great importance that MCM loading be limited to once during any given cell cycle. As such, Cdt1 activity is restricted to only G1-phase of the cell cycle and this activity is tightly regulated. In addition to Geminin, Cdt1 is also controlled by several other mechanisms that regulate its stability (Fujita 2006). Cyclin dependent kinases (CDKs) represent another layer of Cdt1 control (Fujita 2006). As S-phase begins, there is an increase in CDK activity (Bell and Dutta 2002) which results in the phosphorylation of Cdt1 on its N-terminal cyclin-binding motif by cyclin A-dependent kinases (Liu et al. 2004; Sugimoto et al. 2004). This phosphorylation triggers Cdt1 polyubiquitination by the SCF^{Skp2} E3 ubiquitin ligase complex and subsequent targeting to proteasome-mediated degradation during S-phase and G2 (Li et al. 2003; Nishitani et al. 2006). Moreover, phosphorylated Cdt1 is also impaired in its ability to bind DNA, demonstrating multiple mechanisms by which CDKs are able to control Cdt1 function (Sugimoto et al. 2004). Cdt1 is further regulated by a second E3 ubiquitin ligase

pathway, independent of SCF^{Skp2}. During S-phase or after DNA damage, Cdt1 is targeted for proteasomal degradation by binding to PCNA via an N-terminal motif, which results in ubiquitination by the Cul4/Ddb1 E3 ligase (Hu et al. 2004; Hu and Xiong 2006; Nishitani et al. 2006; Senga et al. 2006) (Fig. 4).

The multiple levels of control over Cdt1 are important factors in the overall regulation of replication licensing. Controlling MCM chromatin loading is of critical importance to the cell in that it allows one and only one round of DNA replication to occur, and prevents the inappropriate reloading of MCMs and subsequent re-replication (i.e., relicensing) that can cause genomic instability. Improper overexpression of Cdt1 (Vaziri et al. 2003) or Geminin depletion (Melixetian et al. 2004; Zhu et al. 2004) can lead to re-replication, demonstrating the importance of Cdt1 regulation. In fact, Cdt1 can act as an oncogene and is found to be overexpressed in several human cancers including some carcinomas, melanomas, leukemias, and lymphomas (Arentson et al. 2002; Seo et al. 2005; Liontos et al. 2007). Cdt1 overexpression alone in NIH3T3 cells can cause tumor formation in nude mice (Arentson et al. 2002).

Whereas Cdc6 has been proposed to function as an MCM clamp loader (Perkins and Diffley 1998), the mechanisms by which Cdt1 promotes MCM loading are less clear. Unlike other components of the preRC, Cdt1 does not possess any known enzymatic motifs and its actual biochemical role in the loading of the MCM complex is unknown. Instead, Cdt1 seems to act as a scaffolding protein and its interactions with other proteins determine its physiologic roles (Sugimoto et al. 2008). The work contained within this

dissertation describes a novel mechanism of Cdt1 function that utilizes cell cycle time dependent interactions with specific enzymes to mediate the loading of MCMs.

MCM Complex

The MCM2-7 (mini-chromosome maintenance) family of proteins were grouped together based on sequence similarity and initially identified in genetic screens for mutations defective for plasmid maintenance or cell cycle progression (Dutta and Bell 1997). MCM deletion results in loss of cell viability in *S. cerevisiae* and the loading of the Mcm2-7 complex onto chromatin represents the final step of preRC assembly (Hua and Newport 1998; Mendez and Stillman 2000). The heterohexameric Mcm2-7 complex is thought to form a ring structure once loaded, with the DNA passing through the long, central channel (Remus et al. 2009).

The MCM complex is largely believed to act as the replicative helicase, either alone or as a part of a larger complex with GINS and Cdc45, unwinding the DNA double helix allowing for DNA polymerase to gain access to its substrate and to accommodate replication fork progression (Chong et al. 2000; Sato et al. 2000; Shechter et al. 2000; You et al. 2002; Moyer et al. 2006) (Fig. 3). All members of this heterohexamer are ATPases and members of the AAA+ family (Ying and Gautier 2005). As discussed above, ORC, Cdc6, and Cdt1 are temporally regulated to prevent improper loading of MCMs. The MCM complex itself is also regulated in a cell cycle-dependent manner. MCMs can only associate with DNA in the absence of CDK activity, since CDK activity is required at the onset of S-phase, MCMs are prevented from re-associating with

chromatin after replication has begun (Hua et al. 1997). The purpose of this research project was to elucidate a novel form of MCM loading control that involves the modulation of chromatin structure as a mechanism of replication licensing.

Pre-Replication Complex Quantification

Little is known about mammalian preRC stoichiometry, the number of preRCs on chromosomes, and how this relates to replicon size and usage. Our laboratory has found that, on average, each 100-kb of the mammalian genome contains a preRC composed of one ORC hexamer, 4-5 MCM hexamers, 2 Cdc6 proteins, and 0.35 Cdc45 proteins. Thus, based on ORC availability, mammalian cells, whose diploid genomes are approximately $7x10^9$ bp, contain approximately 70,000 preRCs of this average total stoichiometry. However, except for ORC, the chromatin-bound complement of preRC subunits is even lower.

Cdc45 is highly stable, and the same limited number of stable Cdc45 molecules are present from the beginning of S-phase to its completion. Microinjection of excess purified Cdc45 into S-phase nuclei activates additional replicons by three-fold, indicating that Cdc45 functions to activate dormant preRCs and is rate-limiting for replicon usage and activation. This low density of preRCs, each containing only a few MCMs that compete for limiting amounts of Cdc45, provides a molecular explanation why somatic replicons are large in average size. The stable, continuous, and rate-limiting nature of Cdc45 suggests that Cdc45 contributes to the staggering of replicon usage, and that

replicon activation requires reutilization of existing Cdc45 during S-phase (Wong et al. manuscript submitted 2010).

Chromatin

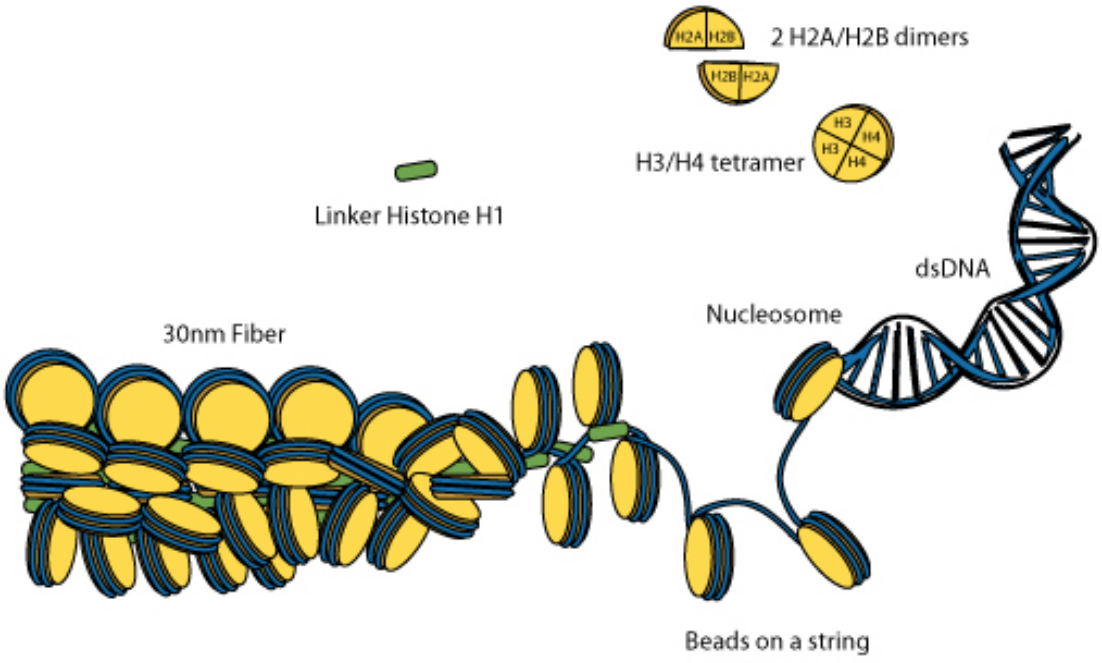


Fig. 5 Structure of Chromatin. Double stranded DNA does not and cannot exist as a lone molecule within the cell, instead DNA is packaged within the nucleus by winding around a core histone octamer to form a nucleosome. The nucleosomes are organized into higher-order chromatin structures, including a 30nm fiber "solenoid".

DNA does not exist within the nucleus as a naked molecule. Instead, it is wrapped tightly around a positively charged histone core octamer that is comprised of two histone H2A/H2B dimers and a histone H3/H4 tetramer (Wolffe and Hayes 1999). This DNA-histone structure is known as a nucleosome, and represents the most basic unit of chromatin (de Ruijter et al. 2003) and are formed during DNA replication (Saha et al. 2006). Nucleosomes are connected with linker DNA of variable lengths, forming a beads-on-a-string structure, which organize into a more compact 30 nm fiber (Wu et al. 2007). 30 nm fiber structures are further condensed into higher-order chromatin structures within the nucleus.

This packaging of DNA creates a physical barrier for processes that require DNA as a substrate, which leads to a fundamental accessibility issue for proteins involved in transcription, DNA repair, and DNA replication (Demeret et al. 2001). Areas of the genome, known as euchromatin, are less tightly compacted, therefore these regions are more actively transcribed and replicate earlier in S-phase compared to condensed chromatin regions known as heterochromatin. Thus, manipulation of chromatin structure represents an important level of spatio-temporal control for transcription and DNA replication (Demeret et al. 2001).

Two major classes of proteins are able to affect the condensation state of chromatin: ATP-dependent chromatin remodelers and histone modifying enzymes (Sengupta and Seto 2004). ATP-dependent remodelers utilize the energy of ATP to mediate chromatin remodeling by physically altering the position of nucleosomes on the

genome (Vignali et al. 2000). ATP-dependent remodelers are organized into five major families: SWI/SNF, ISWI, CHD, SWR1, and INO80 (Saha et al. 2006; Hayashi and Masukata 2010). Post-translational covalent modification of core histones, to both tail and globular domain residues, and linker histone H1 are also an important mechanism to facilitate chromatin remodeling. A diverse array of modifications, including acetylation, methylation, phosphorylation, ribosylation, and ubiquitination, can bring about changes in chromatin fluidity either themselves by altering the histone-DNA interaction or by creating a pattern of modifications that are recognized by downstream, effector proteins (Strahl and Allis 2000). The recognition of one or more sequential modifications by effector proteins or protein complexes is known as the "histone code".

Histone Acetyltransferases

An acetylation reaction describes the process of adding an acetyl group (-$COCH_3$) to a molecule, which is catalyzed in biological systems by histone acetyltransferases, or HATs. HATs, are divided into five large families: Gcn5-related acetyltransferases, MYST family HATs, p300/CBP HATs, general transcription factor HATs, and the hormone receptor-related HATs (Carrozza et al. 2003b). HATs often function as part of large, multi-subunit complexes that transfer acetyl groups onto lysine residues of histone tails and, though less frequently, histone globular domains.

Genomic regions containing acetylated histones are generally associated with increased transcriptional activity. The widely accepted consequence of histone acetylation is the partial neutralization of the positive charge on histones, which in turn,

diminishes its association with negatively charged DNA, thus loosening the chromatin structure (Sengupta and Seto 2004). Common acetylated residues associated with transcriptionally active, open chromatin include acetylated histone H4K5, 8, 12, and 16, acetylated histone H3K9, 14, 18, and 23, acetylated histone H2AK5 and 9, and acetylated histone H2BK5, 12, 15, and 20 (Strahl and Allis 2000; Rice and Allis 2001). Though this is not mutually exclusive of a role in the histone code mode of function for acetylation where acetylated lysine residues are recognized by downstream effectors, such as bromodomain containing proteins (Yang and Seto 2007).

In addition to effects on histones and chromatin structure, acetylation also represents an important post-translational modification for non-histone proteins (Glozak et al. 2005). Reversible acetylation of non-histone proteins alters the electrostatic properties of the protein and can subsequently influence protein stability, protein-protein interactions, protein localization, and DNA binding (Glozak et al. 2005; Minucci and Pelicci 2006). Therefore, reversible acetylation plays a role in a diverse array of cellular processes, including affecting proteins involved in gene expression, replication, DNA repair, translation, cell signaling, apoptosis, the cytoskeleton, and metabolism (Yang and Seto 2007). Recently, global, high-resolution mass spectrometry analysis has demonstrated that the regulatory scope of the acetylome is comparable to the diverse spectra of protein phosphorylation (Choudhary et al. 2009).

HBO1

HBO1 (histone acetyltransferase binding to ORC, also known as KAT7 or MYST2) is a MYST family HAT that was first identified by screening a HeLa cDNA library in a yeast two-hybrid system using human ORC1 as bait (Iizuka and Stillman 1999). The HBO1 HAT complex contains JADE1/2/3, Eaf6, and tumor suppressor proteins ING4/5. JADE1 positively influences the acetyltransferase activity of HBO1 (Foy et al. 2008) and the PHD finger domains contained within the ING4/5 and JADE subunits influence HBO1 HAT complex targeting through preferential associations with methylated histone H3 (Saksouk et al. 2009).

HBO1 is primarily responsible for the acetylation of lysines 5, 8, and 12 of histone H4 (Doyon et al. 2006) and plays a role in preRC licensing (Iizuka et al. 2006). Furthermore, HBO1 binds to mammalian origins through a physical interaction with Cdt1 and acetylates histone H4 tails at origin regions during G1-phase, which is required for MCM helicase recruitment (Miotto and Struhl 2008; Miotto and Struhl 2010). Acetylation by HBO1 is also inhibited by Geminin in a mechanism that depends on binding to the HBO1-Cdt1 complex (Miotto and Struhl 2010). p53 binds to HBO1 and inhibits its acetyltransferase activity and the subsequent MCM loading in response to certain cytotoxic shocks (Iizuka et al. 2008). Interestingly, HBO1 has been found to be overexpressed in cancer cell lines and cancer tissues, which corresponds with its necessary role in preRC licensing (Iizuka et al. 2009). The work contained in this research project describes a mechanism by which Cdt1 acts to decondense chromatin to

facilitate MCM loading in a process that requires histone H4 acetylation and is inhibited by coexpression of a catalytically-dead mutant of HBO1.

Histone Deacetylases

Histone deacetylases (HDACs) catalyze the opposite reaction to HATs, namely the removal of the acetyl moiety from ε-amino groups of lysine residues in both histone and non-histone proteins. By opposing the reaction of HATs, HDACs inherently play a fundamental role in the many biological processes that acetylation affects as described previously. Since histone acetylation neutralizes that positive charge on the histone and decreases its affinity with DNA, deacetylation has the converse effect and restores the positive charge on the histone, which results in a tighter, more compact chromatin structure (de Ruijter et al. 2003; Sengupta and Seto 2004). As shown in Figure 6, mammalian HDACs are organized into two major families and four subclasses based on their sequence homology to yeast counterparts and cofactor dependencies (Yang and Seto 2007).

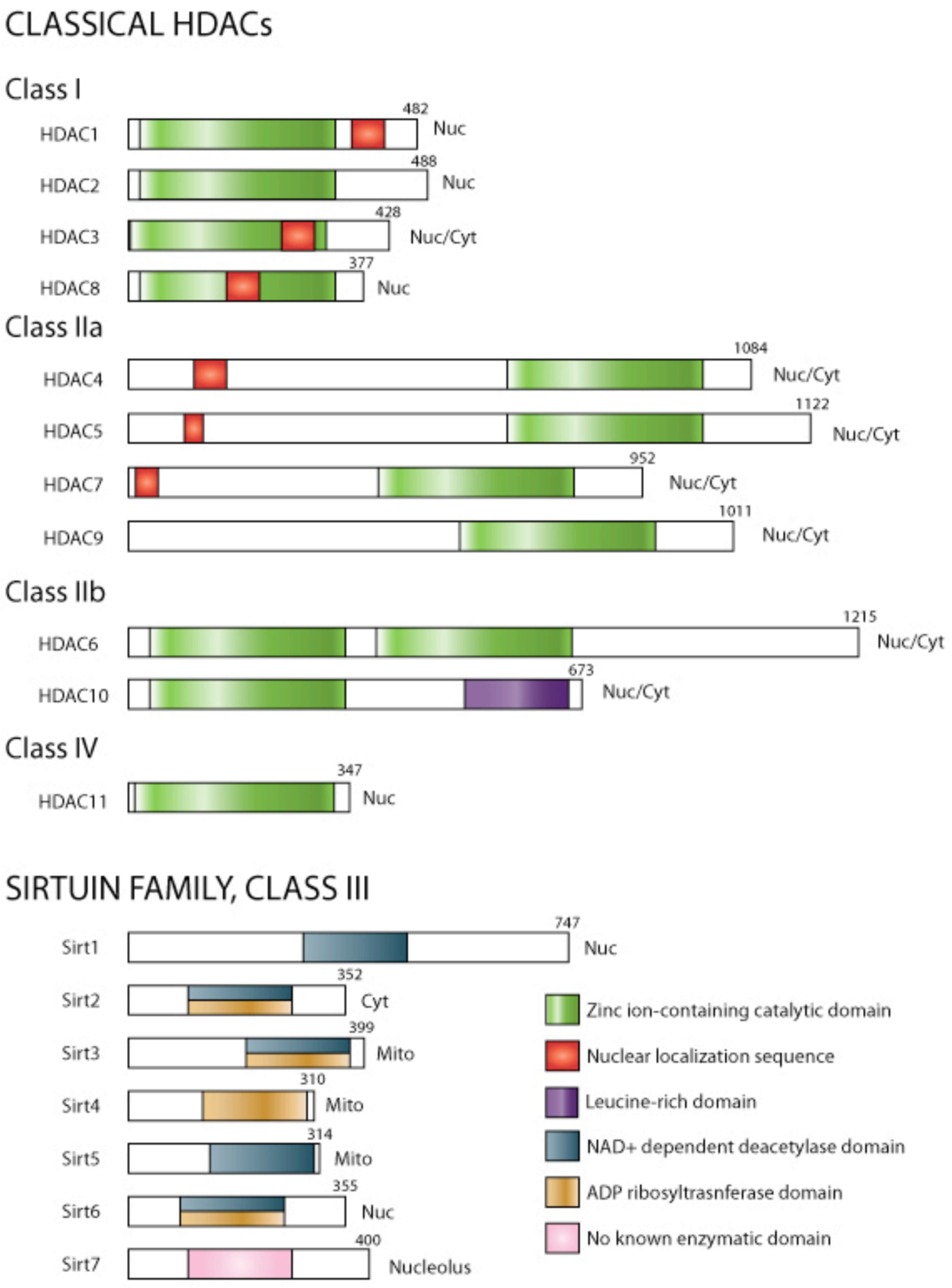


Fig. 6 Mammalian HDAC Family Organization.

Class I HDACs

Mammalian class I HDACs show sequence homology to yeast RPD3, contain an N-terminal deacetylase domain and a C-terminal tail, and include HDAC1, HDAC2, HDAC3, and HDAC8 (Yang and Seto 2008). Class I HDACs appear to be expressed in most cells and are almost exclusively found in the nucleus (de Ruijter et al. 2003). The conserved deacetylase core is shared by classical HDACs and encompasses approximately 390 amino acids. Within this conserved deacetylase domain exists a tube-like pocket that houses the zinc ion cofactor (Finnin et al. 1999). The Zn^{2+} ion serves as a part of a charge-relay system that also utilizes adjacent histidine residues and two aspartic residues to catalyze the removal of acetyl groups (Finnin et al. 1999; de Ruijter et al. 2003).

The classical HDACs are all inhibited by trichostatin A (TSA) and suberoyl anilide bishydroxamide (SAHA) that act by inserting into the deacetylase pocket and forming a coordinate interaction with the Zn^{2+} ion and active site residues to impede the charge-relay system (Finnin et al. 1999). All class I members, with the exception of HDAC8, function within larger nuclear complexes that serve to repress transcription and modulate the epigenetic landscape. For example, HDAC1 and HDAC2 are both found in Sin3, Mi-2/NuRD, and CoREST complexes and HDAC3 is a part of the N-CoR/SMRT complex, all of which act to silence transcription (Yang and Seto 2008).

Class II HDACs

Class II HDACs show sequence homology to yeast Hda1 and depend on Zn^{2+} ion as a cofactor for their deacetylase function. Like class I HDACs, class II HDACs are inhibited by TSA and SAHA. While class I HDACs are widely expressed in mammalian cells, class II HDACs appear to be more restricted in terms of expression in different cell types, suggesting they may play a role in differentiation and development (de Ruijter et al. 2003). The class IIa members, comprised of HDAC4, HDAC5, HDAC7, and HDAC9 all share a conserved, long N-terminal extension in addition to their similar deacetylase domains (Yang and Seto 2008).

Contained on these class IIa N-terminal extensions are binding sites for 14-3-3 and myocyte enhancer factor 2 (MEF2) (de Ruijter et al. 2003). The binding of these proteins to the class IIa HDACs affects their subcellular localization with MEF2 promoting nuclear localization whereas 14-3-3 promotes cytoplasmic retention. This dynamic nuclear/cytoplasmic transport of class IIa HDACs makes them unique signal transducers as they are actively shuttled in response to multiple cellular signals. HDAC6, a class IIb HDAC, is unique in that it is predominantly cytoplasmic and contains two active deacetylase domains. Given its subcellular localization, it serves as an important cytoplasmic, non-histone protein deacetylase and plays a role in a variety of cellular functions including cellular motility, cellular adhesion, activation of certain kinases, and regulating some immunologic functions (Yang and Seto 2008).

Class III HDACs

Class III HDACs, termed Sirtuins, represent a distinct family from the classical HDACs and are homologous to the *S. cerevisiae* SIR2 (Silent Information Regulator 2). Seven human sirtuins, numbered Sirt1-7, have been identified to date and not all possess the ability to act as protein deacetylases, instead some act as mono-ADP ribosyltransferases. Sirtuins differ from classical HDACs in terms of their enzymatic mechanism, subcellular localization, structure, sequence, and function (Michan and Sinclair 2007). Instead of utilizing Zn^{2+} ion as a cofactor, Sirtuins employ NAD^{+} as a co-substrate for the enzymatic removal of acetyl groups. Sirt1, Sirt2, Sirt3, Sirt5, and Sirt6 display deacetylase activity, while Sirt4 possesses only the ability to act as a ribosyltransferase. Interestingly, Sirt2, Sirt3, and Sirt6 have the ability to catalyze both deacetylase reactions and ribosyltransferase reactions (Frye 1999; Liszt et al. 2005).

Since their mechanism of action is so different than the classical HDACs, it is no surprise that TSA and SAHA are incapable of acting as inhibitors to the class III HDACs, instead sirtuins are inhibited by nicotinamide, a byproduct of their deacetylation reaction (Landry et al. 2000). Sirt1, Sirt6, and Sirt7 reside predominantly in the nucleus, while the others are cytoplasmic proteins. Interestingly, Sirt3, Sirt4, and Sirt5 reside in the mitochondria. Sirtuins represent a relatively new area of HDAC research and many strides are being made to elucidate their impact on biological functions (Michan and Sinclair 2007).

Class IV HDAC: HDAC11

Identified in 2002, HDAC11 is the lone member of the class IV HDAC grouping. HDAC11 possesses similarity to class I HDACs and, to a lesser degree, class II HDACs, however its sequence similarity was too low to designate it as a member of either class (Gao et al. 2002; de Ruijter et al. 2003). HDAC11 does exhibit the qualities of a classical HDAC in that it requires Zn^{2+} ion as a cofactor for its deacetylase activity and is inhibited by TSA. It is primarily localized to the nucleus, however it has been shown to co-immunoprecipitate with the cytosolic HDAC6 protein (Gao et al. 2002). HDAC11 is conserved from *C. elegans*, *Drosophila*, bacteria, plants, to humans (Yang and Seto 2008). This high level of evolutionary conservation suggests HDAC11 possesses an important function in a diverse range of organisms.

Expression of HDAC11 in Antigen Presenting Cells (APCs) functions to inhibit IL-10 expression and induced inflammatory APCs to prime naive T cells and restore the responsiveness of tolerant T helper cells (Villagra et al. 2009). HDAC11 is highly expressed in murine brain cells (Liu et al. 2008) and seems to play a role in the regulation of oligodendrocyte-specific protein gene expression and oligodendrocyte development (Liu et al. 2009). Recently, an S-phase, direct interaction between HDAC11 and Cdt1 has been identified (Glozak and Seto 2009). Cdt1 was also found to be acetylated at its N-terminus by the HATs PCAF and p300, and overexpression of HDAC11 correlated with reduction in acetylated Cdt1. This reversible acetylation protects Cdt1 from ubiquitination and resulting proteasomal degradation, thus representing a potential

mechanism to regulate Cdt1 stability (Glozak and Seto 2009). Due to its recent discovery, further biological roles of HDAC11 have yet to be fully elucidated, particularly with respect to regulation of DNA replication. The research contained within this dissertation describes a novel role for HDAC11, in which it plays a role in regulating MCM loading by Cdt1 by manipulating chromatin structure.

Chromatin Remodeling in DNA Replication

As described above, the condensation of the DNA substrate into nucleosomes and higher-order chromatin structures poses a fundamental problem for proteins and enzymes that require access to the DNA in order to function and represents an important regulatory component. Although this issue of chromatin accessibility is a highly-studied topic in the field of transcriptional control (Wolffe and Hayes 1999; Hassan et al. 2001; Carrozza et al. 2003a), very little is known about how chromatin influences DNA replication, in terms of both initiation and elongation. Just as in transcription, it is easily predicted that access to the DNA within the context of chromatin by the replication machinery represents an important regulatory step. In addition to creating access to DNA for preRC formation prior to S-phase, specific DNA-histone interactions need to be disrupted and recreated during the cell cycle to maintain faithful duplication of the genome and its chromatin structures (Falbo and Shen 2006).

Experiments in yeast, where ORC binding is more specific than higher eukaryotes, have demonstrated that nucleosomal positioning can affect ORC binding and preRC assembly (Falbo and Shen 2006; Hayashi and Masukata 2010). When ARS DNA

is repositioned from an accessible area to one masked by a nucleosomal core particle, DNA replication is inhibited (Simpson 1990). In support of this, replication origins, i.e. poly(dA-dT) regions, and ORC localization in *S. cerevisiae* yeast maps to nucleosome-free regions using high-throughput sequencing (Eaton et al. 2010). While similar genome-wide nucleosome positioning mapping in *S. pombe* resulted in differing nucleosome-depleted region patterns, nucleosome depletion was also detected over high efficiency origins (Lantermann et al. 2010). Interestingly, others have shown that nucleosomal positioning adjacent to ARS1 can enhance replication initiation, suggesting ORC binding and preRC formation efficiency may be dependent on chromatin context (Lipford and Bell 2001). Together, these data suggest nucleosomal positioning plays an important role in determining origin efficiency in eukaryotic cells.

It has previously been shown that, during replication fork elongation, Cdc45 recruits Cdk2 to forks and leads to phosphorylation of the linker histone H1 (Alexandrow and Hamlin 2005). This histone H1 phosphorylation causes higher-order chromatin structures to be opened and for fork progression to occur. Significantly, such findings produced a novel explanation as to how the replication machinery is able to progress through higher-order chromatin, particularly heterochromatic areas, which contain highly-condensed and largely inaccessible DNA.

With respect to chromatin remodeling at sites of replication initiation, initial studies have shown acetylation to be an important post-translational modification in determining origin fitness. In yeast, deletion of the HDAC Rpd3 leads to overall genome replication initiating earlier in S-phase, as well as causing several normally late-firing

origins to fire earlier (Vogelauer et al. 2000; Vogelauer et al. 2002). Conversely, targeting the Gcn5 HAT to a late-firing origin causes it to fire earlier than normally observed in wild type cells (Vogelauer et al. 2000). Very similar results were obtained with an origin studied in *Drosophila* as Rpd3 deletion resulted in increased genomic replication (Aggarwal and Calvi 2004). Targeting the *Drosophila* homolog of Rpd3 to a specific origin of the chorion locus reduced replicative activity, whereas targeting the Chameau acetyltransferase resulted in increased origin activity (Aggarwal and Calvi 2004). Furthermore, in mammalian cells, firing of the β-globin origin is influenced by local acetylation state (Goren et al. 2008). Targeting of HATs to this region brings about a shift to earlier replication, that depends on the acetyltransferase activity as a CBP catalytically dead mutant did not result in the same level of S-phase time reduction. In contrast, targeting an HDAC to β-globin in erythroblasts, which normally replicate this region early in S-phase, results in a shift to late replication (Goren et al. 2008). Consistent with this, HBO1 deletion inhibits preRC licensing and DNA replication as discussed above (Iizuka et al. 2006). Together, these date describe a situation in which histone acetylation plays an important role in DNA replication initiation, however none full demonstrate a direct link between this acetylation event, chromatin remodeling, and DNA replication.

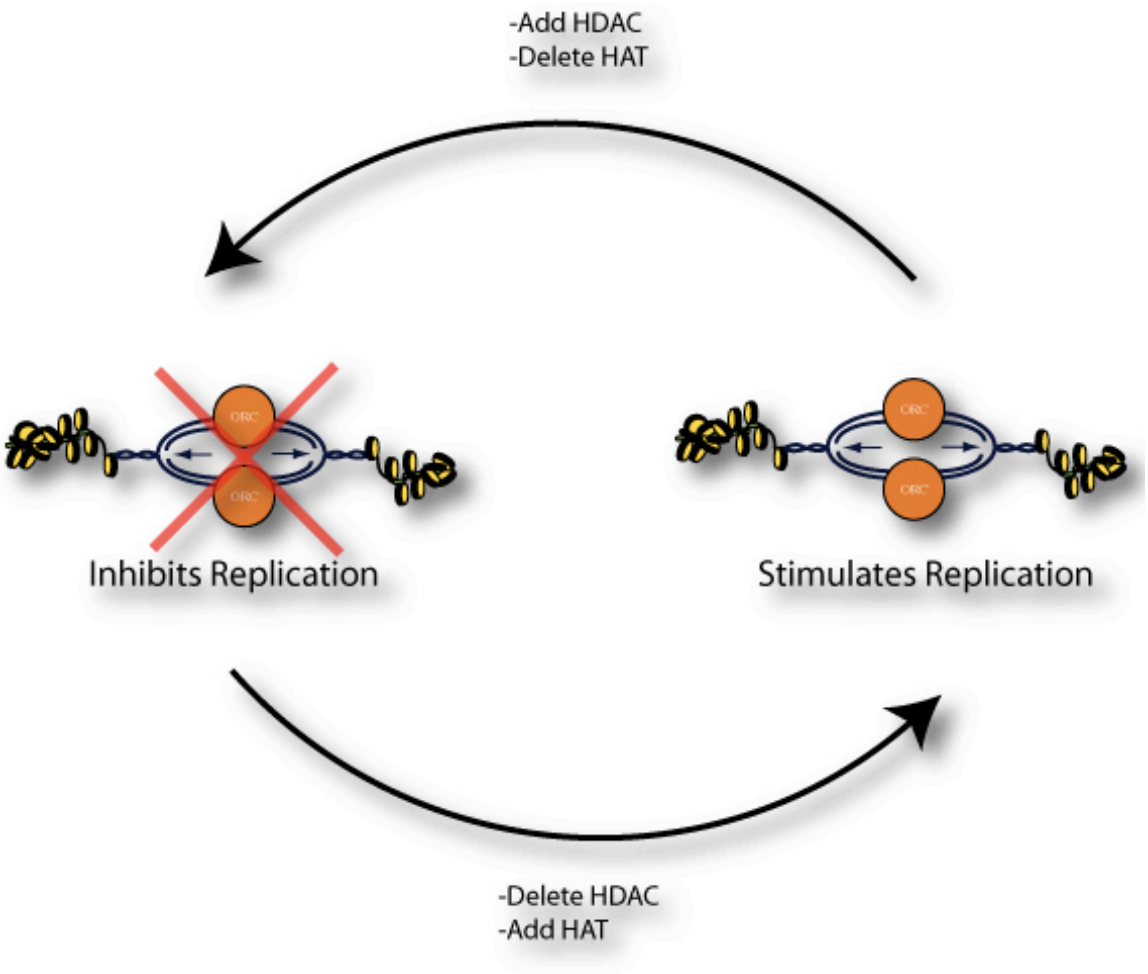


Fig. 7 Acetylation Affects DNA Replication Initiation. In yeast, global deletion of the HDAC Rpd3 results in earlier replication firing, while targeting the Gcn5 HAT results in earlier origin firing. In flies, global disruption of Rpd3 resulted in increased replication. Targeting Rpd3 to a specific origin reduced replicative activity whereas tethering the Chameau acetyltransferase resulted in increased origin activity. In mammalian cells, targeting a HAT stimulates earlier origin firing while targeting an HDAC causes delayed origin activity. Global deletion of the HAT HBO1 also disrupts DNA replication and preRC licensing.

CHAPTER 2

MATERIALS AND METHODS

Cell Culture

CHO, A03_1, and HeLa cell lines were maintained in Minimum Essential Medium, MEM, (Cellgro) supplemented with 10% Fetal Clone II (HyClone) and 0.1% Gentamicin (Gibco). HaCaT and 293T cell lines were maintained in Dulbecco's Minimum Essential Medium, DMEM, (Cellgro) supplemented with 10% FBS (HyClone) and 0.1% Gentamicin (Gibco). A03_1 cell medium also contained 0.3 μM methotrexate. Cells were synchronized by isoleucine deprivation (CHO) or serum deprivation (HaCaT) as described (Alexandrow and Hamlin 2005; Mukherjee et al. 2010). Replicating DNA was labeled with 15 μM BrdU for 30 minutes at 37^{o}C.

Transfections

Transfections were performed for 24 hours with FuGene-6 (Roche) or by treatment with polyethylenimine, PEI, as described (Reed et al. 2006). Briefly, freeze-thawed PEI reagent was diluted in 150 mM NaCl before plasmid DNA addition. Mixtures were vortexed and incubated at room temperature for 30 minutes before adding

directly to cell media. Initial transfection efficiencies were determined by transfection of Zsgreen plasmid for 24 hours and subsequent analysis by fluorescent microscopy. Adenoviral infection assays were performed as described (Vaziri et al. 2003). Colony forming assays used pTK-Hygro co-transfected and hygromycin selection (400 μg/ml).

Antibodies

Anti-LacI (Stratagene or Upstate); anti-BrdU (Roche); anti-H1P (provided by C. Mizzen, University of Illinois); anti-HBO1 (provided by M. Smith, University of Virginia); anti-Geminin, anti-Myc (S. Cruz Biotech); anti-HDAC11, anti-Flag, anti-actin (Sigma); anti-PCNA, anti-tubulin (Calbiochem); anti-HA (Covance); anti-Cdt1 (provided by H. Nishitani, Kyushu University, Japan); anti-Mcm2, anti-Mcm4, and anti-Mcm7 were generated by Covance or Aves Labs. Anti-mouse, anti-rabbit, and anti-chicken HRP-conjugated secondary antibodies (Jackson) were used. Anti-mouse, anti-rabbit, and anti-chicken Texas Red or FITC-conjugated secondaries (Jackson) for immunofluorescence were used.

Plasmids and cDNAs

HsCdt1, HsGeminin, and pEBG-GST were provided by A. Dutta (University of Virginia). HsCdt1, CgCdc45, CgCdc6, BRCA1(6c-w mutant), HsHDAC1, and HsHDAC11 were expressed using pRcLac (Alexandrow and Hamlin 2005). No NLS sequence was added to any LacI construct. LacI-VP16 was provided by A. Belmont (University of Illinois). HBO1-wt and HBO1-G485A were provided by M. Smith

(University of Virginia). Geminin, Cdc6, HBO1-wt, and HBO1-G485A were expressed from pcDNA3-HA, and HBO1-wt, HDAC1, and HDAC11 were expressed from pcDNA3-Flag. Cdt1 was expressed using pcDNA3-6xMyc. Set8-HBD was generated by proofreading PCR and expressed using pcDNA3-HA-NLS.

Protein Chemistry

Immunoprecipitations (IP) were performed in TNN (50 mM Tris, pH7.4, 250 mM NaCl, 0.1% Igepal CA-630, and phosphatase and protease inhibitors). Immune complexes were washed with lysis buffer 3X. For immunoblots, equal numbers of cells were lysed and boiled in loading dye (for total lysates [TCE]) or were separated into detergent-resistant (chromatin) or detergent-soluble fractions as described (Mendez and Stillman 2000; Alexandrow and Hamlin 2005). PreRC subunits present in the chromatin fraction are sensitive to nuclease digestion (Mendez and Stillman 2000). TCE, soluble, and/or chromatin samples representing equivalent cell numbers were analyzed by standard immunoblotting and ECL. Briefly, samples were loaded onto 10% SDS-PAGE gels and then transferred to Immobilon PVDF Transfer Membranes (Millipore) with subsequent probing with appropriate antibodies. For gel filtration, Myc-Cdt1, Flag-HDAC11 and HA-geminin were co-transfected into 293T cells using Lipofectamine 2000. Cells were collected and lysed with sonication in PBS lysis buffer containing 0.2% NP-40. Lysates were purified over an anti-Flag column (Sigma). Flag-HDAC11 and co-purifying proteins were eluted using a Flag peptide (Sigma). Eluates were subjected to a size exclusion column (Superdex 200 HR 10/30 column, GE) using FPLC, and 0.3 mL

fractions were collected and analyzed by immunoblotting.

Immunofluorescence

Cells were analyzed by IF and BrdU incorporation as described (Alexandrow and Hamlin 2005; Winter et al. 2009). Briefly, cells grown on square microscope coverslips were rinsed 1x with RT PBS-pH7.4 and then fixed with either 2% formaldehyde (Fisher) for 15 minutes at room temperature or with 100% methanol (Fisher) for 5 minutes at -20^{o}C. Cells were then washed 2x with PBS-7.4 and then permeabilized with PBS-7.4 containing 1% Normal Donkey Serum (NDS, from Jackson) and 0.2% Triton X-100 (Promega), then washed 2x with PBS containing 1% NDS. If replicating DNA was labelled with BrdU, DNA was then denatured with 1.5N HCl (in H_2O) for 30 minutes at RT and washed with PBS-7.4. Cells were then incubated for 1 hour at RT in a humidified chamber with the appropriate primary antibody dilution in PBS-7.4 with 1% NDS. Cells were then washed 2x at RT with PBS-7.4 with 1% NDS. Cells were then incubated again for 1 hour at RT in a humidified chamber with the appropriate fluorescent dye-conjugated secondary antibody diluted in PBS-7.4 with 1%NDS. Cells were then washed 2x at RT in PBS then stained for 5 minutes with 0.5 μg/mL DAPI (4',6-diamidino-2-phenylindole) and washed. Coverslips were then mounted on slides using 50 μL of Prolong Antifade reagent (Molecular Probes). Photographs of cells were obtained with a Zeiss Automated Upright Fluorescence Microscope in the Moffitt Cancer Center Analytic Microscopy Core, and images were merged using Adobe Photoshop.

Flow Cytometry

For flow analysis of DNA re-replication, cells were trypsinized, washed with PBS-7.4, and then initially fixed with 1% formaldehyde (Fisher). Cells were then washed in PBS-7.4 and then fixed and stored in 70% ethanol (Fisher) at -20^{o}C overnight. After centrifugation to remove ethanol, cells were washed in PBS-7.4 and then resuspended in PBS-7.4 containing 30 μg/mL propidium iodide (PI), 0.1% Triton X-100, and 200 μg/mL RNase A. Sample data were collected at the Moffitt Cancer Center Flow Cytometry Core Facility with a FACScan bench-top analyzer and data was analyzed using ModFit LT (VSH) and FlowJo software for percentage of cells containing greater than 4N DNA content.

ChIP Assays and qPCR

Synchronized HaCaT cells were fixed with 1% formaldehyde for 10 min at room temperature. Crosslinked chromatin was sonicated in 10 mM Tris-HCl (pH8), 1 mM EDTA, 0.5 mM EGTA, 1% SDS (plus phosphatase and protease inhibitors) to an average length of ~500 bp. Samples were adjusted to 5 mM Tris-HCl (pH8), 30 mM NaCl, 0.2% Triton X-100, 0.2% SDS, 0.8% BSA, 0.4 mM EDTA, 0.1 mM EGTA, and chromatin from 5 x 10^{6} cells was used for IP with anti-HDAC11 or control IgG (4^{o}C overnight). Immune complexes were precipitated with anti-rabbit agarose, washed, and eluted in 10 mM Tris-HCl (pH8), 1 mM EDTA, 1% SDS at 65^{o}C. Crosslinks were reversed at 65^{o}C overnight, and samples were treated with Proteinase K for 3 hr at 50^{o}C. Resulting DNA

was purified using phenol/chloroform extractions, and subjected to quantitative PCR (qPCR) in triplicate using a BioRad MyIQ detection system with TaqMan primers and FAM probes against previously described origin sequences (Ladenburger et al. 2002; Sibani et al. 2005). Primers used were as follows:

Lamin B2 origin probe: 5‘-TTAGACATCCGCTTCATTAGGGCAGAGG-3’

Lamin B2 origin forward: 5’-GCTACACTAGCCAGTGACCTT-3’

Lamin B2 origin reverse: 5’-GTTCTGCCTCTGAGTTTATTCCTG-3’

Lamin B2 exon probe: 5’-CTGAACTGGGATCTGACACCCACCA-3’

Lamin B2 exon forward: 5’-AGAAGAGACCAGGGTTCACAGA-3’

Lamin B2 exon reverse: 5’-GTGTTAACAGTCAGGCGCAT-3’

MCM4 origin probe: 5’-ACCCAAACTACCTCCGCAGGTCAGACGT-3’

MCM4 origin forward: 5’-TGGCCCGAATCAACATGGAA-3’

MCM4 origin reverse: 5’-AGCCAAGTCCAACACCAAGT-3’

MCM4 exon 9 probe: 5’-CCCACCGCAGCTCCCTACATTCCTT-3’

MCM4 exon 9 forward: 5’-TCCTCGACCCTGCTTTATGA-3’

MCM4 exon 9 reverse: 5’-TGCTGCAACAGACAGCAACA-3’

As previously described (Birch et al. 2009), the enrichment of specific genomic DNA sequences was determined based on the threshold cycle (C_t) for each PCR product and analyzed according to the formula $2^{-\Delta[Ct(IP)-Ct(input)]}$-$2^{-\Delta[Ct(control\ IgG)-Ct(input)]}$. Using this method, DNA relative to input and immunoprecipitated by anti-HDAC11 was normalized to DNA immunoprecipitated by control IgG. P values were obtained using the Student's two-tailed T-test.

DNase I Accessibility Assays

Chromatin was isolated in a buffer containing 10 mM Tris-HCl (pH7.5), 5 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM KCl, 300 mM sucrose and 0.1% Triton X-100 for 5 min on ice, then washed and resuspended with the same buffer lacking detergent. One third of the chromatin from a 10 cm plate of cells was digested with DNase I (Promega) at 3 Units/100 μl for 10 min at RT. Another third was treated identically, but without DNase I (used for nomalization; untreated control). Reactions were stopped by addition of 10 mM EDTA/2 mM EGTA and incubated at 65^{o}C for 10 min. DNA was lightly sonicated, then purified and analyzed using TaqMan-based qPCR as described for ChIP assays.

CHAPTER 3

RESULTS

HDAC11 Associates with Replication Origins, Inhibits Cdt1-Induced Re-Replication, and Suppresses MCM Loading

HBO1 interacts with Cdt1 at origins specifically during G1 and acetylates H4 tails, which is required for MCM loading (Miotto and Struhl 2008; Miotto and Struhl 2010). The acetylation diminishes during S-phase, a time when MCM loading is normally prevented (Miotto and Struhl 2010), suggesting that a histone deacetylase may be involved in negatively regulating MCM loading. HDAC11 interacts directly with Cdt1 in S-phase (Glozak and Seto 2009) and can deacetylate H4 tails (Gao et al. 2002; Villagra et al. 2009), but is poorly understood in terms of its physiological function in cells. As such, we asked if HDAC11 could bind to origins in S-phase and negatively influence MCM loading and DNA replication. Chromatin immunoprecipitation (ChIP) analyses performed on two origins previously studied for HBO1 interactions (Miotto and Struhl 2008) demonstrated that HDAC11 becomes bound to MCM4 and Lamin B2 origins in S-phase but not in G1, whereas nearby chromosomal regions show a small, but

non-significant increase in HDAC11 (Fig. 8). Therefore, HDAC11 interacts with Cdt1 and associates with chromosomal origins with the opposite kinetics of HBO1 (*i.e.*, during S-phase), providing an explanation for why, in addition to reduced HBO1 activity, the H4 acetylation diminishes during S-phase (Miotto and Struhl 2010).

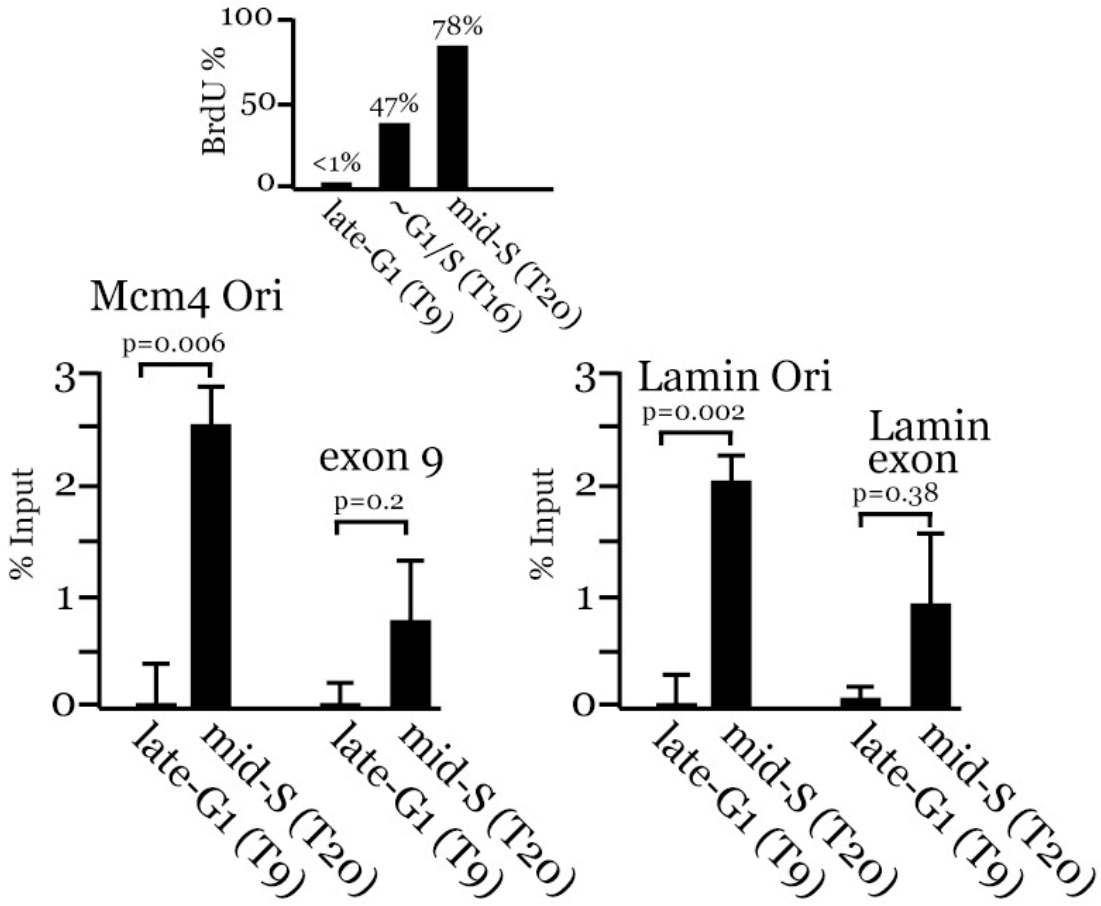


Fig. 8 HDAC11 Chromatin Immunoprecipitation. Synchronized HaCaT cells (as verified by BrdU incorporation and subsequent analysis by fluorescent microscopy, quantified results graphically shown in top panel) were subjected to anti-HDAC11 ChIP and qPCR analysis at the indicated time points to determine interactions to origin sequences (MCM4 Ori and Lamin Ori) and non-origin sequences (MCM4 exon 9 and Lamin exon).

Co-expression of HBO1 has been shown to enhance Cdt1-induced re-replication (Miotto and Struhl 2008). Given that HDAC11 associates with Cdt1 and origins in S-phase and our observation that HDAC11 overexpression in CHO by transient transfection suppresses BrdU incorporation compared to non-transfected cells (data not shown), we reasoned that HDAC11 might act in an opposite manner to HBO1 and suppress Cdt1-induced re-replication. Adenoviruses were used to overexpress Cdt1 (Vaziri et al. 2003) at three different levels, which produced a dose-dependent increase in the percentage of cells with >4N DNA content as determined by propidium iodide staining and subsequent FACS analysis (Fig. 9). Overexpression of HDAC11 alone did not result in changes to the distribution of cells within the cell cycle (data not shown), but co-expression of HDAC11 with Cdt1 caused a significant reduction in the number of re-replicating cells (Fig. 10, top panels). Interestingly, expressing more Cdt1 diminishes the inhibitory effects of HDAC11 (Fig. 10, bottom panels). This indicates that the suppression of DNA replication by HDAC11 is derived from a stoichiometric relationship that exists between the amount of Cdt1 and HDAC11 that is co-expressed. Furthermore, these results suggest that the inhibitory effect of HDAC11 is not due to an unrelated block to cell cycle progression into S-phase. Higher levels of Cdt1 overexpression not only increases the proportion of cells exhibiting >4N DNA content, but also appears to shift the distribution of cells within the cell cycle resulting in a diminished G1 population of cells, but an increased number of cells in S and G2-phases (Fig. 9). This may be caused by subpopulations of cells that have re-replicated only parts of their genome, but have not

accumulated enough re-replication to be detected by this FACS procedure. Staining by PI measures DNA content within a cell and this procedure is only able to detect re-replication in the subpopulation of cells that accumulates in the >4N DNA FACS population, which ignores lesser levels of re-replication that have not generated sufficient PI staining (Dorn et al. 2009). To circumvent this underestimation of re-replication levels, future experiments to analyze Cdt1-induced re-replication and suppression by HDAC11 could utilize the more sensitive single molecule DNA fiber analysis, or fiber spreading method. Analysis of re-replication using this technique will allow for the measurement of re-replication at the single replication fork level, which could more accurately provide physiologically relevant data regarding generation of re-replication events and the suppression of such events (Dorn et al. 2009).

Overexpression of HDAC11 alone suppresses the loading of Mcm2 on chromatin (Fig. 11), but has no affect on the total levels of Mcm2 or Cdt1. These results demonstrate that HDAC11 localizes to chromosomal origins in S-phase and inhibits the ability of Cdt1 to promote DNA replication and MCM loading. As such, and given the positive role of HBO1 in these processes during G1 (Iizuka et al. 2008; Miotto and Struhl 2008), HDAC11 temporally opposes the function of HBO1 in regulating replication licensing via Cdt1 interactions in S-phase.

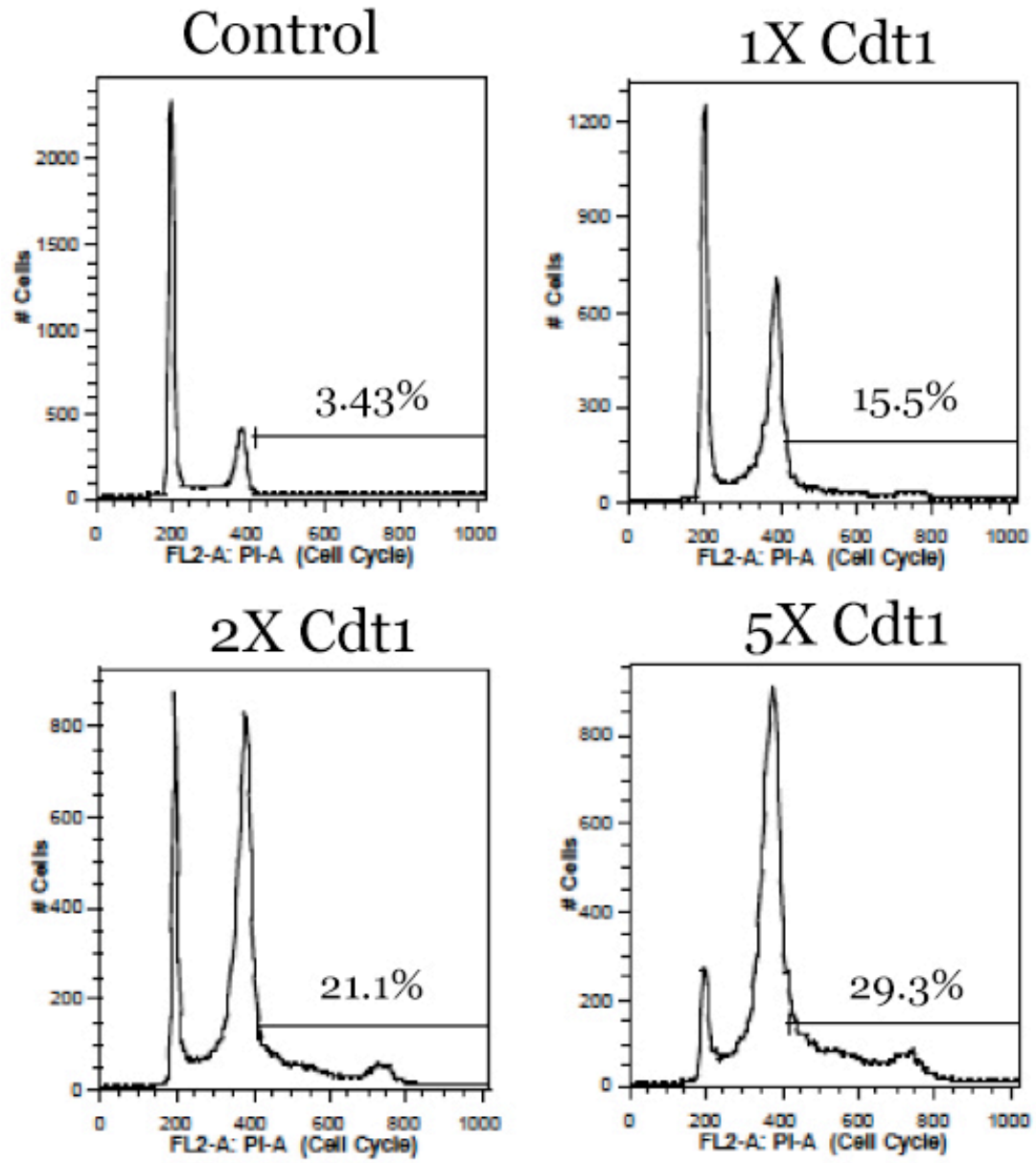


Fig. 9 Cdt1 Overexpression Causes Re-Replication. HeLa cells were infected for 48 hours with increasing amounts of adenovirus expressing Cdt1. Samples were processed for flow cytometric analysis and data was analyzed with FlowJo software. Relative amounts of each virus used to infect cells are shown and percentages indicate proportion of cells with >4N DNA content.

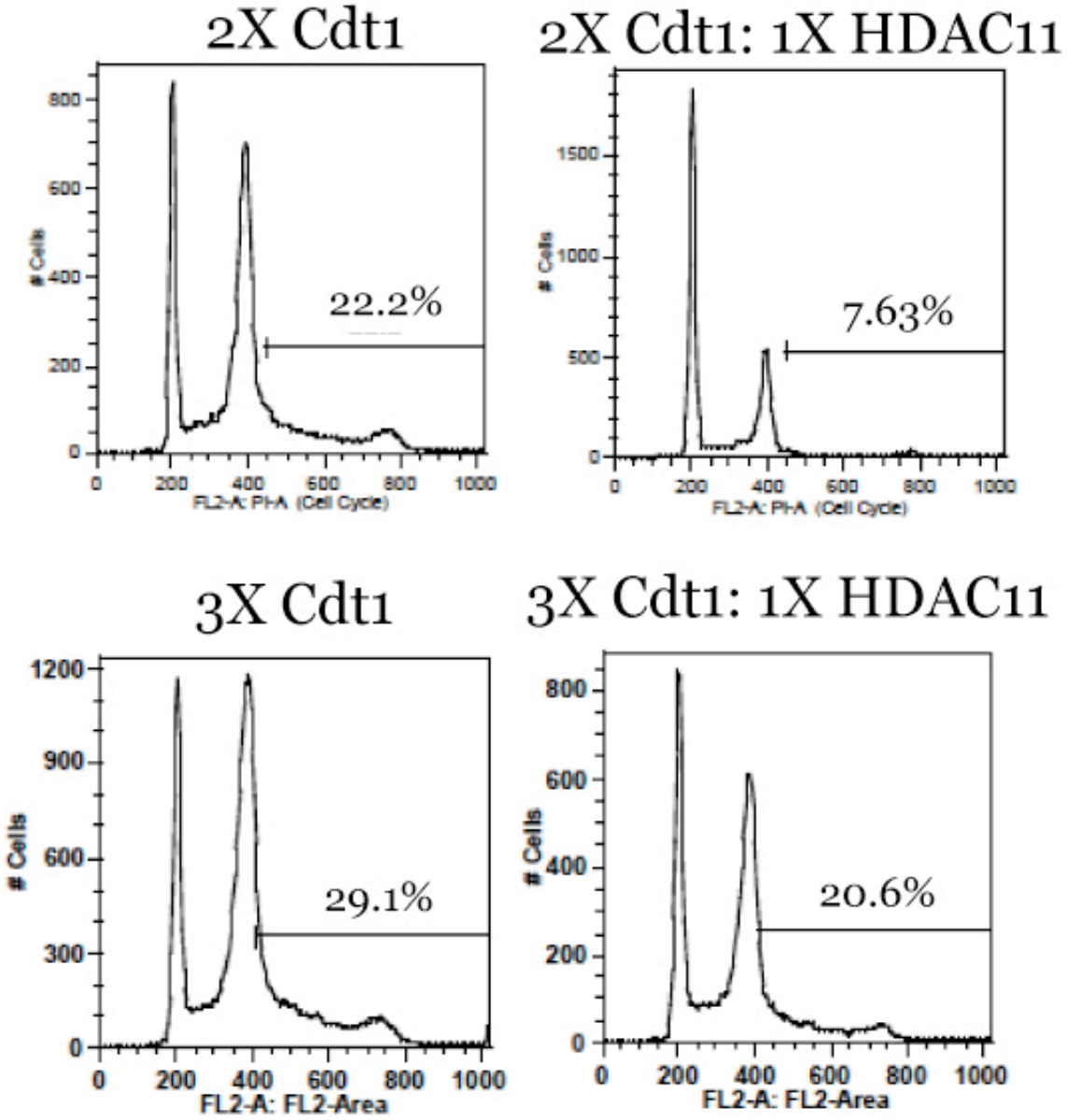


Fig. 10 HDAC11 Suppresses Cdt1-Induced Re-Replication. HeLa cells were infected for 48 hours with increasing amounts of adenovirus expressing Cdt1 or Cdt1 and HDAC11. Samples were processed for flow cytometric analysis and data was analyzed with FlowJo software. Relative amounts of each virus used to infect cells are shown and percentages indicate proportion of cells with >4N DNA content.

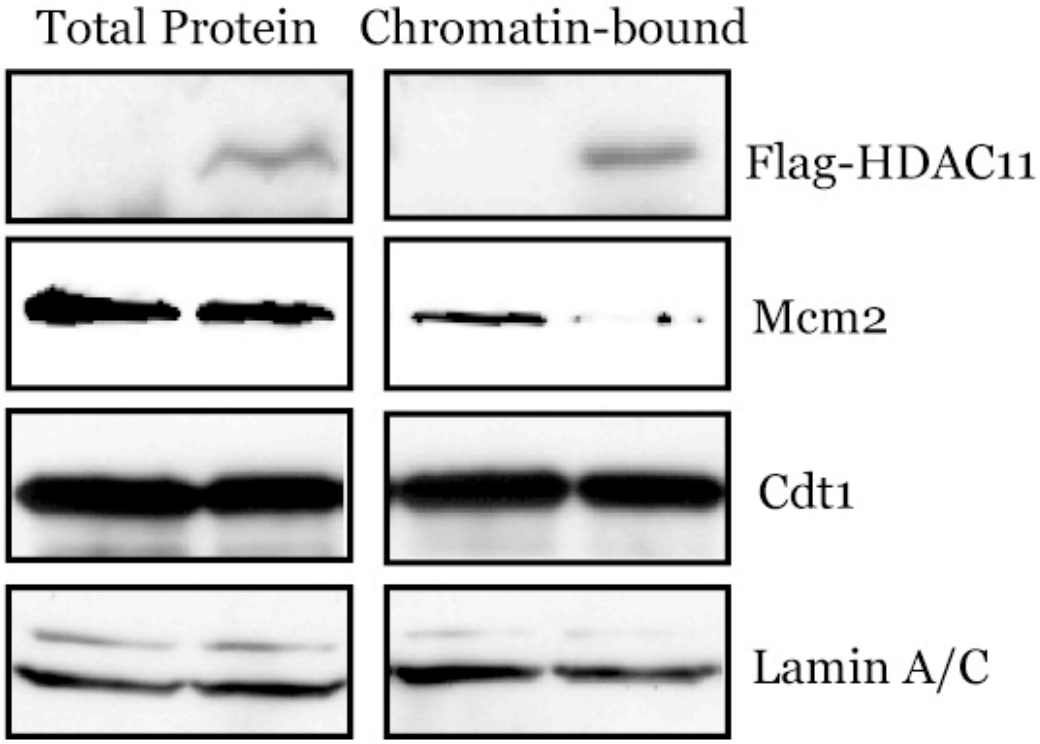


Fig. 11 HDAC11 Overexpression Inhibits MCM Binding to Chromatin. HeLa cells were infected for 24 hours with adenovirus expressing HDAC11 or control GFP. Cells were harvested and separated into soluble and chromatin-bound fractions. Immunoblots were performed with indicated antibodies.

Geminin Facilitates the Binding of HDAC11 to Cdt1

The ability of HDAC11 to bind to Cdt1, negatively influence MCM loading, and suppress DNA replication is similar to the biological effects of Geminin (Wohlschlegel et al. 2000; Saxena et al. 2004). This suggested a relationship might exist between Geminin and HDAC11 in regulating Cdt1 function. We obtained an anti-HDAC11 antibody that recognizes two isoforms of HDAC11, indicated here as bands A&B (Fig. 12 and 13).

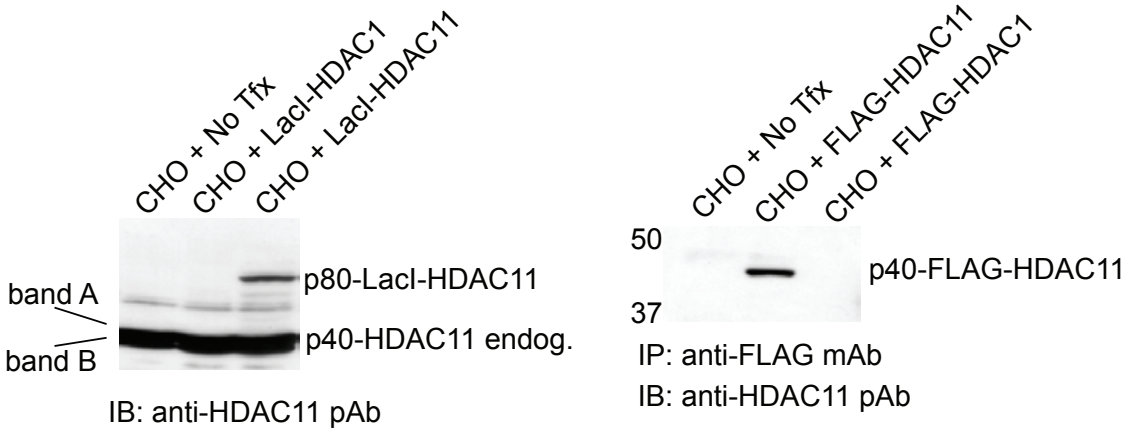


Fig. 12 Verification of anti-HDAC11 Antibody. CHO cells were transfected with LacI-HDAC1 or LacI-HDAC11 (left panel). The left panel immunoblot demonstrates the recognition of an endogenous HDAC11 doublet, denoted as band A and band B and the specific recognition of an overexpressed LacI-HDAC11 fusion protein with shifted molecular weight, but not a LacI-HDAC1 fusion protein. The right panel shows IP-Western analysis of CHO cells transfected with Flag-HDAC1 or Flag-HDAC11. Lysates were subjected to anti-Flag IP then anti-HDAC11 IB.

Using synchronized cell lysates separated into soluble and chromatin bound fractions, we observed that Geminin and the faster migrating band B of HDAC11 become chromatin bound with similar kinetics specifically during S-phase, both of which parallel PCNA binding kinetics (Fig. 13). The slower migrating band A of HDAC11 increases only modestly on chromatin during S-phase. In contrast, HBO1 associates with chromatin earlier in G1 and peaks during the time of MCM loading (6-12 hrs), consistent with the positive role HBO1 enzymatic activity plays in promoting licensing during G1 (Iizuka et al. 2006; Miotto and Struhl 2008; Miotto and Struhl 2010). Cdt1 is chromatin-bound throughout G1 and S-phase, but a slower migrating form (asterisk) becomes visible that overlaps MCM loading kinetics (Fig. 13). The slower migrating Cdt1 is likely to be a ubiquitinylated form of Cdt1 that is known to be degraded (Arias and Walter 2005). Consistent with this, the slower migrating Cdt1 diminishes after its initial appearance. The kinetics for HBO1 and HDAC11 are graphed in Figure 14. These results are consistent with a model in which HBO1 promotes licensing in G1 and HDAC11 prevents re-licensing during S-phase, in both cases through association with Cdt1.

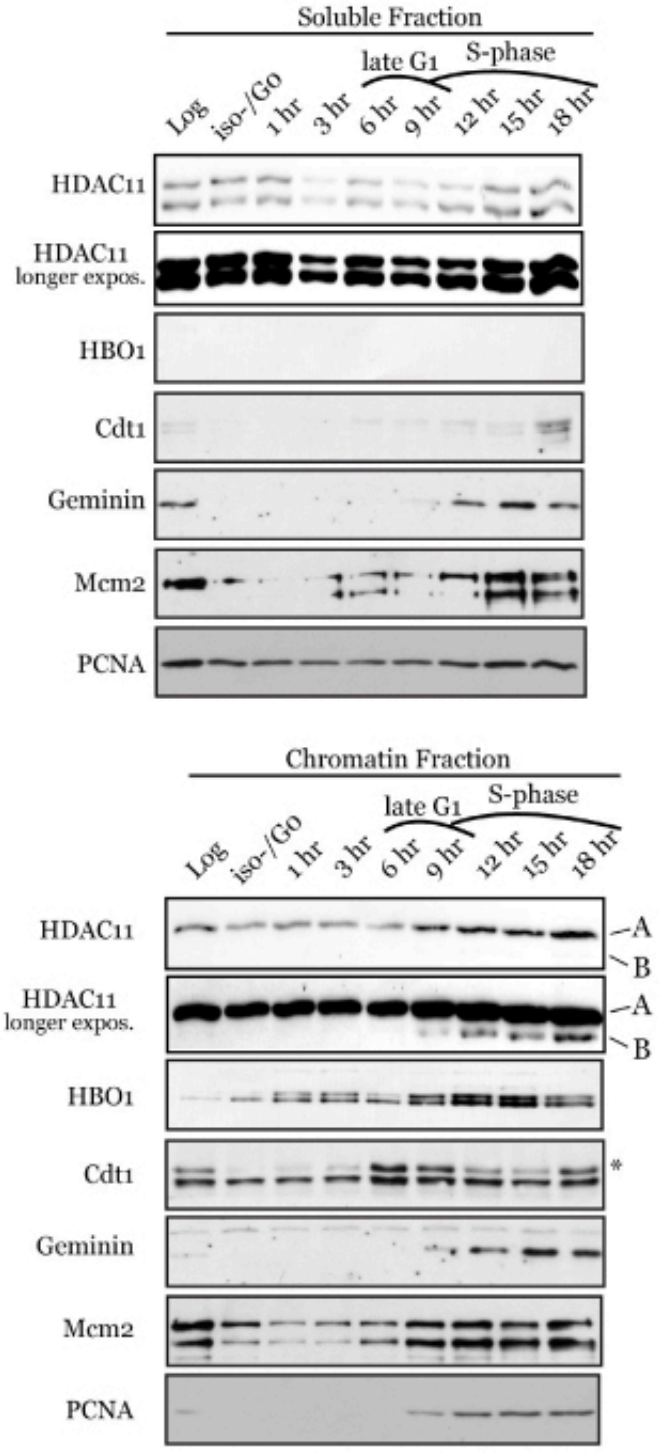


Fig. 13 Chromatin Binding Kinetics of HDAC11. Synchronized CHO cells were separated into soluble and chromatin-bound fractions at the indicated times and subjected to IB with indicated antibodies. BrdU verified synchrony (data not shown).

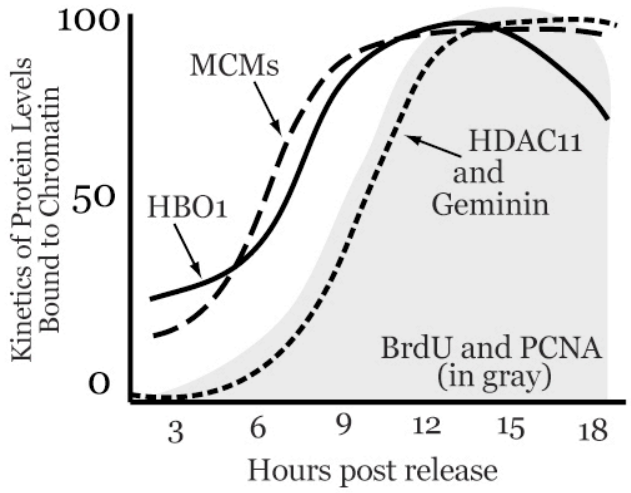


Fig. 14 Graphical Representation of HDAC11 Chromatin Binding Kinetics.

Although Geminin negatively influences the acetyltransferase activity of HBO1, it does not affect the physical interaction of HBO1 with Cdt1 (Miotto and Struhl 2008). Given the similar chromatin binding kinetics between Geminin and HDAC11, we next determined if Geminin influenced the interaction of HDAC11 with Cdt1. Geminin and HDAC11 can independently bind Cdt1 *in vivo* and *in vitro* (Saxena et al. 2004; Glozak and Seto 2009), indicating that neither protein requires the other to directly bind Cdt1. HDAC11, Geminin, and Cdt1 were transiently expressed in several combinations, and immunoprecipitations (IP) were performed against HDAC11 or Cdt1, followed by immunoblotting (IB) for the presence of the other expressed proteins in the IP complexes. Without Geminin, HDAC11 and Cdt1 interact to a small degree when either protein is pulled down in the IP step (Fig. 15, lane 5, rows A&C). Similarly, Geminin can bind Cdt1 in the absence of HDAC11 (Fig. 15, lane 6, row D). However, when all three proteins are co-expressed, there is a noticeable increase in the amount of HDAC11 that

interacts with Cdt1, when either Cdt1 or HDAC11 is pulled down in the IP step (Fig. 15, compare lanes 5&8 on rows A&C). The amount of Geminin that interacts with Cdt1 is not influenced by HDAC11, indicating that the converse is not true (Fig. 15, compare lanes 6&8, row D). These results demonstrate that Geminin increases the efficiency of the HDAC11-Cdt1 interaction.

We next asked if Geminin, HDAC11, and Cdt1 could form a trimeric complex in cells, or coexist together in a larger, multi-protein complex. Cdt1, Geminin, and HDAC11 were co-expressed and complexes containing Flag-HDAC11 were purified and separated by a size-exclusion column. All three proteins co-elute in an ~700 kDa size range (Fig. 16 fractions 15&16). Such an elution profile could be due to two separate, but similarly-sized, large complexes in which HDAC11 is present with Geminin in one and with Cdt1 in the other. However, this is highly unlikely given that Geminin and Cdt1 interact efficiently in cells on their own (Wohlschlegel et al. 2000; Saxena et al. 2004). Therefore, these results indicate that all three proteins reside together in one complex (that contains other unknown proteins), which is consistent with the fact that HDAC11 and Geminin both associate with Cdt1 *in vivo* during S-phase under physiologic conditions (Wohlschlegel et al. 2000; Glozak and Seto 2009). Since Geminin and HDAC11 do not reduce the efficiency with which either protein can bind Cdt1 (Fig. 15), Geminin and HDAC11 do not compete for binding to Cdt1 and can interact with Cdt1 simultaneously. These results suggest that one function of Geminin in negatively regulating DNA replication may derive from an inherent ability of Geminin to facilitate HDAC11 binding to Cdt1, leading to decreased MCM loading.

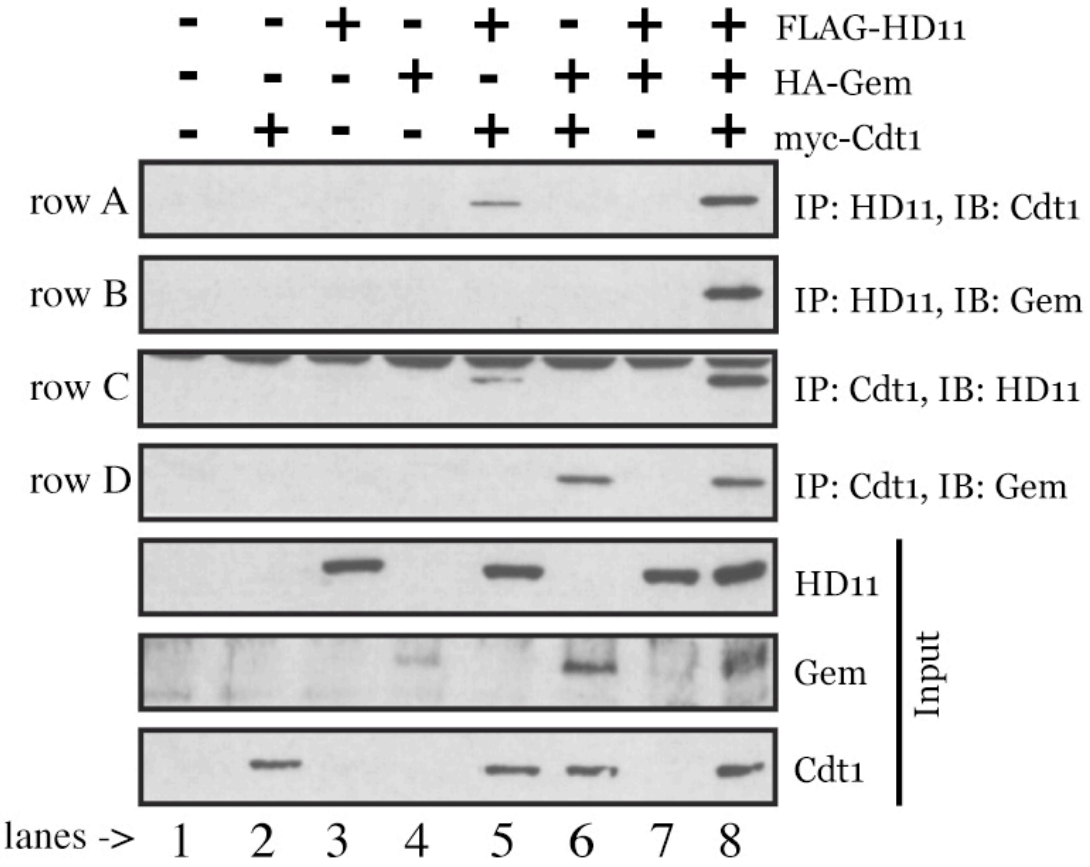


Fig. 15 Geminin Enhances the Cdt1-HDAC11 Interaction. Indicated proteins (top) were expressed in 293T cells and subjected to immunoprecipitation (IP) and immunoblotting (IB) analysis as indicated on right. IP and IB analyses were performed with anti-tag antibodies. Representative of three separate experiments with similar results.

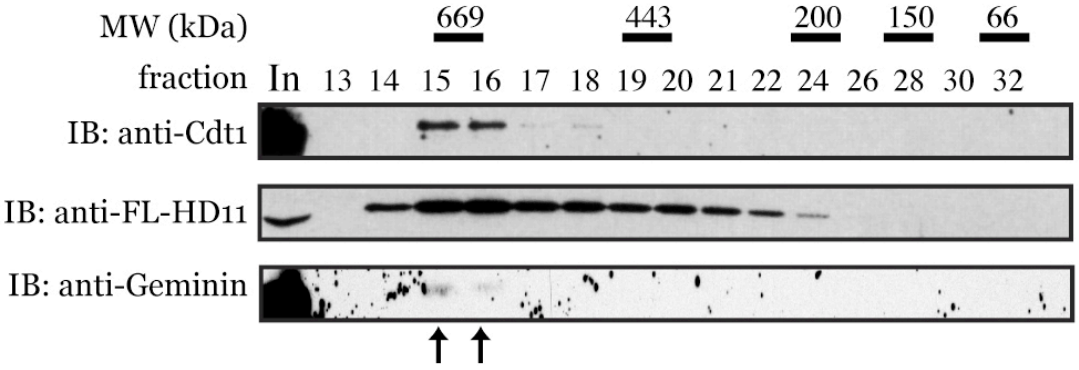


Fig. 16 Geminin, HDAC11, and Cdt1 Interact in a Single Complex. Flag-HDAC11, HA-Geminin, and Myc-Cdt1 were co-expressed in 293T cells followed by anti-Flag purification. Eluates containing Flag-HDAC11 complexes were separated on a size exclusion column and analyzed by IB.

Cdt1 Targeting Induces Large-Scale Chromatin Decondensation

Cdt1 recruits two histone modifying enzymes, HBO1 and HDAC11, that regulate MCM loading and DNA replication in an opposing manner. The timing of the association of these enzymes with replication origins coincides with the presence or absence, respectively, of acetylated histone H4 (Miotto and Struhl 2010). Although the

H4 acetylation is known to be required for MCM recruitment (Miotto and Struhl 2010), the mechanism by which it facilitates this is unknown. We hypothesized that the ability of Cdt1 to differentially recruit these enzymes produced higher-order chromatin structural changes that facilitate or inhibit MCM recruitment via altered chromatin accessibility. Currently, there is no technological means to assess changes to higher-order chromatin structure at chromosomal origins. However, to test this concept, we employed an innovative chromatin remodeling system that assesses the ability of proteins to generate changes to higher-order chromatin structure (Tumbar et al. 1999; Alexandrow and Hamlin 2005). This system utilizes a CHO-derived cell line (A03_1) that contains a 90 Mb homogeneous staining region (HSR) that was engineered through stable insertion and amplification of a Lac-operator(LacO)/DHFR vector (final HSR contains ~1600 such vectors). The presence of LacO sites throughout the HSR allows for microscopic visualization of chromatin structural changes that occur following targeting of LacI-fused proteins of interest (Fig. 17). In its normal unperturbed state, the HSR adopts a condensed dot-like structure that is heterochromatic in nature (Li et al. 1998). However, targeting proteins that recruit chromatin remodeling enzymes elicits dramatic changes in the HSR structure, resulting in clearly observable, highly decondensed HSRs occupying large portions of the nucleus (Tumbar et al. 1999; Alexandrow and Hamlin 2005). This system provides insight into regulation of higher-order chromatin dynamics that cannot be analyzed by any other current experimental means.

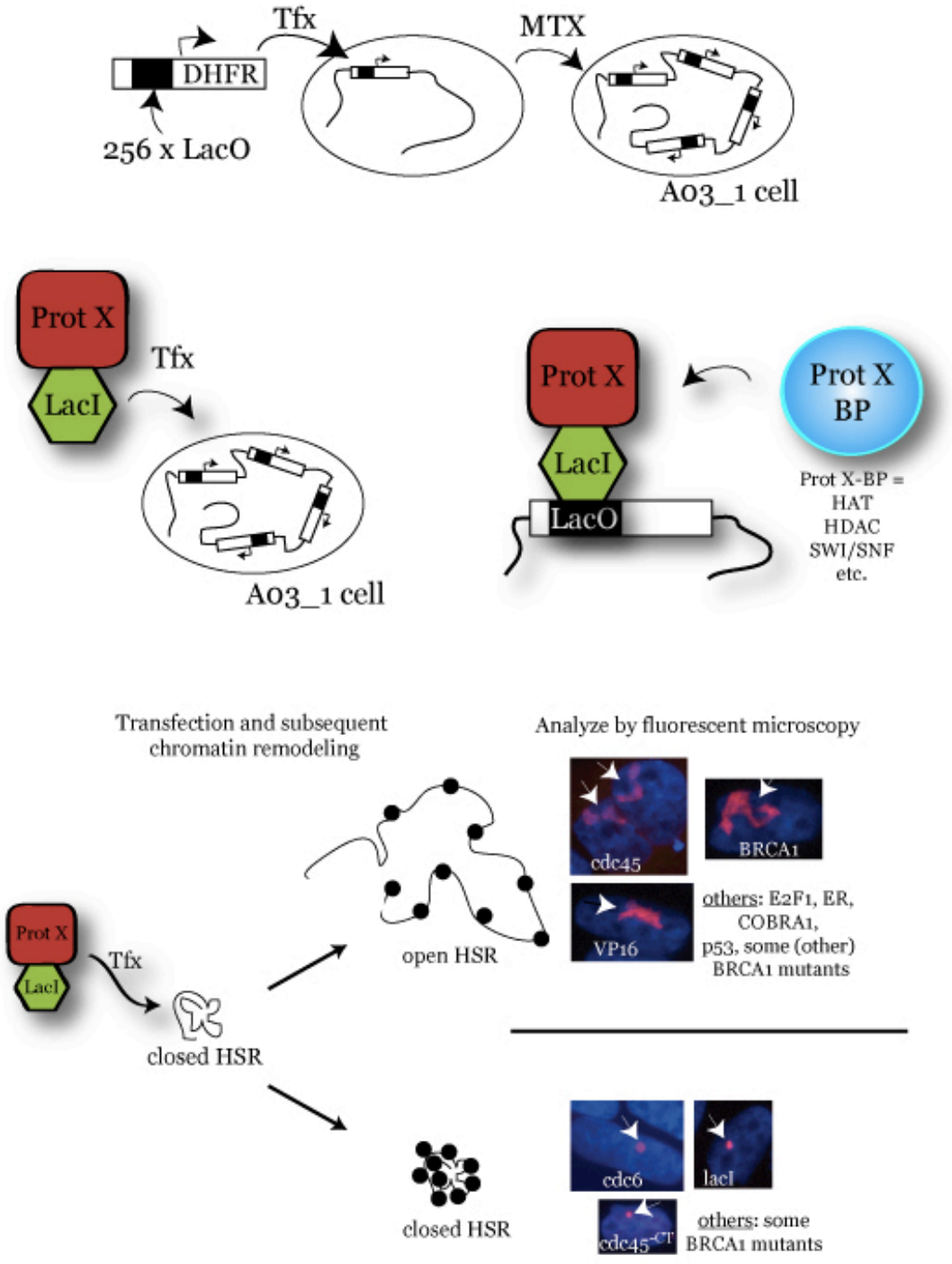


Fig. 17 Schematic of *in vivo* Chromatin Remodeling System.

The mechanisms underlying chromatin remodeling in this system derive from specific, physiologically relevant events involved in altering chromatin structure by targeted proteins. Several transcription factors, including p53, E2F1, BRCA1, VP16, and ER, promote decondensation via histone acetylation, H2AX phosphorylation, and recruitment of chromatin modifying enzymes (Tumbar et al. 1999; Ye et al. 2001; Nye et al. 2002). The replication protein, Cdc45, promotes decondensation via Cdk2 recruitment and H1 phosphorylation(Alexandrow and Hamlin 2005). In contrast, some proteins promote condensation (Verschure et al. 2005), while others produce no changes to the HSR structure (remains condensed).

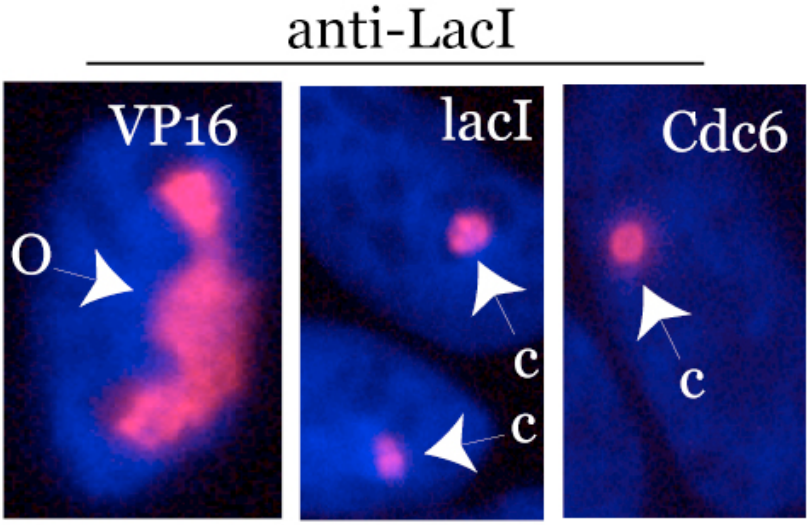


Fig. 18 VP16 induces chromatin remodeling, but Cdc6 and LacI do not. LacI-VP16, LacI alone, or LacI-Cdc6 were transiently expressed in A03_1 cells, followed by IF with anti-LacI and Texas Red to detect open/decondensed ('O') or closed/condensed ('C') HSRs. Nuclei were stained with DAPI.

To determine if Cdt1 can promote large-scale decondensation of the HSR, Cdt1 was fused to LacI and transfected into A03_1 cells. As controls, LacI-VP16, LacI-Cdc6, or the LacI DNA binding domain (DBD) alone were also expressed. Figure 18 shows that LacI-VP16 promotes large-scale decondensation, while LacI-Cdc6 and LacI-DBD do not, consistent with previous findings (Tumbar et al. 1999; Alexandrow and Hamlin 2005). Targeting Cdt1 to the HSRs produces a dramatic decondensation of the chromatin (Fig. 19).

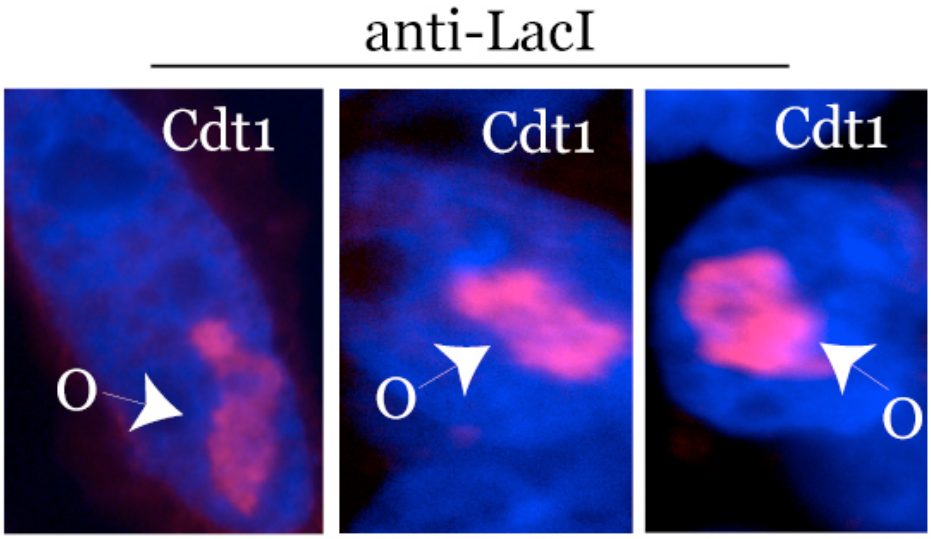


Fig. 19 Cdt1 Induces Chromatin Remodeling. LacI-Cdt1 was transiently expressed in A03_1 cells, followed by IF with anti-LacI and Texas Red to detect open/decondensed ('O') or closed/condensed ('C') HSRs. Nuclei were stained with DAPI.

All proteins express similarly (Fig. 20), and the fact that LacI alone expresses significantly higher indicates that targeting proteins does not itself elicit changes to the HSR due to crowding or related effects. As described previously (Alexandrow and Hamlin 2005), we assigned 'open' versus 'closed' status to the visual appearance of the HSRs using objective criteria. Open structures clearly display large, decondensed HSRs that occupy more than 10% of the nuclear area. Closed HSRs are obvious condensed structures that failed to unfold and typically cover less than 5% of the nuclear area. In all analyses, some HSRs are visible that are dot-like in appearance, but somewhat larger in size (~5-10% of nuclear area). We refer to the latter as Indeterminate, since classifying such HSRs is highly subjective. Using these objective criteria, ~2/3 of LacI-Cdt1 targeted HSRs become decondensed, similar to that for VP16 (Table I). In addition to being enriched at the HSRs due to LacI targeting, the LacI-Cdt1 protein is also localized throughout the nucleus and not in the cytoplasm (Fig. 21), demonstrating that the localization of ectopic Cdt1 is regulated by physiologic mechanisms. We conclude from these results that targeting Cdt1, but not Cdc6, to chromosomal regions *in vivo* produces a clearly observable and robust decondensation of higher-order chromatin structure.

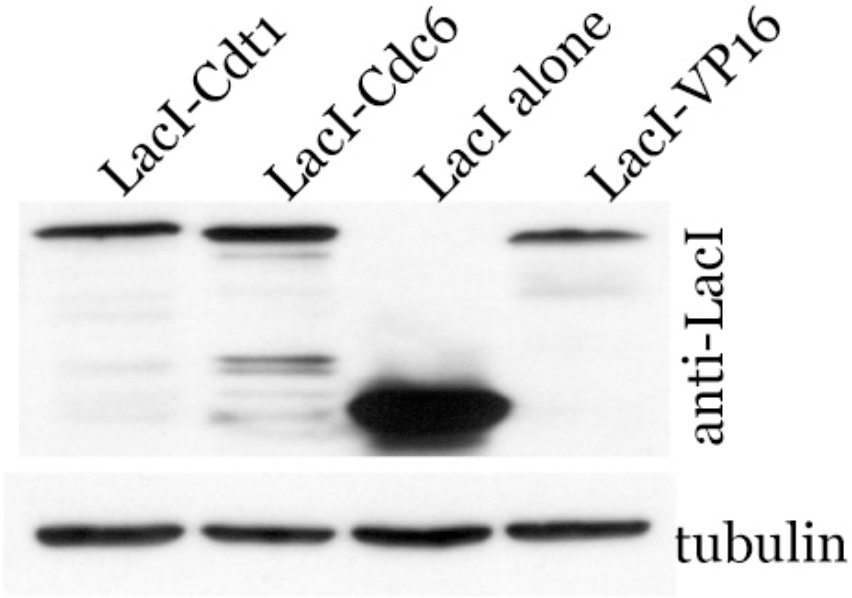


Fig. 20 Expression Levels of LacI-Fusion Proteins. Immunoblot of LacI-fusion protein expression for the results in Table 1.

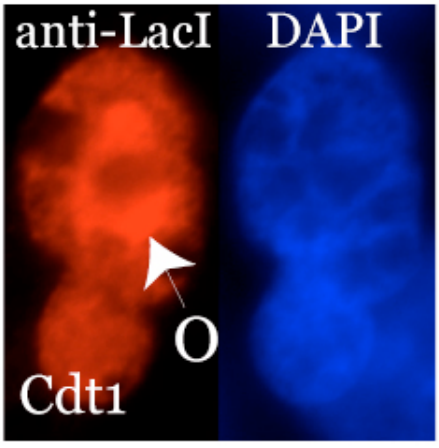


Fig. 21 LacI-Cdt1 Localizes to the Nucleus. Anti-LacI immunofluorescence separated from DAPI showing LacI-Cdt1 present throughout the nucleus.

Table I: Quantification of open and closed chromatin structures/HSRs

Proteins Expressed	Decondensed (open) chromatin	Condensed (closed) chromatin	Indeterminate
LacI-Cdt1	65% (+/-3%)	30% (+/-2%)	5% (+/-1%)
LacI-Cdc6	7% (+/-2%)	41% (+/-3%)	36%[a],16% (+/-2%)
LacI-DBD alone	7% (+/-2%)	73% (+/-3%)	20% (+/-3%)
LacI-VP16	57% (+/-3%)	36% (+/-3%)	7% (+/-1%)
LacI-Cdt1 + HA-Geminin (1Gem:1Cdt1)	58% (+/-3%)	34% (+/-2%)	8% (+/-1%)
LacI-Cdt1 + HA-Geminin (5Gem:1Cdt1)	12% (+/-2%)	80% (+/-4%)	8% (+/-1%)
LacI-Cdc45 + pcDNA3	74% (+/-4%)	23% (+/-2%)	3% (+/-1%)
LacI-Cdc45 + HA-Geminin (5Gem:1Cdc45)	73% (+/-4%)	25% (+/-2%)	2% (+/-1%)
LacI-VP16 + pcDNA3	63% (+/-3%)	33% (+/-3%)	4% (+/-1%)
LacI-VP16 + HA-Geminin (5Gem:1VP16)	60% (+/-3%)	33% (+/-3%)	7% (+/-1%)

In each case, at least 150 transfected cells were scored. The results depicted are representative of at least three experiments performed for each condition, with similar outcomes (used to produce errors, SD).

[a] The amount of LacI-Cdc6-expressing cells that displayed a homogenous nuclear stain with no closed or open HSRs present.

Cdt1-Induced Chromatin Unfolding Occurs During G1

We reasoned that, if chromatin unfolding induced by Cdt1 were physiologically involved in creating chromatin access for loading MCMs, then such unfolding should occur during G1. We determined the cell cycle phase at the time when decondensation occurred after LacI-Cdt1 targeting. To indicate S-phase cells, BrdU staining was used, and cells that were in G2 and/or M-phases were identified by anti-H1-phospho (H1-P) staining, since H1-P levels are highest at these times (Lu et al. 1994; Alexandrow and Hamlin 2005). LacI-Cdt1-induced open HSRs were found almost exclusively in transfected cells that neither displayed BrdU nor H1-P staining (Fig. 22). These results indicate that the cells are primarily in G1 (but early S-phase is also possible) at the same time that the transient LacI-Cdt1 protein is expressed and open HSRs are being generated. Interestingly, closed HSRs correlated in the opposite manner (*i.e.*, with S, G2, or M-phase cells). Thus, chromatin unfolding by Cdt1 occurs in G1, when Cdt1 is known to function in MCM loading.

	BrdU neg.	BrdU pos.
Cdt1 open	89%	11%
Cdt1 closed	31%	69%
	H1-P neg.	H1-P pos.
Cdt1 open	91%	9%
Cdt1 closed	45%	55%

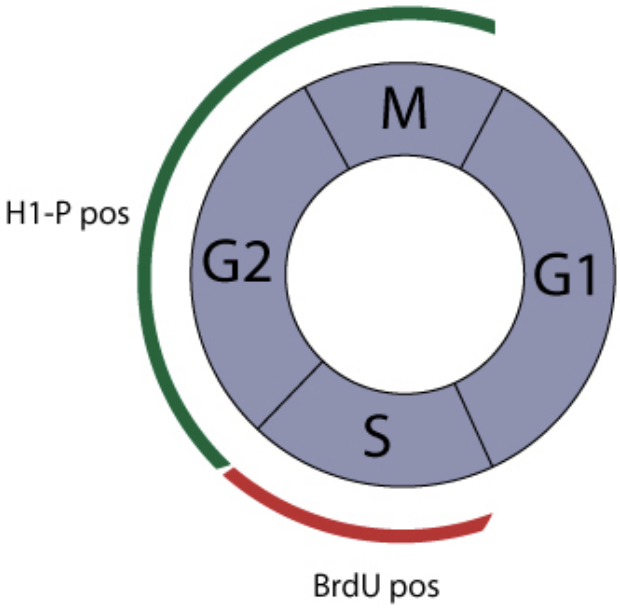


Fig. 22 Cdt1-Induced Chromatin Decondensation Occurs in G1-phase. LacI-Cdt1 was expressed in A03_1 cells for 24 hours, then pulsed with BrdU. Anti-BrdU and anti-H1-P staining was used to relate the index of BrdU-negative and H1-P-positive cells to the open or closed HSR status.

Geminin Efficiently and Specifically Suppresses

Cdt1-Induced Chromatin Unfolding

Since Geminin is a physiologic inhibitor of Cdt1 at high Geminin:Cdt1 ratios (Lutzmann et al. 2006), we asked if the decondensation by Cdt1 were Geminin sensitive. Chromatin decondensation assays were performed using a 1:1 ratio of Geminin:Cdt1 vectors, or a higher 5:1 ratio. Relative protein expression is shown in Figure 23.

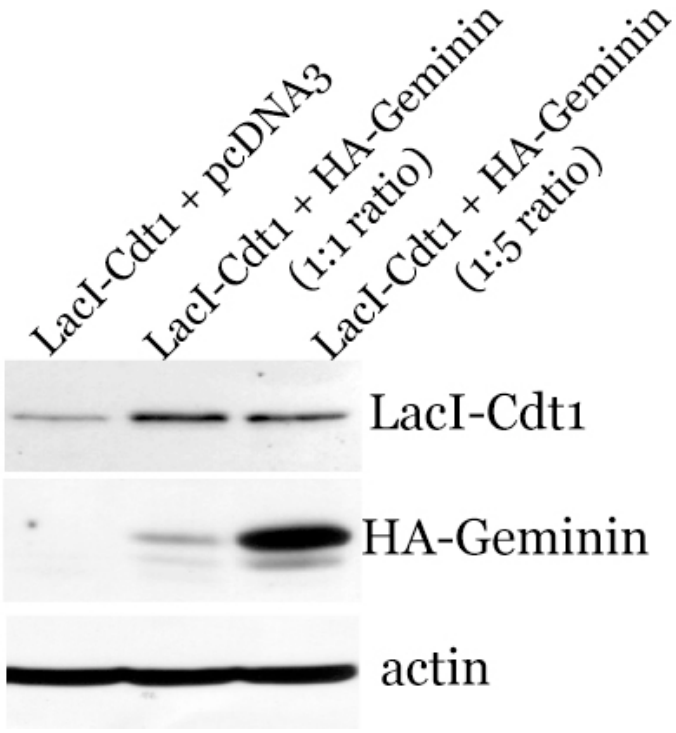


Fig. 23 Expression Levels of LacI-Cdt1 and HA-Geminin. HA-Geminin was transfected to a 5:1 or 1:1 plasmid ratio with LacI-Cdt1 and relative protein expression was verified by IB.

Compared to LacI-Cdt1 + pcDNA3 control, 1:1 ratios of Geminin:Cdt1 did not alter the amount of decondensation produced by Cdt1 (Table I). However, co-expression of Geminin at a 5:1 ratio significantly suppressed the ability of Cdt1 to decondense chromatin (Table I and Fig. 24, top panels). Under these conditions, we noticed the appearance of a number of very small, but slightly decondensed HSRs, which we define as 'small-open' (Fig. 24, top left panel). We considered these 'small-open' HSRs as closed, since they have clearly not succeeded in becoming large decondensed HSRs that are normally seen with Cdt1 expressed alone (compare Fig. 24, top panels vs. bottom panels). Chromatin unfolding induced by Cdc45 or VP16 was not sensitive to inhibition by Geminin (Table I), indicating that the inhibitory effect of Geminin toward Cdt1 is specific and is not due to global effects that suppress chromatin remodeling mechanisms. These results demonstrate a novel effect of Geminin in modulating chromatin accessibility through its interaction with Cdt1.

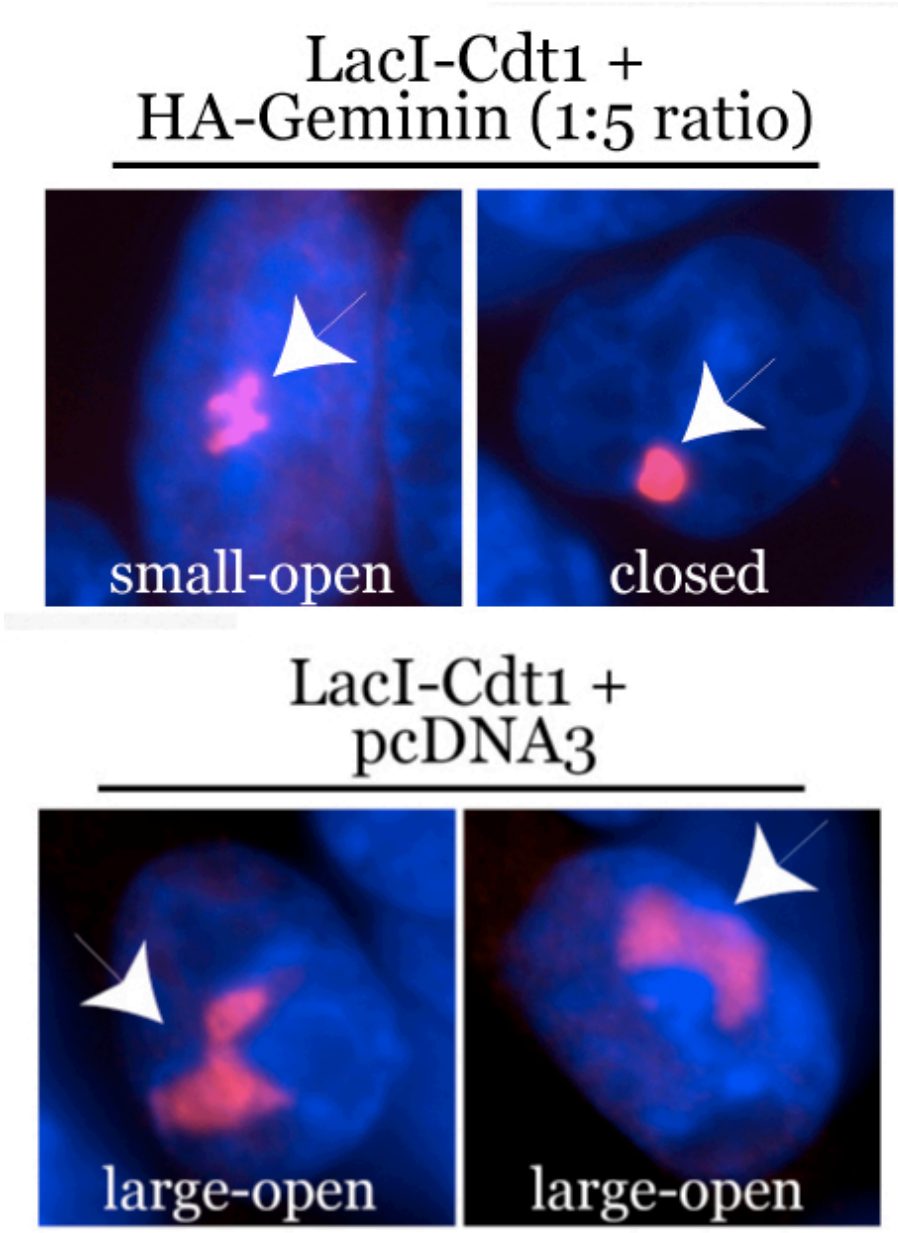


Fig. 24 Geminin Inhibits Cdt1-Induced Chromatin Unfolding. Examples of small-open and closed HSRs resulting from transfection of a 5:1 plasmid ratio of HA-Geminin:LacI-Cdt1.

Chromatin Unfolding by Cdt1 is Required for Cell Proliferation and Efficient DNA Re-Replication

We next determined the region within Cdt1 that is required for promoting chromatin unfolding, and then tested for biological effects of loss of this domain. Carboxy-terminal truncations of Cdt1 were generated and tested for chromatin unfolding ability, and it was found that a region in the middle of Cdt1 is required for chromatin decondensation. A deletion mutant of Cdt1 was made that lacked specifically this region (Fig. 25) and was deficient for chromatin unfolding (Fig. 26).

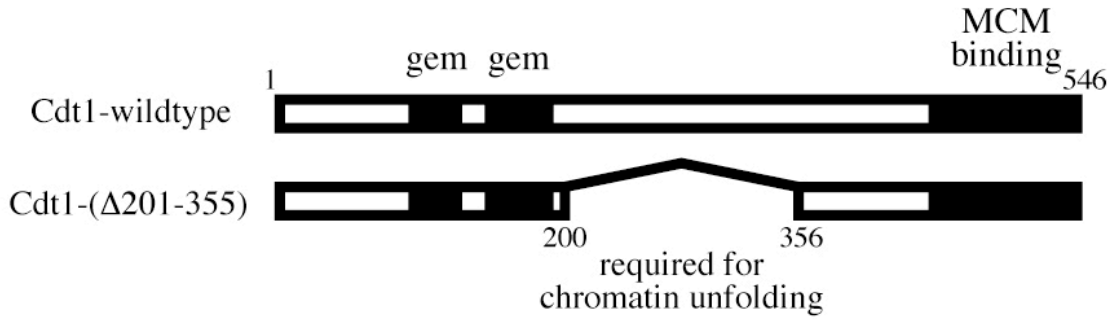
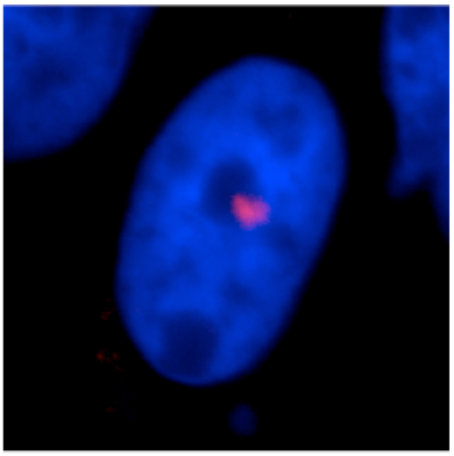


Fig. 25 Deletion Mutant of Cdt1 that is Defective for Chromatin Remodeling. Diagram showing location of Cdt1 chromatin remodeling domain.



Cdt1-(Δ201-355)

Fig. 26 Cdt1 Deletion Mutant Does Not Cause Chromatin Unfolding. Chromatin unfolding ability of Cdt1-(Δ201-355) was tested. LacI-Cdt1-(Δ201-355) was expressed in A03_1 cells for 24 hours before analysis.

Stable expression of Cdt1-(Δ201-355) was found to significantly inhibit the ability of cells to proliferate relative to wt-Cdt1 (Fig. 27). Intriguingly, a previous report analyzing Cdt1 mutant alleles found that Cdt1 lacking this region is 25-60% less efficient at promoting re-replication versus multiple Cdt1 alleles that contain this region (Teer and Dutta 2008). In agreement with this prior study, Cdt1-(Δ201-355) is 25-50% reduced in re-replication ability versus wt-Cdt1 (Fig. 28). The reason Cdt1 re-replication is not completely diminished is because all alleles containing the amino-terminus of Cdt1 will induce re-replication due to dilution of Cdt1 degradation machinery by competitive binding, allowing endogenous Cdt1 to induce re-replication in addition to the exogenous protein being tested (Teer and Dutta 2008). As such, we conclude from these experiments that the chromatin unfolding function of Cdt1 is required for sustained cell cycle progression due at least in part to a necessity for this region to promote efficient DNA replication.

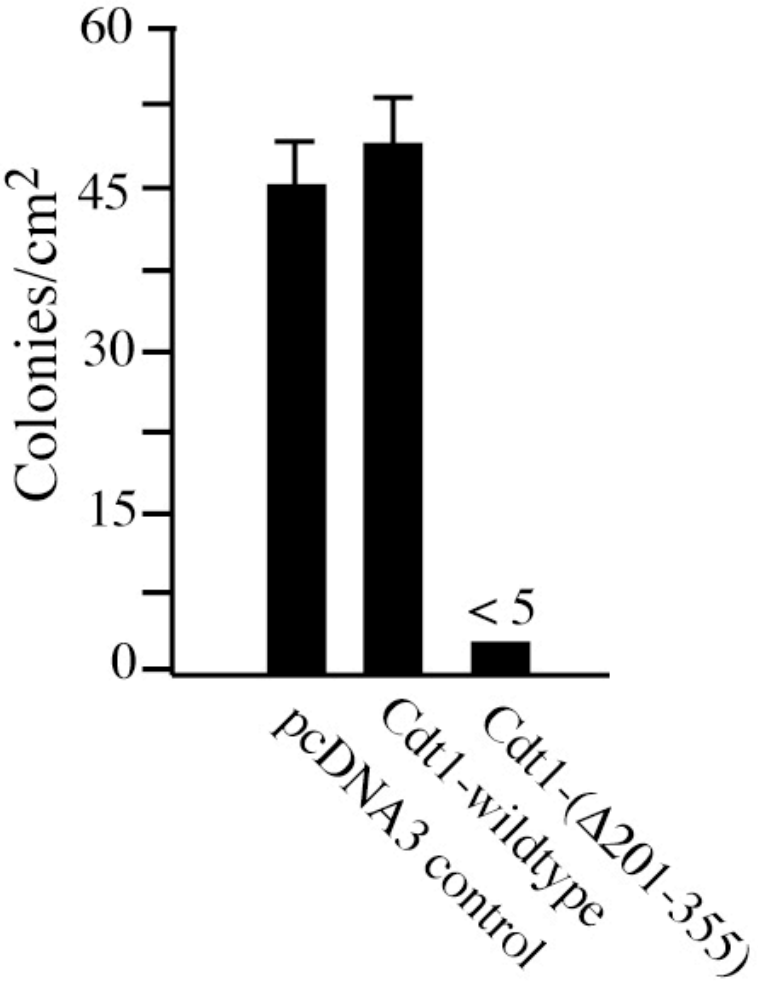


Fig. 27 Cdt1 Deletion Mutant Inhibits Cell Survival. Colony forming assays were performed in CHO cells to test the ability of wt-Cdt1 and Cdt1-(Δ201-355) to suppress colony growth. Stable selection for protein expression lasted 14 days, followed by Giemsa staining.

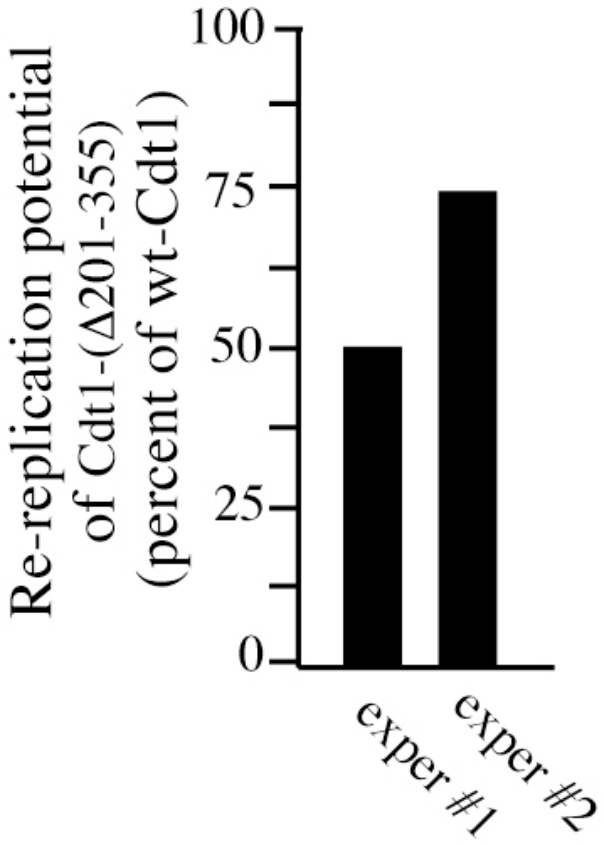


Fig. 28 Cdt1 Deletion Mutant is Inhibited in Causing Re-Replication. HeLa cells were used as in Figure 8 to determine the re-replication induction ability of Cdt1-(Δ201-355) compared to wt-Cdt1, except 48 hour transfections were used. Results from two experiments are shown, compared to wt-Cdt1 normalized to 100% re-replication ability.

Chromatin Decondensation by Cdt1 Stimulates MCM Recruitment

We next asked if chromatin decondensation by Cdt1 stimulated the recruitment of endogenous MCMs. Chromatin unfolding assays were performed in which LacI-Cdt1 was expressed, followed by co-staining against LacI (to identify open or closed HSRs)

and Mcm4 or Mcm7. Figure 29 shows that endogenous Mcm4 and Mcm7 both become noticeably enriched at Cdt1-decondensed HSRs. In contrast, HSRs decondensed by BRCA1 or VP16 did not enhance Mcm7 recruitment (Fig. 30). We also found that PCNA became enriched at Cdt1-decondensed HSRs (Fig. 31), but the effect was not dramatic and only occurred in a small percentage of such samples (<10%, data not shown).

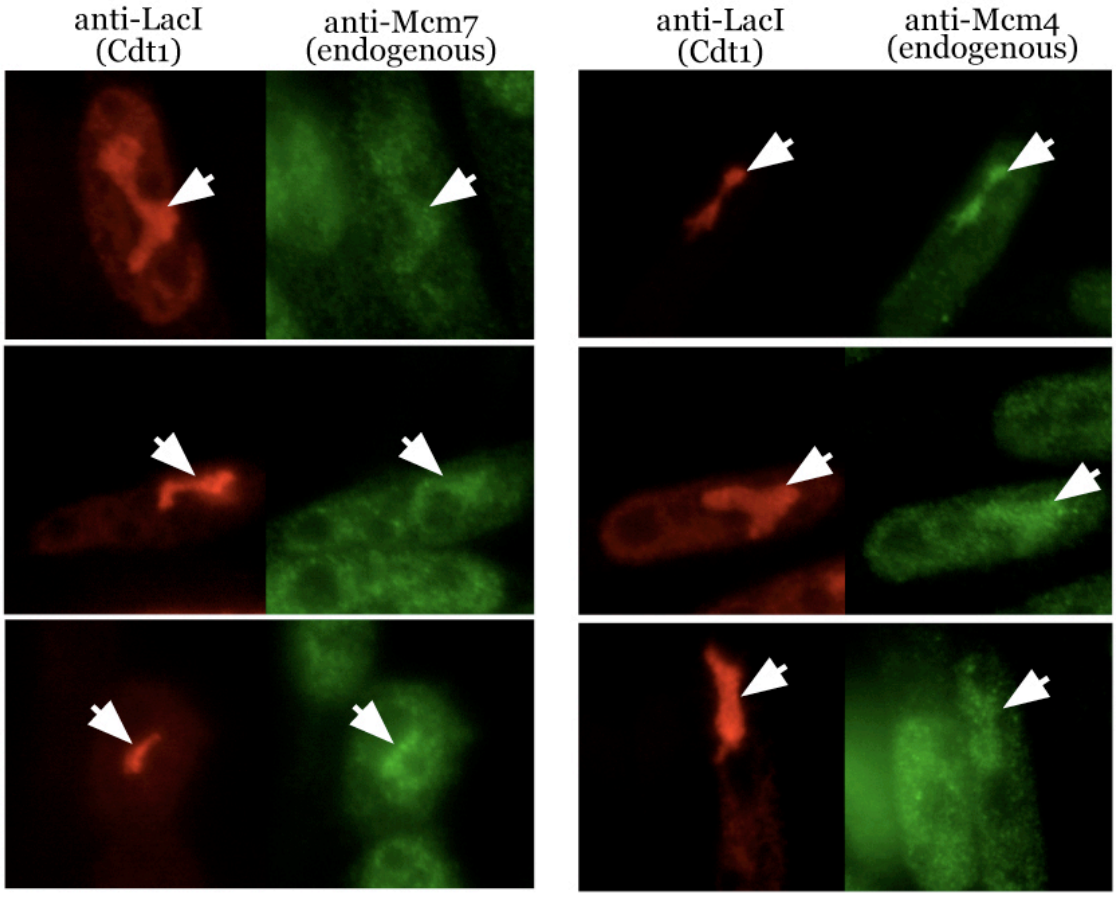


Fig. 29 Cdt1-Induced Chromatin Unfolding Stimulates MCM Recruitment. Open HSRs following LacI-Cdt1 expression were co-stained by IF with antibodies to LacI, Mcm7, or Mcm4. Arrows indicate open HSRs and enrichment of endogenous MCMs. A03_1 cells were used and transfections lasted 24 hours.

Relative to BRCA1 and VP16, where MCM co-localization was far less frequent and not dependent on chromatin decondensation, ~1/3 of Cdt1-decondensed HSRs displayed enriched MCM recruitment (Fig. 30). Only a small number (5%) of Cdt1-bound HSRs that failed to open recruited MCMs. This result was obtained in more than 6 separate experiments (data not shown, but see below). This consistent observation probably derives from our necessary use of asynchronous populations for these analyses. The machinery involved in MCM loading is only available during a certain period of time in the cell cycle, and in cells released from quiescence, MCM loading occurs in the latter ~1/3 of G1 (Mukherjee et al. 2009). Cdt1-induced decondensation occurs in G1 (Fig. 22) and MCM recruitment is seen in only ~1/3 of these, which correlates with such a prediction.

A simple explanation for why MCMs are enriched at the HSRs upon Cdt1 targeting could derive from the fact that Cdt1 can bind to MCMs (Tanaka and Diffley 2002; Yanagi et al. 2002; Teer and Dutta 2008). However, Cdt1-bound HSRs that fail to open are not efficiently enriched with MCMs (Fig. 32), indicating that the presence of Cdt1 alone at these chromosomal sites is not sufficient for MCM recruitment. Since a significant number of Cdt1-decondensed HSRs are not enriched for MCMs, the recruitment of MCMs does not itself produce a crowding effect that causes the unfolding. We conclude from these results that Cdt1-induced decondensation is a prerequisite for stimulating MCM recruitment.

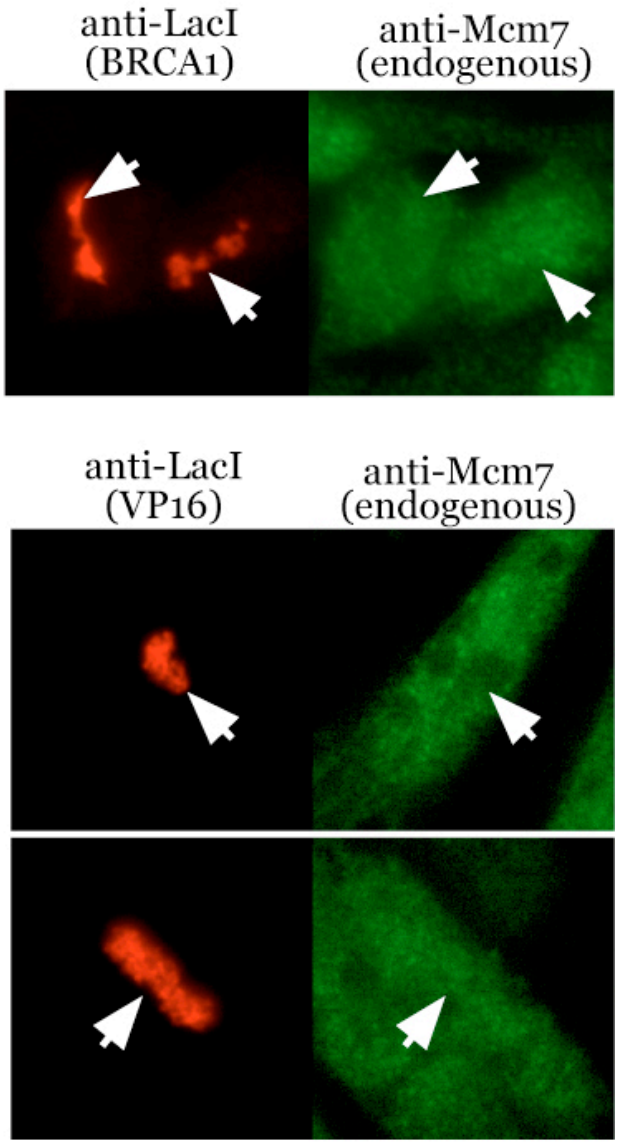


Fig. 30 BRCA1 and VP16 Chromatin Remodeling Does Not Recruit Endogenous MCMs. Open HSRs following LacI-BRCA1 or LacI-VP16 expression were co-stained by IF with antibodies to LacI or Mcm7. Arrows indicate open HSRs and enrichment of endogenous MCMs. A03_1 cells were used and transfections lasted 24 hours.

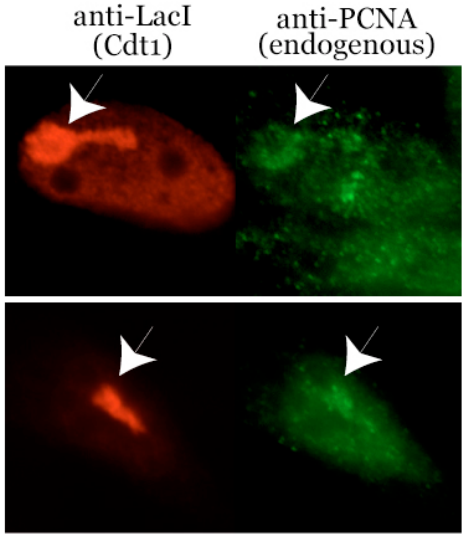


Fig. 31 PCNA can be Recruited to Chromatin Unfolded by Cdt1. Open HSRs following LacI-Cdt1 expression were co-stained by IF with antibodies to LacI and PCNA. Arrows indicate open HSRs and enrichment of endogenous PCNA. A03_1 cells were used and transfections lasted 24 hours.

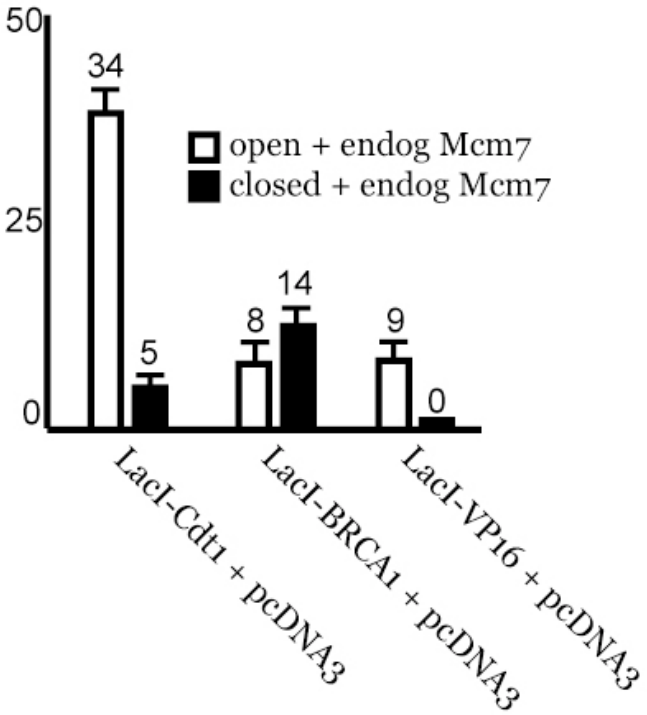


Fig. 32 Quantification of Endogenous Mcm7 Recruitment. This graph depicts the proportion of HSRs that demonstrated colocalization with endogenous Mcm7 as a result of LacI-Cdt1, LacI-BRCA1, or LacI-VP16 targeting.

HBO1 and HDAC11 Regulate Cdt1-Induced Chromatin Unfolding

Given that HBO1 and HDAC11 are known histone/chromatin modifiers (Gao et al. 2002; Doyon et al. 2006), we asked whether these factors could modulate Cdt1-induced chromatin decondensation. We verified that HDAC11 and Cdt1 interact *in vivo* in reciprocal co-IP experiments (Fig. 33, left). Similarly, HBO1 and Cdt1 interact *in vivo* (Fig. 33, right).

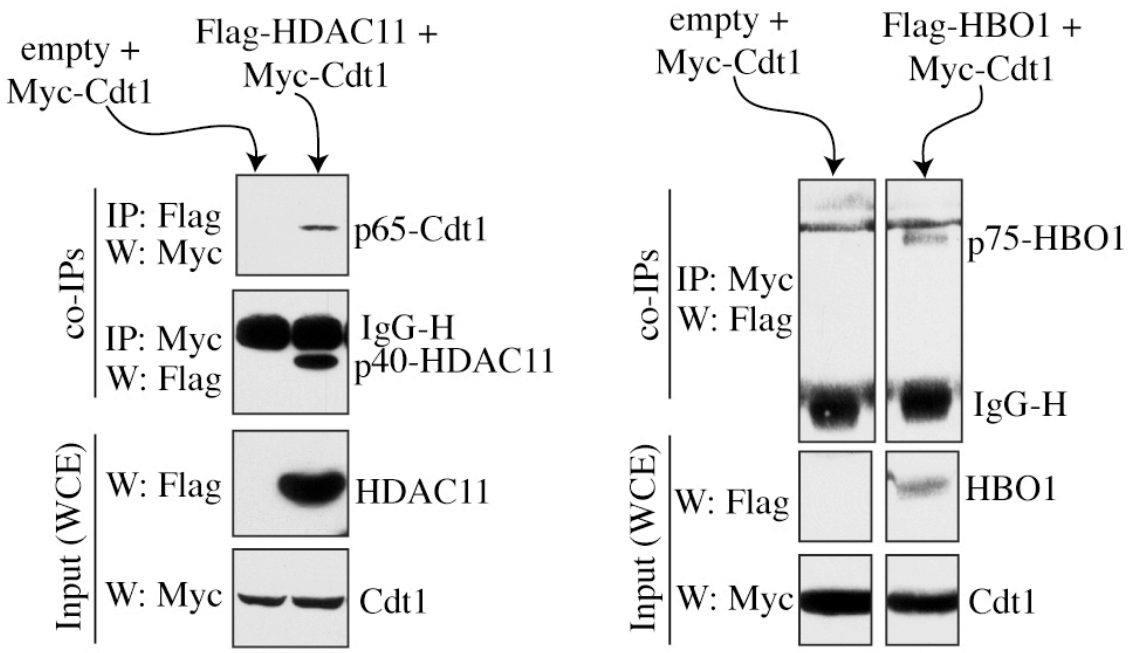


Fig. 33 Cdt1 Interacts with HDAC11 and HBO1 *in vivo*. IP-Western assays were performed using the indicated proteins and anti-tag antibodies. 293T cells were used and transfections lasted 24 hours.

LacI-Cdt1 was co-expressed with HDAC1, HDAC11, HBO1-wt, or HBO1^{G485A} (catalytically-inactive), and the decondensation potential of Cdt1 was determined for each condition. Similar amounts of LacI-Cdt1 expression were achieved, but more LacI-Cdt1 was expressed with HBO1^{G485A} (Fig. 34). Similar expression of HDAC1 and HDAC11 was achieved, while HBO1^{G485A} expressed slightly less well compared to HBO1-wt.

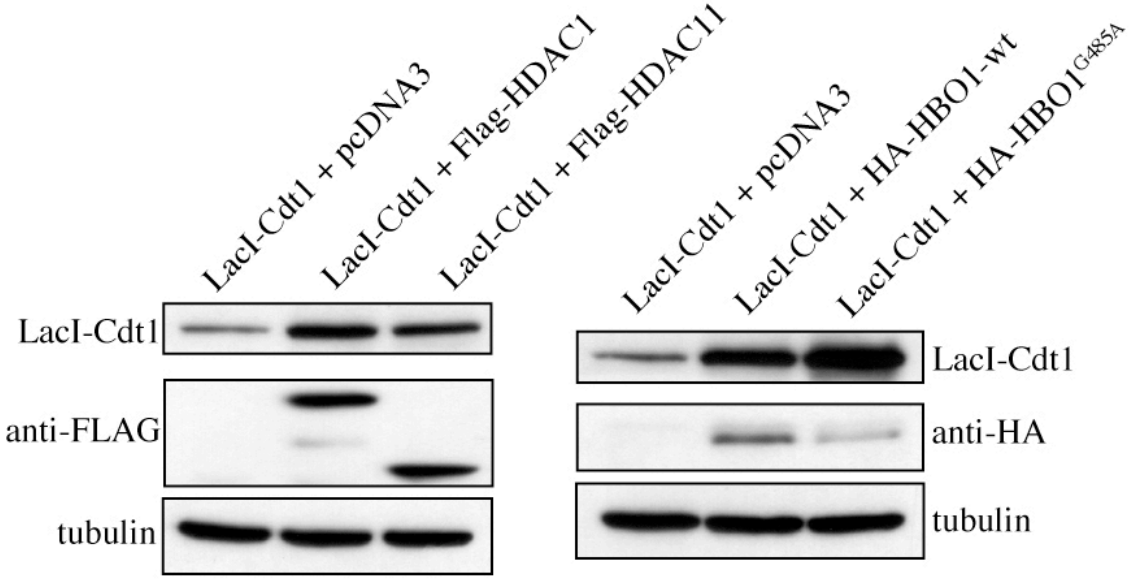


Fig. 34 Expression Levels of LacI-Cdt1 with Flag-HDAC1, Flag-HDAC11, HA-HBO1-wt, and HA-HBO1-Mutant. Immunoblot of indicated proteins showing their relative protein expression for the results in Table 2 and 3.

HDAC1 and HBO1-wt do not alter the ability of Cdt1 to induce chromatin unfolding (Table II). However, HDAC11 dramatically suppresses the ability of Cdt1 to cause decondensation, producing a concomitant increase in closed HSRs. Despite being expressed at lower levels relative to HBO1-wt, and in the presence of higher amounts of LacI-Cdt1, HBO1^{G485A} also suppresses Cdt1-induced decondensation. In comparison, HDAC1, HDAC11, HBO1-wt, and HBO1^{G485A} do not affect VP16-induced decondensation (Table II). We conclude from these results that HBO1 normally performs a positive role specifically in Cdt1-induced chromatin unfolding, while HDAC11 is a strong and specific inhibitor of the decondensation by Cdt1. Furthermore, these results indicate that the effects of HBO1 and HDAC11 on chromatin remodeling by Cdt1 are not due to global cellular changes that affect chromatin remodeling in general.

Table II: Quantification of effects of HBO1, HDAC11, and Set8-HBD on chromatin unfolding

Proteins Expressed	Decondensed (open) chromatin	Condensed (closed) chromatin	Indeterminate
LacI-Cdt1 + pcDNA3	62% (+/-5%)	32% (+/-2%)	6%
LacI-Cdt1 + Flag-HDAC1	58% (+/-3%)	35% (+/-2%)	7%
LacI-Cdt1 + Flag-HDAC11	11% (+/-2%)	69% (+/-3%)	20%
LacI-Cdt1 + Flag-HBO1-wt	65% (+/-3%)	35% (+/-2%)	0%
LacI-Cdt1 + Flag-HBO1^{G485A}	41% (+/-3%)	58% (+/-3%)	1%
LacI-VP16 + pcDNA3	64% (+/-3%)	33% (+/-2%)	3%
LacI-VP16 + Flag-HDAC1	60% (+/-3%)	35% (+/-2%)	5%
LacI-VP16 + Flag-HDAC11	60% (+/-3%)	34% (+/-2%)	6%
LacI-VP16 + Flag-HBO1-wt	62% (+/-3%)	32% (+/-2%)	6%
LacI-VP16 + Flag-HBO1^{G485A}	61% (+/-3%)	29% (+/-2%)	10%
LacI-Cdt1 + Flag-HDAC1	67% (+/-2%)	30% (+/-3%)	3%
LacI-Cdt1 + Flag-Set8-HBD	41% (+/-2%)	51% (+/-2%)	8%
LacI-Cdt1 + GST	62% (+/-2%)	29% (+/-3%)	9%

In each case, at least 150 transfected cells were scored. The results depicted are representative of three experiments performed for each condition, with similar outcomes (used for errors, SD; Indeterminate errors were all less than 2%).

HBO1 and HDAC11 Influence MCM Recruitment to Cdt1-Targeted HSRs

We next asked if HBO1 or HDAC11 influenced the level of MCM recruitment to Cdt1-targeted HSRs. Chromatin remodeling assays were performed as above, but co-stained and quantified for enrichment of endogenous Mcm4 or Mcm7. HDAC1 and HBO1-wt again had no effect on the ability of Cdt1 to cause chromatin decondensation (data not shown), nor did either protein significantly alter the amount of Mcm4 or Mcm7 that was enriched overall (Fig. 35 and Table III). In both cases, MCM enrichment was primarily associated with HSRs that had undergone a decondensation event (Fig. 35). In contrast, HDAC11 and HBO1^{G485A} again inhibited the ability of Cdt1 to cause decondensation (producing closed HSRs; data not shown), and this was associated with a significant reduction in total MCM enrichment (Fig. 35 and Table III). For both HDAC11 and HBO1^{G485A}, any enrichment of MCMs was almost exclusively associated with the small percentage of HSRs that had unfolded under these conditions (data not shown). We conclude from these results that HBO1 is normally required for efficient chromatin unfolding and MCM recruitment by Cdt1, while HDAC11 is a potent and specific inhibitor of the ability of Cdt1 to cause decondensation and MCM recruitment.

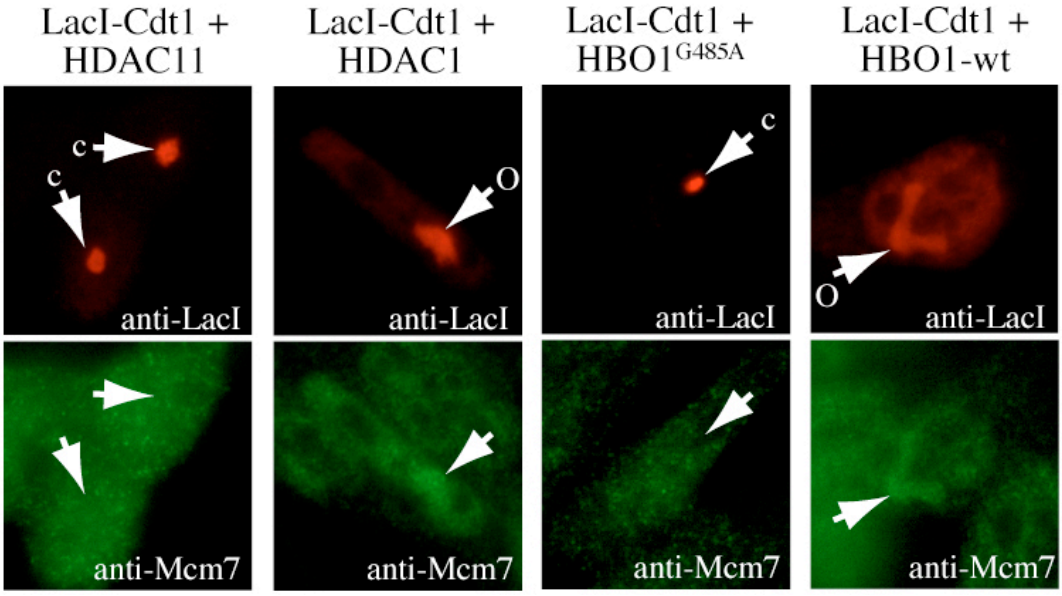


Fig. 35 HDAC11 and HBO1-Mutant Inhibit Cdt1-Induced Chromatin Unfolding and Subsequent Endogenous MCM Recruitment. Examples of co-localizing Mcm7 (or lack thereof) in cells expressing indicated combination of proteins. Samples were processed by IF with indicated antibodies as in Figure 29. Open/decondensed ('O'), closed/condensed ('C') HSRs. Quantitative results are presented in Table 3.

Chromatin Decondensation and MCM Recruitment by Cdt1 Involve Histone H4 Acetylation

Recruitment of MCMs to chromosomal origins depends on HBO1 acetyltransferase activity toward histone H4 (Miotto and Struhl 2010). We reasoned that histone H4 modifications played a role in the Cdt1-induced decondensation and MCM recruitment to the HSRs due to the involvement of HBO1 and HDAC11 in this process.

Although we predicted that H4 acetylation on residues K5, K8, or K12 should be increased at the decondensed HSRs following Cdt1 targeting, we observed no stable association of such modifications with the unfolded HSRs (data not shown). H4 acetylation is known to be a transient event at origins (Miotto and Struhl 2010), which likely explains our inability to observe stable H4 modifications. However, to show that H4 acetylation does play a functional role in the decondensation process, we took advantage of the ability of the Set8 histone methylase H4 binding domain (HBD) to interact with H4 tails and block their acetylation (Yin et al. 2008; Miotto and Struhl 2010).

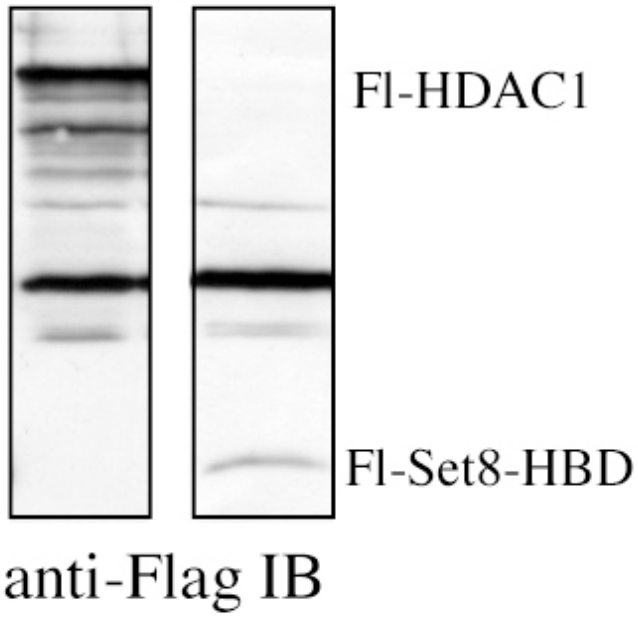


Fig. 36 Expression of Flag-HDAC1 and Flag-Set8-HBD. Anti-Flag immunoblot showing the relative transient expression of Flag-HDAC1 versus Flag-Set8-HBD. Panels are from the same immunoblot/exposure.

Set8-HBD, HDAC1, or GST was co-expressed with LacI-Cdt1, and the ability of Cdt1 to promote chromatin decondensation and MCM recruitment was determined. As described above, HDAC1 does not affect the ability of Cdt1 to unfold chromatin and promote MCM recruitment. However, although Set8-HBD expresses less efficiently than HDAC1 (Fig. 36), co-expression of Set8-HBD significantly reduces the ability of Cdt1 to promote decondensation relative to HDAC1 (Figs. 35&37 and Table II). Co-expression of GST similarly has no effect on Cdt1-induced chromatin unfolding (Fig. 37 and Table II) and MCM recruitment (Fig. 37 and Table III). These results strongly suggest that, at least in a transient manner, acetylation of H4 tails is necessary for Cdt1 to induce chromatin unfolding and stimulate MCM recruitment *in vivo*. These findings are consistent with prior studies showing that HBO1 catalytic activity and the resultant H4 acetylation at origins are required for MCM recruitment by Cdt1 (Miotto and Struhl 2010), but now provide mechanistic evidence that such H4 acetylation promotes chromatin accessibility and fluidity that facilitates the loading of the MCM complex. Intriguingly, the amount of suppression elicited by Set8-HBD is very similar to that caused by HBO1^{G485A} (Table II), as expected if H4 acetylation by HBO1 plays a functional role in Cdt1-induced chromatin unfolding. However, neither Set8-HBD nor HBO1^{G485A} are as potent as HDAC11 at suppressing Cdt1-induced unfolding, suggesting that additional modifications, perhaps on other histone subunits, are likely involved in this process.

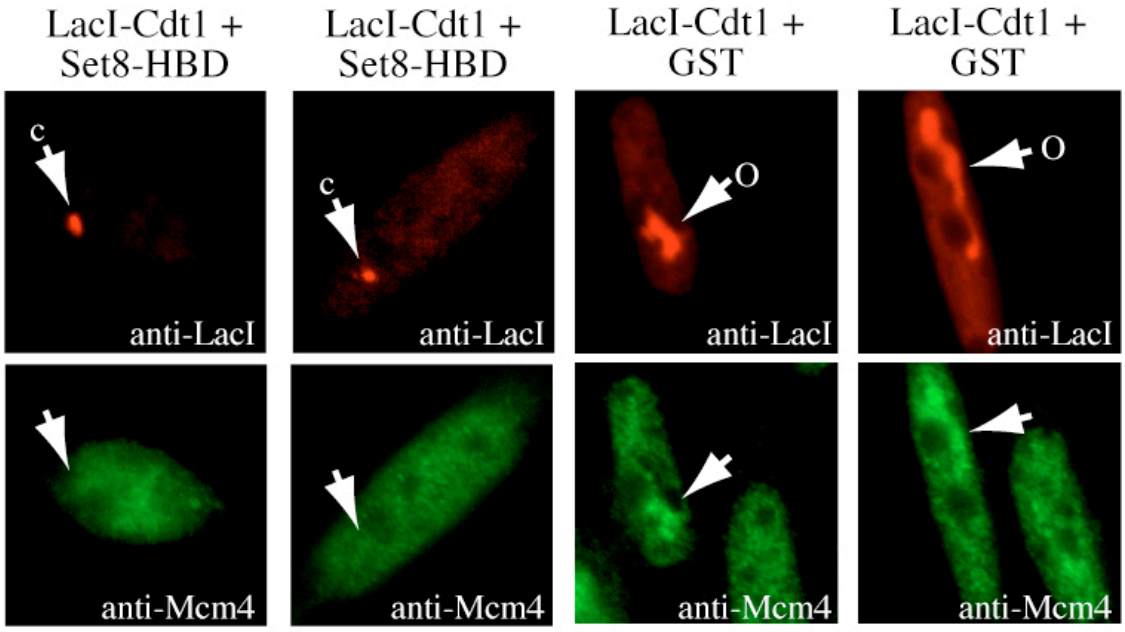


Fig. 37 Blocking Histone H4 Acetylation Prevents Cdt1-Induced Chromatin Decondensation and Endogenous MCM Recruitment. Examples of co-localizing Mcm4 (or lack thereof) in A03_1 cells expressing indicated proteins. Open/decondensed ('O'), closed/condensed ('C') HSRs. Quantitative results are presented in Table 3.

Table III: Quantification of MCM colocalization with HSRs

Proteins Expressed	Percent Overall Change in MCM Enrichment
Cdt1 + pcDNA3	baseline Mcm7 or Mcm4
Cdt1 + HDAC1	+4% Mcm7
Cdt1 + HDAC11	-71% Mcm7
Cdt1 + HBO1-wt	0% Mcm7
Cdt1 + $HBO1^{G485A}$	-42% Mcm7
Cdt1 + HDAC1	-12% Mcm4
Cdt1 + HDAC11	-79% Mcm4
Cdt1 + HBO1-wt	+8% Mcm4
Cdt1 + $HBO1^{G485A}$	-54% Mcm4
Cdt1 + GST	baseline Mcm4
Cdt1 + Set8-HBD	-45% Mcm4

In each case, at least 100 transfected cells were scored.
The results depicted are representative of at least two
experiments performed for each condition, with similar outcome
Errors for changes in MCM enrichment were 1-3% SD for each.

Chromatin at Endogenous Origins of DNA Replication is More Accessible during G1 versus S-phase

Our results suggest that chromatin at origins of DNA replication will be more accessible in G1 when MCMs are loading, due to Cdt1 and HBO1 activities, but less accessible during S-phase, due to HDAC11 recruitment by Cdt1. Intriguingly, at least three reports in the literature have shown this situation to be true at higher eukaryotic origins. The chromatin at the GAS41 origin in chicken cells and the β-globin origin in human cells displays increased DNase I hypersensitivity during G1, but becomes less accessible to nuclease digestion in S-phase (Djeliova et al. 2002; Zimmermann et al. 2007). Similarly, chromatin at the ori-β and ori-γ origins in CHO cells is more accessible and sensitive to micrococcal nuclease in G1 versus S-phase (Pemov et al. 1998).

We determined if the same were true at the MCM4 and Lamin B2 origins in human cells. HaCaT cells were synchronized and released into the cell cycle, and intact chromatin was isolated in late-G1 and S-phase and subjected to controlled DNase I digestion followed by qPCR analysis (Fig. 38). Less accessible chromatin at these origins reduces DNase I digestion, resulting in more substrate available for qPCR. Relative to late-G1, the MCM4 and Lamin B2 origins are both less accessible to DNase I in S-phase as indicated by the increased qPCR substrate availability from these time points. Thus, six higher eukaryotic endogenous replication origins analyzed by different methods (indirect end labeling or qPCR) display increased chromatin accessibility in G1, but less accessibility during S-phase. Our results now provide a molecular explanation for this differential chromatin accessibility at replication origins that involves Cdt1-

modulated control over higher-order chromatin structure via temporal recruitment of HBO1 and HDAC11.

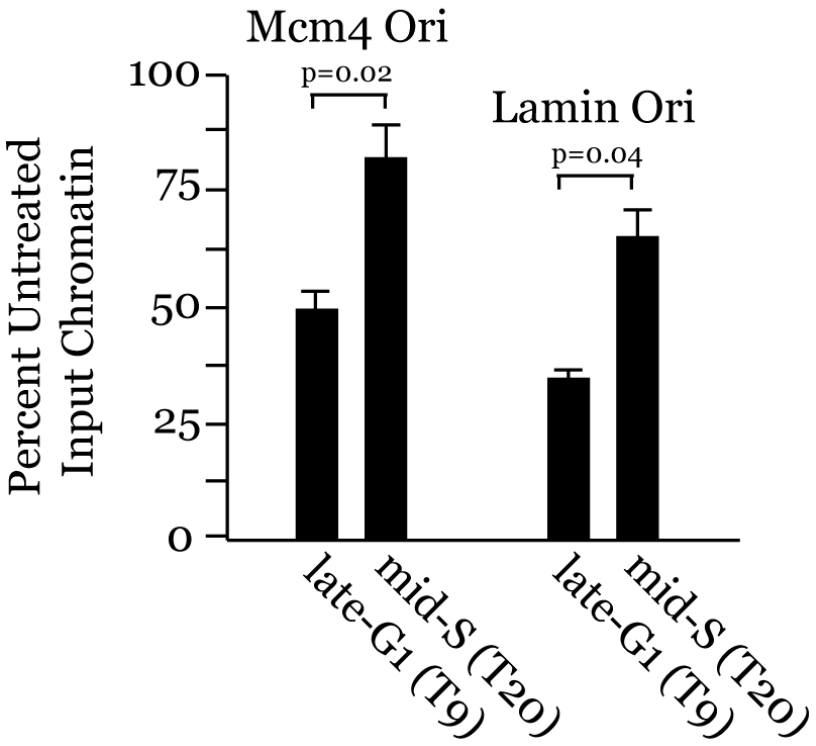


Fig. 38 Chromatin at Endogenous DNA Replication Origins is More Accessible During G1-phase than in S-phase. HaCaT cells were synchronized by serum deprivation and verified by BrdU incorporation as in Figure 8. qPCR was performed on DNase I treated chromatin samples from the indicated time points. Results were normalized against input chromatin from each time point that was not treated with DNase I, to account for increases in DNA levels during S-phase. Assays were performed in triplicate to generate error bars.

CHAPTER 4

DISCUSSION AND FUTURE DIRECTIONS

Cdt1-Induced Chromatin Decondensation Mediates MCM Loading

We present evidence for a novel form of replication licensing control that involves the ability of Cdt1 to modulate chromatin accessibility through the temporal recruitment of HBO1 and HDAC11 (modeled in Fig. 39). In G1, Cdt1 (by ORC interaction) recruits HBO1 to replication origins, resulting in acetylation of H4 within the origin regions (Miotto and Struhl 2008; Miotto and Struhl 2010). We show here that at least one effect of this acetylation is an increase in chromatin accessibility that is required for MCM recruitment. HBO1 catalytic activity is required for MCM loading at origins (Miotto and Struhl 2010), and HBO1 stimulates Cdt1-dependent re-replication (Miotto and Struhl 2008). Upon entering S-phase, *de novo* MCM recruitment is blocked, and we show here that HDAC11 contributes to this process. HDAC11 interacts with Cdt1 and localizes to replication origins specifically in S-phase, and HDAC11 is capable of catalyzing the removal of acetylation from H4 (Gao et al. 2002). Consistent with this, H4 acetylation decreases at replication origins during S-phase (Miotto and Struhl 2010). HDAC11

potently inhibits the ability of Cdt1 to cause chromatin decondensation, suppresses the recruitment of MCMs, and blocks Cdt1-induced re-replication. As such, HDAC11 directly opposes the functions of Cdt1 and HBO1 in promoting replication licensing, thereby producing a 'yin-yang' relationship between HBO1 and HDAC11. The mechanism underlying this relationship derives from the ability of HBO1 to promote chromatin decondensation for MCM loading in G1, followed by the recruitment of HDAC11 in S-phase, which produces chromatin inaccessibility and prevents MCM loading. Such a model is supported by temporal changes in chromatin accessibility at endogenous origins in higher eukaryotic cells shown here and by others (Pemov et al. 1998; Djeliova et al. 2002; Zimmermann et al. 2007), where origins are more accessible in G1 and transition to less accessible chromatin organization in S-phase.

Geminin is a physiologic inhibitor of Cdt1 during S-phase (Wohlschlegel et al. 2000). While the binding of Geminin to Cdt1 reduces the ability of Cdt1 to interact with the MCM complex (Yanagi et al. 2002; Cook et al. 2004), Geminin has been found to also influence the function of HBO1 in association with Cdt1. Geminin does not alter the interaction of HBO1 with Cdt1 (Miotto and Struhl 2008), but instead inhibits the acetyltransferase activity of HBO1 (Miotto and Struhl 2010). We present evidence here that another mechanism whereby Geminin modulates Cdt1 function is through enhanced HDAC11 recruitment to Cdt1 (modeled in Fig. 39). Thus, Geminin indirectly suppresses H4 acetylation at origins by inhibiting HBO1 acetyltransferase activity and by promoting the recruitment of HDAC11. As a result, Geminin produces decreased chromatin accessibility that blocks MCM loading, which is supported by our observation that

Geminin potently and specifically suppresses chromatin decondensation induced by Cdt1.

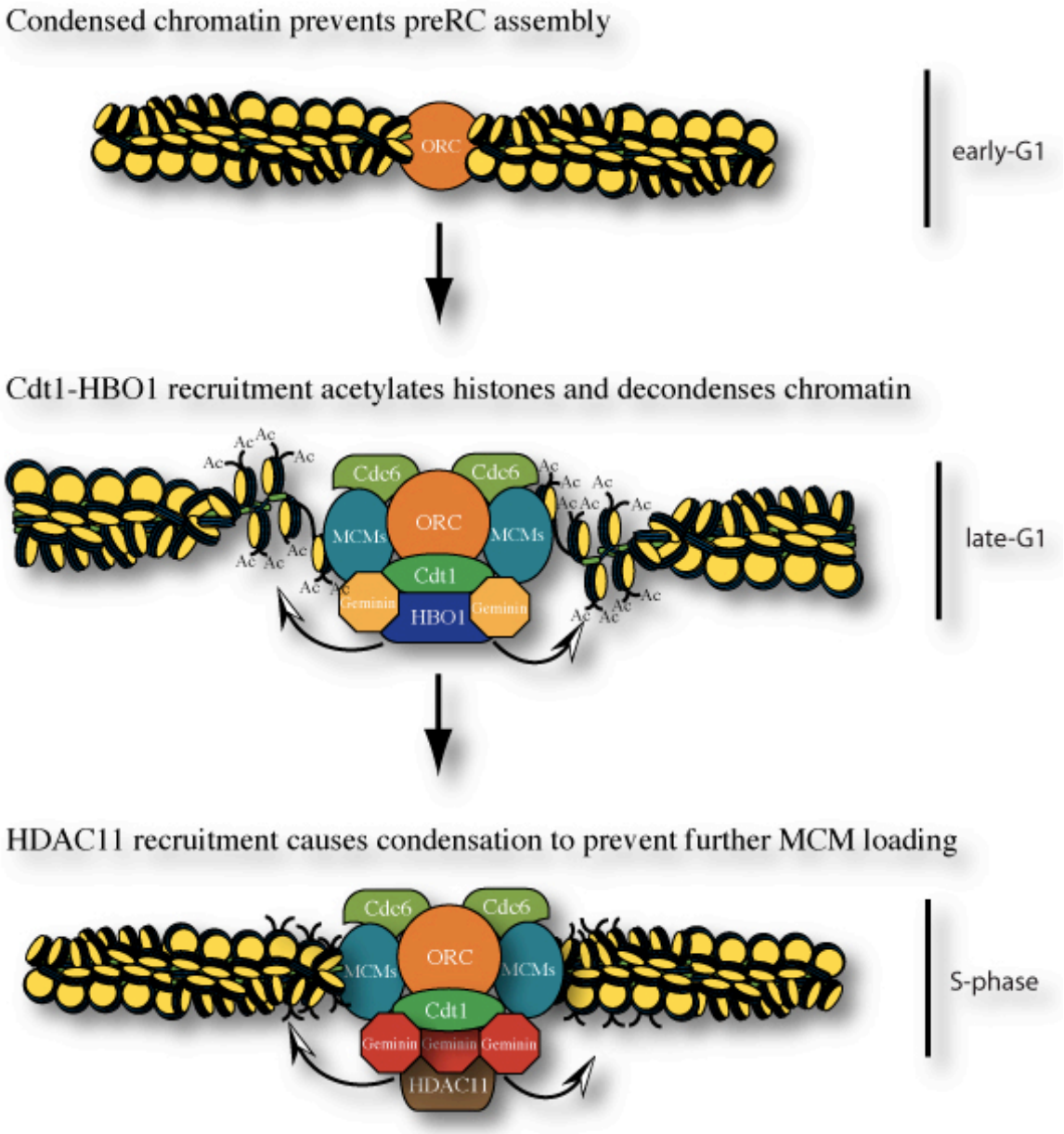


Fig. 39 Working Model.

Currently, we do not know how Geminin modulates HBO1 HAT activity or HDAC11 association with Cdt1. One possibility is that Geminin directly influences HBO1 activity and interactions of HDAC11 with Cdt1, although Geminin does not compete with either protein for Cdt1 binding. However, an alternative explanation may derive from Geminin-regulated recruitment of unknown factors that themselves control these events. Clearly, further investigation is required to answer these questions.

There is currently no technological means to observe large-scale chromatin structural changes at specific single genomic loci in mammalian cells (*i.e.*, origins). Although we have utilized an innovative, but engineered, chromatin remodeling system to address this question, several lines of evidence indicate that the events observed using this system recapitulate those occurring at origins, but at a macroscopic level. Chromatin decondensation induced by Cdt1 occurs during G1 and is sensitive to Geminin in a highly specific manner. Cdt1-induced decondensation involves H4 acetylation during the process of unfolding, is dependent on HBO1 function, and is sensitive to HDAC11 inhibition. In both cases, these enzymes elicit their effects specifically for chromatin decondensation derived from Cdt1. MCM recruitment is clearly observed as a specific result of Cdt1-induced decondensation, and is inhibited by mutant HBO1 and HDAC11 in a specific manner. As described above, Cdt1, HBO1, and H4 acetylation dependency for MCM recruitment is also true at origins specifically during G1. HDAC11 associates with origins during S-phase (and not in G1), when H4 acetylation decreases and origin access would be predicted to be blocked, and HDAC11 reduces Cdt1-induced re-

replication potential and suppresses genomic MCM loading. Most importantly, and highly consistent with our findings, endogenous DNA replication origins display temporal changes in chromatin organization that produce more open and accessible conditions during G1, but transition to a less accessible chromatin state in S-phase (results herein, and references (Pemov et al. 1998; Djeliova et al. 2002; Zimmermann et al. 2007). Collectively, such results provide a strong argument that our observations *in vivo* at this engineered locus represent physiologic events occurring during replication licensing that cannot be seen by any other current experimental approach. Importantly, these results indicate that chromatin accessibility is at least one mechanism whereby Cdt1, HBO1, HDAC11, and Geminin regulate replication origins via H4 acetylation changes.

Chromatin Remodeling in DNA Replication

In yeast and flies, the HBO1 homologs GCN5 and Chameau, respectively, induce acetylation of histones globally and near origins, which promotes origin firing (Vogelauer et al. 2002; Aggarwal and Calvi 2004). In contrast, Rpd3, which is homologous to HDAC11, decreases acetylation and reduces origin activity (Vogelauer et al. 2002; Aggarwal and Calvi 2004). Similarly, the timing of activation of the β-globin origin in mammalian cells is influenced by its acetylation state. Whereas acetylation of the β-globin is associated with earlier firing, targeting HDAC2, which is related to HDAC11, renders the origin late-firing (Goren et al. 2008). Our results provide a mechanistic explanation for these studies of replication origin control by histone acetylation in which

the acetylation influences chromatin accessibility for MCM loading. Although we do not know biochemically how H4 acetylation promotes chromatin unfolding, at least two possibilities are likely. First, histone H4 acetylation has been shown to directly enhance chromatin accessibility via structural changes (Shogren-Knaak et al. 2006), consistent with what we have observed herein. Second, histone acetylation may recruit bromodomain containing proteins that facilitate the chromatin unfolding (Strahl and Allis 2000). Neither of these mechanisms is mutually exclusive with the other and it is possible both may contribute to chromatin structural changes at origins. Finally, it is likely that chromatin modifying enzymes other than HBO1 and HDAC11 are involved in regulating replication origins. For example, SNF2H and WSTF have been shown to co-purify with Cdt1 and differentially bind to chromatin depending on histone tail modifications (Hakimi et al. 2002; Sugimoto et al. 2008).

Cdt1 and Cancer

Cdt1 is oncogenic and overexpressed, sometimes via amplification, in several human cancers, including lung and colon carcinomas, melanomas, and some leukemias and lymphomas (Arentson et al. 2002; Seo et al. 2005; Liontos et al. 2007). Overexpression of Cdt1 alone in NIH3T3 fibroblasts leads to the formation of tumors in Rag2 null mice (Arentson et al. 2002). Furthermore, the T-cell-directed overexpression of Cdt1 in the absence of p53 in transgenic mice leads to the development of lymphoblastic lymphoma, further demonstrating the impact of deregulated Cdt1 in

contributing to tumorigenecity (Seo et al. 2005). These observations indicate that increased Cdt1 expression and deregulation of preRC licensing can lead to tumorigenesis.

A normal cell cycle involves a single round of DNA replication, and the oncogenic nature of Cdt1 derives from its ability to promote MCM loading and re-replication, the result of which is an increase in genomic instability (Vaziri et al. 2003; Liontos et al. 2007). Decreasing levels of Geminin, a biological inhibitor of Cdt1, also results in re-replication, suggesting the functional balance of preRC components is important in preventing over-replication of the genome (Saxena and Dutta 2005). The acute overexpression of Cdt1 and Cdc6 leads to re-replication and DNA damage, most notably double strand breaks, which initially result in senescence and apoptosis (Liontos et al. 2007). However, prolonged growth stimulus by Cdt1 and Cdc6 eventually overcomes these antitumor barriers and the induced re-replication leads to recombination and genomic instability that selects for cell populations with aggressive phenotypes (Liontos et al. 2007). Chromosomal analysis of tumor cells resulting from Cdt1 overexpression in NIH3T3 cells demonstrated a multitude of chromosomal abnormalities including chromosome breaks and gaps, polyploidy, and chromosome end associations (Seo et al. 2005). Furthermore, increased Cdt1 and Cdc6 expression in a subset of mantle cell lymphomas was highly associated with the formation of secondary chromosomal abnormalities compared to MCL populations without an unbalanced increase in DNA replication licensing proteins (Pinyol et al. 2006). These findings demonstrate that Cdt1 deregulation result in re-replication and subsequent loss of genome integrity, which correlate with tumorigenesis.

The genetic instability caused by Cdt1-induced re-replication results in the transformation of a cell by disrupting the normal context of chromosomal regulation of genes responsible for growth control, apoptosis, and metastasis. A diverse array of genetic alterations can potentially lead to changes in expression of such proteins that are involved in oncogenesis. Aneuploidy, which results in large changes in genetic material by gains or losses of entire chromosomes or chromosomal domains, is one such result of genomic instability. The loss of large genomic regions could result in the deletion of tumor suppressor proteins, while chromosomal duplication has the potential to increase gene dosage of oncogenes (Lengauer et al. 1998; van Gent et al. 2001). Re-replication can also lead to the amplification of specific genomic regions, potentially resulting in the onion-skin model of amplification (Bostock 1986). The result of this type of re-replication is increased gene copy number, which would have an impact in the tumorigenic potential of a cell if oncogenes were amplified (Green et al. 2010). Other ramifications of these re-replication events include DNA damage and double strand breakage, which can perpetuate genomic instability (Green and Li 2005). Errors in double stranded break repair, normally performed by non-allelic homologous recombination and non-homologous end joining, can lead to compromised genetic stability culminating in loss or gains of genomic material and potentially chromosomal translocations (Ferguson et al. 2000; Ferguson and Alt 2001; Khanna and Jackson 2001). Translocations represent gross genomic alterations that can potentially give rise to tumorigenesis (Ferguson and Alt 2001). In this process, chromosomes are rearranged, whereby large chromosomal regions are exchanged and fused, which can deregulate

oncogenes or tumor suppressors and cause transformation (Lengauer et al. 1998; van Gent et al. 2001)

Our results suggest that one molecular mechanism mediating Cdt1's ability to promote re-replication is the temporal recruitment of histone modifying enzymes that alter chromatin structure and thereby modulate chromatin accessibility. Excessive levels of Cdt1 will inappropriately cause chromatin decondensation cycles at origins, allowing re-loading of MCMs within one cell cycle. The resultant re-initiation of DNA replication within S-phase produces genomic instability, and as such, provides a novel molecular explanation for how tumorigenesis can occur due to changes in chromatin accessibility at replication origins. This model of deregulated chromatin access is supported by the findings that the expression of the histone acetyltransferase, HBO1, is elevated in several human carcinomas (Iizuka et al. 2009).

Since the overexpression of licensing factors, in particular Cdt1, can lead to oncogenic transformation, an important question then arises with respect to HDAC11 and its potential role as a tumor suppressor. Since we have demonstrated that HDAC11 is recruited to replication origins in S-phase and acts to inhibit Cdt1 function, it stands to reason that disruption of HDAC11 expression or function would lead to Cdt1-induced re-replication and subsequent genomic instability. To test this hypothesis, acute knockdown of HDAC11 protein levels by RNAi (or the utilization of a catalytically-dead HDAC11 mutant) would be useful to assess its ability to regulate the re-replication caused by Cdt1 as determined by FACS or single molecule DNA fiber analysis. Stable knockdown of HDAC11, for example by shRNA or generation of a HDAC11 null mouse, would also

provide great insight into its ability to act as a tumor suppressor. One may expect that such cells lacking HDAC11 could be more prone to tumorigenesis. Long-term suppression of HDAC11 expression could potentially lead to cytogenetically abnormal cells harboring chromosomal aberrations reflective of long-lived genomic instability, which would be identified by FISH analysis. Furthermore, such stable knockdowns could also be analyzed by the single molecule DNA fiber analysis to measure low level re-replication. Since Cdt1 is regulated, both in terms of function and stability, by multiple mechanisms in addition to HDAC11, it is possible that HDAC11 impairment alone would not cause re-replication as the redundancy in Cdt1 regulation would be sufficient in preventing this. To this end, it would be beneficial to analyze HDAC11 knockdowns with respect to their tolerance of Cdt1 overexpression when compared with control, normal HDAC11 cells. For example, HDAC11 null cells may be more susceptible to Cdt1-induced re-replication than normal cells. Moreover, since Cdt1 overexpression in NIH3T3 cells and p53 null transgenic mice lead to tumorigenesis (Arentson et al. 2002; Seo et al. 2005), and we observe that HDAC11 inhibits Cdt1-induced re-replication, co-overexpression of HDAC11 with Cdt1 in the NIH3T3 cells or transgenic mouse model system may be inhibited in tumor formation. It would be of great interest to pursue these investigations to determine if HDAC11 can indeed function as a tumor suppressor.

Two mechanisms of cell cycle arrest previously thought to be independent, were recently shown to be causally related. Replicative stresses and their resultant genomic instability trigger DNA damage responses and cell cycle senescence via ATM and Chk2

(Gorgoulis et al. 2005; Liontos et al. 2007), while oncogene activation leads to oncogene-induced senescence through $p16^{INK4A}$ and ARF (Braig and Schmitt 2006). Instead of being distinct mechanisms, two groups recently found that oncogene-induced senescence utilizes DNA damage repair mechanisms to elicit senescence in addition to $p16^{INK4A}$ and ARF (Bartkova et al. 2006; Di Micco et al. 2006). These reports demonstrate that the expression of activated oncogenes results in senescence that is dependent on both DNA replication and DNA damage repair, and that inhibition of these processes inhibits the observed oncogene-induced senescence. The expression of oncogenic Ras was shown to directly lead to hyper-replication and increased replicon activation (Di Micco et al. 2006) while the expression of other oncogenes, including *mos*, *cdc6*, and *cycline E* led to senescence dependent on functioning DNA damage repair machinery, which is normally activated as a consequence of double strand breaks reflective of re-replication and genomic instability (Bartkova et al. 2006). Together, these findings suggest that oncogene overexpression induces re-replication and genomic instability. Such a result could be due to increased preRC component expression as well as increased licensing of preRCs. Furthermore, several histone modifying enzymes have been shown to be upregulated as a result of oncogene overexpression, which could lead to aberrant chromatin access being granted and subsequent, improper MCM loading and preRC re-licensing as we have shown chromatin fluidity to be a likely regulator of preRC assembly. Activated Ras has been associated with increased Gcn5 and PCAF histone acetyltransferase expression that is associated with the epithelial-mesenchymal transition in carcinoma cells (Pelaez et al. 2010), while increased Her2 oncogene expression in

certain breast cancer cells is associated with an increased copy number of the HBO1 gene (Hu et al. 2009).

If the induction of the DNA damage repair pathway observed with oncogene overexpression is indeed dependent on re-replication resulting from the generation of improper chromatin access and preRC assembly, modulation of chromatin modifying enzymes could result in inhibition or exacerbation of senescence. Reduction of proteins that facilitate the creation of chromatin access, such as HATs, could potentially abrogate the hyper-replication and senescence that accompanies activated Ras expression. For example, acute downregulation of HBO1 by RNAi along with Ras overexpression may diminish the observed hyper-replication by DNA combing and senescence by SA-β-gal staining compared to when control RNAi is used with Ras. Conversely, overexpression of HBO1 with Ras could lead to increased levels of hyper-replication and increased number of senescent cells. Furthermore, overexpression of HDAC11 with activated Ras may serve to prevent or inhibit hyper-replication and subsequent induction of senescence. Undertaking such experimental procedures that serve to correlate chromatin access with oncogene-induced hyper-replication would add further support to the model in which we propose chromatin fluidity as a mechanism of replication licensing.

Future Directions

The findings described here define a novel form of replication licensing involving the generation of chromatin access by Cdt1 to facilitate MCM loading. This control is mediated by two Cdt1-interacting enzymes: HBO1 and HDAC11. The mechanisms of

control elucidated in this research project establish a new paradigm for Cdt1 function, but many questions must still be answered in order to develop a thorough understanding of this chromatin decondensation-based licensing mechanism. For instance, though HBO1 and HDAC11 are integral players in this Cdt1 driven chromatin remodeling, are other histone modifying enzymes or ATP-dependent chromatin remodelers involved? By what mechanisms does Geminin affect the Cdt1-HDAC11 interaction? What domains of Cdt1 are responsible for HDAC11 and HBO1 interaction? What effect does HDAC11 disruption have on replication? Further investigation is required to elucidate a more complete and fluid comprehension of this novel mode of licensing.

Cdt1-Induced Remodeling and Histone Modifications

We have shown that histone H4 acetylation plays a role in Cdt1-mediated chromatin decondensation (Fig. 37). While intriguing, it is unlikely that H4 acetylation is the only modification on which chromatin remodeling at origins and MCM loading is based. Interestingly, we have found that a small proportion of Cdt1 opened HSRs colocalizes with dimethylated histone H3 lysine 9. This finding is important as it suggests other histone modifications and other enzymes are likely involved in the remodeling. Furthermore, the methylation state of histone H3 is known to influence HBO1 HAT complex binding, though not through H3K9 (Saksouk et al. 2009). To further our understanding of the histone modification status of Cdt1-induced remodeling, we propose to utilize a broader panel of antibodies to modified histones to analyze in conjunction with HSR decondensation.

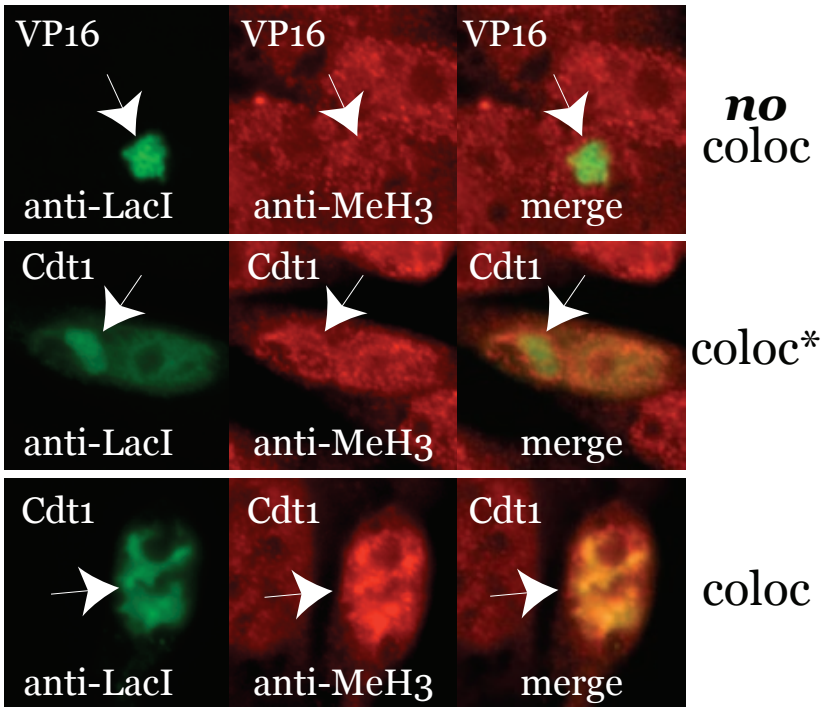


Fig. 40 Cdt1-Induced Remodeling Colocalizes with Methylated Histones. A03_1 cells were transfected with LacI-Cdt1 or LacI-VP16 and co-stained for anti-LacI or endogenous anti-diMethyl histone H3 lysine 9. Arrows indicate location of HSR. *denotes a modified histone ring circumscribing the HSR for this example.

In order to identify novel Cdt1-binding proteins, Sugimoto et al. performed a large-scale Cdt1-complex purification by affinity chromatography and liquid chromatography, followed by tandem mass spectrometry analysis (Sugimoto et al. 2008). To identify other proteins modulating Cdt1-induced chromatin remodeling, it may be prudent to perform such large-scale Cdt1 protein purifications in synchronized cell populations, as other labs have found important Cdt1 interactions to be cell cycle-dependent (Miotto and Struhl 2008; Glozak and Seto 2009). As such, Cdt1 purifications

in asynchronous populations may result in the dilution of important binding partners with more short-lived interactions. Such a pursuit could serve to dissect out separate Cdt1-containing complexes that exist during preRC licensing (i.e. G1) and after MCM loading is actively suppressed (i.e. S-phase). This investigation has the potential to uncover other chromatin modifying enzymes (i.e. histone methyltransferases, other HATs, etc.) or ATP-dependent remodelers as active players in preRC licensing.

Cdt1 and Geminin-HDAC11

We have demonstrated that Cdt1, Geminin, and HDAC11 exist in a single, large complex (Fig. 16) and that Geminin enhances the Cdt1-HDAC11 interaction (Fig. 15). To further investigate the role of each protein in the dynamics of a large, multi-subunit complex *in vivo*, we propose to utilize the Bimolecular Fluorescence Complementation system *(Hu et al. 2002)*, which involves tagging a protein of interest with half of the enhanced yellow fluorescent protein (EYFP) and tagging a potential binding partner with the other half of EYFP. Upon co-transfection, stably interacting proteins will bring the two half tags within close proximity, thereby recapitulating the full length EYFP protein, resulting in yellow fluorescence detectable by microscopy. We have generated functional parental vectors based on this system and have demonstrated effective fluorescence complementation between Cdt1 and Geminin (Fig. 41).

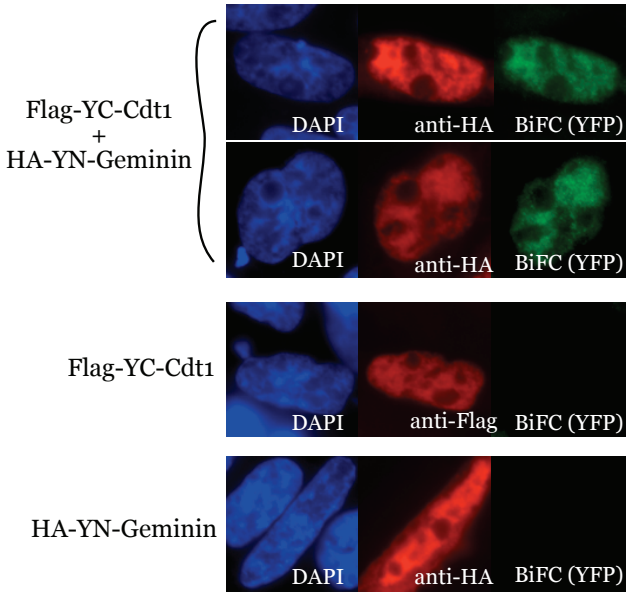
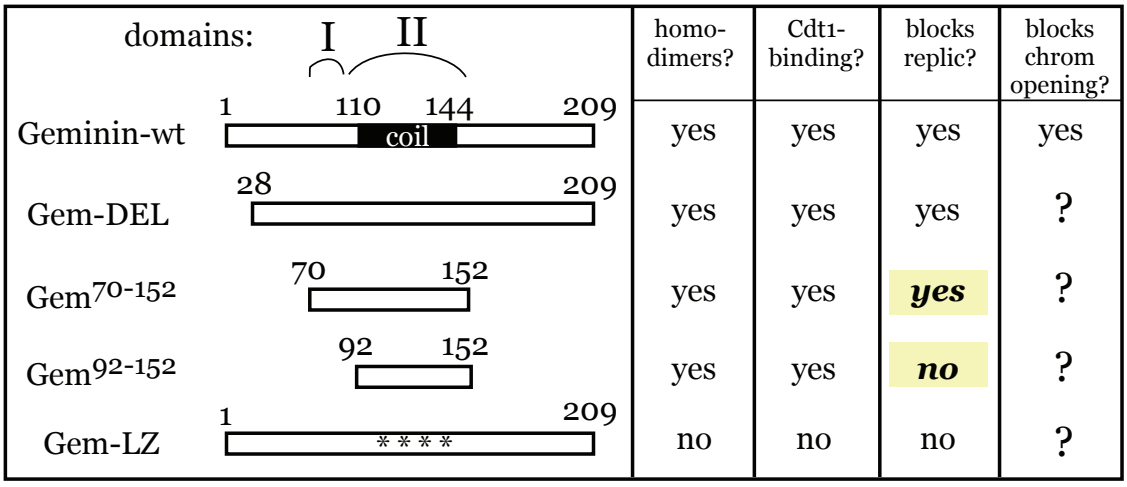


Fig. 41 Bimolecular Fluorescence Complementation between Cdt1 and Geminin. CHO cells transfected with both Cdt1 and Geminin, each fused with a half YFP tag generates fluorescence (top panels). CHO cells transfected with only Cdt1 with its half tag or Geminin with its half tag do not emit light (bottom panels).

We propose to clone HDAC11 into the BiFC vector complementary to Cdt1 to determine if this protein pair can generate successful complementation in this system, and the subcellular localization of this association. BiFC interactions between Cdt1-Geminin and Cdt1-HDAC11 can then be used to determine if titration of overexpressed HDAC11 or overexpressed Geminin, respectively, have any effect in modulating the fluorescent complementations, either in intensity or localization.

To further assess Geminin's role in Cdt1-induced chromatin remodeling, we propose to utilize a series of Geminin mutants (Fig. 42). As previously reported *(Saxena et al. 2004)*, several Geminin mutants have been analyzed in terms of their ability to homodimerize, bind Cdt1, and block replication, however the ability of these mutants to disrupt Cdt1-induced chromatin remodeling has yet to be investigated. We intend to co-

transfect each mutant in a titratable manner to test their ability to affect chromatin unfolding by Cdt1. Furthermore, we propose to determine each mutant's ability to modulate the Cdt1-HDAC11 interaction by performing co-IPs and utilizing the BiFC system as described above. Utilizing the mutants along with the BiFC system will provide a clearer understanding of Geminin's ability to enhance the Cdt1-HDAC11 interaction. Perhaps Geminin serves as a molecular switch at the onset of S-phase when the Geminin:Cdt1 ratio increases *(Lutzmann et al. 2006)*, which could potentially trigger HDAC11 and Cdt1 to bind.



homo-dimers?	Cdt1-binding?	blocks replic?	blocks chrom opening?
yes	yes	yes	yes
yes	yes	yes	?
yes	yes	***yes***	?
yes	yes	***no***	?
no	no	no	?

Fig. 42 Geminin Mutants. Schematic of the Geminin mutants to be analyzed.

Modulation of Cdt1 Function by HBO1 and HDAC11

We have shown that HBO1 and HDAC11 are crucial regulators of Cdt1-dependent chromatin remodeling, and others have demonstrated the importance of HBO1 activity to replication licensing (Iizuka et al. 2006; Miotto and Struhl 2010). HBO1 and HDAC11 both directly interact with Cdt1 (Glozak and Seto 2009; Miotto and Struhl 2010), however the precise domain or region of Cdt1 where these interactions take place have yet to be clearly identified. To this end, we plan to perform co-immunoprecipitation assays using full length HBO1 and HDAC11 with a panel of Cdt1 deletion mutants that we have already generated (Fig. 43). We have analyzed these mutants in terms of their ability to mediate chromatin unfolding (data not shown) and have found Cdt1 Δ201-355 to be dysfunctional in not only chromatin remodeling capability (Fig. 26), but also impaired in its ability to maintain survival in long-term colony growth assays (Fig. 27) and in its ability to stimulate re-replication (Fig. 28). However, identifying specific regions on Cdt1 responsible for HBO1 and/or HDAC11 binding represents an important step in understanding the molecular basis of these interactions and the potential influence other proteins (i.e. Geminin) may have in modulating them.

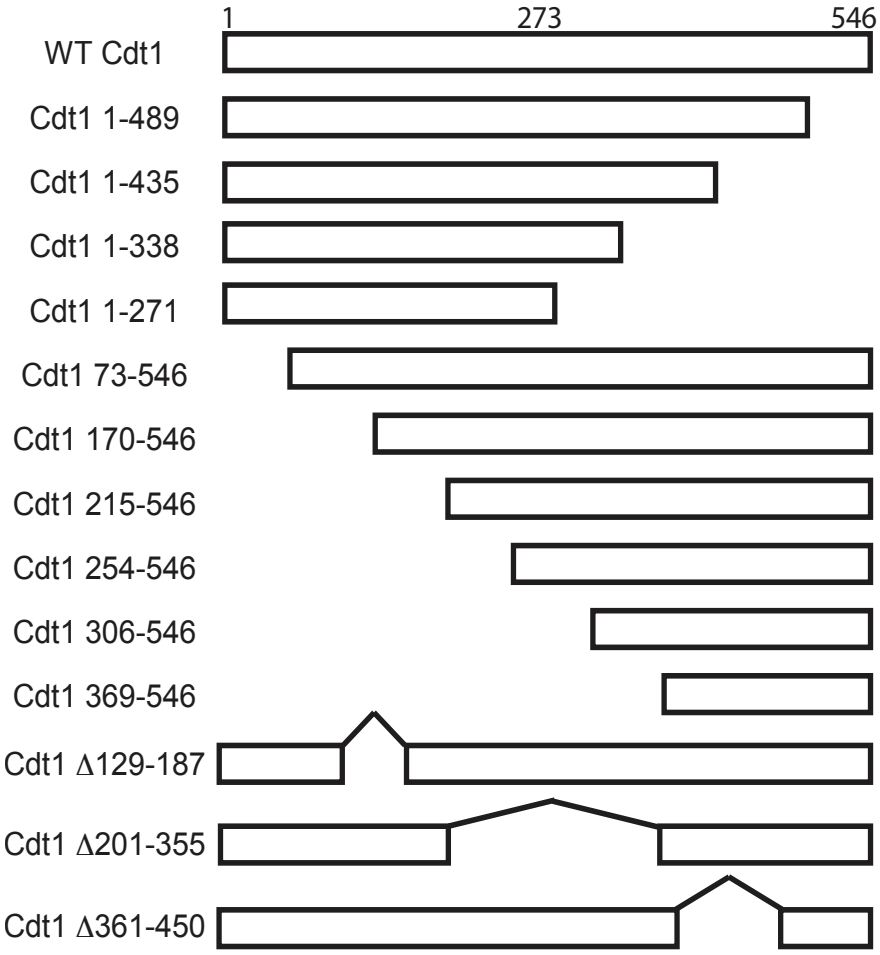


Fig. 43 Cdt1 Deletion Mutants.

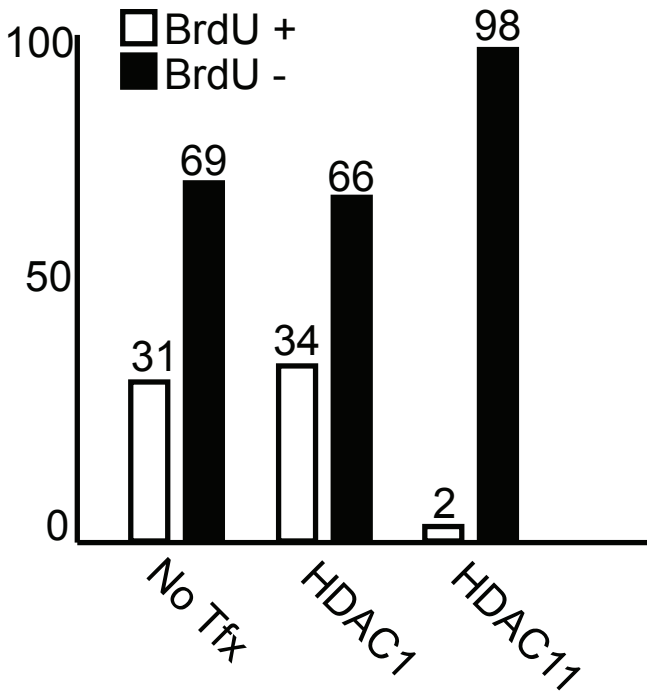


Fig. 44 HDAC11 Specifically Blocks S-phase. HDAC1 or HDAC11 (or none) were expressed for 24 hours in CHO cells. BrdU was pulsed for the last 30 minutes. BrdU labeling indices were determined for each condition.

To further our understanding of HDAC11's role in licensing control, it will be important to examine the effects of HDAC11 enzymatic disruption on HDAC11 inhibition of Cdt1-induced chromatin remodeling, HDAC11 inhibition of Cdt1-induced re-replication (Fig. 10), and HDAC11 inhibition of replication (Figs. 11&44). RNAi knockdown of HDAC11, drug-mediated enzymatic inhibition (eg TSA), or generation of

a catalytically dead HDAC11 mutant all represent viable methods by which to induce HDAC11 dysfunction. If HDAC11 is an essential regulator of Cdt1 function, disrupting this negative control could potentially result in a similar manner to overexpressing Cdt1 alone in each assay.

Overall, the field of replication licensing and G1-S control represent an important avenue of basic cancer research, both in terms of understanding fundamental disease biology and in the identification of potential drug targets. Precise coordination of the events leading up to DNA replication initiation is absolutely crucial to maintaining genomic stability. As such, multiple levels of control exist to regulate the cell cycle machinery entrusted with copying the genetic information of a cell in its entirety, without error. The complexity and the elegance of such a vital network of molecular signals is awe-inspiring. As research in this field progresses, the details of this intricate regulatory mechanism will slowly be revealed and I can only hope that the full scope of chromatin fluidity-based regulation of replication licensing will be unearthed.

CHAPTER 5

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