# EPIGENETIC FACTORS ARE DYNAMICALLY REGULATED THROUGHOUT THE CELL CYCLE AND ARE REQUIRED FOR EFFICIENT DNA REPLICATION

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ABSTRACT

LINDSAY FAIRCLOTH RIZZARDI: Epigenetic Factors Are Dynamically Regulated Throughout The Cell Cycle And Are Required For Efficient DNA Replication
(Under the direction of Dr. Jeanette Gowen Cook)

Chromatin consists of DNA wrapped around a core of histone proteins that can be modified to elicit distinct cellular responses. All DNA-templated processes including DNA replication must function within the context of chromatin. The focus of this study is the interaction between the replication machinery and the chromatin structure it must traverse to faithfully replicate the genome. Individual histone modifications can promote recruitment of replication proteins directly or recruit chromatin remodelers that increase the accessibility of the underlying DNA. Our studies in budding yeast, *Saccharomyces cerevisiae,* have identified a specific histone modification, H3K4me2, that promotes efficient DNA replication; although, the mechanism of this regulation remains unclear. Additionally, we found that the enzyme responsible for depositing H3K4me2 is cell cycle regulated, being most abundant during G1 when cells prepare the genome for DNA replication.

Another histone modification required during G1 for proper DNA replication is H4K20me1. As this modification is not found in budding yeast, our studies were undertaken in a mammalian cell culture system. The enzyme responsible for this modification had previously been identified as Set8 (a.k.a. PR-Set7) and we have begun investigating the regulation of this enzyme

throughout the cell cycle and during a cellular stress response. This enzyme is degraded both during S phase and after UV-induced DNA damage by the $CRL4^{Cdt2}$ E3 ubiquitin ligase complex. We discovered that this degradation after DNA damage is abrogated during mitosis and after osmotic stress. This mechanism is conserved for at least one other $CRL4^{Cdt2}$ substrate, Cdt1, that is an essential replication factor. Protection from UV-induced degradation requires active Cdk1 during mitosis as inhibition of this kinase leads to degradation of both Set8 and Cdt1. We are currently investigating whether this is a direct or indirect effect. During osmotic stress in asynchronous cells, the stress MAPKs p38 and JNK may play a more important role than Cdk1. It is clear that the histone modifications and the enzymes responsible for their deposition are critical components ensuring proper DNA replication and as such are tightly regulated. Future work will elucidate the exact mechanisms by which these chromatin factors influence DNA replication.

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ACS – ARS consensus sequence

APC – Anaphase promoting complex

ARS – Autonomously replicating sequence

ATP – Adenosine triphosphate

BAH – Bromo-adjacent homology

Cdc6 – Cell division cycle 6

CDK – Cyclin-dependent kinase

Cdt1 – Cdc10 dependent transcript 1

Cdt2 – Cdc10 dependent transcript 2

ChIP – Chromatin immunoprecipitation

COMPASS – Complex associated with Set1

CPT – Camptothecin

CRL4 – Cullin 4A-RING E3 ubiquitin ligase

CTD – C-terminal domain (of RNA polymerase II)

DDK – Dbf4-dependent kinase

DMT – Demethyltransferase

DNA – Deoxyribonucleic acid

DUE – DNA unwinding element

GINS – Go-Ichi-Ni-San complex

HAT – Histone acetyltransferase

HBO1 – Histone acetyltransferase binding to ORC

HDAC – Histone deacetylase

HECT – Homologous to the E6-AP carboxyl terminus

HMT – Histone methyltransferase

HU – Hydroxyurea

HUWE-1 – HECT, UBA, and WWE domain containing 1

JNK – Jun N-terminal kinase

LPS – Lipopolysaccharide

MAPK – Mitogen-activated protein kinase

MCM – Minichromosome maintenance complex

NHF – Normal human fibroblast

NFR – Nucleosome-free region

NLS – Nuclear localization sequence

ORC – Origin recognition complex

ORCA – ORC associated

PCNA – Proliferating cell nuclear antigen

PHD – Plant homeo domain

PIC – Pre-initiation complex

PIP – PCNA interacting protein

PKA – Protein kinase A

Plk1 – Polo-like kinase 1

Pol II – RNA polymerase II

preRC – Pre-replication complex

PTM – Posttranslational modification

RNA – Ribonucleic acid

SAGA – Spt-Ada-Gcn5 acetyltransferase

SCF – Skp, Cullin, F-box containing complex

siRNA – Small interfering RNA

TNFα – Tumor necrosis factor alpha

TSS – Transcription start site

UTR – Untranslated region

UV – Ultraviolet radiation

# CHAPTER 1

## Introduction

The cell cycle is a tightly regulated process that directs cell growth, ensures accurate duplication of the genome, and produces two identical daughter cells. Maintaining genome integrity is a top priority throughout this process. To accomplish this feat, every facet of DNA replication is under multiple layers of regulatory surveillance ensuring that the genome is prepared for replication in G1, that replication is completed error-free during S phase, and that the chromosomes segregate properly during mitosis. When any aspect of this process goes awry, DNA can become damaged resulting in gene mutation, chromosome rearrangements, and aneuploidy, all of which are hallmarks of cancer (1). Replicating the genome, like all DNA-templated processes, involves navigating the three-dimensional nuclear space to gain access to the DNA; this access is often restricted by the compaction of DNA into chromatin.

Chromatin consists of 147 bp of DNA wrapped around an octamer of histone proteins (2 each of histones H2A, H2B, H3, and H4) that form nucleosomes. Histone proteins possess unstructured N-terminal tail regions that

can be post-translationally modified by phosphorylation, acetylation, methylation, ubiquitination, etc. (2). In the case of methylation, up to three methyl groups can be added to a single lysine residue, and there is evidence that these methylation states are functionally distinct (3). These post-translational modifications (PTMs) can alter DNA accessibility, serve as recognition sites for other proteins, and stabilize protein complexes (reviewed in reference 4). High levels of histone acetylation exist in easily accessible, highly transcribed euchromatin, while the much more compact heterochromatin tends to be hypoacetylated. It has been proposed that various combinations of histone modifications act as a "histone code" that provides epigenetic information to regulate DNA processes (5). It is likely that some aspect of this histone code is involved in the regulation of DNA replication.

## Origins of replication

### *Sequence determinants*

DNA replication is initiated at sites in the genome known as origins of replication. These sites were first identified in budding yeast, *Saccharomyces cerevisiae,* by isolating DNA sequences that were capable of directing plasmid replication. This work led to the identification of a conserved sequence element termed the autonomously replicating sequence (ARS). The ARS contains a highly conserved but degenerate 11-bp ARS consensus sequence [ACS; (T/A)TTTAT(A/G)TTT(T/A)] along with three poorly defined B elements (B1, B2, and B3) that are nonetheless important for origin function (reviewed in reference

6). These B elements, while not conserved, can serve as binding sites for transcription factors or DNA unwinding elements (DUE) (7).

Identifying replication origins in eukaryotic systems other than yeast has been much more difficult for two main reasons. The first is that there is no conserved sequence element common to replication origins in other eukaryotes. While these regions tend to be AT-rich there is no defined sequence that confers origin activity (8). The second is the lack of sensitive and stringent methods for isolating mammalian origins on a genome-wide scale. Origin identification can become complicated by the fact that origins are not uniformly active or efficient making it difficult to confidently confirm any but the most robust origins. Until recently, *S. cerevisiae* was the only organism in which all of the origins had been functionally identified. There have since been many technological advances that have successfully identified origins in more complex eukaryotes; however, these data sets often show very little overlap (<40%) (reviewed in reference 9). The techniques utilized include short nascent strand sequencing, ChIP-seq, Repli-Seq, or combinations of these, and have allowed identification of origins even in human cells (10-12). Massive deep sequencing of short nascent strands from four different human cells lines has recently yielded what is now regarded as a complete map of replication origins in human cells. Approximately 250,000 origins of replication were mapped nearly ten times the number of origins that had previously been predicted to exist (13). Despite this new information, the underlying factors regulating origin position in the genome are still unclear. Even in *S. cerevisiae* the ACS element is not the sole determinant of origin activity

because this sequence occurs in excess of 12,000 times throughout the genome and only ~ 400 functional origins exist (14). In addition, the aptly named origin recognition complex (ORC) binds specifically to the ACS, but is still unable to define active origins as it binds to more than 400 loci (15). In metazoans where no sequence element has been identified, ORC prefers to bind AT-rich sequences and metazoan origins tend to be 70-80% AT-rich (8). It is clear that neither DNA sequence nor ORC binding are sufficient to define origins of replication. This fact has lead researchers to investigate epigenetic determinants of origin identity, specifically the role of chromatin in origin determination. Recent studies have shown the importance of the chromatin environment in regulating many cellular processes including DNA replication (16-18).

*Chromatin structure*

Genome-wide analysis of nucleosome positioning in yeast and mammalian cells have shown that early replicating origins exist in nucleosome-free regions (NFRs) that are easily accessed by the replication machinery (19-21). Origins in all organisms tend to be AT-rich and these poly-A stretches resist bending, thus excluding nucleosomes (8,22). Interestingly, ORC helps to maintain NFRs and promotes the proper phasing of nearby nucleosomes that is needed for efficient DNA replication (23). Proper nucleosome phasing and nucleosome position relative to the origin of replication is important for optimal origin function. Shifting nucleosomes either towards or away from the origin NFR negatively impacts origin activity. Origins are frequently found near transcription start sites (TSS) that are also nucleosome free and are flanked by nucleosomes

rich in “activating” histone modifications (such as H3K4me2, 3 and H3 and H4 acetylation). Additionally, origin firing time is correlated with transcription (24-26). In transcriptionally inactive regions, histone-modifying enzymes are recruited to origins to facilitate binding of the replication machinery. In *Drosophila* S2 cells for example, the histone acetyltransferase complex SAGA (Spt-Ada-Gcn5 acetyltranferase) and the chromatin remodeler Brahma were found to promote ORC recruitment to these heterochromatic regions (27).

When our work began, little was known about how the chromatin environment influenced origin activity or position in the genome. However, it was demonstrated by many groups (predominately in yeast and *Drosophila melanogaster*) that manipulating histone acetylation could alter the timing of replication initiation from origins, or origin “firing”, in S phase. Origins located in compact, hypoacetylated heterochromatin tended to fire late in S phase, but moving these origins to more “open”, euchromatic regions allowed them to fire early in S phase (28). Similarly, increasing global histone acetylation by deleting a histone deacetylase (Rpd3) resulted in a shorter S phase due to earlier origin firing genome-wide (29). Conversely, tethering a histone acetyltransferase (Gcn5) to a normally late-firing origin caused it to fire much earlier in S phase. In budding yeast, examination of the dynamics of histone acetylation at an individual origin of replication (ARS1) revealed that H3 and H4 tail acetylation was lowest during G1 and increased dramatically during S phase, consistent with a role for histone acetylation in origin firing (30). However, this could be a consequence of histone turnover as newly synthesized histones in all eukaryotic

cells are acetylated at H4K5 and K12; although, why these acetyl marks are important for new histone incorporation is unclear since unmodified H3-H4 dimers are readily incorporated into new nucleosomes *in vitro* (31-33). Work from many groups has extended these findings and revealed that high levels of histone acetylation are correlated with origin activity even in human cells (25,30,34-36).

*Histone PTMs*

Researchers have struggled to identify a distinct set of histone modifications (or a chromatin signature) that determines the location of replication origins in the genome. While this identification has proven to be quite difficult, histone modifications that are enriched at origins have been identified, and functional studies have shown that individual histone modifications can regulate origin activity (Table 1.1). Through these efforts, a picture is emerging of the chromatin structure surrounding origins. Mass spectrometry analysis of an isolated yeast origin identified several previously unknown histone modifications that were specific to origins: H4K79ac, H3K23ac, H3K37me1, and H2AS15 phosphorylation (30). This study also identified a novel histone modification that was specifically absent from origins, H2BK111me. Interestingly, H3K37me1 was entirely absent during S phase and was always found in conjunction with H3K36me1 on the same histone tail. However, another modification enriched at origins, H3K27ac, was never found on the same tail as H3K36me1, or me2. H3K36me1 was previously shown to promote association of the replication

initiation factor Cdc45 with origins (37). Work to define the role of H3K37me1 in replication is currently underway in the Kouzarides lab.

Many other histone modifications are also enriched at origins and influence DNA replication. In *S.cerevisiae*, H3T45 is phosphorylated by the Cdc7-Dbf4-dependent kinase (DDK) during S phase, and mutation of T45 to alanine results in reduced H3K36me3 levels and sensitivity to replication stress caused by hydroxyurea (HU) and camptothecin (CPT) (38,39). In human cells proliferating cell nuclear antigen (PCNA) specifically recognizes and binds to monomethylated H3K56 (methylated by G9a) during G1 (40). In Tetrahymena, H3K27me1, but not di- or tri-methylated H3K27, was found to play an important role in replication fork progression, and the methyltransferase responsible, TRX1, was also shown to interact with PCNA (41). Another histone modification, H3K79me2 is highly enriched at origins in human cells and spreads during S phase, possibly acting as a marker of replicated regions (42-45). Interestingly, depletion of the H3K79 methyltransferase DOT1L leads to low levels of rereplication suggesting that H3K79me2 could play a role in preventing aberrant replication. A new computational analysis of histone modifications in *S. cerevisiae* has synthesized an “alphabet” of nucleosome characteristics associated with different functional elements (46). Origins of replication were characterized by low nucleosome occupancy with minimal histone modifications (low levels of asymmetric H3R2me2), near non-expressed or lowly expressed genes, as had been noted by previous researchers. It is important to note that

**Table 1.1 Origin-associated histone modifications involved in DNA replication**

| PTM | Writer | Relevant Reader | Proposed Function in Replication | Reference |
|---|---|---|---|---|
| *S. cerevisiae* | | | | |
| H3K4me2,3 | Set1 | ? | promotes origin firing | This study |
| H2BK123ub | Bre1/Rad6 | ? | promotes fork progression | (233) |
| H3K36me1 | Set2 | Cdc45 | promotes formation of PIC | (37) |
| H3T45phos. | DDK | ? | promotes replication fidelity | (38) |
| H3K37me1 | ? | ? | ? | (30) |
| *H. sapiens* | | | | |
| H3K56me1 | G9a | PCNA | promotes PCNA association at origin | (40) |
| H3K79me2 | DOT1L | ? | potentially inhibits ORC association | (43) |
| H4K20me1 | Set8 | L3MBTL1 | potentially recruits initiation factors | (115) |
| H4K20me2 | Suv4-20h1/2 | Orc1 | promotes preRC formation | (76) |
| H4K20me3 | Suv4-20h1/2 | ORCA | promotes preRC formation | (77) |

this work was done on data collected from asynchronous cell populations and that many histone modifications at origins are dynamic and cell cycle regulated.

Many replication proteins influence, and are influenced by, chromatin modifying enzymes that are active at origins of replication. These interactions can serve to regulate origin activity during all stages of the cell cycle. As described in detail below, origins become licensed in G1 phase, fire in S phase while origin licensing is inhibited, and are “reset” as cells exit mitosis into the subsequent G1 phase. The chromatin structure influences each aspect of this process and will be discussed further in the following sections.

**Origin licensing and firing**

For DNA replication to successfully duplicate the entire genome, a sufficient number of replication origins must initiate replication in S phase. If too few origins fire, individual replication forks must travel longer distances making them susceptible to fork collapse and subsequent DNA damage. Conversely, if origins fire more than once during the cell cycle (a process termed rereplication) then replication forks can collide leading to DNA damage (Figure 1.1A). To prevent these destructive events, origins of replication are tightly regulated. During G1, origins are prepared, or “licensed”, for DNA replication by the assembly of pre-replication complexes (preRCs). Origin licensing is complete once the replicative helicase of mini-chromosome maintenance (MCM) complexes has been loaded onto chromatin at origins through the activity of the canonical preRC components (Figure 1.1B). Replication origins are recognized and bound by ORC (the first preRC component recruited) that remains

associated with origins throughout the cell cycle. The next component, cell division cycle 6 (Cdc6), is recruited to origins through direct interaction with ORC. In the cytoplasm, Cdc10 protein-dependent transcript 1 (Cdt1) interacts with MCM complexes by binding to the Mcm2 and Mcm6 subunits to facilitate their import into the nucleus (47-50) At least two Cdt1-MCM complexes are then recruited to origins in a step-wise manner through Cdt1 interaction with both Cdc6 (49) and Orc6 (51-53). In contrast to ORC and Cdc6, Cdt1 lacks any enzymatic activity but is absolutely required for MCM recruitment to origins. It is through the ATPase activity of ORC and Cdc6 that the MCM helicase ring is opened and loaded onto chromatin while also promoting release of Cdt1 (54). While ATP hydrolysis occurs rapidly upon association of the Cdt1-MCM complex, actual loading of the MCM double-hexamer onto the DNA occurs some time later (55). In human cells, an origin licensing checkpoint exists so that cells will not enter S phase or initiate replication unless a sufficient number of origins have been licensed in G1 (56,57). While this checkpoint relies on p53 activity and hyperphosphorylation of Rb, the exact mechanism by which cells sense how many origins have been licensed is still undefined.

Once MCM complexes have been loaded, the origins are licensed for replication and ORC, Cdc6, and Cdt1 are no longer required in order for replication to commence in S phase (58,59) However, the loaded MCM complexes are not yet active as DNA helicases. As cells reach the G1/S transition, a host of replication initiation factors are recruited and form the pre-initiation complex (PIC) (Figure 1.1C) (60,61). Prior to S-phase cyclin-dependent

**Figure 1.1**

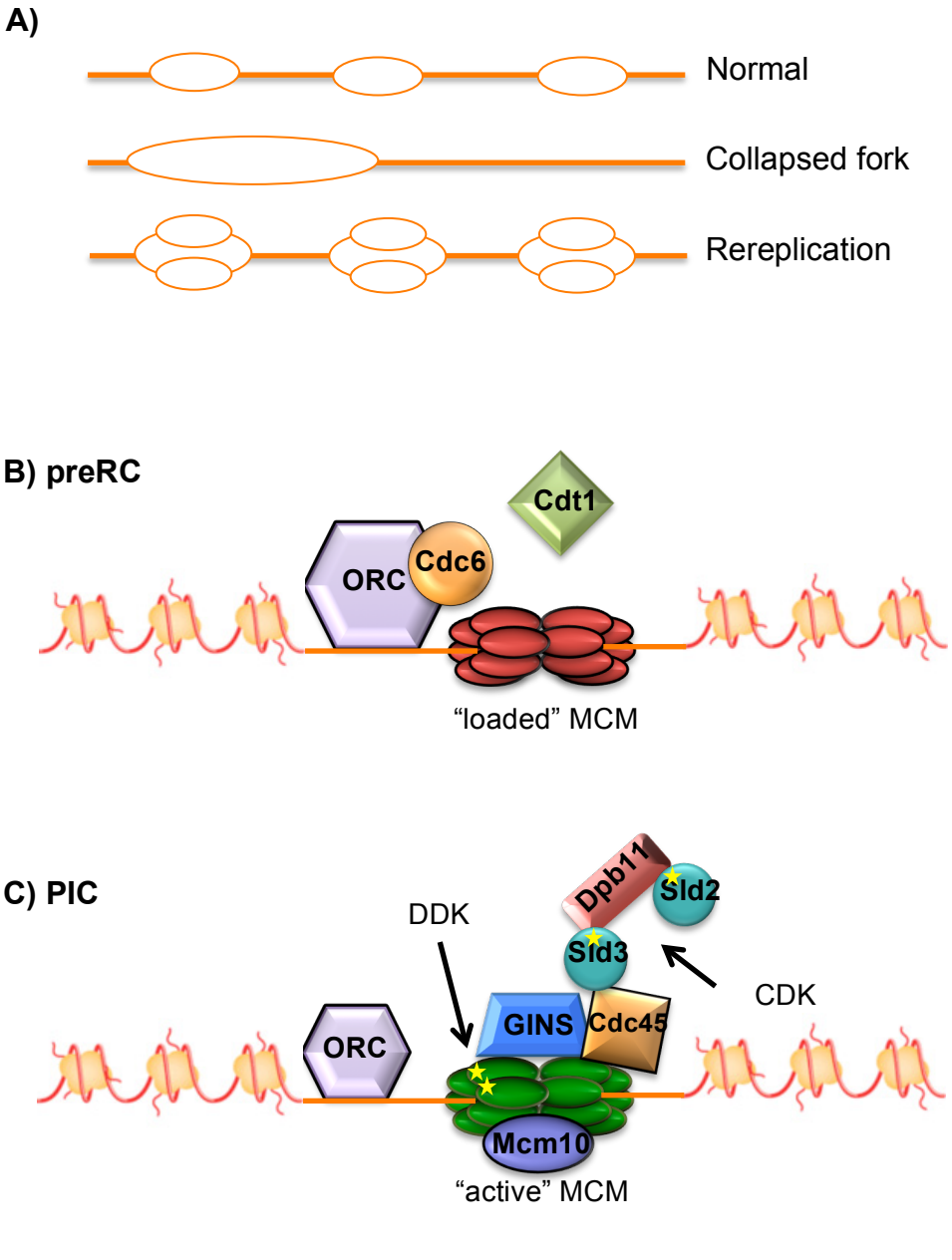
A)
Normal
Collapsed fork
Rereplication
B) preRC
Cdt1
ORC
Cdc6
"loaded" MCM
C) PIC
DDK
Dpb11
Sld2
Sld3
CDK
GINS
Cdc45
ORC
Mcm10
"active" MCM

**Figure 1.1 DNA replication is initiated at origins of replication and is tightly regulated to prevent spurious replication initiation.** A) Normal replication requires a sufficient number of origins to fire in S phase. If too few origins fire, the DNA may not be fully replicated leading to increased DNA damage and genome instability. DNA damage can also occur if replication origins fire multiple times during a single cell cycle (rereplication). B) PreRC assembly is restricted to the G1 phase of the cell cycle. ORC is the first complex member recruited to origins and directly interacts with Cdc6 and Cdt1. Cdt1 recruits the MCM DNA helicases and through the ATPase activity of ORC and Cdc6 the MCM complexes are loaded onto the DNA. After MCM loading origins are considered to be licensed for replication and the other preRC components are not required for subsequent DNA replication in S phase. C) As cells transition from G1 to S phase, replication initiation factors are recruited and form the PIC. This complex functions to activate the MCM DNA helicase and commence replication. During late G1, the Dbf4-dependent kinase (DDK) phosphorylates Mcm4 and Mcm6 to promote recruitment of Cdc45 and Sld3. As cells enter S phase, CDK activity increases and promotes the recruitment of GINS that stabilizes the Cdc45-MCM complex (often referred to as the CMG complex). This complex in addition to CDK phosphorylation of Sld2 and Sld3 helps to stably recruit Mcm10, Sld2, and Dpb11 to form the complete PIC.

kinase (CDK) activation, DDK phosphorylates Mcm4 and Mcm6 to promote the recruitment of Cdc45 and Sld3 during G1 (62). As CDK activity increases at the G1/S transition, the Go-Ichi-Ni-San (GINS) complex is recruited and stabilizes the Cdc45-MCM interaction. In *S. cerevisiae* this complex forms the basis for recruiting Sld2, Dpb11, and Mcm10 that are then able to activate the MCM helicase. In metazoans, TopBP1 and RecQ4 are the homologs of Dpb11 and Sld2, respectively. Once the MCM helicase becomes active, the replicative DNA polymerases and other DNA processing factors are recruited resulting in formation of the complete replisome and commencement of DNA replication in S phase (62).

Origin licensing is tightly regulated to restrict DNA replication to once and only once per cell cycle. MCM loading outside of G1 phase must be inhibited because new licensing during S and G2 phases can lead to rereplication. The consequences of rereplication include gene amplification, chromosomal translocation, and aneuploidy that are drivers of oncogenesis (1). Origin licensing is restricted to G1 through many non-redundant mechanisms including transcriptional regulation, protein re-localization, expression of protein inhibitors, and ubiquitin-mediated proteolysis of both licensing components and cell cycle regulators. These mechanisms are discussed in detail as they pertain to each replication factor in the following sections.

*ORC and ORCA*

ORC is currently recognized as the first critical determinant of origin identity and is recruited to origins during late M phase. ORC consists of the core

complex of Orc2-5 with Orc1 and Orc6 more weakly associated. Orc1 is the largest ORC subunit and is essential for preRC formation while Orc6 is dispensable for preRC formation *in vitro* (63,64). ATP hydrolysis by Orc1 releases the MCM complex from ORC to complete MCM loading onto chromatin (54,65). While in yeast all ORC subunits are stably bound throughout the cell cycle, in metazoans Orc1 is cell cycle regulated. Orc1 is bound to origins during G1, but is largely degraded by the $SCF^{Skp2}$ ubiquitin ligase complex during S phase and reaccumulates as cells transition from mitosis to G1 (66). Studies in multiple organisms revealed that ORC is regulated through phosphorylation by CDKs (reviewed in reference 8). In budding yeast, Cyclin B-CDK binds to the RXL motif of Orc6 and phosphorylates both Orc6 and Orc2 at the G1/S transition preventing association of other preRC components (67,68). In mammalian systems, Orc1 is phosphorylated by mitotic cyclin-CDKs also resulting in inactivation of the complex to prevent rereplication.

ORC recruitment is relatively straightforward in *S. cerevisiae*, as it is the only organism in which ORC recognizes and binds a conserved sequence element, the ACS. However, as previously mentioned, ORC binding sites and ACS elements are much more abundant than origins suggesting that other factors must be involved in defining origins. Several histone modifications influence the recruitment of ORC. The Orc1 subunit contains a conserved bromo-adjacent homology (BAH) domain that has been shown to promote nucleosome binding in eukaryotic cells (23). In metazoans, this domain is able to recognize both mono- and di-methylated H4K20 (69). H4K20 is first mono-methylated by

Set8 then subsequently di- and tri-methylated by Suv4-20h1 or Suv4-20h2 (70). If recognition of H4K20me2 by Orc1 is required for ORC recruitment, then mono-methylation of H4K20 by Set8 is an essential first step in determining origin identity (discussed further below). ORC can also influence deposition of histone modifications. In *Drosophila*, H4K12 and H3K56 acetylation are dependent on ORC binding at origins (34,71). Consistent with this fact, Orc1 (as well as Mcm2, Cdt1, and geminin) physically interacts with the HAT responsible for H4K12 (and H4K5) acetylation in human cells, HBO1 (discussed further below) (35,72-74).

Another factor involved in origin licensing is the WD40 repeat domain-containing protein ORC-associated (ORCA; also known as LRWD1) (75). ORCA is required at many origins to stabilize the chromatin association of ORC. ORCA binds to Orc1 and Cdt1 during G1 phase and Orc2 throughout the cell cycle (75,76). Similar to Orc1, ORCA recognizes and binds methylated H4K20, specifically H4K20me3 (77). ORCA is most abundant during G1 and levels decrease as cells transition into S phase helping to prevent origin licensing outside of G1 (76). While ORCA interacts with the ubiquitin ligase $CRL4^{DDB1}$, recent studies suggest that multiple ubiquitin ligases likely target ORCA for degradation in S phase (78). Polyubiquitinated ORCA was detected at the G1/S transition, and only non-ubiquitinated ORCA associates specifically with Orc2 suggesting that this interaction may protect ORCA from ubiquitination (75).

*Cdc6*

Cdc6 is another preRC component recruited to origins and is a member of the AAA+ ATPase family. Cdc6 is dependent on ORC for its chromatin

association. Recent structural data on the *S. cerevisiae* proteins suggests that Cdc6 binding actually alters the structure of ORC such that the BAH domain of Orc1 is properly positioned (79). The ATPase activity of both ORC and Cdc6 likely cause destabilization of the MCM ring to allow its loading onto the DNA (80,81). During early G1, Cdc6 is targeted for degradation by $APC^{Cdh1}$ (82). $APC^{Cdh1}$ prevents Cdc6 accumulation until late in G1 when Cdc6 is phosphorylated at serine 54 by Cyclin E-Cdk2 disrupting the interaction between Cdc6 and Cdh1 (83). During S phase $APC^{Cdh1}$ is inactivated and Cdc6 is exported from the nucleus through a two-step mechanism. First, Gcn5 acetylates Cdc6 at three lysine residues (lysines 92, 105, and 109) (84). Cdc6 acetylation is required for subsequent phosphorylation at serine 106 by Cyclin A-Cdk2 that promotes the nuclear export of Cdc6, preventing its participation in origin licensing (85,86). Cdc6 persists in the cytoplasm and is degraded by $APC^{Cdh1}$ in the next G1 phase. A recent report suggests that in cycling cells Cdc6 is present in late mitosis after anaphase and the bulk of origin licensing occurs during this window; in contrast, cells coming out of quiescence must wait until Cdc6 accumulates during G1 before licensing can occur (87). This tight regulation of Cdc6 ensures that preRC formation is restricted to the very narrow windows in late mitosis and G1 when Cdc6 is present in the nucleus.

*Cdt1*

Cdt1 is the only preRC component that lacks enzymatic activity, but it is critical for origin licensing, as it is responsible for the recruitment of the MCM helicase at origins of replication. An auto-inhibitory domain in Mcm6 prevents

recruitment of the MCM complex to ORC-Cdc6, but through interaction with Cdt1, this inhibition is relieved. Deleting this auto-inhibitory region allows Cdt1-independent MCM recruitment to ORC-Cdc6 *in vitro*, but not helicase loading (55,88). Cdt1 has also been suggested to facilitate origin licensing by enhancing chromatin accessibility (72). Cdt1 directly interacts with HBO1 (89) and histone acetylation by HBO1 is critical for origin licensing (35,73). During G1, histone H4 acetylation by HBO1 positively influences origin licensing by promoting large-scale chromatin decompaction as evidenced by an elegant immunofluorescence assay (72). During S phase this process is reversed through binding of HDAC11 to Cdt1-geminin complexes resulting in decreased histone acetylation. This "chromatin reset" provides an additional mechanism to restrict origin licensing to the G1 phase of the cell cycle. Clearly, Cdt1 is at the center of many regulatory mechanisms that ensure DNA replication takes place once and only once during the cell cycle.

Degradation of Cdt1 can be prevented by acetylation at lysines 24 and 29 by p300 and PCAF in asynchronously dividing 293T cells (90). These acetylated lysines were detected during early G1. During S phase however, HDAC11 associated with and deacetylated Cdt1 promoting Cdt1 degradation (90). It is unclear which E3 ubiquitin ligase Cdt1 is protected from by acetylation, but the proximity of K24 to T29 suggests that it could interfere with Skp2 binding, although this has not yet been tested. Further work is needed to fully understand the importance of Cdt1 acetylation/deacetylation during G1 and S phases.

*MCM DNA helicase*

The MCM complex is a heterohexamer that functions as the replicative helicase. Recent data has demonstrated that two MCM complexes are loaded at origins in a head-to-head conformation (51). While these two are sufficient to commence replication, it has been shown that between 10-20 MCM complexes can be loaded at origins in vertebrates (91). Mcm3 stimulates the ATPase activity of ORC and Cdc6 to promote multiple rounds of ATP hydrolysis allowing the sequential loading of multiple MCM complexes onto origin DNA (88). The chromatin decompaction that occurs during preRC formation could “make room” for loading additional MCM complexes (72). These extra MCM complexes can initiate replication from dormant origins when cells experience replication stress, thus protecting the DNA from becoming damaged (92,93). On its own, the MCM complex is a poor helicase but as cells transition from G1 to S phase, replication initiation factors (Cdc45 and GINS) are recruited and, after phosphorylation by Cyclin B-CDKs and DDK, activate the MCM helicase (60).

Localization of MCM complexes is tightly regulated in *S. cerevisiae*, but not in other eukaryotes, such that they are only present in the nucleus during G1 and S phase (reviewed in reference 6). After translation in the cytoplasm, only intact MCM heterohexamers can enter the nucleus due to the nuclear localization sequences (NLS) being present only in Mcm2 and Mcm3. During S phase as MCMs are displaced from the DNA, Cyclin B-CDK activity promotes nuclear export of Mcm4 (94). Levels of MCMs in the nucleus of other eukaryotes remains unchanged throughout the cell cycle (reviewed in reference 95).

The preRC components discussed above represent the well-established

complex that is sufficient for *in vitro* origin licensing. However, two other factors were recently found to be both necessary for origin licensing and sufficient to promote recruitment of the canonical preRC components. These two factors are both involved in the post-translational modification of histone proteins. They are histone acetyltransferase binding to ORC (HBO1) and the histone methyltransferase Set8 (a.k.a. PR-Set7).

*HBO1*

HBO1 is a member of the MYST domain family of histone acetyltransferases (HATs) that acetylates histone H4 primarily at K5 and K12. It is the catalytic subunit of a HAT complex consisting of JADE1 (scaffold protein), hEaf6, and ING4 or ING5 (96-98). The two plant homeo domain (PHD) fingers of JADE1 are required for chromatin binding while the PHD finger of ING5 can direct binding specifically to regions with H3K4me2, 3 (96,97). A recent study of the lamin B2 origin in human cells revealed that H3K4me3 peaks during mid-G1 at this origin and is required for HBO1 recruitment and subsequent tetra-acetylation of H4 (at K5, 8, 12, and 16) (99).

HBO1 interacts with Orc1, Mcm2, and Cdt1 (73,74,89). When HBO1 was depleted in human cells, MCMs failed to load even though the chromatin association of ORC, Cdc6, and Cdt1 was unaffected (89,100). Histone H4 acetylation by HBO1 is not required for ORC binding but is critical for MCM loading (35). While HBO1 is not present in *S. cerevisiae*, a related MYST domain family member, Sas2, has been shown to genetically interact with ORC suggesting that the HAT-ORC interaction is conserved (101). The interaction of

HBO1 with Cdt1 seems to stimulate Cdt1-dependent rereplication suggesting it functions as a coactivator of Cdt1 (89). HBO1 interacts with Cdt1 during G1, but in S phase two mechanisms counteract HBO1 activity. First, HDAC11 associates with Cdt1 resulting in deacetylation of H4, which prevents re-loading of MCMs (72). Second, HBO1 HAT activity is inhibited when Cdt1 is bound by its inhibitor geminin. Geminin actually strengthens the interaction of HDAC11 with Cdt1 (35,72).

During mitosis HBO1 is bound and phosphorylated at serine 57 by Polo-like kinase 1 (Plk1). This phosphorylation seems to be required for its role in promoting MCM loading in G1 such that when this site is mutated cells arrest in G1 and fail to load MCMs (102). In fact, tethering the *Drosophila* HBO1 homolog, Chameau, to an artificial origin resulted in increased origin activity (36). Additionally, tethering HBO1 to an inactivated c-myc origin in human cells was sufficient to activate the origin, as was tethering of Cdt1, Orc2, or E2F1 (103). During cellular stress, the interaction between HBO1 and Cdt1 seems to be disrupted due to Jun N-terminal kinase (JNK)-dependent phosphorylation (discussed in Chapter 3) (104). HBO1 is clearly an important chromatin modifier that regulates origin function and whose recruitment to origins could be attributed to interactions with both preRC components and methylated H3K4.

*Set8*

Set8 (a.k.a. PR-Set7) is the sole methyltransferase responsible for the monomethylation of histone H4 at lysine 20 (H4K20me1) and has emerged as a critical player in origin licensing (105-107). This protein is highly conserved in

eukaryotes, but is not found in *S. cerevisiae.* Interestingly, in *Schizosaccharomyces pombe* the Set8 homolog, Set9, is responsible for the deposition of all three H4K20 methyl states. Set8 methylation of H4K20 is impaired when H4K16 is acetylated (108-110). This obstruction is overcome through the association of the HDAC SirT2 with Set8. SirT2 not only deacetylates H4K16 to allow H4K20 methylation by Set8, but it also deacetylates Set8 at lysine 90 resulting in the spread of H4K20me1 (110). The HAT responsible for Set8 K90 acetylation has not yet been identified.

Set8 plays a critical role in origin licensing and its regulation is as important as that of Cdt1 for preventing licensing outside of G1 phase. H4K20me1 is enriched at origins of replication in *Drosophila* (111). Knockdown of Set8 in human cells results in decreased chromatin association of ORC, Cdc6, and MCM complexes (112). Conversely, tethering human Set8 to a non-origin genomic locus is sufficient to promote the chromatin-association of ORC and MCM complexes (112). A recent mass spectrometry study identified Orc3 as a potential interacting partner of Set8 (113). In addition, loss of *SetD8* in mice results in embryonic lethality and indicates the essential requirement for Set8 during development (112). The role of H4K20me1 in DNA replication is likely exerted through effector proteins that specifically bind this modification. One such protein is L3MBTL1, a human homolog of the *Drosophila* polycomb tumor suppressor that may facilitate replication protein recruitment to chromatin through its interaction with components of the replication machinery (Cdc45, PCNA, and MCM) (114,115). Alternatively, H4K20me1 could promote origin licensing in G1

by serving as the template for di- and tri-methylation by both Suv4-20h1 and Suv4-20h2 methyltransferases at origins of replication (70,116,117). The BAH domain of Orc1 was recently shown to bind H4K20me2 while the preRC-associated protein, ORCA, specifically binds to H4K20me3 (76,77). The decrease in H4K20me1 during S phase could thus be due in part to the transition to these higher methylation states, although these marks have not yet been specifically examined at replication origins genome-wide. Loss of both Suv4-20h1 and Suv4-20h2 causes defects in S phase entry, but the effects are less severe than loss of Set8 suggesting additional roles for Set8 (and H4K20me1) in the G1/S transition (106,117).

As with many origin licensing factors, Set8 abundance is regulated both transcriptionally and post-translationally. *SETD8* gene expression fluctuates throughout the cell cycle, peaking in mitosis while Set8 protein levels drop during S phase even when *SETD8* is expressed from a constitutive promoter (118,119). Recently, the 3'UTR of Set8 was identified as a target of microRNA-7 in breast cancer cells (120). Ubiquitin-mediated proteolysis by $SCF^{Skp2}$, $CRL4^{Cdt2}$ and $APC^{Cdh1}$ is largely responsible for regulating Set8 protein abundance (109). Both $SCF^{Skp2}$ and $CRL4^{Cdt2}$ target Set8 for degradation during S phase (118,121). Aberrant Set8 accumulation during this cell cycle phase results in rereplication that is dependent on the catalytic activity of Set8 (112,122). The ability of a stabilized Set8 to induce rereplication further highlights its role in promoting origin licensing and the importance of its proper regulation. As cells enter mitosis, Set8 is phosphorylated at serine 29 by Cyclin B-Cdk1 preventing ubiquitination of Set8

by $APC^{Cdh1}$. During early anaphase however, Set8 is dephosphorylated by the Cdc14 phosphatases resulting in $APC^{Cdh1}$-mediated proteolysis and reduced protein levels during early G1 (123). As a result, Set8 and H4K20me1 levels peak in G2/M, are dramatically reduced during mid to late G1, and remain low during S phase.

The absence of the Set8 methyltransferase can only result in corresponding changes in histone methylation if histone H4K20me1 is simultaneously converted either to unmethylated H4K20 by a demethylase (PHF8) or to H4K20me2, 3. PHF8 plays an important role in the G1/S transition by demethylating H4K20me1 at promoters of E2F-dependent genes during G1 (124). PHF8 is recruited to these sites through the interaction of its PHD domain with H3K4me2 and me3. Just as PHF8 is recruited to promoters through interaction with H3K4me2, 3, it could also be targeted to origins of replication by the same mechanism since H3K4me2 and me3 are also present at those loci (25).

An alternative mechanism for Set8's role in DNA replication could be the methylation of an as yet unidentified non-histone protein required for progression from G1 to S phase. Currently the only non-histone substrates of Set8 that have been identified are p53 and Numb (125,126). These three proteins form a complex and when p53 and Numb are methylated, target gene expression is reduced and Numb dissociates from p53 resulting in increased p53 degradation (125,126). However, it is unlikely that p53/Numb methylation is responsible for the replication defects caused by Set8 depletion because the replication

phenotypes upon depletion of Set8 are not entirely p53 dependent, and p53 null cells are viable (111,127,128). The precise mechanism by which Set8 promotes cell cycle progression is therefore still unclear.

**Preventing rereplication**

In summary, origin licensing is a highly orchestrated event and there are many mechanisms in place to prevent spurious origin activation and subsequent rereplication that ultimately results in genome instability. PreRC formation is restricted to the G1 phase of the cell cycle by many non-redundant mechanisms. The first is inactivation of the ORC complex by S-phase CDK phosphorylation of Orc2 and Orc6. The second involves regulation of Cdc6 levels. In *S. cerevisiae*, Cdc6 is degraded by the $SCF^{Ccd4}$ E3 ubiquitin ligase complex when it becomes active during S phase. However, in metazoans a fraction of Cdc6 is exported to the cytoplasm after phosphorylation by Cyclin A-Cdk2. The third mechanism involves inhibition of Cdt1 by geminin and Cdt1 degradation by $SCF^{Skp2}$ and $CRL4^{Cdt2}$. In *S. cerevisiae,* both Cdt1 and the MCM complex are exported from the nucleus in response to CDK-dependent phosphorylation (68,94,129). A fourth mechanism is degradation of Set8 by $CRL4^{Cdt2}$. Fifth, HBO1 acetylation of H4 at origins is prevented outside of G1 through disruption of the HBO1-Cdt1 interaction upon geminin binding, and the association of HDAC11 which deacetylates H4 preventing MCM loading.

**Unanswered questions**

At the time this study was undertaken, many of the histone modifications discussed above were not known to be involved in DNA replication. Our goal was

to identify individual histone modifications that promote or hinder origin licensing to determine a chromatin signature denoting origin locations in the genome. What has become apparent throughout this work is that origins are not all regulated in the same way. Some *S. cerevisiae* origins are highly dependent on the BAH domain of Orc1 interacting with a nearby nucleosome while others function normally when this domain is deleted (23). There is also a small subset of origins that are negatively regulated by the HDAC Sir2 and have a stably positioned nucleosome near or overlapping the B2 element (130-132). A recent study in a near-normal EBV-transformed lymphoblastoid cell line found that early and late firing origins are regulated by very different mechanisms driven primarily by epigenetic factors and influenced only slightly by DNA sequence or transcriptional activity (133). While these findings were not unexpected, they do reveal the importance of chromatin in origin regulation. Further, this high level of complexity and the dynamic nature of these modifications create difficulty in pinpointing the factors that confer origin identity.

The discovery in mammalian cells that H4K20 mono-methylation by Set8 is a likely first step in origin licensing has led us, and others, to ask how this enzyme is recruited to origins. Is it really the first factor recruited to origins, or does it interact with other preRC components (e.g. ORC) such that they are recruited together? Current work in our lab is attempting to answer this question. Additionally, we are interested in how Set8 is regulated during the cell cycle and in response to various cellular insults to maintain genome integrity.

# CHAPTER 2

## H3K4 di-methylation promotes DNA replication origin function in *Saccharomyces cerevisiae*[1]

### INTRODUCTION

DNA replication initiates at discrete genomic loci termed origins of replication. Each eukaryotic chromosome is replicated from many individual origins to ensure complete and precise genome duplication during each cell division cycle. Individual origins vary both in the likelihood that they will initiate replication, or "fire", in any given S phase and in the firing time within S phase (16,134). Highly efficient origins fire in most cell cycles, whereas inefficient origins fire in only some cycles and are usually passively replicated by forks emanating from neighboring efficient origins. Though highly efficient origins that support initiation in most cell cycles have been identified in many genomes, the chromosomal determinants of origin location and function are still incompletely

[1]Modified from Rizzardi LF, Dorn ES, Strahl BD, and Cook JG. Genetics. October 2012, Volume 192, pages 371-384.

understood. Strikingly, while DNA sequence elements can be necessary, it is clear that sequence alone is insufficient to fully specify eukaryotic origin location and activity (135,136).

Like all DNA-templated processes, replication occurs on chromatin. Recent progress in the field has demonstrated that the chromatin structure surrounding origins plays an essential role in controlling origin activity. For instance, the positioning of nucleosomes near origins can either stimulate or inhibit origin function (6,19,20,131,137,138). The major protein components of chromatin, the histone proteins, can also be post-translationally modified by acetylation, methylation, phosphorylation, ubiquitination, and sumoylation (2,139). These modifications can alter DNA accessibility and serve as recognition sites for other proteins. Importantly, several individual histone modifications affect aspects of origin function. For example, acetylation of histones H3 and H4 accelerate the timing of origin firing within S phase and can increase origin efficiency (30,36,139,140). In addition, histone H3 lysine 36 mono-methylation (H3K36me1) by the Set2 methyltransferase has been implicated in the recruitment of the replication initiation protein, Cdc45 (37,141). In metazoan genomes, Set8-catalyzed histone H4 lysine 20 mono-methylation (H4K20me1) stimulates the loading of the core replicative helicase (112,127,140).

It is clear that no single histone modification is absolutely required for origin function since loss of individual histone modifying enzymes does not impact cell viability. This observation suggests that a combination of histone modifications facilitate efficient DNA replication in the form of a “histone code”

similar to the combinations known to regulate transcription (5,142). While some elements of this code have been identified (e.g. H3 and H4 acetylation, H4K20 monomethylation and H3K36 monomethylation), the complexity of DNA replication led us to hypothesize that additional histone modifications that impact origin activity remain to be discovered. We therefore sought to identify those histone modifications and chromatin modifiers that are integral to this process. We conducted a genetic screen to identify histone modifications that are required for the fitness of a hypomorphic replication yeast mutant, *cdc6-1*. This screen revealed a previously unidentified positive role for histone H3 lysine 4 (H3K4) methylation in DNA replication, and our subsequent analysis indicates that H3K4 di-methylation is the relevant modification for this activity. These findings contribute to elucidating the pattern of chromatin features that determine origin activity in eukaryotic genomes.

## MATERIALS AND METHODS

### Yeast strains and growth conditions

The *Saccharomyces cerevisiae* strains used in this study are listed in Table 2.1 and any additional genotype information is available upon request. Construction of *de novo* gene deletion strains was performed by PCR-mediated disruption, and some double mutant construction was performed by mating as indicated in Table 2.1.

### Plasmids

All plasmids used in this study are listed in Table 2.2.

### Synthetic genetic array (SGA) screen

SGA analysis was carried out as previously described (141,143,144). Briefly, 63 deletion strains (Table 2.3) were mated to the temperature-sensitive *cdc6-1* strain (JCY332) and haploids carrying both mutations were isolated by growth on selective media. All of the deletion strains originated from the Yeast Knock Out library (Open Biosystems) except strains lacking *SET1* or *DOT1*; the *set1Δ* strains were created *de novo* while the *dot1Δ* strain had been previously published (134,145). Additionally, *SET1* and *BRE1* deletions were recreated *de novo* in the *cdc6-1* mutant in the BY4741 background (yLF058). All of the resulting double mutants were spotted in 5-fold serial dilutions with an initial $OD_{600}$ of 0.5 onto YPD, grown for 3 days at 32°C, and growth was compared to that of the *cdc6-1* single mutant. Double mutants displaying a synthetic growth phenotype were confirmed by analyzing three independent isolates. The fold change in growth is denoted by a score from 1 to 3 indicating an approximate 5-fold to 125-fold change compared to *cdc6-1* alone. Negative values indicate growth defects, while positive values indicate enhanced growth or rescue. No genetic rescue was observed in any double mutant strain.

**Minichromosome maintenance assays**

Minichromosome (or plasmid) maintenance assays were performed as described previously (135,146,147). Briefly, yeast strains containing YCplac33, YCplac111, or YCplac33+2X*ARS209* were grown to log phase in the appropriate selective media and 100-200 cells were plated on both selective and non-selective media to establish an initial percentage of plasmid-bearing cells. These cultures were also diluted to a concentration of 1 X $10^5$ cells/ mL in 5 mL of non-

**Table 2.1 Strains used in this study**

| STRAIN | GENOTYPE | SOURCE |
|---|---|---|
| [a]BY4741 | *MATa met15Δ0* | (225) |
| [a]yLF058 | *MATa met15Δ0, cdc6-1::hph* | This study |
| [a]yLF063 | *MATa met15Δ0, cdc6-1::hph, set1Δ::HIS3MX6* | This study |
| [a]yLF089 | *MATa met15Δ0, set1Δ::HIS3MX6* | This study |
| [a]yLF059 | *MATa met15Δ0, bre1Δ::kanMX* | This study |
| [a]yLF062 | *MATa met15Δ0, set1Δ::kanMX* | This study |
| [a]yLF060 | *MATa met15Δ0, bre2Δ::kanMX* | This study |
| [a]yLF061 | *MATa met15Δ0, swd1Δ::kanMX* | This study |
| [c]YMS196 | *Matα can1Δ::STE2$_p$_Sp.his5$^+$, lyp1Δ::STE3$_p$_LEU2* | (227) |
| [c]JCY332 | *Matα can1Δ::STE2$_p$_Sp.his5$^+$, lyp1Δ::STE3$_p$_LEU2, cdc6-1::hph* | This study |
| [a,e]yLF114 | *Matα lyp1Δ::STE3$_p$_LEU2, cdc6-1::hph, swd1Δ::kanMX* | This study |
| [a,e]yLF120 | *Matα lyp1Δ::STE3$_p$_LEU2, cdc6-1::hph, bre2Δ::kanMX* | This study |
| [a,e]yLF117 | *Matα lyp1Δ::STE3$_p$_LEU2, cdc6-1::hph, rad6Δ::kanMX* | This study |
| [a]yLF154 | *MATa met15Δ0, rad6Δ::kanMX* | OpenBiosystems |
| [a]yLF150 | *MATa met15Δ0, cdc6-1::hph, bre1Δ::kanMX* | This study |
| [a]TSQ131 | *MATα can1Δ::STE2pr-Sp_his5, lyp1Δ::STE3pr-LEU2, cdc7-4::natMX* | (226) |
| [a,e]yLF096 | *MATα set1Δ::kanMX* | This study |
| [a,e]yLF097 | *MATa* | This study |
| [a,e]yLF098 | *MATα lyp1Δ::STE3pr_LEU2, cdc7-4::natMX* | This study |
| [a,e]yLF099 | *MATa can1Δ::STE2pr_Sp-his5, cdc7-4::natMX, set1Δ::kanMX* | This study |
| [a]TSQ880 | *MATα can1Δ::STE2pr-Sp_his5, lyp1Δ::STE3pr-LEU2, cdc7-1::natR* | (226) |
| [a,e]yLF138 | *MATa can1Δ::STE2pr-Sp_his5, lyp1Δ::STE3pr-LEU2, cdc7-1::natMX, set1Δ::kanMX* | This study |

**Table 2.1 Strains used in this study (continued)**

| STRAIN | GENOTYPE | SOURCE |
|---|---|---|
| [a,e]yLF139 | *MATa cdc7-1::natMX* | This study |
| [a,e]yLF140 | *MATα* | This study |
| [a,e]yLF141 | *MATα set1Δ::kanMX* | This study |
| [a]TSQ694 | *MATα can1Δ::STE2pr-Sp_his5, lyp1Δ::STE3pr-LEU2, cdc45-27::natMX* | (226) |
| [e]yLF142 | *MATα his3Δ1, ura3Δ0* | This study |
| [e]yLF143 | *MATα his3Δ1, ura3Δ0, set1Δ::kanMX* | This study |
| [e]yLF144 | *MATa his3Δ1, ura3Δ0, set1Δ::kanMX, cdc45-27::natMX* | This study |
| [e]yLF145 | *MATa his3Δ1, ura3Δ0, cdc45-27::natMX* | This study |
| [b]DY7803 | *MATa hht1-hhf1::LEU2, hht2-hhf2::kanMX3*, [YCp-*URA3*(*HHT2-HHF2*)] | (228) |
| [b,e]yLF155 | *MATa hht1-hhf1::LEU2, hht2-hhf2::kanMX3, cdc6-1::hph,* [YCp-*URA3*(*HHT2-HHF2*)] | This study |
| [b]RUY121 | *MATα* | Fred Cross |
| [b]RUY028 | *MATα mfa::MFA1$_p$_HIS3, ORC6-rxl::LEU2, URA3::GAL$_p$_CDC6ΔNT-HA* | (145) |
| [b,e]yLF049 | *MATα mfa::MFA1$_p$_HIS3, ORC6-rxl::LEU2, URA3::GAL$_p$_CDC6ΔNT-HA, swd1Δ::kanMX* | This study |
| [b,e]yLF050 | *MATα mfa::MFA1$_p$_HIS3, ORC6-rxl::LEU2, URA3::GAL$_p$_CDC6ΔNT-HA, bre1Δ::kanMX* | This study |
| [b]yLF052 | *MATα bre1Δ::kanMX* | This study |
| [b]YNL037 | *MATα dot1Δ::kanMX* | (134) |
| [d]FY406 | *MATa hta1-htb1::LEU2, hta2-htb2::TRP1* [pSAB6 *HTA1-HTB1 URA3*] | (229) |

[a]Additional genotype: *his3Δ1, leu2Δ0, ura3Δ0*
[b]Additional genotype: *can1-100, his3-11,15, leu2-3,112, ura3-1, lys2, trp1-1, ade2-1*
[c]Additional genotype: *his3Δ1, leu2Δ0, ura3Δ0, met15Δ0, lys2Δ0, LYS2+, cyh2*
[d]Additional genotype: *his3Δ200, trp1Δ63, lys2-128δ, ura3-52, leu2Δ1*
[e]Generated by mating and only markers listed were confirmed

**Table 2.2 Plasmids used in this study**

| PLASMID NAME | FEATURES | SOURCE |
|---|---|---|
| YCplac33 | *ARS1, CEN4, URA3* | (230) |
| YCplac111 | *ARS1, CEN4, LEU2* | (230) |
| YCplac33+2X*ARS209* | *ARS1* and 2 copies of *ARS209, CEN4, LEU2* | This study |
| pRS316-*CDC6* | *CDC6, CEN6* | This study |
| pGLx2 | *GAL1-LexA, 2μ, URA3* | This study |
| pGLx2-*SET1* | *GAL1-LexA-SET1, 2μ, URA3* | This study |
| pGLx2-*set1-ΔRRM* | *GAL1-LexA-set1-ΔRRM, 2μ, URA3* | This study |
| pGLx2-*set1-H1017K* | *GAL1-LexA-set1-H1017K, 2μ, URA3* | This study |
| pRS315-9XMyc-*BRE1* | pRS315-9XMyc-*BRE1, LEU2* | A. Shilatifard |
| pRS315-9XMyc-*bre1-H665K* | pRS315-9XMyc-*bre1-H665K, LEU2* | A. Shilatifard |
| pRS415-3XMyc-*SET1* | prS415-3XMyc-*SET1, LEU* | (231) |
| pZS145 | *HTA1*-Flag-*HTB1, CEN6, HIS3* | Z-W. Sun |
| pZS146 | *HTA1*-Flag-*htb1-K123R, CEN6, HIS3* | Z-W. Sun |
| pZS145-R119A | *HTA1*-Flag-*htb1-R119A, CEN, HIS3* | (232) |
| pZS473 | *HTA1*-Flag-*htb1-R119D, CEN6, HIS3* | Z-W. Sun |
| WT H3 | YCp-*TRP1*(*HHT2-HHF2*) | B. Strahl |
| H3K4R | YCp-*TRP1*(*hht2-K4R-HHF2*) | B. Strahl |
| H3K79R | YCp-*TRP1*(*hht2-K79R-HHF2*) | B. Strahl |

selective media and grown for 8-10 generations before once again plating on both selective and non-selective media. Precise generation numbers were calculated using the following formula: $n = \log(C_F/C_I) / \log(2)$, where $C_F$ represents the final number of cells as measured by $OD_{600}$ and $C_I$ represents the starting cell number of $10^5$ cells/mL. After 2 days of growth, colonies were counted and the plasmid loss rate (L) per generation (n) was calculated using the following formula: $L = 1-(\%F / \%I)^{(1/n)}$, where %F is the final percentage of cells that retained the plasmid and %I is the initial percentage of cells that contain the plasmid.

**Immunoblotting**

Whole cell extracts were prepared by extraction with trichloroacetic acid (TCA). Cell growth was halted by the addition of TCA to a final concentration of 5% and the cell pellets were frozen at -80°C. Pellets were resuspended in 200 μL TCA buffer (10 mM Tris-HCl, pH 8.0, 10% TCA, 25 mM $NH_4OAc$, 1 mM EDTA) and broken by glass bead lysis. Proteins were precipitated by centrifugation, resuspended in 100 μL resuspension buffer (0.1 M Tris-HCl, pH 11.0, 3% SDS), and boiled for 5 min. Samples were centrifuged, and the supernatant was quantified using the Dc Assay (BioRad). Equal concentrations of lysates were loaded onto 15% SDS-PAGE gels and transferred onto PVDF. The following antibodies were used: anti-H3 (1:10,000; ActiveMotif 39163), anti-H3K4me1 (1:2000; Millipore 07-436), anti-H3K4me2 (1:2000; abcam 32356), anti-H3K4me3 (1:10,000; gift from M. Bedford), anti-H2B (1:5000; ActiveMotif 39237), anti-Myc (9E10; 1:1000; Santa cruz sc-40), and anti-LexA (1:5000; Millipore 06-719).

**Chromatin immunoprecipitation**

Yeast strains were crosslinked with 1% formaldehyde for 15 min at RT and quenched with 250 mM glycine for 5 min at RT. Forty $OD_{600}$ units of crosslinked cells were harvested by centrifugation, washed thoroughly, and the pellets were stored at -80°C. The cell pellets were resuspended in 300 mM FA-lysis buffer (50 mM HEPES-KOH, pH 7.5, 300 mM NaCl, 1 mM EDTA, 1% TritonX-100, 0.1% Na-deoxycholate) with protease inhibitors, broken by glass bead lysis, and fixed chromatin was sheared by sonication using a Branson sonifier 250. Average DNA fragment lengths were 100-300 bp determined by gel analysis. After centrifugation and quantification via Bradford Assay (BioRad), 0.5 mg of soluble chromatin was incubated with 2 μL of antibody (anti-H3 (ActiveMotif), anti-H3K4me2 (Abcam) or anti-H3K4me3 (Millipore)) in 1.5 mL tubes overnight at 4°C and immunoprecipitated with 10 μL Protein A Dynabeads (Invitrogen) for 1 h at 4°C. The beads were washed sequentially with 300 mM FA-lysis buffer, twice with 500 mM FA-lysis buffer (50 mM HEPES-KOH, pH 7.5, 500 mM NaCl, 1 mM EDTA, 1% TritonX-100, 0.1% Na-deoxycholate), once with LiCl solution (10 mM Tris-HCl, pH 8.0, 250 mM LiCl, 0.5% NP-40, 0.5% Na-deoxycholate, 1 mM EDTA), and once with TE, pH 8.0. After washing, the chromatin was eluted from the beads in 200 μL elution buffer (0.1 M $NaHCO_3$, 1% SDS) for 30 min at RT. The eluted material was treated with RNAse A and Proteinase K before de-crosslinking at 65°C overnight. The DNA was purified using Genesee UPrep spin columns and eluted in 100 μL water. IP samples and IP controls (*set1Δ*) were used undiluted while input samples were diluted 1:10.

Samples were analyzed by qPCR on the ABI 7900 HT (AppliedBiosystems) using SYBR Green master mix with Rox (Fermentas). Primer sequences are available upon request. Signals from the immunoprecipitates are reported as a percentage of the input and normalized to H3. Error bars represent the standard deviations of the average signals between experiments (n ≥ 3).

## RESULTS

### Identification of histone modifiers that promote DNA replication

DNA replication origins in the budding yeast *Saccharomyces cerevisiae* are defined by both sequence elements and local chromatin structure. Although DNA replication is essential for cell proliferation, the majority of histone modifications and chromatin-modifying enzymes are not individually required for yeast cell viability. This observation supports the model that a *combination* of histone modifications supports replication origin function. To identify new histone modifications that contribute to this combination, we conducted a genetic screen. We reasoned that individual chromatin elements that influence replication activity would be revealed as genetic suppressors or enhancers of cell growth in a strain bearing a hypomorphic mutation in an essential replication gene, *CDC6*.

The Cdc6 ATPase plays an essential role at origins in loading the replicative helicase complex composed of MCM2-7 (6,137,148). The *cdc6-1* mutant harbors a G260D mutation in the catalytic domain resulting in failure to load MCMs at restrictive temperatures (139,147). Yeast cells harboring the *cdc6-1* mutation produce a Cdc6 protein that functions normally at 29°C, is nonfunctional at temperatures above 34°C, but retains partial function at

intermediate temperatures between 30°C and 33°C (139,149). To identify suppressors or enhancers of *cdc6-1,* we deleted most of the known histone modifiers, chromatin remodelers, and histone chaperones (63 total; see Table 2.3) in the *cdc6-1* temperature-sensitive replication mutant strain. Double mutant strains were tested for fitness at semi-permissive temperatures and compared to the parent single mutant strains (141,150). The majority of double mutant strains grew neither better nor worse than their respective parents under any of the tested growth conditions, and no null alleles improved the growth of the *cdc6-1* mutant (Table 2.3). Although a role for the HAT Gcn5 in DNA replication has been shown (37,140,151,152), it was not included in this screen because the null mutant strain has a slow growth phenotype that would complicate interpretation of the double mutant phenotype. In contrast, 21 of the null alleles (including a positive control *tom1Δ*) impaired growth in the *cdc6-1* strain but had little effect in otherwise wild-type backgrounds. These genes represent a wide array of chromatin factors including histone acetyltransferases (HATs), histone deacetylases (HDACs) and histone methyltransferases (HMTs) (Table 2.3). Interestingly, many of these factors contributed either directly or indirectly to a single histone modification, H3K4 methylation, which is deposited by Set1, the catalytic subunit of the COMPASS complex (Figure 2.1A).

**COMPASS activity and H3K4 methylation promote DNA replication**

We confirmed the enhancer phenotype of the *SET1* deletion strain by constructing a *set1Δ* allele *de novo* in the *cdc6-1* parent strain. Growth of the *cdc6-1* strain was only slightly impaired at 31°C compared to wild-type or the

**Table 2.3 Results from SGA screen for genetic interaction with *cdc6-1***

| DELETION | SCORE[a] | FUNCTION/COMPLEX |
|---|---|---|
| *bre1*[b,c] | -3 | E3 ubiquitin ligase |
| *chd1*[b] | -3 | Remodeler/SAGA |
| *rad6*[b,c] | -3 | E2 ubiquitin-conjugating enzyme |
| *spt8*[b] | -3 | SAGA HAT |
| *bre2*[b,c] | -2 | COMPASS |
| *cdc73*[b] | -2 | Paf1C |
| *hst1*[b] | -2 | HDAC |
| *hst3*[b] | -2 | HDAC |
| *isw1*[b] | -2 | Remodeler |
| *rph1*[b] | -2 | H3K36 DMT |
| *rtf1*[b] | -2 | Paf1C |
| *sap30*[b] | -2 | HDAC |
| *set1*[b,c] | -2 | H3K4 HMT |
| *swd1*[b,c] | -2 | COMPASS |
| *swd3*[b,c] | -2 | COMPASS |
| *tom1* | -2 | E3 ubiquitin ligase/Positive Control |
| *ctk1* | -1 | Kinase |
| *hat1* | -1 | HAT |
| *hda1* | -1 | HDAC |
| *sdc1*[b,c] | -1 | COMPASS |
| *snf5* | -1 | Remodeler |
| *asf1* | 0 | Histone Chaperone |
| *dot1*[b,c] | 0 | H3K79 HMT |
| *eaf1* | 0 | NuA4 HAT |
| *eaf3* | 0 | NuA4 HAT |
| *eaf6* | 0 | NuA3/4 HAT |
| *ecm5* | 0 | JmjC DMT |
| *gis1* | 0 | JmjC DMT |
| *hap2* | 0 | Elongator HAT |
| *hir1* | 0 | Remodeler |
| *hir2* | 0 | Remodeler |
| *hir3* | 0 | Remodeler |
| *HO* | 0 | Endonuclease/Negative Control |
| *hos1* | 0 | HDAC |
| *hos2* | 0 | HDAC |
| *jhd1* | 0 | H3K36 DMT |
| *jhd2* | 0 | H3K4 DMT |
| *nto1* | 0 | NuA3 HAT |
| *rco1* | 0 | Rpd3(S) HDAC |
| *rpd3* | 0 | Rpd3(S)/(L) HDAC |
| *rtt102* | 0 | Remodeler |
| *rtt109* | 0 | H3K56 HAT |
| *rub1* | 0 | Ubiquitin-like |
| *sas2* | 0 | SAS HAT |

**Table 2.3 Results from SGA screen for genetic interaction with *cdc6-1* (continued)**

| DELETION | SCORE[a] | FUNCTION/COMPLEX |
|---|---|---|
| *sas3* | 0 | NuA3 HAT |
| *sds3* | 0 | Rpd3(L) HDAC |
| *set2* | 0 | H3K36 HMT |
| *sgf73* | 0 | SAGA HAT |
| *shg1*[b,c] | 0 | COMPASS |
| *snf1* | 0 | Kinase |
| *snf6* | 0 | Remodeler |
| *spp1*[b,c] | 0 | COMPASS |
| *sps1* | 0 | Kinase |
| *spt5* | 0 | PolII Associated |
| *spt7* | 0 | SAGA HAT |
| *ste20* | 0 | Kinase |
| *swi2/snf2* | 0 | Remodeler |
| *swr1* | 0 | Remodeler |
| *tel1* | 0 | Repair |

[a]Score represents change in growth corresponding to approximate 5-fold differences (1=5-fold, 2=25-fold, etc.)
[b]Strains in a YMS196 *cdc6-1* background confirmed in three independent isolates
[c]Strains created *de novo*

*cdc6-1* strain harboring wild-type *CDC6* on a plasmid, but growth was substantially impaired when *SET1* was deleted in this strain (Figure 2.1B). Expression of wild-type *SET1*, but not the catalytically dead mutant *set1-H1017K*, rescued the synthetic growth defect of the *set1Δ cdc6-1* strain (Figure 2.1B). Importantly, the synthetic growth defect of the *set1Δ cdc6-1* strain was recapitulated in a *cdc6-1* strain in which the only copy of histone H3 bears the K4R (unmethylatable) mutation (Figure 2.1C). These findings indicate that the catalytic activity of Set1 is important for robust growth of the *cdc6-1* replication mutant.

Set1 functions as the catalytic subunit of the COMPASS complex. We hypothesized that other members of this complex would display similar phenotypes when deleted in the *cdc6-1* strain. Deletion of *BRE2*, *SDC1*, *SWD1*, and *SWD3* each impaired the growth of the *cdc6-1* mutant at semi-permissive temperatures, but not deletion of *SPP1* or *SHG1* (Figure 2.1D and Table 2.3). Bre2, Sdc1, Swd1, Swd2, and Swd3 (along with Set1) are the core structural components of the COMPASS complex required for full activity (142,153,154). These results further support the conclusion that COMPASS enzymatic activity and H3K4 methylation are important for proliferation when Cdc6 is crippled.

The poor growth of these double mutant strains could be due to a general exacerbation of the replication defect caused by Cdc6 perturbation, or it could reflect a specific interaction between Cdc6 and H3K4 methylation. If Set1 and H3K4 methylation are generally important for efficient DNA replication, then we expect similar proliferation defects from deleting *SET1* in other replication mutant

**Figure 2.1**

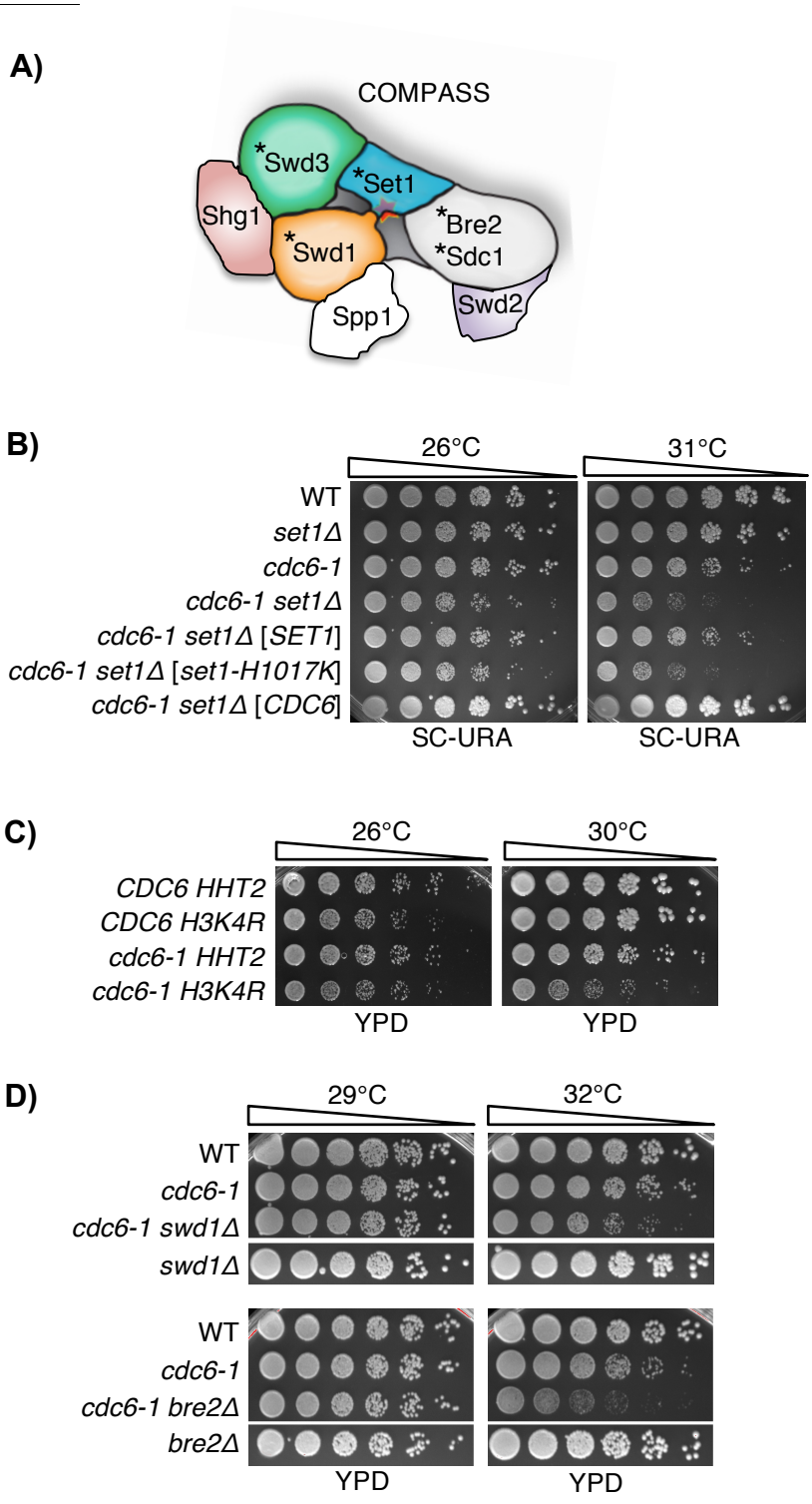
A)
COMPASS
*Swd3
*Set1
Shg1
*Swd1
*Bre2
*Sdc1
Spp1
Swd2
B)
26°C
31°C
WT
set1Δ
cdc6-1
cdc6-1 set1Δ
cdc6-1 set1Δ [SET1]
cdc6-1 set1Δ [set1-H1017K]
cdc6-1 set1Δ [CDC6]
SC-URA
SC-URA
C)
26°C
30°C
CDC6 HHT2
CDC6 H3K4R
cdc6-1 HHT2
cdc6-1 H3K4R
YPD
YPD
D)
29°C
32°C
WT
cdc6-1
cdc6-1 swd1Δ
swd1Δ
WT
cdc6-1
cdc6-1 bre2Δ
bre2Δ
YPD
YPD

**FIGURE 2.1. COMPASS and H3K4 are required for robust growth of the temperature-sensitive *cdc6-1* replication mutant.** A) COMPASS complex model adapted from (142). (* denotes complex member that genetically interacts with *cdc6-1*) B) Wild-type (BY4741), *cdc6-1* (yLF058), *set1Δ* (yLF062), and *cdc6-1 set1Δ* (yLF063) were transformed with an empty vector [pGLx2], a vector producing LexA-tagged wild-type Set1 [pGLx2-*SET1*], or catalytically dead Set1 [pGLx2-*set1-H1017K*], from a *GAL1* promoter construct, or a vector producing normal Cdc6 [pRS316-*CDC6*], from the *CDC6* promoter as indicated. Five-fold serial dilutions were spotted onto SC-URA containing 1% galactose/2% raffinose and grown at the indicated temperatures for 4 days. C) The *cdc6-1* mutation was introduced into the H3-H4 "shuffle" strain (DY7803) transformed with *HHT2* or *hht2-K4R* plasmids. Five-fold serial dilutions were spotted onto YPD and grown at the indicated temperatures for 3 days. D) Five-fold serial dilutions of wild-type (YMS196), *cdc6-1* (JCY332), *cdc6-1 swd1Δ* (yLF036), *cdc6-1 bre2Δ* (yLF037), *bre2Δ* (yLF060), or *swd1Δ* (yLF061) were spotted onto YPD and grown at the indicated temperatures for 3 days.

strains. To test this prediction, *SET1* was deleted in two temperature-sensitive *cdc7* mutants and one temperature-sensitive *cdc45* mutant. These replication factors function at the origin firing step at the G1/S transition, downstream of Cdc6 activity (142-144). Similar to the effect of deleting *SET1* in the *cdc6-1* strain, loss of *SET1* in the *cdc7-1, cdc7-4,* and *cdc45-27* mutants impaired growth at semi-permissive temperatures (Figure 2.2A). In addition, cells lacking *SET1* were also sensitive to pharmacological replication stress induced by hydroxyurea (HU) (Figure 2.2C).

The poor growth of *cdc6-1 set1Δ* cells suggests that Set1 promotes replication; we thus predicted that loss of H3K4 methylation in a hypermorphic replication mutant would at least partially rescue the adverse phenotypes of that mutant. To test this hypothesis, we introduced the *set1* null allele into the hypermorphic replication mutant, RUY028. This yeast strain harbors two mutations that de-regulate replication origin licensing resulting in re-replication, an aberrant phenomenon in which some origins fire more than once per cell cycle leading to DNA damage and poor cell growth. The *ORC6-rxl* mutation prevents inhibitory phosphorylation of the Orc6 subunit of the Origin Recognition Complex (ORC) by CDK, and the *GAL1* pr-*CDC6-ΔNT* allele produces a hyperstable Cdc6 protein (145,155). In this strain, re-replication is induced during growth on galactose, which induces transcription of the *CDC6-ΔNT* allele. As expected, loss of H3K4 methylation upon deletion of *SWD1* or *BRE1* partially rescues the poor growth of the re-replicating strain on galactose (Figure 2.2B).

**Figure 2.2**

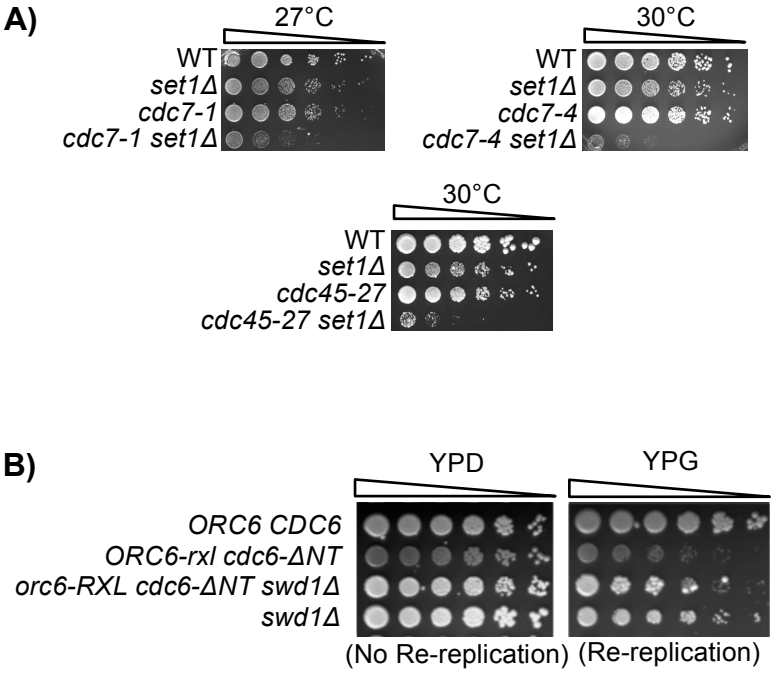
A)
27°C
WT
set1Δ
cdc7-1
cdc7-1 set1Δ
30°C
WT
set1Δ
cdc7-4
cdc7-4 set1Δ
30°C
WT
set1Δ
cdc45-27
cdc45-27 set1Δ
B)
YPD
YPG
ORC6 CDC6
ORC6-rxl cdc6-ΔNT
orc6-RXL cdc6-ΔNT swd1Δ
swd1Δ
(No Re-replication)
(Re-replication)
C)
0 mM
200 mM
HU
WT
set1Δ

**FIGURE 2.2. H3K4 methylation is required for robust growth of multiple replication mutants.** A) Five-fold serial dilutions of the meiotic progeny from the cross of *cdc7-1* (TSQ880), *cdc7-4* (TSQ131), or *cdc45-27* (TSQ694) with *set1Δ* (yLF062) were spotted onto YPD and grown at the indicated temperatures for 3 days. B) Five-fold serial dilutions of wild-type (RUY121), *ORC6-rxl CDC6-ΔNT* (RUY028), *swd1Δ* (yLF051), and *ORC6-rxl CDC6-ΔNT swd1Δ* (yLF049) were spotted onto YP containing 2% dextrose (no re-replication) or galactose (re-replication induced) and grown for 2 days at 30°C. C) Five-fold serial dilutions of wild-type (BY4741) or *set1Δ* (yLF062) were spotted on YPD containing the indicated concentrations of HU and grown for 2 days at 30°C.

Together, these data provide strong evidence that H3K4 methylation promotes efficient DNA replication, most likely through regulation of origin activity.

A positive role for H3K4 methylation in replication suggests that this histone modification is enriched near origins of replication. To test this idea directly, we performed chromatin immunoprecipitation (ChIP) assays for both di- and tri-methylated H3K4 in asynchronous wild-type yeast cultures. Analysis of several genomic loci revealed that both H3K4 methylation states are enriched at both an early-and late-firing origin of replication (*ARS315* and *ARS822,* respectively) relative to a non-origin, telomere-proximal region (Figure 2.3A and B). In addition, if Set1 activity were important for origin licensing during G1, we would expect Set1 protein levels to be high during this cell cycle phase. Cells expressing a 3XMyc-tagged Set1 were arrested in G1 using alpha factor and samples were taken at the indicated times after release (Figure 2.3C). Surprisingly, Set1 was only detectable during G1 supporting a role for this HMT in promoting origin licensing.

**The Rad6/Bre1 ubiquitin ligase complex promotes DNA replication**

Methylation of histone H3K4 by the COMPASS complex requires prior monoubiquitination of histone H2B at lysine 123 (H2BK123) by the Rad6/Bre1 ubiquitin E3 ligase (146,147,153). Our original screen detected a strong synthetic growth defect when the *bre1Δ* library mutant was crossed with the *cdc6-1* parent strain. *De novo* deletion of either *BRE1* or *RAD6* in the *cdc6-1* mutant resulted in an approximate 125-fold decrease in growth at semi-permissive temperatures, validating this genetic interaction (Figures 2.4A and B). Ectopic expression of

**Figure 2.3**

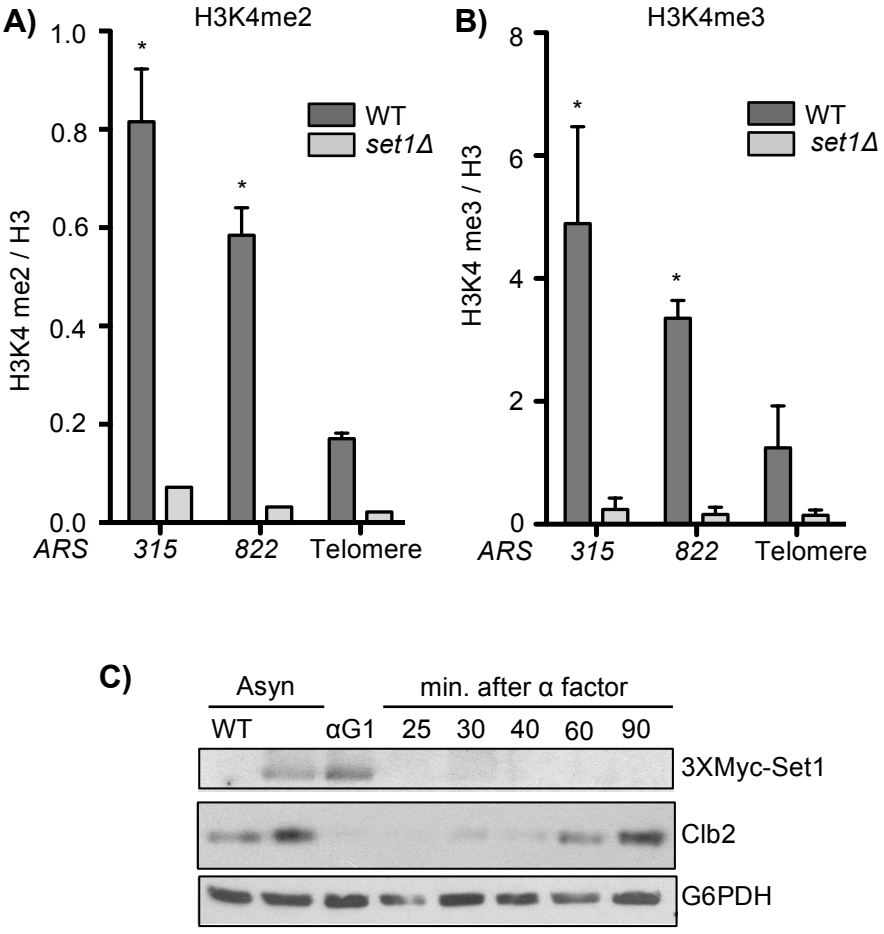
A)
H3K4me2
H3K4 me2 / H3
1.0
0.8
0.6
0.4
0.2
0.0
WT
set1Δ
*
*
ARS
315
822
Telomere
B)
H3K4me3
H3K4 me3 / H3
8
6
4
2
0
WT
set1Δ
*
*
ARS
315
822
Telomere
C)
Asyn
min. after α factor
WT
αG1
25
30
40
60
90
3XMyc-Set1
Clb2
G6PDH

**FIGURE 2.3. H3K4 methylation is present at replication origins and Set1 is most abundant during G1.** Chromatin immunoprecipitation (ChIP) experiments were performed on asynchronous wild-type (BY4741) or *set1Δ* (yLF062) strains grown at 30°C. Immunoprecipitates using antibodies to total histone H3, di-methylated H3K4 (H3K4me2) in A and tri-methylated H3K4 (H3K4me3) in B were analyzed by quantitative PCR for chromosomal DNA fragments from a region near telomere VI-R and two replication origins, *ARS315* and *ARS822.* Error bars represent the standard deviations of $n \geq 3$ biological replicates. Significant enrichment of H3K4 methylation at origins compared to telomere VI-R was determined by Student's unpaired t-test. (*$p<0.05$) C) Wild-type (BY4741) yeast strains expressing 3XMyc-tagged Set1 were synchronized in G1 and cells were harvested at the indicated times after alpha factor release.

wild-type *BRE1* under control of its own transcriptional promoter fully rescued the exacerbated growth phenotype, but expression of a catalytically dead Bre1 (*bre1-H665A*) did not (Figure 2.4A). Moreover, *BRE1* deletion also partially rescued the growth impairment of the re-replicating RUY028 strain similar to what was observed after *SET1* deletion (Figure 2.4C). These data suggest that the Rad6/Bre1 ubiquitin ligase complex is also required for efficient DNA replication, likely through its involvement in promoting H3K4 methylation.

**H3K4 methylation is required for efficient minichromosome maintenance**

To assess DNA replication more specifically than general cell proliferation, we used a plasmid loss assay that measures the ability of cells to maintain a minichromosome bearing a centromere and a single replication origin (148,153). Growth in non-selective medium for several cell divisions allows cells that failed to initiate replication of the minichromosome to survive, and these cells are then counted as colonies on non-selective medium. Strains lacking either *SET1* or *BRE1* displayed significantly higher plasmid loss rates per cell division than wild-type strains did (Figure 2.5A, $p<0.05$). Similar to the phenotype resulting from *SET1* deletion, cells expressing the mutant histone H3K4R displayed a significant increase in plasmid loss rate (Figure 2.5B).

Of note, H2B monoubiquitination by Rad6/Bre1 is not only a pre-requisite for H3K4 methylation, but also for H3K79 methylation (147,153). Neither deletion of *DOT1*, the histone H3K79 methyltransferase, nor mutation of H3K79 to arginine had any effect on plasmid maintenance (Figure 2.5A and B). Moreover,

**Figure 2.4**

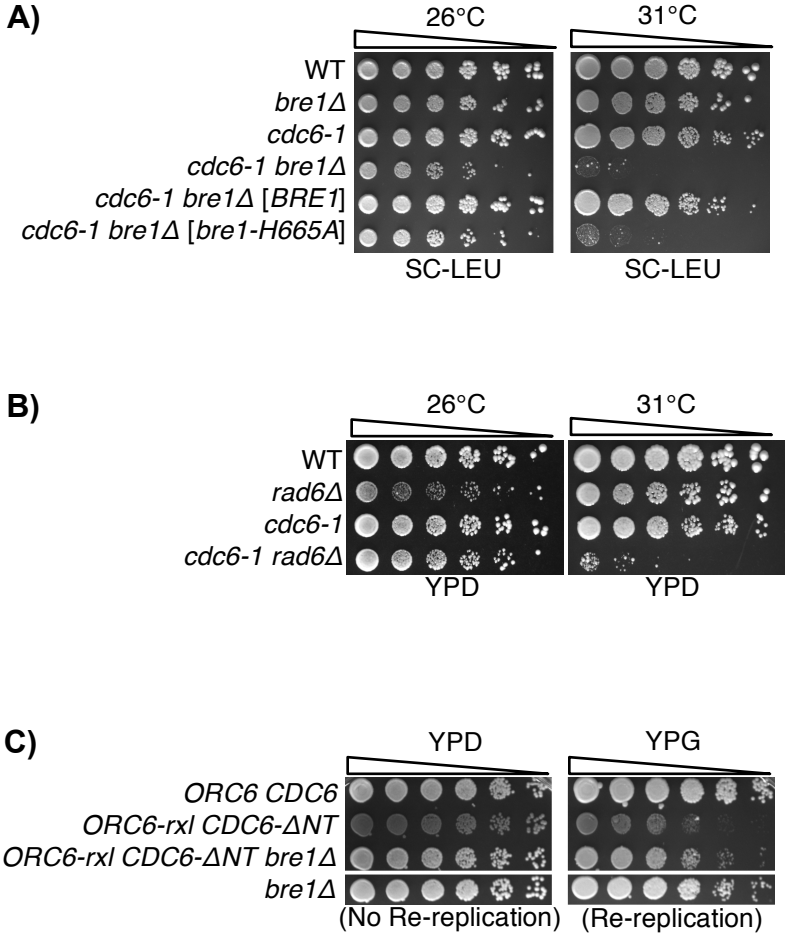
A)
26°C
31°C
WT
bre1Δ
cdc6-1
cdc6-1 bre1Δ
cdc6-1 bre1Δ [BRE1]
cdc6-1 bre1Δ [bre1-H665A]
SC-LEU
SC-LEU
B)
26°C
31°C
WT
rad6Δ
cdc6-1
cdc6-1 rad6Δ
YPD
YPD
C)
YPD
YPG
ORC6 CDC6
ORC6-rxl CDC6-ΔNT
ORC6-rxl CDC6-ΔNT bre1Δ
bre1Δ
(No Re-replication)
(Re-replication)

**FIGURE 2.4. H2BK123 mono-ubiquitination promotes robust growth of the temperature-sensitive *cdc6-1* replication mutant.** A) Wild-type (BY4741), *bre1Δ* (yLF151), *cdc6-1* (yLF058), and *cdc6-1 bre1Δ* (yLF150) were transformed with either an empty vector [pRS315], a vector expressing *BRE1* (pRS315-*9xMyc-BRE1*), or *bre1-H665A* (pRS315-*9xMyc-bre1-H665A*) from the native *BRE1* promoter. Five-fold serial dilutions were spotted onto SCD-LEU and grown for 4 days at the indicated temperatures. B) Five-fold serial dilutions of wild-type (YMS196), *cdc6-1* (JCY332), *rad6Δ* (yLF154), and *cdc6-1 rad6Δ* (yLF117) were spotted onto YPD and grown for 3 days at the indicated temperatures.
C) Wild-type (RUY121), *ORC6-rxl CDC6-ΔNT* (RUY028), *bre1Δ* (yLF052), and *ORC6-rxl CDC6-ΔNT bre1Δ* (yLF050) were spotted onto YP containing 2% dextrose or 1% galactose (re-replication induced) and grown for 3 days at 30°C.

loss of Dot1 did not impair the growth of the *cdc6-1* strain (Table 2.3). Efficient growth and plasmid maintenance by these strains indicate that the elevated plasmid loss rate of the *BRE1* null strain is likely due to the subsequent loss of H3K4 methylation rather than loss of H3K79 methylation. Further, these results demonstrate that inefficient minichromosome maintenance is not a universal phenotype of strains lacking histone-modifying enzymes or expressing mutant histones.

If these minichromosome maintenance defects are specific to perturbations of origin function and not chromosome segregation or expression of the selectable marker, then adding multiple origins to the test plasmid should rescue the elevated loss rates of the *set1Δ* and *bre1Δ* strains. Additional origins multiply the chances for a successful origin initiation event on the plasmid, and success at any one origin allows replication and transmission to both daughter cells. This property has been used by others to validate origin-specific phenotypes (149,156). We modified the single *ARS1*-containing plasmid by adding two copies of *ARS209*. As before, the plasmid harboring only one origin was lost more frequently from *set1Δ* and *bre1Δ* strains than from wild-type strains, but this effect was reversed with the addition of multiple origins (Figure 2.5C). This result supports the conclusion that H2BK123 monoubiquitination by Bre1 and the consequent H3K4 methylation by Set1 promote DNA replication origin function in yeast.

**Figure 2.5**

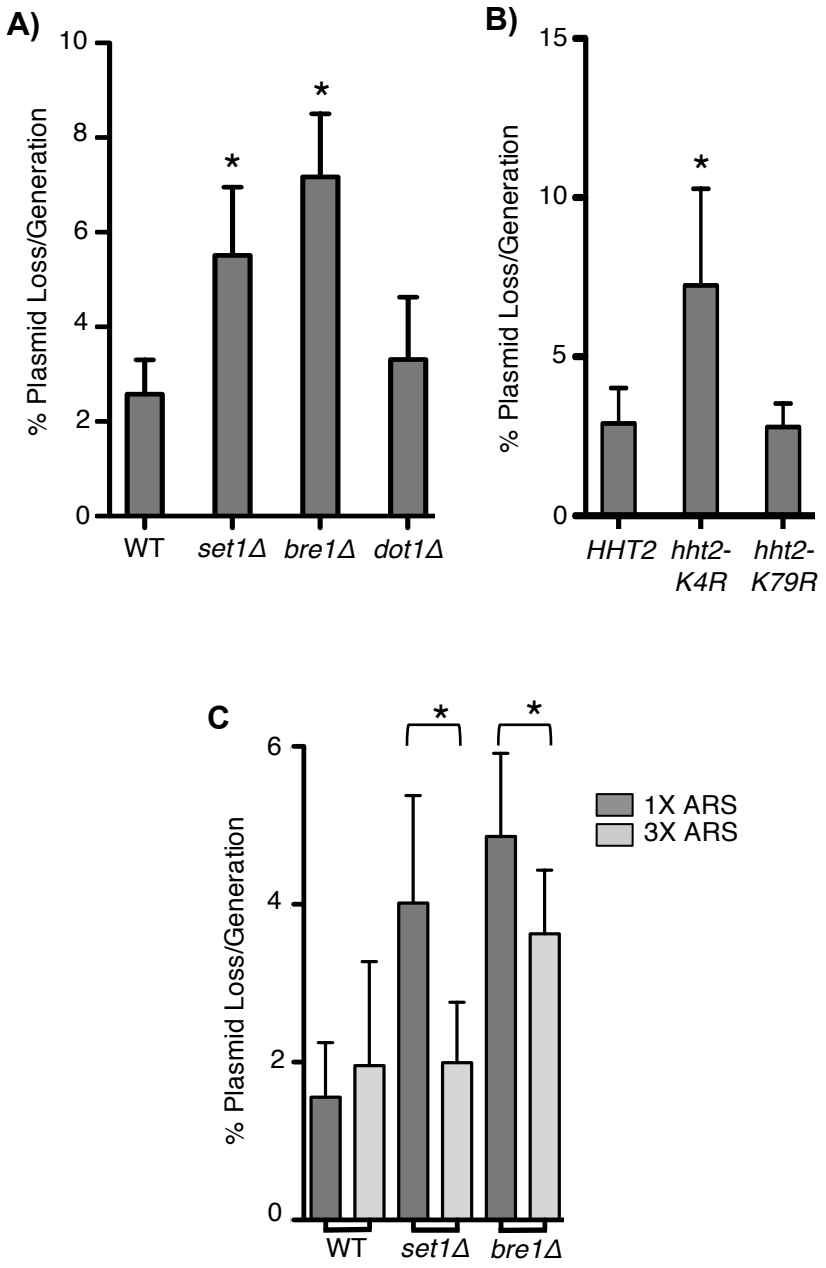
A)
% Plasmid Loss/Generation
0
2
4
6
8
10
*
*
WT
set1Δ
bre1Δ
dot1Δ
B)
% Plasmid Loss/Generation
0
5
10
15
*
HHT2
hht2-K4R
hht2-K79R
C
% Plasmid Loss/Generation
0
2
4
6
*
*
1X ARS
3X ARS
WT
set1Δ
bre1Δ

**FIGURE 2.5. H3K4 methylation is required for efficient origin-dependent minichromosome maintenance.** A) Plasmid loss rates of a single origin bearing plasmid [YCpLac33] were measured in a wild-type strain (BY4741) and strains lacking *SET1* (yLF089), *BRE1* (yLF151), or *DOT1* (YNL037). Loss rates are reported per cell division. B) Plasmid loss rates of a single origin bearing plasmid [YCpLac111] were measured in the histone "shuffle" strain (DY7803) transformed with plasmids expressing wild-type *HHT2*, *hht2-K4R,* or *hht2-K79R.* C) Plasmid loss rates of plasmids bearing either a single origin [YCpLac33] or three origins [YCpLac33 + 2X *ARS209*] were measured. For all experiments, the average loss rates were obtained from at least three independent transformants, and the error bars indicate standard deviations. Statistics were performed using the Student's unpaired t-test. (*$p<0.05$)

**H3K4 di-methylation is required for efficient origin function**

The Set1/COMPASS complex is responsible for mono-, di-, and tri-methylation of H3K4. These methylation states are differentially distributed over gene bodies (150,157,158) and several studies have shown that different methylation states of H3K4 and H3K36 can have different functions (37,151,152,159). The individual H3K4 methylation states depend on other histone residues and are influenced by the presence or absence of individual COMPASS subunits (153,154,160). To gain insight into which H3K4 methylation states were required for efficient DNA replication, we measured plasmid loss rates in strains lacking different COMPASS complex members. Like the *set1Δ* strain (Figure 2.5A) both *swd1Δ* and *bre2Δ* strains displayed significantly elevated plasmid loss compared to their isogenic wild-type counterparts (Figure 2.6A). These two proteins are both required for Set1 stability (142,161), and in their absence no H3K4 methylation is detectable (Figure 2.6B). Unlike Swd1 and Bre2, Spp1 is a COMPASS subunit that is required for H3K4 tri-methylation, but not mono- or di-methylation (Figure 2.6B) (5,155,162); loss of *SPP1* had no effect on plasmid maintenance (Figure 2.6A). This result is consistent with our earlier observation that Spp1 loss did not affect proliferation of the *cdc6-1* strain (Table 2.3). Taken together, these data suggest that H3K4 di-methylation is sufficient for proper origin function and that H3K4 tri-methylation is dispensable.

To examine the role of H3K4 di-methylation without perturbing the COMPASS complex, we took advantage of H2B mutants that were previously shown to differentially affect H3K4 methylation (92,153,163,164). Mutation of

H2BK123 to arginine abolished monoubiquitination of this residue and consequently eliminated both H3K4 di- and tri-methylation (Figure 2.6D). Importantly, similar to loss of *BRE1*, this mutation induced a significantly higher plasmid loss rate than wild-type (Figure 2.6C). This result, in combination with the failure of the Bre1 catalytically dead mutant to complement the growth defect of the *bre1Δ cdc6-1* mutant, strongly argues that the H2BK123 ubiquitination function of Bre1 is required for full origin function. Additionally, it supports the importance of H3K4 di-methylation in this process.

A previous study reported that mutational alteration of H2BR119 to alanine does not prevent H2BK123 monoubiquitination, but does affect the degree of H3K4 methylation (37,153). As previously reported, changing H2BR119 to alanine (H2BR119A) reduces the chromatin association of Spp1 and therefore H3K4 tri-methylation, whereas changing H2BR119 to aspartic acid (H2BR119D) results in loss of H3K4 di- and tri-methylation to the same extent as eliminating H2BK123 monoubiquitination (23,153). Our analysis of these histone H2B mutants revealed that strains expressing *htb1-R119D* displayed elevated plasmid loss rates similar to those of the *htb1-K123R* strain (Figure 2.6C). Mutation of R119 to alanine abolished H3K4 tri-methylation as expected, while having only a moderate effect on H3K4 di-methylation (Figure 2.6D). This H2B mutant showed a plasmid loss rate similar to the wild-type *HTB1* strain. The strict correlation between the ability to produce H3K4 di-methylation and normal plasmid maintenance underscores the importance of H3K4 di-methylation, but not necessarily H3K4 tri-methylation, for origin function (Figure 2.6C).

**Figure 2.6**

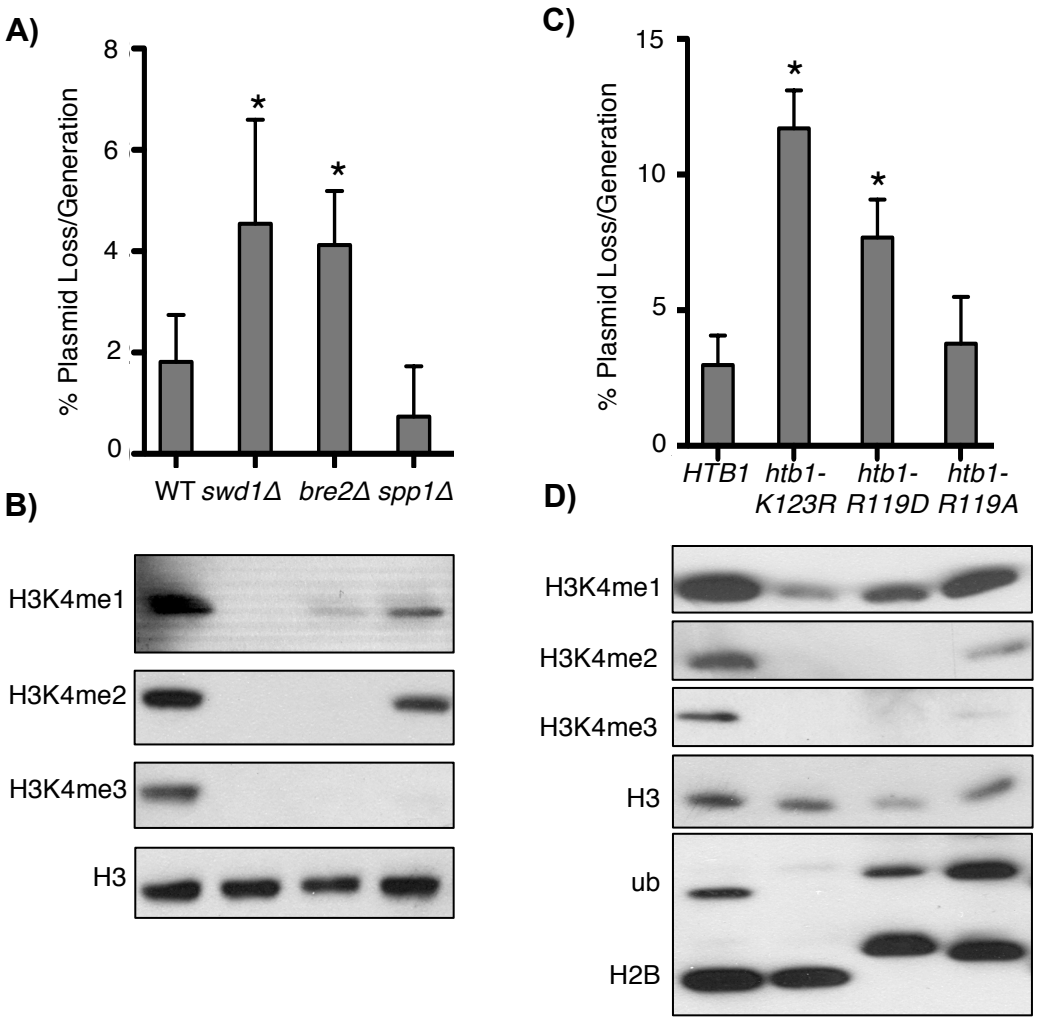
A)
% Plasmid Loss/Generation
8
6
4
2
0
*
*
WT swd1Δ bre2Δ spp1Δ
B)
H3K4me1
H3K4me2
H3K4me3
H3
C)
% Plasmid Loss/Generation
15
10
5
0
*
*
HTB1 htb1-K123R htb1-R119D htb1-R119A
D)
H3K4me1
H3K4me2
H3K4me3
H3
ub
H2B

**FIGURE 2.6. H3K4 di-methylation promotes efficient minichromosome maintenance.** A) Plasmid loss rates of the single origin bearing plasmid [YCpLac33] were measured for wild-type (BY4741), *swd1Δ* (yLF061), *bre2Δ* (yLF060), and *spp1Δ* (yLF153). B) Immunoblot analysis of whole cell extracts (from strains shown in A) using antibodies to total H3, H3K4me1, H3K4me2, and H3K4me3. C) Plasmid loss rates of the single origin bearing plasmid [YCpLac111] were measured for the H2B" shuffle" strain (FY406) transformed with plasmids expressing *HTB1* [pZS145], *htb1-K123R* [pZS146], *htb1-R119D* [pZS473], or *htb1-R119A* [pZS145-*R119A*]. D) Immunoblot analysis of whole cell extracts (from strains shown in C) using antibodies specific for total H2B, H3, and H3K4 methylation as in B. All plasmid loss data represent the mean and standard deviation of at least three independent transformants. Statistics were performed using the Student's unpaired t-test. (*$p<0.05$)

Our initial observation that loss of *SET1* in a *cdc6-1* mutant strain resulted in a proliferation defect, coupled with the requirement for Set1 for proper minichromosome maintenance, clearly indicates that H3K4 methylation is necessary for robust DNA replication. To determine if H3K4 di-methylation is sufficient for DNA replication, we transformed the *cdc6-1 set1Δ* strain with plasmids directing the production of wild-type Set1, catalytically dead Set1 (Set1H1017K), or a Set1 protein lacking the RRM domain. The latter mutant can only di-methylate H3K4 (29,156,165). Each of these Set1 proteins was fused to a LexA tag for detection on immunoblots (Figure 2.7B) and expressed from the *GAL1* promoter on galactose-containing medium. Immunoblot analysis of the three H3K4 methylation states confirmed that the fusions generated the expected methylation states (Figure 2.7B). As before, production of active Set1 suppressed the growth phenotype caused by deleting *SET1* in the *cdc6-1* strain (Figure 2.7A). Importantly, production of Set1ΔRRM also fully rescued this proliferation defect without the ability to produce H3K4 tri-methylation (Figure 2.7B). These data demonstrate that H3K4 di-methylation is both necessary and sufficient for robust growth of the *cdc6-1* mutant and suggest that this histone modification plays an important positive role in origin function.

**DISCUSSION**

This study documents a novel role for H3K4 di-methylation in DNA replication origin function. Yeast strains with hypomorphic mutations in multiple replication genes are highly dependent on H3K4 di-methylation for robust growth. In these replication mutants (*cdc6-1, cdc7-1, cdc7-4, cdc45-27*) under semi-

**Figure 2.7**

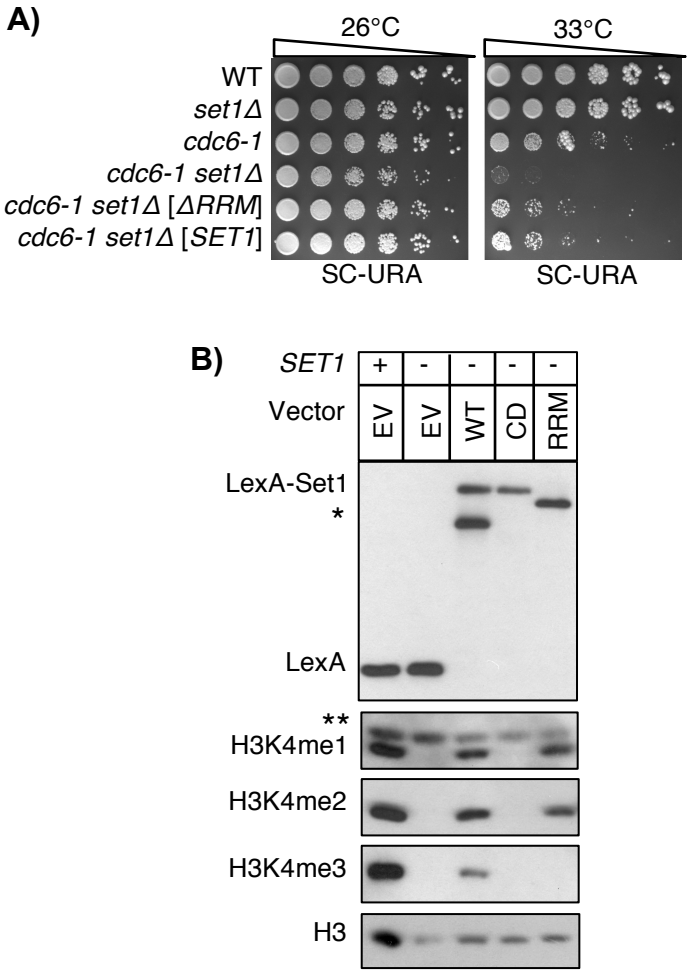
A)
26°C
33°C
WT
set1Δ
cdc6-1
cdc6-1 set1Δ
cdc6-1 set1Δ [ΔRRM]
cdc6-1 set1Δ [SET1]
SC-URA
SC-URA
B)
SET1
+
-
-
-
-
Vector
EV
EV
WT
CD
RRM
LexA-Set1
*
LexA
**
H3K4me1
H3K4me2
H3K4me3
H3

**FIGURE 2.7. H3K4 di-methylation is sufficient for robust growth of the *cdc6-1* mutant.** A) Wild-type (BY4741), *cdc6-1* (yLF058), *set1Δ* (yLF062), and *cdc6-1 set1Δ* (yLF063) were transformed with an empty vector [pGLx2], a vector producing LexA-tagged wild-type Set1 [pGLx2-*SET1*], or Set1 lacking the RRM domain [pGLx2-*set1-ΔRRM*] from the *GAL1* promoter. Five-fold serial dilutions were spotted onto SC-URA containing 1% galactose and grown at the indicated temperatures for 5 days. B) Immunoblot analysis of whole cell extracts from either wild-type (BY4741) or *set1Δ* (yLF062) strains transformed with empty vector [pGLx2] or vectors producing LexA-tagged normal ("WT") Set1 [pGLx2-*SET1*], catalytically dead ("CD") Set1 [pGLx2-*set1-H1017K*], or Set1 lacking the RRM domain ("RRM") [pGLx2-*set1-ΔRRM*] from the *GAL1* promoter. Blots were probed with antibodies specific for LexA, total H3, H3K4me3, H3K4me2, and H3K4me1 (* represents a likely degradation product unique to the positive control construct; ** represents a non-specific band detected by the H3K4me1 antibody).

permissive conditions, replication activity is reduced to the minimum needed for normal growth, and any further reduction in active origins caused by loss of H3K4 di-methylation results in severely impaired proliferation. Additionally, normal propagation of a minichromosome containing a single origin requires H3K4 di-methylation even in an otherwise wild-type strain. Every mutation that prevents H3K4 di-methylation, including loss of the pre-requisite histone H2BK123 monoubiquitination, causes a similar replication phenotype. These data provide clear evidence that H3K4 di-methylation is important for full origin function.

The Set1 histone methyltransferase and the Bre1 ubiquitin E3 ligase have well-established roles in regulating gene expression (157,158,166). Nonetheless, several lines of evidence suggest that the phenotypes we have detected are due to faulty replication as opposed to altered gene expression. First, many chromatin-modifying enzymes showed no synthetic growth phenotype when combined with the *cdc6-1* mutation. Some of these non-interacting genes include those with much more profound effects on patterns of gene expression than Set1, suggesting that replication phenotypes are not a general outcome of perturbed gene expression control. Interestingly, even the Rpd3 histone deacetylase that regulates origin firing time within S phase (159,167) did not genetically interact with *cdc6-1* (Table 2.3), implying that the synthetic growth phenotypes observed here are relatively specific for origin function and not origin timing. Second, a genome-wide analysis identified only 55 transcripts that changed significantly in a *set1Δ* strain compared to a wild-type strain, and none of those genes are predicted to directly affect origin activity (160,168). Third, the mini-chromosome

maintenance phenotypes associated with loss of H3K4 di-methylation were largely suppressed by the addition of extra origins to the test plasmid, indicating that the phenotypes are closely tied to origin function and not other biological parameters. Fourth, we directly detected H3K4 di- and tri-methylation at two yeast origins. Moreover, our analysis of a published genome-wide H3K4 tri-methylation dataset identified peaks of methylation distinct from nearby transcription-associated peaks (161,169, and our unpublished observations). Finally, our observation that loss of H3K4 methylation exacerbates poor growth of a replication hypomorphic strain but *suppresses* the poor growth of an origin firing hypermorphic strain indicates that the replication phenotypes reported here are most likely direct positive effects of H3K4 di-methylation at origins. Taken together, these data provide strong evidence that the role of H3K4 di-methylation in origin function is direct and separate from any indirect transcriptional effects.

DNA replication is an essential process for proliferation, yet neither Set1 nor Bre1 are essential gene products. Interestingly, very few null mutations in yeast chromatin-modifying enzymes show significant growth defects despite their importance for several essential processes, such as transcription, replication, and repair. The function of histone modifications in transcriptional control has been described as a “histone code” in which combinations of post-translational modifications promote or repress transcription at a given locus (5,151,162,170-172). In this model, no single histone modification generates an active or inactive promoter, and thus the effects of mutations that alter local chromatin structure are cumulative. We propose that the same concept applies to chromatin structure

at DNA replication origins. Consistent with the idea that, like promoters, origins can accommodate elimination of a single positive element of chromatin structure, the kinetics of S phase progression are normal in a *set1Δ* strain grown in standard conditions (data not shown). Also analogous to transcriptional control at promoters, we propose that histone H3K4 di-methylation is an important element of origin chromatin structure, but its absence alone does not severely inhibit origin function. In fact, origin activity is robust enough that even substantial reductions in expression of MCM proteins or deletion of many origins from a yeast chromosome causes no growth defects (37,92,163,164). Nevertheless, the effect of H3K4 di-methylation loss over several cell cycles or in combination with other replication perturbations causes a significant loss of replication fitness.

How does H3K4 di-methylation promote DNA replication? We propose two models by which H3K4 di-methylation could facilitate DNA replication (Figure 2.8). The first is a recruitment model whereby a replication factor directly interacts with di-methylated H3K4 to associate with replication origins. This possibility is supported by precedent since it has previously been suggested that mono-methylation of H3K36 can recruit the replication initiation factor, Cdc45 (37,173). In addition, a member of the origin recognition complex, Orc1, contains a bromo-adjacent homology (BAH) domain that mediates nucleosome binding; this domain is known to be important for the association of ORC with origins (23,30). Therefore, domains in other replication factors may bind to nucleosomes or specific histone modifications, or as yet unidentified bridging proteins could link H3K4 di-methylation to recruitment of the core replication machinery at origins.

Illuminating the molecular mechanism(s) by which H3K4 di-methylation is connected to replication proteins at origins will require additional studies.

The second model suggests that H3K4 di-methylation near the origin recruits another chromatin modifier such as a histone acetyltransferase (HAT). The HAT could then acetylate nearby nucleosomes, resulting in increased accessibility to the origin to allow more efficient association of replication factors (Figure 8). Acetylation is already known to affect the efficiency and timing of origin firing (29,165). Additionally, several HAT complexes contain subunits that specifically recognize H3K4 methylation. The HAT complex SAGA contains the catalytic subunit Gcn5 as well as two proteins, Chd1 and Spt8, that genetically interact with *cdc6-1* (Table 2.3). Chd1 is a chromatin remodeler whose mammalian counterpart is capable of binding H3K4 methylation (166) while Spt8 impacts transcription by directly recruiting TBP (167). SAGA also contains the Sgf29 subunit that has recently been reported to bind di- and tri-methylated H3K4 to facilitate SAGA recruitment to some promoters (168). SAGA is responsible for acetylation of multiple H3 residues as well as H4K8 and H2BK11 and K16 (169). Two other HAT complexes were also identified in our screen, NuA3 and NuA4; both contain subunits that bind H3K4 methylation, Yng1 and Yng2, respectively (151,170-172). Further examination of these and other H3K4 methylation readers will shed light on the role of H3K4 methylation in the context of origin-specific chromatin.

Prior research has identified other histone modifications that impact origin function. Mono-methylation of H3K36 by Set2 facilitates recruitment of the

**Figure 2.8**

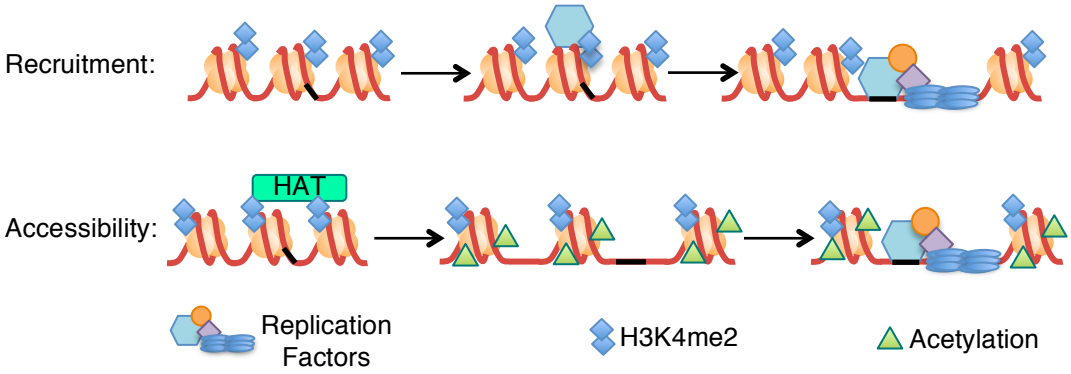
Recruitment:
Accessibility:
HAT
Replication Factors
H3K4me2
Acetylation

**FIGURE 2.8. Two models by which H3K4 di-methylation could promote efficient DNA replication.** The recruitment model suggests either direct or indirect recruitment of replication factors by H3K4 di-methylation, while the accessibility model suggests recruitment of a HAT that acetylates nucleosomes near the origin resulting in an open chromatin state that allows replication factors to access and bind the origin.

replication initiation factor, Cdc45 in yeast (37). In addition to methylation, H3K4 can also be acetylated by both Gcn5 and Rtt109 (173). At promoters, this acetylation is typically found just upstream from the peak of H3K4 tri-methylation. Whether this modification is also found at origins is currently unknown. Multi-acetylated histones H3 and H4 have been shown by several groups to impact the ability of origins to fire (30) and the timing of origin firing in yeast, *Drosophila*, and mammalian cells (16,24,29,174). Additionally, the HATs Hat1 and Gcn5 physically interact with the replication machinery (136,140,175). In mammalian cells, Gcn5 acetylates Cdc6 at several lysine residues and this acetylation is required for subsequent phosphorylation by cyclin/CDKs (19,20,84,131,138). In vertebrates, the H4-specific HAT, HBO1, physically interacts with the replication machinery (2,73,74,100), and the acetylation of histones by HBO1 is essential for origin licensing (30,35,36,140). In metazoans, methylation of H4K20 by Set8 (a.k.a. PR-Set7) is cell cycle regulated (37,112,176) and is required for proper S phase initiation and prevention of re-replication (112,127,177). In *S. cerevisiae* histone H3 is phosphorylated at threonine 45 in a cell cycle-dependent manner by the Cdc7-Dbf4 kinase. Mutating this residue to alanine causes sensitivity to hydroxyurea and camptothecin indicating its importance in proper DNA replication (5,38). Because all organisms except *S. cerevisiae* lack common nucleotide sequence motifs at origins, discovering a chromatin signature that promotes origin function is essential to understand the location and activity of replication zones in higher eukaryotes. Given the complexity of metazoan genomes, it may be that several different chromatin signatures specify origins in

different chromosomal domains. Our discovery that H3K4 di-methylation is able to promote DNA replication adds to growing number of histone modifications that could function together to confer origin function at discrete genomic loci. Further work will determine which suite of histone modifications are common to all origins or are required only at specific genomic loci to successfully promote DNA replication.

# CHAPTER 3

## Regulation of Set8 and Cdt1 by phosphorylation throughout the cell cycle

### INTRODUCTION

DNA replication is an essential cellular process that is highly regulated to maintain genome integrity. During G1, sites in the genome where replication initiates (origins of replication) are prepared or “licensed” for replication through formation of the preRC complex. The preRC complex consists of ORC, Cdc6, and Cdt1 that together recruit and load the replicative helicase of MCM proteins. Recently, the lysine methyltransferase Set8 was found to be a key factor promoting preRC formation (reviewed in reference 176). To prevent re-firing of origins, or rereplication, preRC formation must not occur outside of G1 phase. There are many mechanisms in place to restrict the activity of these proteins outside of G1; the most significant is the proteolytic degradation of the replication proteins Cdc6, Cdt1, and Set8.

Both Cdt1 and Set8 are targeted for proteasomal degradation in S phase by the E3 ubiquitin ligase complexes, $SCF^{Skp2}$ and $CRL4^{Cdt2}$ (178). $SCF^{Skp2}$

functions in S phase to inhibit spurious origin licensing through polyubiquitination of Cdt1 and Set8. $SCF^{Skp2}$-dependent Cdt1 degradation requires prior phosphorylation of Cdt1 at T29 by Cyclin E/A-Cdk2 that facilitates association with Skp2 (178,179). A Cy-motif present in Cdt1 mediates its interaction with and phosphorylation by these Cyclin-CDK complexes (178). No similar mechanism regulating Set8 degradation by $SCF^{Skp2}$ has been reported.

$CRL4^{Cdt2}$-mediated degradation of cell cycle regulators also ensures that replication occurs once and only once per cell cycle. The Cdt2 (Cdc10-dependent transcript 2) substrate receptor is unique in that its interaction with substrates is dependent on the substrate first interacting with DNA-loaded PCNA. PCNA becomes loaded at replication forks during S phase and during the DNA synthesis steps of DNA repair (180-183). Proteins that interact with PCNA contain a motif known as a PCNA-interacting protein box (PIP box). $CRL4^{Cdt2}$ substrates contain a specialized PIP box known as the PIP degron. The PIP degron differs from the canonical PIP box in two ways. First, a TD motif is present within the PIP box that strengthens the interaction with PCNA (184-187). Second, the PIP degron contains a conserved basic residue located four amino acids downstream from the PIP box that is required for interaction with Cdt2 (184,187,188). All *bona fide* substrates of $CRL4^{Cdt2}$ contain this PIP degron. Interestingly, PCNA must be loaded onto chromatin to promote interaction between the substrate and Cdt2, though the basis for substrates to distinguish soluble PCNA from chromatin-loaded PCNA is not yet clear (187,189). Through the requirement for substrate interaction with PCNA, $CRL4^{Cdt2}$-mediated

proteolysis is coupled to DNA replication during S phase and after DNA damage. Cdt1 harbors a PIP degron at its N-terminus through which it interacts with both Cdt2 and PCNA, while Set8's PIP box is more centrally located. Failure to degrade either Cdt1 or Set8 at the onset of S phase results in multiple rounds of origin firing, or rereplication, that causes DNA damage and genome instability (112,122,179,190-195).

Recently we have suggested that Cdt1 is phosphorylated by stress-induced mitogen-activated protein kinases (MAPK) (104,196). Addition of cellular stressors, such as salt, lipopolysaccharide (LPS), or tumor necrosis factor alpha (TNF-α), activate the stress MAP kinases Jun N-terminal kinase (JNK) and p38 (197). Our lab mapped five residues in the C-terminal third of Cdt1 that are phosphorylated by both JNK and p38 (196). Both the JNK and p38 stress MAP kinases phosphorylate these amino acids not only in response to exogenous stress, but also during unperturbed G2 and M phases when JNK and p38 are normally active. Phosphorylated Cdt1 is often detected as a slower-migrating isoform that is present during G2/M, cellular quiescence, or upon activation of stress MAPKs. However, phosphomimetic mutation of the five C-terminal phosphorylated residues of Cdt1 ($Cdt1_{2E3D}$) cannot complement knockdown of endogenous Cdt1 due to impaired MCM loading (196).

Cdt1 is phosphorylated during mitosis and our lab has shown that Cdt1 is required at kinetochores for proper microtubule attachment (198). Cdt1 must be dephosphorylated upon mitotic exit to promote origin licensing during G1 because C-terminal phosphorylation of Cdt1 impairs MCM loading (196). Another

phosphorylated residue also negatively impacts origin licensing. Cdt1 phosphorylation at T29 by JNK impairs HBO1 association with replication origins (104). In contrast to T29 phosphorylation by Cdk2 (199), JNK-mediated phosphorylation of this site has no effect on Skp2 binding or Cdt1 stability (104). Our lab has shown that JNK phosphorylates at least five C-terminal residues, so perhaps these additional sites prevent association of Cdt1 with Skp2 leading to what appears to be two different effects of T29 phosphorylation.

Set8 degradation is also influenced by phosphorylation. Set8 is phosphorylated by Cdk1 at S29 during mitosis, and phosphorylation at this site protects Set8 from $APC^{Cdh1}$-mediated proteolysis (123). This modification must be removed by the Cdc14 phosphatase to degrade Set8 and allow timely progression through mitosis (123). Whether this phosphorylation or novel phosphorylation sites also protect Set8 from $CRL4^{Cdt2}$-mediated degradation is the focus of this study.

Here, we investigate how $CRL4^{Cdt2}$-mediated degradation of Cdt1 and Set8 is regulated in response to cellular stress and cell cycle position. We have observed that both Set8 and Cdt1 are protected from UV-induced degradation during a cellular stress response and distinct cell cycle phases. We will determine if phosphorylation of these substrates under these conditions is the mechanism preventing their degradation and identify the kinase(s) responsible. If phosphorylation is protective, does it prevent association of these substrates with the E3 ligase? If so, does this regulatory mechanism extend to all known $CRL4^{Cdt2}$ substrates? Additionally, what are the biological consequences if this

protection is disrupted? We provide evidence that Cdt1 is protected from UV-induced, Cdt2-dependent degradation during mitosis and cellular quiescence while Set8 is only protected during mitosis. Further, we show that protection during mitosis can be reversed upon inhibition of Cdk1. We continue to investigate the mechanism(s) and significance of this protection for maintaining genome integrity.

## MATERIALS AND METHODS

### Cell culture and manipulations

HCT116, HeLa, T98G, and NHF-hTERT cells were cultured in Dulbecco modified Eagle medium (DMEM) (Difco) supplemented with 10% fetal calf serum (Sigma). Cells were synchronized in prometaphase by treatment with 2 mM thymidine for 18 h followed by release into 100 nM nocodazole for 10 h (or 12 h for T98G cells). T98G human glioblastoma cells were serum starved (DMEM only) for 48 h to promote cell cycle exit into quiescence (or $G_0$). NHF cells were grown in DMEM supplemented with 0.05% serum for 72 h to promote cell cycle exit. Cellular stress was induced by the addition of 250 mM NaCl 15 min prior to induction of DNA damage either by UV irradiation (20 J/$m^2$) or addition of 100 μM $H_2O_2$. MAP kinase inhibitors were used at the following concentrations: c-Jun N-terminal kinase (JNK) inhibitor II (SP600125; 100 μM), JNK inhibitor VIII (10 μM), and p38 (SB203580; 30 μM). The Cdk1 inhibitor, RO-3306 (Sigma), was used at 10 μM and the CDK inhibitor roscovitine was used at 25 μM. Cells were transfected with plasmids using 0.05% polyethylenimine (Sigma). Small interfering RNA (siRNA) transfections were performed with 100 nM each of

siRNA duplex using Dharmafect 1 reagent (Dharmacon) according to the manufacturer's guidelines for depletion of luciferase (control) and p38α/β. JNK1/2 siRNA was used at 250 nM. Synthetic duplexed RNA oligonucleotides were synthesized by Invitrogen; their sequences are provided in Table 3.1.

**Antibodies**

Antibodies were purchased from the following sources: p38, phospho-p38, Jun N-terminal kinase (JNK), phospho-JNK, MAPKAP kinase 2, phospho-MAPKAP kinase 2, and Set8 from Cell Signaling Technologies; hemagglutinin (HA) from Roche; green fluorescence protein (GFP) (JL-8) from Clonetech; alpha tubulin (DM1A) from Sigma; Cdt2 antibody was a gift from A. Dutta. Antibodies to human Cdt1 have been previously described (49).

**Plasmids and protein lysate preparation**

Plasmids for transient transfection were generated by recombination (Gateway LR clonase; Invitrogen) of pENTR-Set8 derivatives with a Gateway-compatible pLX302 lentiviral vector. Whole-cell lysates were prepared in CSK buffer (200) (supplemented with protease and phosphatase inhibitors) and containing S7 micrococcal nuclease and $CaCl_2$ to release chromatin-bound material into the soluble pool and clarified by centrifugation. Whole-cell lysates were alternatively prepared by direct lysis of equal cell numbers in 2X Laemmli sample buffer with 10% β-mercaptoethanol.

**Table 3.1 siRNA sequences used in this study**

| Target | Sequence | Reference |
|---|---|---|
| Luciferase | 5'-UCGAAGUACUCAGCGUAAG-3' | This study |
| p38α | 5 '-GCAUUACAACCAGACAGUUGAUAUU-3' | (196) |
| p38β | 5'-CAACCACCAGGUGUCAAAUGAGAAA-3' | (196) |
| JNK1/2 | 5′-TGAAAGAATGTCCTACCTT-3′ | (224) |

## RESULTS

### Set8 and Cdt1 are protected from CRL4$^{Cdt2}$-dependent degradation in the presence of cellular stress.

Degradation of Cdt1 and Set8 after DNA damage is dependent on the E3 ubiquitin ligase CRL4$^{Cdt2}$. We have previously shown that these proteins can be protected from CRL4$^{Cdt2}$-mediated degradation under conditions of cellular stress and recapitulated those results here (Figure 3.1) (196). Addition of an osmoticstressor, in this case NaCl, results in stabilization of both Cdt1 and Set8 after UV irradiation or $H_2O_2$ treatment (Figure 3.1A and B). Our lab previously reported that this stabilization of Cdt1 was due to phosphorylation by JNK and p38 stress-activated MAPKs; inhibition of both kinases restored Cdt1 (and Set8) degradation after UV irradiation (196). Treatment with these inhibitors also reversed the mobility shift representative of phosphorylated Cdt1. To explore the relationship between stress MAPK activity and degradation of Set8 and Cdt1, HCT116 cells were treated with siRNAs against p38α/β and JNK1/2 both separately and in combination (Figure 3.1C). After inducing osmotic stress, we irradiated cells with UV to induce the degradation of CRL4$^{Cdt2}$ substrates. When cells were treated with a control siRNA (Figure 3.1C; lanes 1 and 2), both Cdt1 and Set8 were degraded after UV irradiation. Prior osmotic stress (Figure 3.1C; lane 3) prevented this degradation as was previously seen with sorbitol pretreatment. This protection is reversed upon addition of both p38α/β and JNK1/2 siRNAs (Figure 3.1C; lane 4), but not with either siRNA alone (Figure 3.1C; lanes 5 and 6). Although difficult to discern, Set8 is still present in lanes 5

**Figure 3.1**

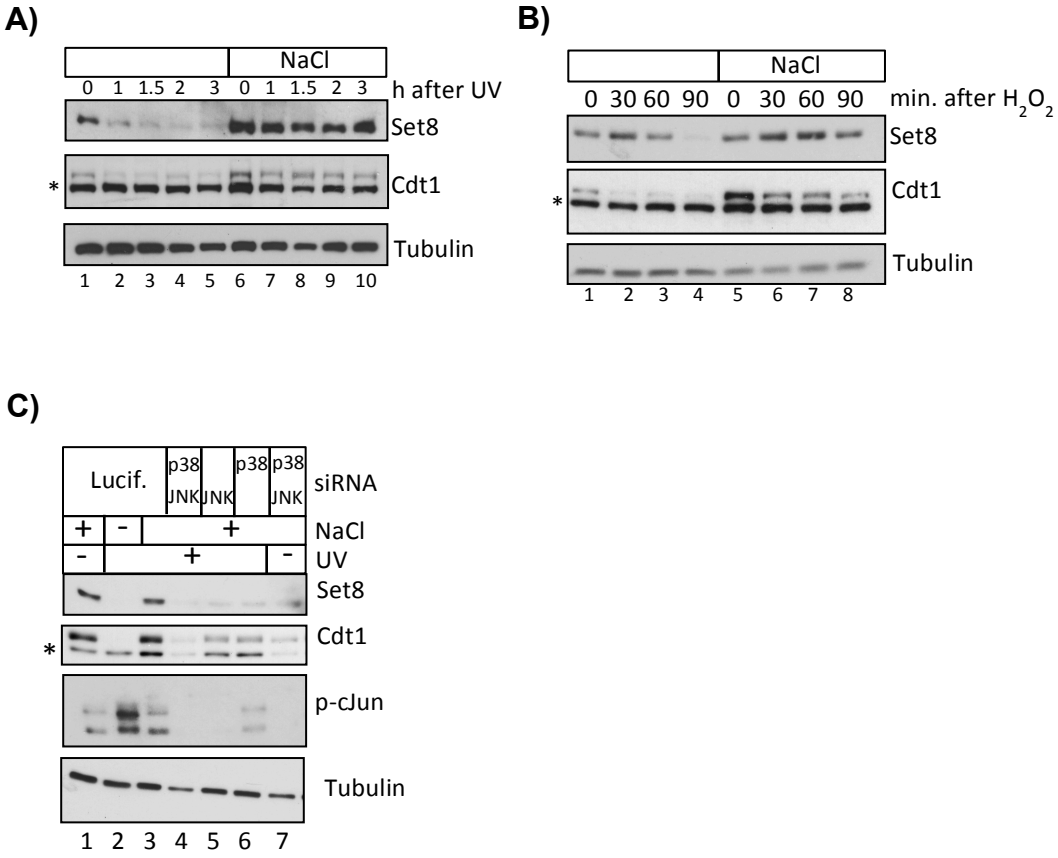
A)
NaCl
0 1 1.5 2 3 0 1 1.5 2 3
h after UV
Set8
*
Cdt1
Tubulin
1 2 3 4 5 6 7 8 9 10
B)
NaCl
0 30 60 90 0 30 60 90
min. after H2O2
Set8
*
Cdt1
Tubulin
1 2 3 4 5 6 7 8
C)
Lucif.
p38
JNK
JNK
p38
p38
JNK
siRNA
+ - +
NaCl
- + -
UV
Set8
*
Cdt1
p-cJun
Tubulin
1 2 3 4 5 6 7

**Figure 3.1. Cellular stress prevents the DNA damage-induced degradation of Set8 and Cdt1.** A) Both Set8 and Cdt1 are degraded after UV treatment, but this degradation is prevented when 250 mM NaCl is added to HCT116 cells. B) They are also degraded when DNA damage is induced by 100 nM $H_2O_2$. This is again reversed by the addition of 250 mM NaCl. C) HCT116 cells were transfected with control small interfering RNA (siRNA) targeting Luciferase (lanes 1-3), siRNA molecules targeting p38α and p38β (lane 6), JNK1/2 (lane 5), or all four stress-activated MAPK isoforms (lanes 4 and 7) for 52 h and then treated with 250 mM NaCl 15 min prior to UV irradiation as indicated. Endogenous Set8, Cdt1, phoshpo-cJun, phospho-MAPKAPK2, p38α/β, JNK1/2 were detected by immunoblotting. The p38α and p38β isoforms are recognized by the same antibody and migrate together by SDS-PAGE. The * denotes non-specific background bands.

and 6 while completely absent in lane 4. However, treatment with siRNAs to both MAP kinases caused a reduction in the Set8 and Cdt1 levels in the absence of UV irradiation (Figure 3.1C; lane 7), but not to the same degree as treatment with both siRNAs with UV (Figure 3.1C; lane 4) or the absence of osmotic stress (Figure 3.1C; lane 2). Further experiments using different siRNA conditions should resolve this ambiguity as these siRNAs have been utilized previously without affecting Cdt1 protein levels (196). These data suggest that, in asynchronous cells, both p38α/β and JNK1/2 can contribute to the stabilization of Cdt1 and Set8.

**Set8 and Cdt1 are protected from CRL4$^{Cdt2}$-mediated degradation in nocodazole-arrested cells.**

Given that Set8 and Cdt1 are both phosphorylated during mitosis and that phosphorylation can prevent ubiquitin-mediated degradation of Cdt1 and Set8, we investigated whether Cdt1 and Set8 were protected from CRL4$^{Cdt2}$-mediated degradation during mitosis (Figure 3.2) (123,196). JNK, p38, and Cdk1 are all active during mitosis. In asynchronous cells, Cdt1 and Set8 are degraded after UV irradiation as expected (Figure 3.2A; compare lanes 1 and 2), but surprisingly both were protected from degradation in nocodazole-arrested cells (Figure 3.2A; compare lanes 3 and 4). This observation was initially made by J. Hall and K. Coleman (unpublished observations). As previously reported, phosphorylated Cdt1 migrates slower on the gel and this isoform is present during the nocodazole arrest (Figure 3.2A; compare lanes 1 and 3). Two hours after release

**Figure 3.2**

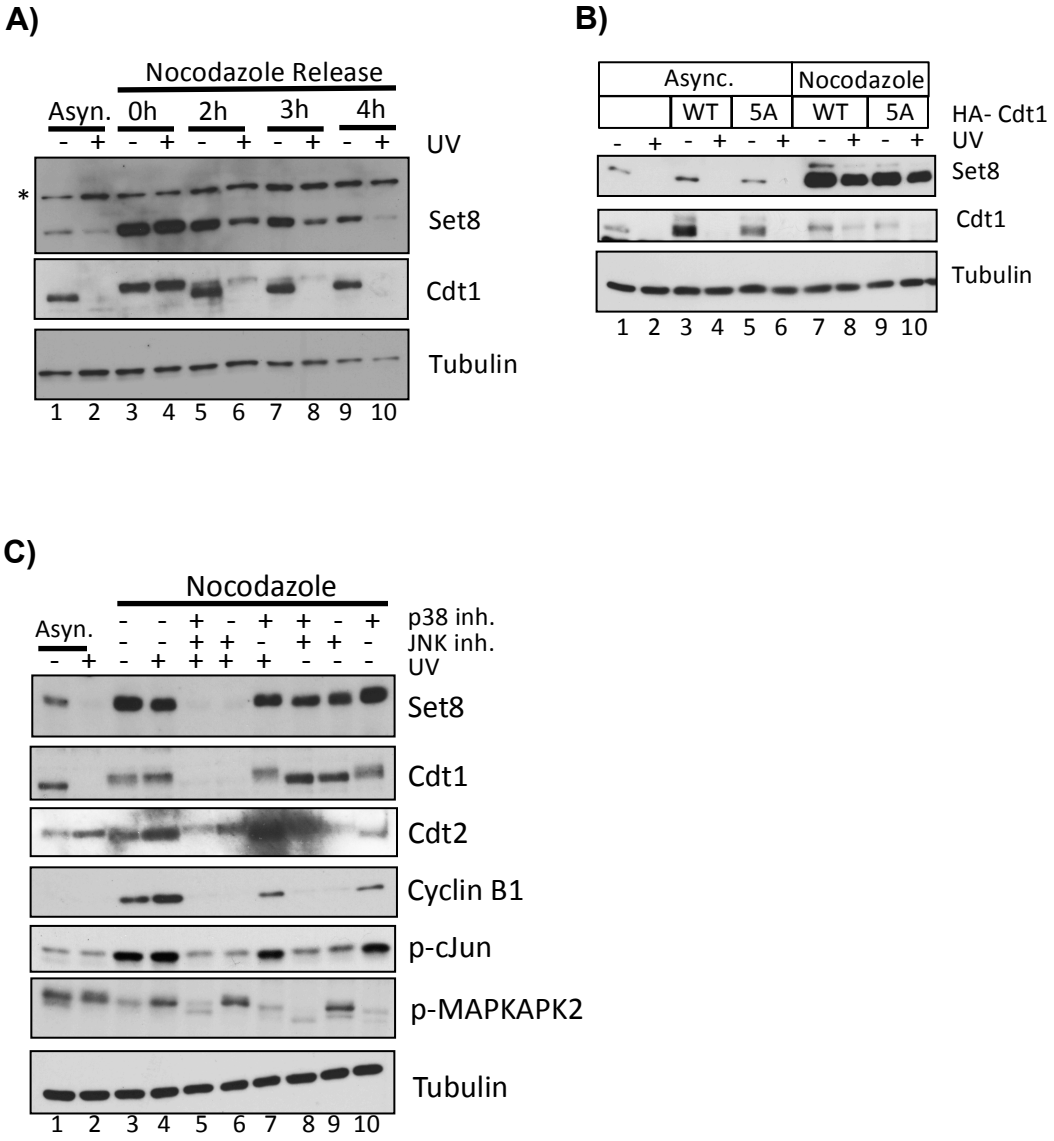
A)
Nocodazole Release
Asyn.
0h
2h
3h
4h
UV
*
Set8
Cdt1
Tubulin
1 2 3 4 5 6 7 8 9 10
B)
Async.
Nocodazole
WT
5A
WT
5A
HA- Cdt1
UV
Set8
Cdt1
Tubulin
1 2 3 4 5 6 7 8 9 10
C)
Nocodazole
Asyn.
p38 inh.
JNK inh.
UV
Set8
Cdt1
Cdt2
Cyclin B1
p-cJun
p-MAPKAPK2
Tubulin
1 2 3 4 5 6 7 8 9 10

**Figure 3.2. Set8 and Cdt1 are protected from UV-induced degradation in G2/M.** A) HeLa cells were arrested in nocodazole, released into fresh media, and irradiated with UV at the indicated times. Cells were harvested 1 h after UV irradiation. B) Asynchronous and nocodazole arrested T98G cells carrying HA-tagged wild-type Cdt1 or $Cdt1_{5A}$ were UV irradiated and harvested after 2 h. C) HCT116 cells were arrested in nocodazole. Cycloheximide and MAPK inhibitors were added 15 min before UV irradiation. (The p38 inhibitor was SB203580, and the JNK inhibitor was SP600125.) Cells were harvested 90 min after UV irradiation.

from nocodazole, the majority of Cdt1 detected is in the unphosphorylated form (Figure 3.2A; lane 5, lower band) and is degraded after UV irradiation (Figure 3.2A; lane 6) while the phosphorylated upper band can still be detected until 3-4 h after release from nocodazole even after treatment with UV (Figure 3.2A; lanes 7 – 9). Similarly, Set8 also becomes susceptible to degradation 2 h after release from nocodazole (Figure 3.2A; compare lanes 9 and 10). These results demonstrate that both Set8 and Cdt1 are again subject to UV-induced degradation upon exiting mitosis. Further experiments comparing the timing of degradation with activation of JNK, p38, and CDK are ongoing.

We previously mapped five phosphorylation sites on Cdt1 that were required for MAPK-mediated protection from UV-induced degradation (196). To confirm that the E3 ligase $CRL4^{Cdt2}$ was still present and active in nocodazole-arrested cells, we mutated all five of these residues to alanine ($Cdt1_{5A}$) rendering Cdt1 susceptible to degradation. We compared the degradation of this HA-tagged $Cdt1_{5A}$ with HA-tagged $Cdt1_{WT}$ (Figure 3.2B). Both constructs are degraded in asynchronous cells after UV irradiation (Figure 3.2B; lanes 4 and 6). In nocodazole-arrested cells, $Cdt1_{5A}$ is degraded while $Cdt1_{WT}$ is protected from degradation (Figure 3.2B; compare lanes 8 and 10). This result indicates that the $CRL4^{Cdt2}$ E3 ligase is still functional in nocodazole-arrested cells and that these substrates are protected during this cell cycle stage. This data indicates that phosphorylation at these five sites is sufficient to protect Cdt1 from $CRL4^{Cdt2}$-mediated degradation. Set8 is known to be phosphorylated at serine 29 during mitosis (123,201), but we have yet to determine if this phosphorylation is

sufficient to protect Set8 from UV-induced degradation. We hypothesize that there could be additional phosphorylated residues that are required for Set8 stabilization. One potential MAPK and/or CDK phosphorylation site is T110; we have mutated both T110 and S29 to either alanine or aspartic acid and experiments with these mutants are ongoing. If our hypothesis is correct, then mutating these sites to alanine to prevent phosphorylation should result in UV-induced degradation of Set8 during mitosis.

Given that phosphorylation of Cdt1 by stress MAPKs protects it from UV-induced, $CRL4^{Cdt2}$-mediated degradation (196), we hypothesized that phosphorylation by MAPKs also occurs during mitosis to protect Cdt1 from degradation. Set8 is phosphorylated during mitosis as well, but Cyclin B-Cdk1 is the only kinase currently known to phosphorylate Set8. Because JNK and p38 are both active during mitosis, we hypothesize that Cdt1 and Set8 are both regulated by MAPKs during this cell cycle phase. Not surprisingly, the protection of both Cdt1 and Set8 observed in nocodazole-arrested cells can be reversed upon pharmacological inhibition of MAPK activity (Figure 3.2C). Inhibiting both p38 and JNK activity (Figure 3.2C; lane 5) as well as inhibiting JNK alone (Figure 3.2C; lane 6) is sufficient to render Cdt1 and Set8 susceptible to UV-mediated degradation. This result is in contrast to what we observed in asynchronous cells treated with osmotic stress in which p38 and JNK were both required for full protection following UV irradiation (Figure 3.1C). These data suggest that during mitosis, only JNK is responsible for regulating the stability of Set8 and Cdt1. One

caveat to this interpretation is that the findings may be a consequence of off-target effects caused by treatment with JNK inhibitor II (a.k.a. SP600125). In addition to inhibiting JNK activity, JNK inhibitor II acts upstream of Aurora-A kinase and polo-like kinase 1 (Plk1) to prevent Cdk1 activation (202). When CDK activity is inhibited in mitosis, $APC^{Cdh1}$ becomes active and degrades Cyclin B. When we treat our cells with JNK inhibitor II, Cyclin B is lost suggesting this off-target effect occurs in our hands as well (Figure 3.2C; lanes 5, 6, 8, and 9). To address the issues with JNK inhibitor II, we are currently performing siRNA treatments in nocodazole-arrested cells to determine if there is a role for JNK in protecting Cdt1 and Set8 from UV-induced degradation. Additionally, we are conducting experiments using another more specific JNK inhibitor, JNK inhibitor VIII. Due to the off-target effects of JNK inhibitor II, our current data suggest that during mitosis, either JNK or CDKs are acting upon Cdt1 and Set8 (either directly or indirectly) to protect them from UV-induced degradation.

**Inhibition of Cdk1 activity, rather than JNK, reverses Cdt1 and Set8 protection from UV-induced degradation in nocodazole-arrested cells.**

Given the off-target effects of the JNK inhibitor II, we utilized the CDK inhibitor roscovitine to determine if the effects on Cdt1 and Set8 degradation were due to Cdk1 inactivation. We can phenocopy both loss of Cyclin B and degradation of $CRL4^{Cdt2}$ substrates by treating nocodazole-arrested cells with roscovitine (known to cause mitotic exit) (Figure 3.3A; compare lanes 6 and 8). These data indicate that inhibition of CDK activity, rather than JNK activity, is sufficient to render Cdt1 and Set8 sensitive to UV-mediated degradation.

**Figure 3.3**

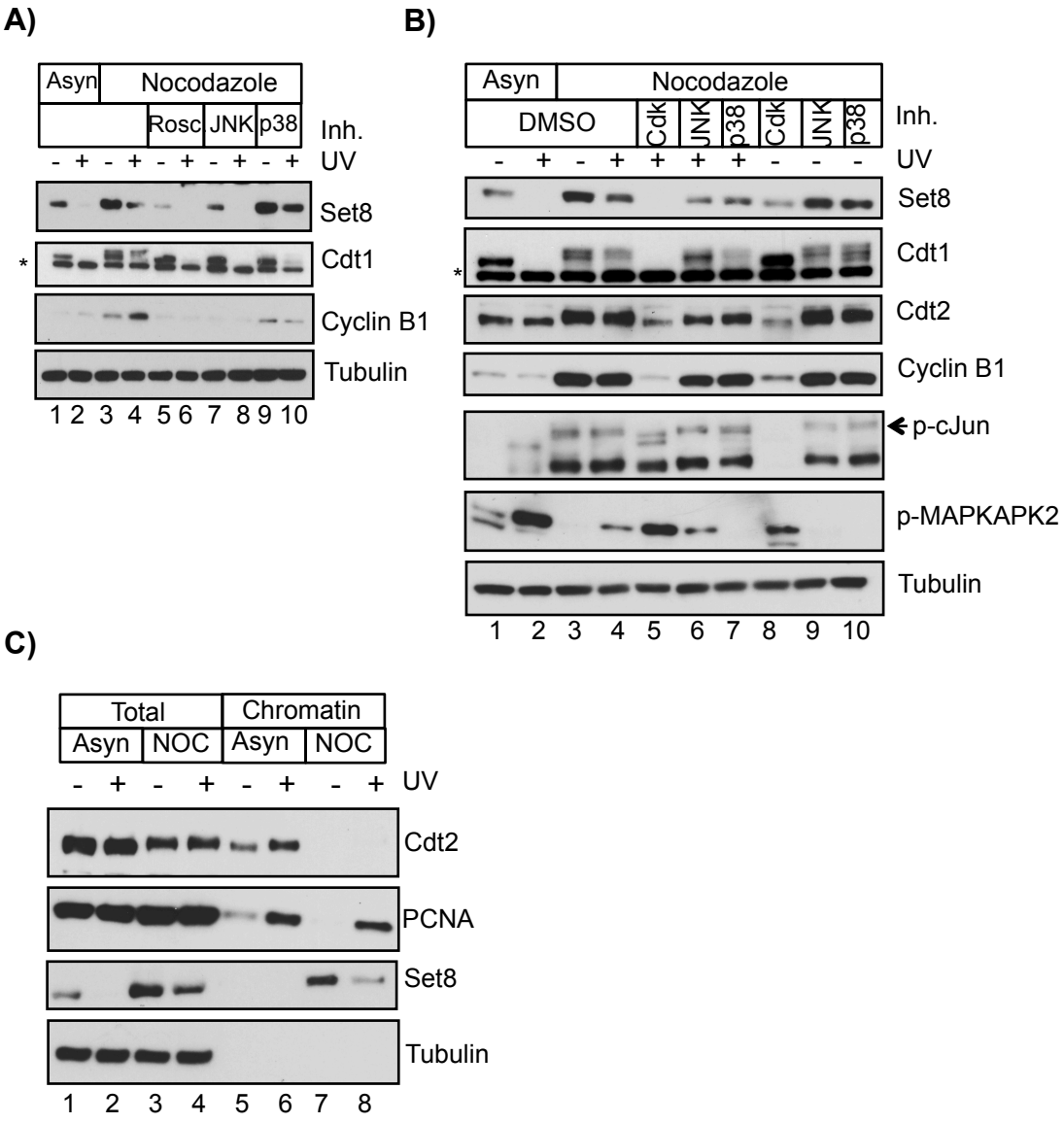
A)
Asyn
Nocodazole
Rosc
JNK
p38
Inh.
- + - + - + - + - + UV
Set8
Cdt1
*
Cyclin B1
Tubulin
1 2 3 4 5 6 7 8 9 10
B)
Asyn
Nocodazole
DMSO
Cdk
JNK
p38
Cdk
JNK
p38
Inh.
- + - + + + + - - - UV
Set8
Cdt1
*
Cdt2
Cyclin B1
p-cJun
p-MAPKAPK2
Tubulin
1 2 3 4 5 6 7 8 9 10
C)
Total
Chromatin
Asyn
NOC
Asyn
NOC
- + - + - + - + UV
Cdt2
PCNA
Set8
Tubulin
1 2 3 4 5 6 7 8

**Figure 3.3. Cdk1, not JNK, is required for protection of Set8 and Cdt1 after UV irradiation in nocodazole-arrested cells.** A) HCT116 cells were arrested in nocodazole and treated with roscovitine, JNK inhibitor II, or p38 inhibitor (SB203580) for 15 min prior to UV irradiation. Cells were harvested 2 h after irradiation. The * denotes a non-specific background band in both A and B. B) HCT116 cells were arrested in nocodazole and treated with Cdk1 inhibitor (RO-3306), JNK inhibitor VIII, or p38 inhibitor (SB203580) for 30 min prior to UV irradiation. Cells were harvested 2 h after irradiation. The arrow indicates the phosphorylated cJun band. C) HCT116 cells were arrested in nocodazole and harvested 2 h after UV irradiation.

Recently, Cyclin B-Cdk1 and Cyclin A-Cdk2 were shown to phosphorylate Cdt2 at T464 during mitosis and protect it from $SCF^{FBXO11}$-mediated degradation (203,204). When cells were treated with roscovitine or a Cdk1-specific inhibitor (RO-3306), the half-life of Cdt2 was greatly reduced. In contrast to these other reports, Cdt2 levels were at best modestly and inconsistently reduced in our cells upon addition of JNK inhibitor II (Figure 3.2C; lanes 5 and 9). However, this reduction in Cdt2 abundance was not sufficient to impede the degradation of Set8 or Cdt1.

To determine if the protection of Set8 and Cdt1 in nocodazole-arrested cells was due solely to Cdk1 activity, we treated nocodazole-arrested cells with the Cdk1 inhibitor, RO-3306, 30 min prior to UV irradiation (Figure 3.3B). Treatment with this drug sensitized Cdt1 and Set8 to degradation in mitosis to the same extent as treatment with either JNK inhibitor II or roscovitine (Figure 3.3B; lane 5). To confirm that JNK activity was not involved, we treated with a more specific JNK inhibitor, JNK inhibitor VIII, and did not see any degradation of Cdt1 or Set8 after UV (Figure 3.3B; lane 6). However, upon examination of phosphorylated cJun levels, JNK inhibitor VIII failed to inhibit JNK activity in this experiment (Figure 3.3B; lanes 5 and 8). As the inhibitor was initially validated in our lab using cells treated with osmotic stress, adjustments to the timing of inhibitor addition may need to be made in nocodazole-arrested cells. Further experiments are currently underway to determine the importance Cdk1 activity, either directly or indirectly, for $CRL4^{Cdt2}$ substrate stability during mitosis.

Additionally, we will repeat our experiments in nocodazole-arrested cells and asynchronous cells plus osmotic stress using JNK inhibitor VIII to determine if JNK activity is important for Cdt1 and Set8 stabilization under those circumstances. Pending these additional results, it remains clear that Cdk1 activity is either directly or indirectly involved in regulating the stability of $CRL4^{Cdt2}$ substrates.

The most obvious possibility for why Set8 and Cdt1 fail to be degraded after UV during mitosis is failure to interact with the $CRL4^{Cdt2}$ E3 ligase. We have already demonstrated that Cdt2 is present in nocodazole-arrested cells (Figures 3.3C and 3.3B); however, we wanted to confirm that Cdt2 could be recruited to chromatin in response to DNA damage during this cell cycle phase. Cells arrested in mitosis were irradiated with UV, harvested 2 h later, and the chromatin was isolated. Cdt2 and PCNA are clearly present in both asynchronous and mitotic cells (Figure 3.3C; lanes 1-4). Additionally, PCNA is loaded onto chromatin in both asynchronous and mitotic cells after UV irradiation (Figure 3.3C; lanes 5-8). Surprisingly, Cdt2 is not properly recruited to chromatin in nocodazole-arrested cells following UV irradiation. This result could be indicative of a failure to interact with its substrates during this cell cycle phase. We are currently conducting immunoprecipitation experiments to test this possibility. We hypothesize that mitotic kinases, likely Cdk1, phosphorylates either Cdt2 to inhibit its activity, or the $CRL4^{Cdt2}$ substrates to prevent their interaction with Cdt1. These possibilities are not mutually exclusive and could both be in effect simultaneously.

**Differential regulation of Cdt1 and Set8 during quiescence.**

Clearly phosphorylation of at least one $CRL4^{Cdt2}$ substrate influences its degradation and it is likely that this regulatory mechanism is conserved for many if not all $CRL4^{Cdt2}$ substrates. While our work has focused predominately on mitosis, Cdt1 is also phosphorylated during quiescence as evidenced by the slower migrating phospho-Cdt1 band (196). Interestingly, quiescence is another cell cycle phase that has high levels of p38, but unlike mitosis, JNK and Cdk1 activities are low (Figure 3.3; p-cJun levels indicative of JNK activity) (205,206). We have utilized two cell lines in this investigation, T98G cells (glioblastoma line) and NHF-hTERT cells (normal human fibroblasts). T98G cells lack detectable levels of endogenous Cdt1 in quiescence so we created a T98G cell line stably expressing Cdt1. Previous work in the lab has shown that in these T98G cells, Cdt1 is protected from UV-induced degradation (K. Coleman; unpublished observation). To further investigate this phenomenon, we examined Cdt1 and Set8 stability after UV irradiation in NHF-hTERT cells arrested in quiescence by serum starvation (Figure 3.4). This cell line does express detectable levels of Cdt1 during quiescence. Interestingly, Cdt1 is protected from degradation as previously reported, but Set8 is not. Previous reports utilizing serum-starved HaCaT and RPE1-hTERT cells indicate that Cdt2 is degraded shortly after serum removal by $SCF^{FBXO11}$ (203). However, this is likely not the case in our quiescent NHF cells (Figure 3.4) because Set8 is degraded after UV irradiation. It is possible that another E3 ubiquitin ligase complex is responsible for Set8 degradation, and we are currently conducting experiments to assess if Cdt2 is functional during quiescence. The mechanism by which Cdt1 is protected from

**Figure 3.4**

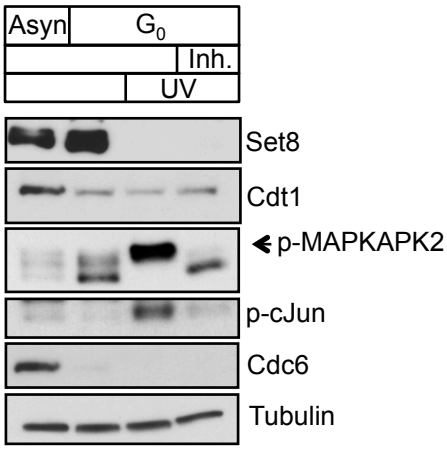

**Figure 3.4. Cdt1 is protected from UV-induced degradation during $G_0$, but Set8 is not.** NHF cells were serum deprived in DMEM plus 0.05% FBS for 72 h. JNK (SP600125) and p38 (SB203580) inhibitors were added 15 min prior to UV irradiation, and cells were harvested 2 h after irradiation. The arrow indicates the phosphorylated form of MAPKAPK2.

degradation is unclear. P38 is active during this phase (as indicated by p-MAPKAPK2); however, treatment with p38 and JNK inhibitors did not result in Cdt1 degradation after UV (Figure 3.4). This finding raises the possibility that another mechanism functions specifically during this cell cycle phase to stabilize Cdt1 but not Set8. Experiments are currently underway to determine the mechanism of this highly specific stabilization.

**DISCUSSION**

The data described above clearly demonstrate that phosphorylation mediates stability of the $CRL4^{Cdt2}$ substrate Cdt1, and likely Set8, during specific cell cycle stages and in response to cellular stress. The work presented here raises some very interesting questions regarding the regulation of preRC components outside of G1 and S phases. Why it is beneficial for the cell to protect these proteins from degradation when their presence outside of G1 could allow rereplication to occur? Clearly rereplication does not occur in an unperturbed cell cycle so there must be safeguards in place to restrict the origin licensing capabilities of these substrates. We know this is the case for Cdt1 because the $Cdt1_{2E3D}$ phosphomimetic mutant cannot fully complement preRC formation when endogenous Cdt1 is knocked down yet, this mutant is protected from UV-dependent degradation (196). However, it is unclear at this time if Set8 is able to perform its origin licensing function when phosphorylated. Phosphorylation of Set8 at serine 29 by Cdk1 has no effect on the ability of Set8 to methylate H4 (123). While we have not definitively shown that phosphorylation

at this site is protecting Set8 from $CRL4^{Cdt2}$-mediated degradation, those experiments are underway.

Cdt1 and Set8 are protected from UV-induced degradation during mitosis, but for Set8, it is unclear why this protection at this cell cycle phase is necessary or favorable for the cell. In the case of Cdt1, we know it has an important role during mitosis to promote stable microtubule-kinetochore attachments (198). Our lab has shown that depletion of Cdt1 during prometaphase results in an arrest at this phase of mitosis. The importance of Set8 stability during mitosis is not quite as clear. Set8 and H4K20me1 are required for chromosome condensation during G2/M (111). H4K20me1 is recognized and bound by condensin II, which is required for chromatin condensation during mitosis (124). Additionally, L3MBTL1 specifically recognizes H4K20me1 and is also important for chromatin compaction (114). However, it is unclear if *de novo* H4K20 methylation by Set8 is required or if Set8 plays another as yet uncharacterized role in mitosis.

There are several possibilities regarding the requirement for Set8 and/or H4K20me1 in mitosis. First, if we consider the need for H4K20me1 for chromatin compaction during G2/M, there are two ways to obtain this modification. The first mechanism, of course, is through *de novo* methylation by Set8 that has accumulated during G2. The second is through demethylation of the higher methylation states, H4K20me2/3, that were deposited as cells transitioned from G1 to S phase. In 2012, PHF2 was identified as the demethylase for H4K20me3; it was previously shown to demethylate H3K9me2 (207). PHF2 activity is dependent on phosphorylation; protein kinase A is the only kinase to date known

to phosphorylate PHF2 and it does so at four C-terminal sites (S757, 899, 954, and 1056) with S1056 being the major site of phosphorylation (208). While PKA is not cell cycle regulated, there could be other as yet unidentified mechanisms regulating PHF2 activity. Interestingly, PHF2 was identified in a global mass spectrometry study as being phosphorylated during mitosis at S37/39 and T41 (motif: **S**A**S**P**T**IP) (201). Cdk1 could be phosphorylating these sites during mitosis since the phosphopeptide most frequently detected was the $S_{39}$/P site. A potential model for restoring H4K20me1 after S phase could involve both increased Set8 levels to promote *de novo* methylation as well as activation of the PHF2 demethylase to remove the H4K20me3 (and perhaps me2) that was deposited at the G1/S transition. It would be interesting to determine if PHF2 protein and/or activity levels are cell cycle regulated and to determine which kinase is responsible for PHF2 activation during mitosis. We are currently conducting experiments to prevent Set8 accumulation only during G2/M and assess the impact on both mitosis and the subsequent G1 phase. It is also possible that Set8 methylates some as yet unidentified protein important for mitotic exit and/or origin licensing in the subsequent G1 phase.

Despite our previous data demonstrating that p38 and JNK phosphorylate Cdt1 in response to osmotic stress, it is still unclear which kinases actually protect Cdt1 and Set8 from proteolytic degradation. The data presented here indicated that JNK rather than p38 is a likely candidate. However, due to the inhibition of Cdk1 activity with JNK inhibitor II, our previously published results about he role of JNK and p38 in regulating Cdt1 during G2/M must be

reinterpreted (196). Current efforts have been unsuccessful in reversing the Cdt1 mobility shift with p38 and the more specific JNK inhibitor VIII, but treatment with the Cdk1 inhibitor RO-3306 is sufficient to reverse the Cdt1 mobility shift (Figure 3.3B; lane 8). These data suggest that both during nocodazole-arrest and after osmotic stress, Cdt1 is phosphorylated in a Cdk1-dependent manner with no direct contribution from JNK or p38 kinases. However, it must be noted that our previous publication reported that upon treatment with siRNAs against JNK and p38, the Cdt1 mobility shift *was* reversed; although, this result was found in asynchronous cell treated with sorbitol and not mitotic cells (196). Efforts to perform this experiment in synchronized cells are underway.

Interestingly, treatment with p38 and JNK inhibitor VIII either singly or in combination failed to restore Cdt1 (or Set8) degradation after UV irradiation (data not shown). In contrast, treatment with the Cdk1 inhibitor is sufficient to render both Set8 and Cdt1 susceptible to UV-induced degradation (Figure 3.3B). Therefore, while it is clear that Cdk1 is important for protecting Cdt1 and Set8 from degradation during mitosis, we still need to determine if this is a direct consequence of Cdk1 phosphorylation or an indirect effect. Interestingly, Cdt2 levels are slightly reduced upon treatment with the Cdk1 inhibitor (Figure 3.3B; lanes 8 and 5). However, Set8 and Cdt1 are still degraded suggesting that this level of Cdt2 is sufficient for their degradation, or that some other degradation mechanism is involved. Performing this experiment with the Cdk1 inhibitor in the presence of Cdt2 siRNA would resolve this matter; if another mechanism were involved, knock down of Cdt2 would have no effect on the degradation of Cdt1

and Set8.

Cdk1 is known to phosphorylate both Cdt1 and Set8 (123,209). Cdt1 can be phosphorylated by Cdk1 or Cdk2 in conjunction with Cyclin A, primarily in S and G2 phases; this phosphorylation promotes Cdt1 degradation by $SCF^{Skp2}$. Phosphorylation by Cyclin B-Cdk1 is restricted to mitosis and while ablation of Cyclin A in *Drosophila* leads to rereplication, loss of Cyclin B does not (210). Set8 is also phosphorylated at serine 29 by Cyclin B-Cdk1, and this modification protects Set8 from degradation by $APC^{Cdh1}$. As cells enter G1 phase, Cdc14 phosphatase removes S29 phosphorylation to allow Set8 degradation (123). We hypothesize that phosphorylation at this site also protects Set8 from $CRL4^{Cdt2}$-mediated degradation and experiments are underway to test this hypothesis.

Perhaps the most interesting finding of this study is that Set8 and Cdt1 are regulated differently during quiescence. Cells enter quiescence after failing to pass the restriction point during G1. During this cell cycle stage, CDK activity is low and $APC^{Cdh1}$ is active. We have found that Cdt1, but not Set8, is protected from degradation after UV irradiation in quiescent cells. This result raises two main questions. The first question is why Set8 is present during quiescence. Presumably, if phosphorylation at S29 protects Set8 from $APC^{Cdh1}$-mediated degradation then, when Cdc14 dephosphorylates this site as cells exit mitosis, Set8 should be degraded and therefore absent during quiescence. Yet, Set8 is clearly present. This could be the result of Cdc14-resistant mitotic Set8 phosphorylation by a kinase other than Cdk1. Set8 would then be protected from $APC^{Cdh1}$, but not from $CRL4^{Cdt2}$ as cells entered quiescence. The second

question is why Set8 is protected from DNA damage-induced degradation during mitosis, but not during quiescence. Several hypotheses have been put forth as to why Set8 is degraded after DNA damage in any cell cycle stage (reviewed in reference 211). Since Set8 is known to be involved in chromatin compaction, it could restrict access to the damaged DNA if not degraded. Alternatively, it is known to methylate p53 and prevent transcription of p53 target genes such as p21 that are needed for halting the cell cycle after DNA damage. It is possible that Set8 is required during quiescence to prepare for G1 phase; yet in the presence of DNA damage it needs to be degraded so as not to inappropriately initiate DNA replication. It is clear that further investigations are required to gain a better understanding of the regulation of Set8 and Cdt1 in this cell cycle phase.

# CHAPTER 4

## Conclusions and Unanswered Questions

Based on our work in *S. cerevisiae*, it is clear that di-methylation of H3K4 by Set1 (as part of the COMPASS complex) is required for efficient origin activity. Loss of this modification results in decreased origin firing and sensitivity to replication stress. Although the mechanism by which H3K4me2 promotes origin function is unclear, there are two likely possibilities that are not mutually exclusive. The first is that H3K4me2 is recognized and bound by a replication factor (such as a preRC component or PIC member). Another possibility is that H3K4me2 recruits another histone modifying enzyme that helps to create a chromatin structure favorable to origin activation. Interestingly, in human cells tri-methylation of H3K4 by MLL1 is essential for HBO1 binding and subsequent H4 hyperacetylation at the lamin B2 origin during G1 (99). We have hypothesized that in budding yeast, the HAT complex SAGA could be recruited by H3K4me2 as a complex member, Sgf29, specifically binds this chromatin modification (168). Illustrating the requirement for dynamic histone modification, H3K4 methylation by MLL1, while favorable for origin licensing in G1, actually prevents association of the PIC member Cdc45 (212). MLL1 is dynamically regulated during the cell cycle, in part to restrict DNA replication to once per cell cycle and to prevent aberrant replication in the presence of DNA damage (212,213). We, too, have

seen that Set1 is dynamically regulated throughout the cell cycle and is detectable only during G1.

**What is the mechanism by which H3K4me2 promotes origin function in *S. cerevisiae*?**

To address this question, we have begun to analyze the replication phenotypes upon deletion of the Sgf29 subunit of SAGA. Preliminary data suggest that this protein has only a small impact on DNA replication, as *sgf29Δ* cells do not quite phenocopy loss of *SET1*, though replication is slightly impaired (data not shown). We have previously attempted to determine if the recruitment of various replication factors to origins was disrupted in cells lacking *SET1*, but were unsuccessful due to technical difficulties; we should revisit these experiments since new information in mammalian cells suggests that the recruitment of these factors is likely affected by H3K4 methylation. We hypothesize that both mechanisms contribute to the positive effect H3K4me2 has on DNA replication.

**How is Set1 recruited to origins of replication?**

Set1 and COMPASS recruitment to chromatin outside of transcription has been largely unexplored. During transcription, Set1 is recruited to promoters through direct interaction with the C-terminal domain (CTD) of RNA polymerase II (Pol II) that is phosphorylated at serine 5 (214). Additionally, the interaction between Set1 and the Paf1 complex also facilitates COMPASS recruitment to chromatin as Paf1 also interacts with elongating Pol II (215). However, less is known about how Set1 is recruited to chromatin outside of transcription. Given

the proximity of many replication origins to promoter regions it is likely that this mechanism of Set1 recruitment could also function at origins. Interestingly, a recent report observed reduced ORC binding at rDNA origins when Pol II was absent (216). Transcription was not required to promote replication, as sites of stalled Pol II were replication-competent. It would be interesting to determine if Pol II CTD S5 phosphorylation is required for H3K4me2 at origins.

In contrast to H3K4 methylation, there is much more data indicating that H4K20me1 (and Set8) is a key regulator of origin licensing. We have shown here that Set8 protein levels are tightly regulated throughout the cell cycle and in response to cell stress and DNA damage. While previous reports have demonstrated that Set8 is degraded after DNA damage by the $CRL4^{Cdt2}$ E3 ubiquitin ligase, we have discovered that events (likely phosphorylation) during G2/M and upon osmotic stress can prevent this degradation. Our lab previously reported similar regulation of the preRC member Cdt1. Data presented here suggest that Cdk1 mediates these events either directly, or indirectly, as protection from UV-induced degradation is reversed upon addition of Cdk1 inhibitors. While there are striking similarities in the regulation of Cdt1 and Set8, during cellular quiescence Set8, but not Cdt1, is susceptible to $CRL4^{Cdt2}$-mediated degradation after DNA damage. These findings raise two very important questions, which are addressed below.

**Which kinase(s) phosphorylate Cdt1 and Set8 to protect them from UV-induced degradation during mitosis?**

Many kinases are active during mitosis and we have begun to consider which of these might be involved in regulating Cdt1 and Set8 stability if the Cdk1 effect we have observed is indirect. A recent paper elucidated a role for Aurora-A kinase in regulating Cdt1 stability in mitosis (217). During mitosis, Aurora-A phosphorylates the Cdt1 inhibitor, geminin, protecting it from $APC^{Cdc20}$-mediated degradation. This protection allows geminin to interact with soluble Cdt1 protecting it from degradation by $SCF^{Skp2}$. This finding is in contrast to interphase cells where geminin depletion causes DNA damage resulting in Cdt1 degradation through $CRL4^{Cdt2}$ (218). As cells finish metaphase both Cyclin B1 and geminin get degraded, and Cdt1 is dephosphorylated and binds chromatin to promote MCM loading. Aurora-A, Plk1, and Cdk1 activity are highly intertwined such that inhibition of one causes inactivation of the others through a variety of mechanisms. Experiments are underway to determine which of these kinases might be directly regulating $CRL4^{Cdt2}$ substrate stability during mitosis.

Interestingly, several kinases were recently found to associate with Set8 including Akt and casein kinase II (CK2) (113). Both of these kinases play important roles in mitotic progression. Akt was recently shown to regulate centrosome composition (219) while CK2 is important for spindle integrity through its phosphorylation of kinetochore components (220). The minimal consensus sequence for CK2 phosphorylation is S/T-$X_1$-$X_2$-D/E (where $X_1$ is not a proline) and there are four such motifs present in Set8 (221). In addition, a

study of the phosphoproteome of Jurkat T cells identified phosphorylated residues at tyrosine 304 and serine 310 in the SET domain of Set8 (222). It is currently unknown which kinase is responsible or what effect these phosphorylations have on Set8 activity or regulation.

**Why are Cdt1 and Set8 regulated differently in quiescent cells and what is the mechanism of this regulation?**

While Cdt1 and Set8 are regulated similarly after UV irradiation in all other cell cycle stages, during quiescence they are quite different. Cdt1 remains protected while Set8 is degraded (Figure 3.4). There is at least one possible reason for this differential regulation. It is possible that there is no difference with regard to protection from $CRL4^{Cdt2}$-mediated degradation, but that another E3 ligase specifically targets Set8, but not Cdt1, for proteolytic degradation. The obvious candidate would be $APC^{Cdh1}$. Set8 is a known target of $APC^{Cdh1}$ but can be protected from APC-mediated degradation through phosphorylation at S29 (123). We are currently investigating whether Set8 is indeed phosphorylated at this residue during quiescence. Set8 could be dephosphorylated upon UV irradiation rendering it susceptible to APC-mediated degradation, but still protected from $CRL4^{Cdt2}$-mediated degradation. While Cdt1 has also been reported to be a substrate of $APC^{Cdh1}$, APC-mediated proteolysis of Cdt1 is not highly robust, and its contribution to Cdt1 regulation may vary depending on cell type (223). For example, quiescent T98G cells have almost undetectable amounts of Cdt1 while Cdt1 in NHF cells is readily detectable (Figure 3.4).

The first question to be answered is whether $CRL4^{Cdt2}$ is functional during quiescence. It was recently reported that Cdt2 is degraded during quiescence by $SCF^{Fbxo11}$ (203). We are currently examining total and chromatin-bound Cdt2 levels before and after UV irradiation in quiescent NHF cells. We could also determine if Cdt1 and Set8 can interact with Cdt2 during quiescence. Assuming Cdt2 is present during quiescence, the next question would be does $APC^{Cdh1}$ promote Set8 degradation in quiescence. We could mutate the S29 residue to alanine or aspartic acid and ask if the phosphomimetic mutant was resistant to UV-induced degradation while the S29A mutant gets degraded. Another possibility is that a separate mechanism functions during quiescence to protect Cdt1, but not Set8 from UV-induced, $CRL4^{Cdt2}$-mediated degradation. There could be a distinct kinase that only phosphorylates Cdt1 protecting it from degradation. Answering these questions could lead us in the right direction to gain a better understanding of this differential regulation in quiescence.

**Do cells need *de novo* H4K20me1 during G2/M for proper origin licensing in the subsequent G1 phase?**

The literature to date has focused predominately on the importance of Set8 methylation of H4K20 during G2/M. Yet, no one has closely examined the consequences of Set8 depletion during this cell cycle phase for the subsequent G1 and origin licensing. It is still possible that Set8 methylates some as yet unidentified non-histone substrate that is important for mitosis, origin licensing in G1, or both. Is *de novo* H4K20me1 deposited at origins during G2/M so that origin licensing can commence upon mitotic exit? Or, are the higher H4K20

methylation states demethylated to generate the H4K20me1 that seems to be required for origin licensing? To begin to answer these questions we will synchronize cells in early S phase when Set8 is degraded and treat with Set8 siRNA to prevent accumulation in G2/M. We will then examine H4K20me1 levels to determine how much demethylation of H4K20me2,3 contributes to H4K20me1 levels in G2/M and the subsequent G1 phase. We will also look for any mitotic phenotypes resulting from loss of Set8. Since Set8 is known to be important for chromatin compaction we will use immunofluorescence to examine DNA compaction in metaphase and chromosome segregation in anaphase. We will also look for any delay in entry or exit from mitosis and G1 using flow cytometry. Using these methods we will begin to determine how H4K20me1 is established at origins and if H4K20 is the only relevant Set8 target for DNA replication.

**Concluding remarks**

When this study was initiated, little was known about how individual histone modifications impacted DNA replication. Our efforts have characterized a role for H3K4me2 in promoting DNA replication and raised some important questions regarding the mechanism of this effect. Other histone modifications have been found that also promote origin activity (see Chapter 1). Additionally, we have shown that Set1 is cell cycle regulated in *S. cerevisiae* with peak expression during G1. This finding corroborates recent data in human cells demonstrating that both $SCF^{Skp2}$ and $APC^{Cdc20}$ regulate MLL (one of six H3K4 HMTs) protein levels to orchestrate proper gene expression and cell cycle transitions (213). The regulation of histone modifying enzymes is an important

area of research that is only now becoming appreciated. It is important to consider not only the regulation of histone modifications themselves, but also of the writers, erasers, and readers of these marks. Future work will highlight the dynamic nature of these enzymes and add yet another layer of complexity to the histone code.

During our studies in yeast, a novel histone modification (H4K20me1) was found to play a key role in origin licensing in metazoans. The breadth of knowledge regarding the role of the H4K20 HMT Set8 in licensing has since expanded rapidly. We became interested in the regulation of this methyltransferase and its function, not just in G1, but throughout the cell cycle. Our current focus is on the degradation of Set8 after DNA damage during different cell cycle stages and during a cellular stress response. We have found that Set8, and other $CRL4^{Cdt2}$ targets, are protected from UV-induced degradation during mitosis and during an osmotic stress response. This protection is mediated through phosphorylation events that we are currently investigating. The kinases responsible for phosphorylating Set8, and other $CRL4^{Cdt2}$ targets, are potentially different between different cell cycle stages and during a stress response. Overall, our efforts in both yeast and mammalian cells have provided further support for the importance of chromatin in regulating DNA replication.

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