DNA Replication Origin Function Is Promoted by H3K4 Di-methylation in *Saccharomyces cerevisiae*

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ABSTRACT DNA replication is a highly regulated process that is initiated from replication origins, but the elements of chromatin structure that contribute to origin activity have not been fully elucidated. To identify histone post-translational modifications important for DNA replication, we initiated a genetic screen to identify interactions between genes encoding chromatin-modifying enzymes and those encoding proteins required for origin function in the budding yeast *Saccharomyces cerevisiae*. We found that enzymes required for histone H3K4 methylation, both the histone methyltransferase Set1 and the E3 ubiquitin ligase Bre1, are required for robust growth of several hypomorphic replication mutants, including *cdc6-1*. Consistent with a role for these enzymes in DNA replication, we found that both Set1 and Bre1 are required for efficient minichromosome maintenance. These phenotypes are recapitulated in yeast strains bearing mutations in the histone substrates (H3K4 and H2BK123). Set1 functions as part of the COMPASS complex to mono-, di-, and tri-methylate H3K4. By analyzing strains lacking specific COMPASS complex members or containing H2B mutations that differentially affect H3K4 methylation states, we determined that these replication defects were due to loss of H3K4 di-methylation. Furthermore, histone H3K4 di-methylation is enriched at chromosomal origins. These data suggest that H3K4 di-methylation is necessary and sufficient for normal origin function. We propose that histone H3K4 di-methylation functions in concert with other histone post-translational modifications to support robust genome duplication.

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 the S phase (Weinreich *et al.* 2004). 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. Although 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 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 (Méchali 2010).

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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 example, the positioning of nucleosomes near origins can either stimulate or inhibit origin function (Simpson 1990; Crampton *et al.* 2008; Berbenetz *et al.* 2010; Eaton *et al.* 2010). The major protein components of chromatin, the histone proteins, can also be post-translationally modified by acetylation, methylation, phosphorylation, ubiquitination, and sumoylation (Kouzarides 2007). 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 accelerates the timing of origin firing within S phase and can increase origin efficiency (Aggarwal and Calvi 2004; Espinosa *et al.* 2010; Unnikrishnan *et al.* 2010). 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 (Pryde *et al.* 2009). In metazoan genomes, PR-Set7-catalyzed histone H4 lysine 20 mono-methylation (H4K20me1)

stimulates the loading of the core replicative helicase (Tardat *et al.* 2007, 2010).

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 facilitates efficient DNA replication in the form of a "histone code" similar to the combinations known to regulate transcription (Strahl and Allis 2000). While some elements of this code have been identified (*e.g.*, H3 and H4 acetylation, H4K20 mono-methylation, and H3K36 mono-methylation), 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 DNA replication role for histone H3 lysine 4 (H3K4) methylation by the COMPASS complex, 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 determines 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 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 1.

Plasmids

All plasmids used in this study are listed in Table 2.

Synthetic genetic array screen

Synthetic genetic array (SGA) analysis was carried out as previously described (Tong and Boone 2006). Briefly, 63 deletion strains (Table 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 was previously published (Gardner *et al.* 2011). 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 fivefold serial dilutions with an initial OD_{600} of 0.5 onto YPD, grown for 3 days at 32°, 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- 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 (Tye 1999). 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 nonselective media to establish an initial percentage of plasmid-bearing cells. These cultures were also diluted to a concentration of 1×10^5 cells/ml in 5 ml of nonselective media and grown for 8–10 generations before once again plating on both selective and nonselective 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°. 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), and anti-LexA (1:5000; Millipore 06-719).

Chromatin immunoprecipitation

Yeast strains were cross-linked with 1% formaldehyde for 15 min at room temperature and quenched with 250 mM glycine for 5 min at room temperature. Forty OD_{600} units of cross-linked cells were harvested by centrifugation and washed thoroughly, and the pellets were stored at −80°. 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

Table 1 Strains used in this study

Strain	Genotype	Source
BY4741[a]	*MAT**a** met15Δ0*	Brachmann *et al.* (1998)
yLF058[a]	*MAT**a** met15Δ0, cdc6-1::hph*	This study
yLF063[a]	*MAT**a** met15Δ0, cdc6-1::hph, set1Δ::HIS3MX6*	This study
yLF089[a]	*MAT**a** met15Δ0, set1Δ::HIS3MX6*	This study
yLF059[a]	*MAT**a** met15Δ0, bre1Δ::kanMX*	This study
yLF062[a]	*MAT**a** met15Δ0, set1Δ::kanMX*	This study
yLF060[a]	*MAT**a** met15Δ0, bre2Δ::kanMX*	This study
yLF061[a]	*MAT**a** met15Δ0, swd1Δ::kanMX*	This study
YMS196[b]	*Matα can1Δ::STE2$_p$_Sp.his5$^+$, lyp1Δ::STE3$_p$_LEU2*	Schuldiner *et al.* (2006)
JCY332[b]	*Matα can1Δ::STE2$_p$_Sp.his5$^+$, lyp1Δ::STE3$_p$_LEU2, cdc6-1::hph*	This study
yLF114[a,c]	*Matα lyp1Δ::STE3$_p$_LEU2, cdc6-1::hph, swd1Δ::kanMX*	This study
yLF120[a,c]	*Matα lyp1Δ::STE3$_p$_LEU2, cdc6-1::hph, bre2Δ::kanMX*	This study
yLF117[a,c]	*Matα lyp1Δ::STE3$_p$_LEU2, cdc6-1::hph, rad6Δ::kanMX*	This study
yLF154[a]	*MAT**a** met15Δ0, rad6Δ::kanMX*	OpenBiosystems
yLF150[a]	*MAT**a** met15Δ0, cdc6-1::hph, bre1Δ::kanMX*	This study
TSQ131[a]	*MATα can1Δ::STE2pr-Sp_his5, lyp1Δ::STE3pr-LEU2, cdc7-4::natMX*	Costanzo *et al.* (2010)
yLF096[a,c]	*MATα set1Δ::kanMX*	This study
yLF097[a,c]	*MAT**a***	This study
yLF098[a,c]	*MATα lyp1Δ::STE3pr_LEU2, cdc7-4::natMX*	This study
yLF099[a,c]	*MAT**a** can1Δ::STE2pr_Sp-his5, cdc7-4::natMX, set1Δ::kanMX*	This study
TSQ880[a]	*MATα can1Δ::STE2pr-Sp_his5, lyp1Δ::STE3pr-LEU2, cdc7-1::natR*	Costanzo *et al.* (2010)
yLF138[a,c]	*MAT**a** can1Δ::STE2pr-Sp_his5, lyp1Δ::STE3pr-LEU2, cdc7-1::natMX, set1Δ::kanMX*	This study
yLF139[a,c]	*MAT**a** cdc7-1::natMX*	This study
yLF140[a,c]	*MATα*	This study
yLF141[a,c]	*MATα set1Δ::kanMX*	This study
TSQ694[a]	*MATα can1Δ::STE2pr-Sp_his5, lyp1Δ::STE3pr-LEU2, cdc45-27::natMX*	Costanzo *et al.* (2010)
yLF142[c]	*MATα his3Δ1, ura3Δ0*	This study
yLF143[c]	*MATα his3Δ1, ura3Δ0, set1Δ::kanMX*	This study
yLF144[c]	*MAT**a** his3Δ1, ura3Δ0, set1Δ::kanMX, cdc45-27::natMX*	This study
yLF145[c]	*MAT**a** his3Δ1, ura3Δ0, cdc45-27::natMX*	This study
DY7803[d]	*MAT**a** hht1-hhf1::LEU2, hht2-hhf2::kanMX3*, [YCp-*URA3*(*HHT2-HHF2*)]	Biswas *et al.* (2006)
yLF155[c,d]	*MAT**a** hht1-hhf1::LEU2, hht2-hhf2::kanMX3, cdc6-1::hph*, [YCp-*URA3*(*HHT2-HHF2*)]	This study
RUY121[d]	*MATα*	Fred Cross
RUY028[d]	*MATα mfa::MFA1$_p$_HIS3, ORC6-rxl::LEU2, URA3::GAL$_p$_CDC6ΔNT-HA*	Archambault *et al.* (2005)
yLF049[c,d]	*MATα mfa::MFA1$_p$_HIS3, ORC6-rxl::LEU2, URA3::GAL$_p$_CDC6ΔNT-HA, swd1Δ::kanMX*	This study
yLF050[c,d]	*MATα mfa::MFA1$_p$_HIS3, ORC6-rxl::LEU2, URA3::GAL$_p$_CDC6ΔNT-HA, bre1Δ::kanMX*	This study
yLF051[d]	*MATα swd1Δ::kanMX*	This study
yLF052[d]	*MATα bre1Δ::kanMX*	This study
YNL037[d]	*MATα dot1Δ::kanMX*	Gardner *et al.* (2011)
FY406[e]	*MAT**a** hta1-htb1::LEU2, hta2-htb2::TRP1* [pSAB6 *HTA1-HTB1 URA3*]	Hirschhorn *et al.* (1995)

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

inhibitors and 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° and immunoprecipitated with 10 μl Protein A Dynabeads (Invitrogen) for 1 h at 4°. 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 room temperature. The eluted material was treated with RNAse A and Proteinase K before de-cross-linking at 65° overnight. The DNA was purified using Genesee UPrep spin columns and eluted in 100 μl water. Immunoprecipitation (IP) samples and IP controls (*set1Δ*) were used undiluted while input samples were diluted 1:10. Samples were analyzed by quantitative PCR 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

Table 2 Plasmids used in this study

Plasmid	Features	Source
YCplac33	*ARS1*, *CEN4*, *URA3*	Geitz and Sugino (1988)
YCplac111	*ARS1*, *CEN4*, *LEU2*	Geitz and Sugino (1988)
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
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*	Nakanishi *et al.* (2008)
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

represent the standard deviations of the average signals between experiments ($n \geq 3$).

Results

Identification of histone modifiers that promote DNA replication

DNA replication origins in the budding yeast *S. 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 (Bell and Dutta 2002; Takahashi *et al.* 2002). The *cdc6-1* mutant harbors a G260D mutation in the catalytic domain resulting in failure to load MCMs at restrictive temperatures (Feng *et al.* 2000). Yeast cells harboring the *cdc6-1* mutation produce a Cdc6 protein that functions normally at 29° and is nonfunctional at temperatures >34°, but retains partial function at intermediate temperatures between 30° and 33° (Feng *et al.* 2000). To identify suppressors or enhancers of *cdc6-1*, we deleted genes for most of the known histone modifiers, chromatin remodelers, and histone chaperones (63 total; see Table 3) in the *cdc6-1* temperature-sensitive replication mutant strain. Double-mutant strains were tested for fitness at semipermissive temperatures and compared to the parent single-mutant strains (Tong and Boone 2006). 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 3). Although a role for the histone acetyltransferase (HAT) Gcn5 in DNA replication has been shown (Espinosa *et al.* 2010), 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 HATs, histone deacetylases (HDACs), and histone methyltransferases (HMTs) (Table 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 1A). H3K4 is mono-, di-, and tri-methylated by Set1 as part of the COMPASS complex. COMPASS consists of seven additional subunits that promote complex integrity (Swd1, Swd2, Swd3, Bre2), regulate catalytic activity (Sdc1 and Spp1), or have unknown function (Shg1) (Takahashi and Shilatifard 2010; Mersman *et al.* 2011; Takahashi *et al.* 2011). Unlike other COMPASS members, Swd2 also functions as part of the APT transcription termination complex, and its role in this complex is essential for cell viability (Soares and Buratowski 2012). For this reason, Swd2 was not included in our screen. The absence of Set1, Swd1, Swd3, Bre2, or Sdc1 impaired the growth of the *cdc6-1* strain (Table 3).

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

Table 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
hos3	0	HDAC
hst2	0	HDAC
htz1	0	H2A.Z
isw2	0	Remodeler
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
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*.

impaired at 31° compared to wild-type or the *cdc6-1* strain harboring wild-type *CDC6* on a plasmid, but growth was substantially impaired when *SET1* was deleted in this strain (Figure 1B). Expression of wild-type *SET1*, but not of the catalytically dead mutant *set1-H1017K*, rescued the synthetic growth defect of the *set1Δ cdc6-1* strain (Figure 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 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. Newly constructed deletions of *BRE2*, *SDC1*, *SWD1*, and *SWD3* each impaired the growth of the *cdc6-1* mutant at semipermissive temperatures, but not deletion of *SPP1* or *SHG1* (Figure 1D and Table 3). Bre2, Sdc1, Swd1, Swd2, and Swd3 (along with Set1) are the core structural components of the COMPASS complex required for full activity (Takahashi *et al.* 2011). 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 would expect similar proliferation defects from deleting *SET1* in other replication mutant strains. To test this prediction, *SET1* was deleted in two temperature-sensitive *cdc7* mutants and one temperature-sensitive *cdc45* mutant. These replication factors are required for origin firing at the G1/S transition, downstream of Cdc6 activity (Tercero *et al.* 2000; Labib 2010) . 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 semipermissive temperatures (Figure 2A).

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 deregulate 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 (Archambault *et al.* 2005). In this strain, re-replication is induced during growth on galactose, which induces transcription of the *CDC6-ΔNT* allele.

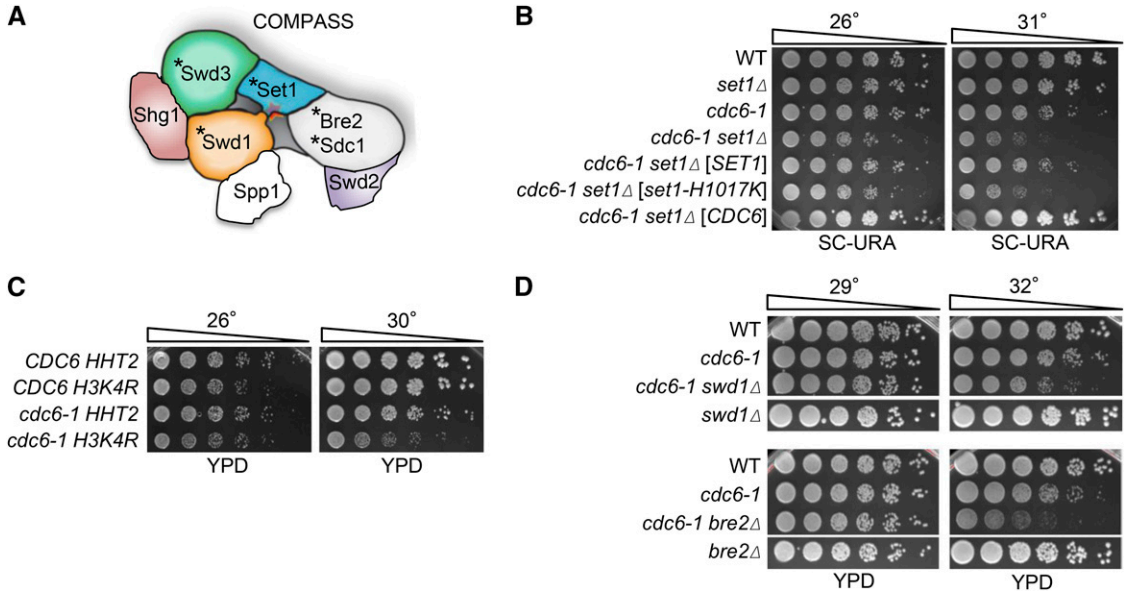

Figure 1 COMPASS and H3K4 are required for robust growth of the temperature-sensitive *cdc6-1* replication mutant. (A) COMPASS complex model adapted from Takahashi *et al.* (2011). An asterisk denotes a complex member that genetically interacts with *cdc6-1*. (B) Wild-type (BY4741), *cdc6-1* (yLF058), *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. Fivefold 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. Fivefold serial dilutions were spotted onto YPD and grown at the indicated temperatures for 3 days. (D) Fivefold serial dilutions of wild-type (YMS196), *cdc6-1* (JCY332), *cdc6-1 swd1Δ* (yLF114), *cdc6-1 bre2Δ* (yLF120), *bre2Δ* (yLF060), or *swd1Δ* (yLF061) were spotted onto YPD and grown at the indicated temperatures for 3 days.

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 2B). 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 assays for both di- and trimethylated 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 a late-firing origin of replication (*ARS315* and *ARS822*, respectively) relative to a nonorigin, telomere-proximal region (Figure 3).

The Rad6/Bre1 ubiquitin ligase complex promotes DNA replication

Methylation of histone H3K4 by the COMPASS complex requires prior mono-ubiquitination of histone H2B at lysine 123 (H2BK123) by the Rad6/Bre1 ubiquitin ligase complex (Lee *et al.* 2007; Nakanishi *et al.* 2009). 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

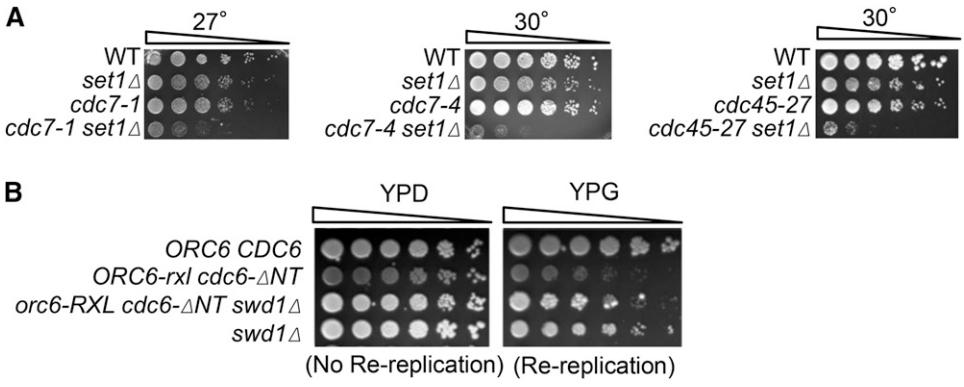

Figure 2 H3K4 methylation is required for robust growth of multiple replication mutants. (A) Fivefold serial dilutions of the meiotic progeny from the cross of *cdc7-1* (TSQ880), *cdc7-4* (TSQ131), or *cdc45-27* (TSQ694) with *set1Δ* (yLF062) were spotted onto YPD and grown at the indicated temperatures for 3 days. (B) Fivefold 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°.

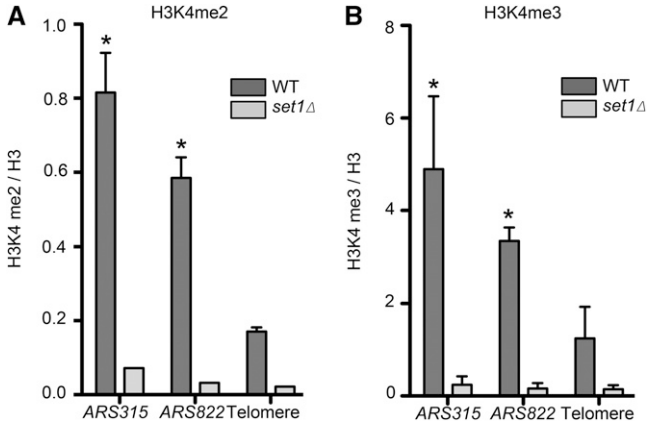

Figure 3 H3K4 methylation is present at replication origins. Chromatin immunoprecipitation experiments were performed on asynchronous wild-type (BY4741) or *set1Δ* (yLF062) strains grown at 30°. Immunoprecipitates using antibodies to total histone H3, di-methylated H3K4 (H3K4me2) in A and tri-methylated 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 using the Student's unpaired *t*-test (*$P < 0.05$).

resulted in an ~125-fold decrease in growth at semipermissive temperatures, validating this genetic interaction (Figures 4, A and B). Ectopic expression of 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 4A). Moreover, *BRE1* deletion also partially rescued the growth impairment of the re-replicating RUY028 strain similar to deletion of *SET1* (Figure 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 (Maine *et al.* 1984). Growth in nonselective 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 nonselective medium. Strains lacking either *SET1* or *BRE1* displayed significantly higher plasmid loss rates per cell division than wild-type strains did (Figure 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 5B).

Notably, H2B mono-ubiquitination by Rad6/Bre1 is a prerequisite not only for H3K4 methylation, but also for H3K79 methylation (Nakanishi *et al.* 2009). Neither deletion of *DOT1*, the histone H3K79 methyltransferase, nor mutation of H3K79 to arginine had any effect on plasmid maintenance (Figure 5, A and B). Moreover, loss of Dot1 did not impair the growth of the *cdc6-1* strain (Table 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. Furthermore, 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 (Hogan and Koshland 1992). 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 5C). This result supports the conclusion that H2BK123 mono-ubiquitination by Bre1 and the consequent H3K4 methylation by Set1 promote DNA replication origin function in yeast.

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 (Pokholok *et al.* 2005), and several studies have shown that the different methylation states of H3K4 and H3K36 can have different functions (Taverna *et al.* 2006; Pinskaya *et al.* 2009; Pryde *et al.* 2009). The individual H3K4 methylation states depend on other histone residues and are influenced by the presence or absence of individual COMPASS subunits (Chandrasekharan *et al.* 2010; Mersman *et al.* 2011). 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 5A), both *swd1Δ* and *bre2Δ* strains displayed significantly elevated plasmid loss compared to their isogenic wild-type counterparts (Figure 6A). These two proteins are both required for Set1 stability (Takahashi *et al.* 2011), and in their absence no H3K4 methylation is detectable (Figure 6B). Unlike Swd1 and Bre2, Spp1 is a COMPASS subunit that is required for H3K4 tri-methylation, but not mono- or di-methylation (Takahashi *et al.* 2009) (Figure 6B); loss of *SPP1* had no effect on plasmid maintenance (Figure 6A). This result is consistent with our earlier observation that Spp1 loss did not affect proliferation of the *cdc6-1* strain (Table 3). Taken together, these data suggest that H3K4

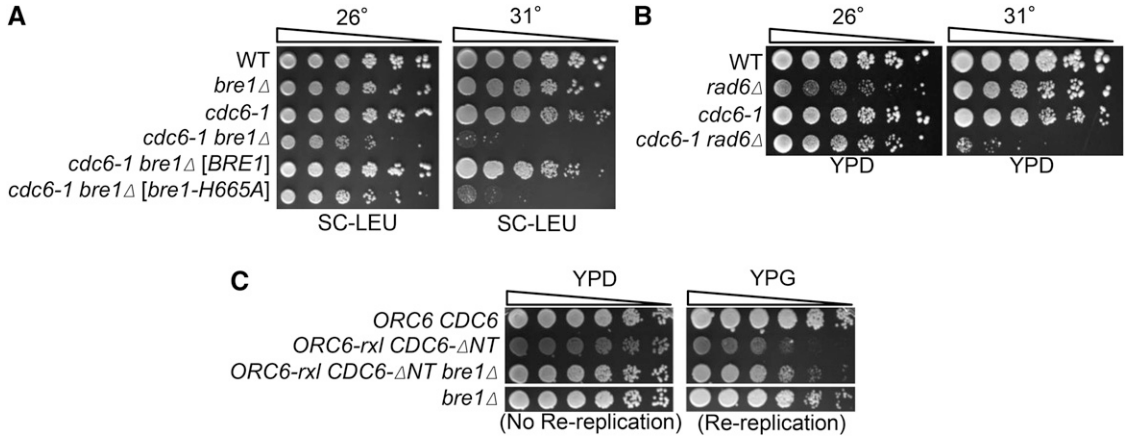

Figure 4 H2BK123 mono-ubiquitination promotes robust growth of the temperature-sensitive *cdc6-1* replication mutant. (A) Wild-type (BY4741), *bre1Δ* (yLF151), *cdc6-1* (yLF058), and *cdc6-1 bre1Δ* (yLF150) were transformed with either an empty vector [pRS315], a vector expressing *BRE1* [pRS315-*9xMyc-BRE1*], or *bre1-H665A* [pRS315-*9xMyc-bre1-H665A*] from the native *BRE1* promoter. Fivefold serial dilutions were spotted onto SCD-LEU and grown for 3 days at the indicated temperatures. (B) Fivefold 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. As previously reported, the *rad6Δ* strain is cold sensitive at temperatures <30° (McDonough *et al.* 1995). (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°.

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 (Chandrasekharan *et al.* 2010). Mutation of H2BK123 to arginine abolished mono-ubiquitination of this residue and consequently eliminated both H3K4 di- and tri-methylation (Figure 6D). Importantly, similar to loss of *BRE1*, this mutation induced a significantly higher plasmid loss rate than wild type (Figure 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 H2B arginine 119 to alanine does not prevent H2BK123 mono-ubiquitination, but does affect the degree of H3K4 methylation (Chandrasekharan *et al.* 2010). As previously reported, changing H2BR119 to alanine (H2BR119A) reduces the chromatin association of Spp1 and therefore H3K4 tri-methylation, whereas changing H2B119 to aspartic acid (H2BR119D) results in loss of H3K4 di- and tri-methylation to the same extent as eliminating H2BK123 mono-ubiquitination (Chandrasekharan *et al.* 2010). 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 6C). Mutation of R119 to alanine abolished H3K4 tri-methylation as expected, while having only a moderate effect on H3K4 di-methylation (Figure 6D). This H2B mutant showed a plasmid loss rate similar to the wild-type *HTB1* strain. The

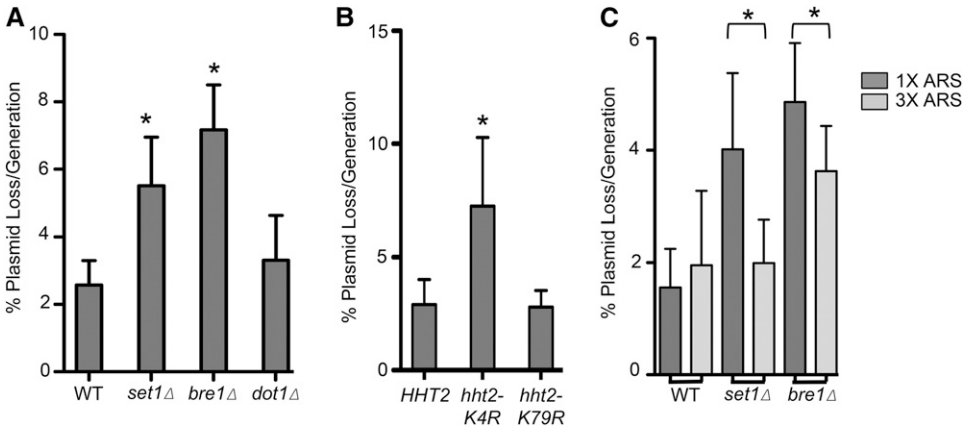

Figure 5 H3K4 methylation is required for efficient origin-dependent mini chromosome maintenance. (A) Plasmid loss rates of a single-origin-bearing plasmid [YCpLac33] were measured in a wild-type strain (BY4741) and in strains lacking *SET1* (yLF089), *BRE1* (yLF151), or *DOT1* (YNL037). Loss rates are 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 (*$P < 0.05$).

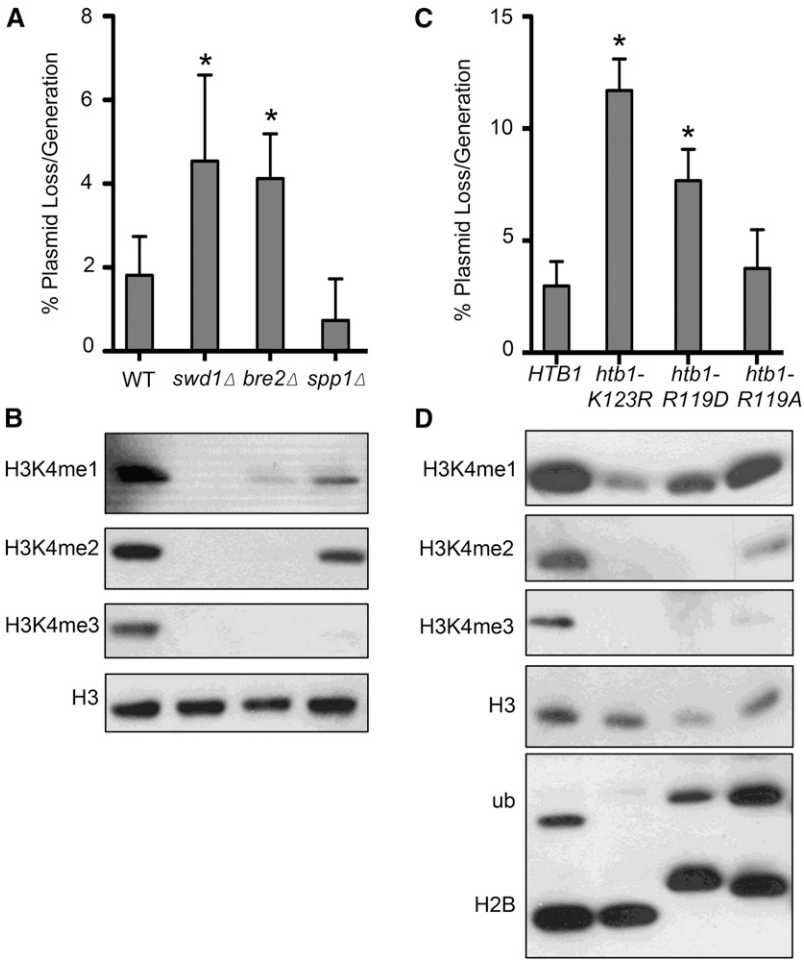

Figure 6 H3K4 di-methylation promotes efficient minichromosome maintenance. (A) Plasmid loss rates of the single-origin-bearing plasmid [YCpLac33] were measured for wild-type (BY4741), *swd1Δ* (yLF061), *bre2Δ* (yLF060), and *spp1Δ* (yLF153). (B) Immunoblot analysis of whole-cell extracts (from strains 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 (*$P < 0.05$).

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 6C).

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 (Schlichter and Cairns 2005). Each of these Set1 proteins was fused to a LexA tag for detection on immunoblots (Figure 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 7B). As before, production of active Set1 suppressed the growth phenotype caused by deleting *SET1* in the *cdc6-1* strain (Figure 7A). Importantly, production of Set1ΔRRM also fully rescued this proliferation defect without the ability to produce H3K4 tri-methylation (Figure 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 semipermissive 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 prerequisite histone H2BK123 mono-ubiquitination, causes a similar replication phenotype. These data provide clear evidence that H3K4 di-methylation is important for full origin function.

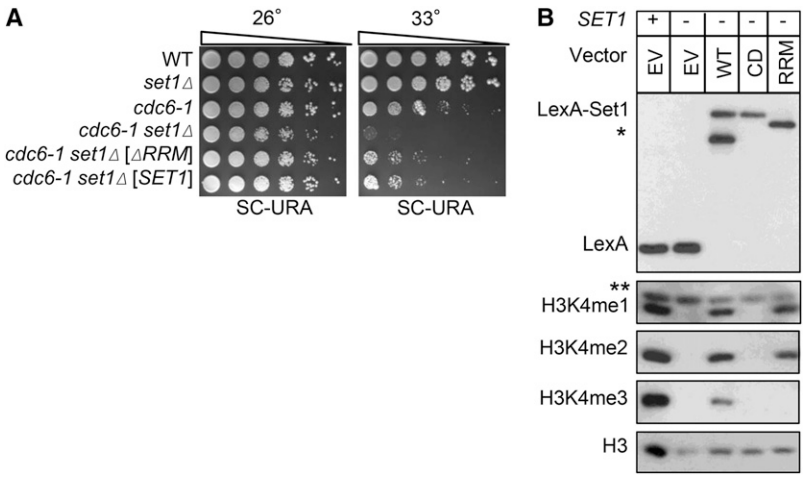

Figure 7 H3K4 di-methylation is sufficient for robust growth of the *cdc6-1* mutant. (A) Wild-type (BY4741), *cdc6-1* (yLF058), *set1Δ* (yLF062), and *cdc6-1 set1Δ* (yLF063) were transformed with an empty vector [pGLx2], a vector producing LexA-tagged Set1 [pGLx2-*SET1*], or Set1 lacking the RRM domain [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. A single asterisk represents a likely degradation product unique to the positive control construct; a double asterisk represents a nonspecific band detected by the H3K4me1 antibody.

The Set1 histone methyltransferase and the Bre1 E3 ubiquitin ligase have well-established roles in regulating gene expression (Shukla *et al.* 2006; Mutiu *et al.* 2007). Nonetheless, several lines of evidence suggest that the phenotypes that we have detected are due to reduced replication activity 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 (Knott *et al.* 2009) did not genetically interact with *cdc6-1* (Table 3), implying that the synthetic growth phenotypes observed here are relatively specific for origin function and not origin firing time. 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 (Lenstra *et al.* 2011). 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 to 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 data set identified peaks of methylation distinct from nearby transcription-associated peaks (Radman-Livaja *et al.* 2010 and our unpublished observations). Finally, our observation that loss of H3K4 methylation exacerbates poor growth of a replication *hypo*morphic strain but *suppresses* the poor growth of an origin-firing *hyper*morphic 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 transcription control has been described as a "histone code" in which combinations of post-translational modifications promote or repress transcription at a given locus (Strahl and Allis 2000; Oliver and Denu 2010). 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 under 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 (Dershowitz *et al.* 2007; Ge *et al.* 2007; Blow *et al.* 2011). 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 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 been suggested that mono-methylation of H3K36 can recruit the replication initiation factor Cdc45 (Pryde *et al.* 2009). In addition, a member of the origin recognition

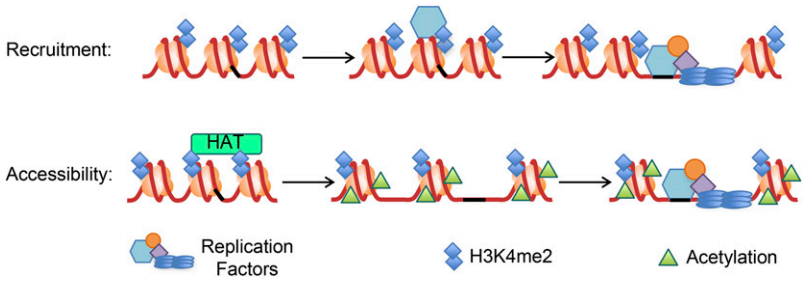

Figure 8 Two models by which H3K4 di-methylation could promote efficient DNA replication. The recruitment model suggests either direct or indirect recruitment of replication factors by H3K4 di-methylation, while the accessibility model suggests recruitment of a HAT that acetylates nucleosomes near the origin, resulting in an open chromatin state that allows replication factors to access and bind the origin.

complex, Orc1, contains a bromo-adjacent homology domain that mediates nucleosome binding in budding yeast and specifically binds to di-methylated H4K20 at origins in human cells (Müller *et al.* 2010; Kuo *et al.* 2012). Such histone-binding domains in core replication factors or as-yet-unidentified bridging proteins could link H3K4 di-methylation to recruitment of the replication machinery at origins.

The second model suggests that H3K4 di-methylation near origins recruits another chromatin modifier such as a HAT. Acetylation of nearby nucleosomes resulting in increased origin accessibility would allow more efficient association of replication factors (Figure 8). Acetylation is already known to affect the efficiency and timing of origin firing (Vogelauer *et al.* 2002; Goren *et al.* 2008). 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 3). Chd1 is a chromatin remodeler whose mammalian counterpart is capable of binding H3K4 methylation (Sims *et al.* 2005) while Spt8 impacts transcription by directly recruiting TATA-binding protein (Sermwittayawong and Tan 2006). 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 (Bian *et al.* 2011). SAGA is responsible for acetylation of multiple H3 residues as well as H4K8 and H2BK11 and K16 (Grant *et al.* 1999). Two other HAT complexes were also identified in our screen, NuA3 and NuA4; both contain subunits, Yng1 and Yng2, respectively, that bind H3K4 methylation (Martin *et al.* 2006a,b; Taverna *et al.* 2006; Shi *et al.* 2007). 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 replication initiation factor Cdc45 in yeast (Pryde *et al.* 2009). In addition to methylation, H3K4 can also be acetylated by both Gcn5 and Rtt109 (Guillemette *et al.* 2011). At promoters, this acetylation is typically found just upstream from the peak of H3K4 tri-methylation. Whether H3K4 acetylation 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 (Unnikrishnan *et al.* 2010) and the timing of origin firing in yeast, *Drosophila*, and mammalian cells (Vogelauer *et al.* 2002; Hiratani *et al.* 2009; Schwaiger *et al.* 2009). Additionally, the HATs Hat1 and Gcn5 physically interact with the replication machinery (Suter *et al.* 2007; Espinosa *et al.* 2010). In mammalian cells, Gcn5 acetylates Cdc6 at several lysine residues, and this acetylation is required for subsequent phosphorylation by cyclin/CDKs (Paolinelli *et al.* 2009). In vertebrates, the H4-specific HAT, Hbo1, binds (Iizuka and Stillman 1999; Burke *et al.* 2001) and acetylates several origin-binding proteins and histone H4 (Iizuka *et al.* 2006; Miotto and Struhl 2010), and this acetyltranferase activity is required for efficient origin licensing. In metazoans, methylation of H4K20 by PR-Set7 (a.k.a. Set8) is cell cycle regulated (Tardat *et al.* 2010; Beck *et al.* 2012) and is required for proper S-phase initiation and prevention of re-replication (Wu and Rice 2011). 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 (Baker *et al.* 2010).

Because all organisms except *S. cerevisiae* lack common nucleotide sequence motifs at origins, discovering a chromatin signature that promotes origin function is essential to understanding 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. In fact, many histone modifications have been found at human replication origins, including H3K4me3, H3K56me, H4K20me1, H4K20me2, and multiple acetylations on H3 and H4 (Rampakakis *et al.* 2009; Miotto and Struhl 2010; Tardat *et al.* 2012; Yu *et al.* 2012). Our discovery that H3K4 di-methylation is able to promote DNA replication adds to the 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 is common to all origins or is required only at specific genomic loci to successfully promote DNA replication.

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