# The NAD$^+$-dependent Sir2p histone deacetylase is a negative regulator of chromosomal DNA replication

**Donald L. Pappas Jr., Ryan Frisch, and Michael Weinreich[1]**

Laboratory of Chromosome Replication, Van Andel Research Institute, Grand Rapids, Michigan 49503, USA

**The establishment of DNA synthesis during the S phase is a multistep process that occurs in several stages beginning in late mitosis. The first step is the formation of a large prereplicative complex (pre-RC) at individual replication origins and occurs during exit from mitosis and entry into G1 phase. To better understand the genetic requirements for pre-RC formation, we selected chromosomal suppressors of a temperature-sensitive *cdc6-4* mutant defective for pre-RC assembly. Loss-of-function mutations in the chromatin-modifying genes *SIR2*, and to a lesser extent in *SIR3* and *SIR4*, suppressed the *cdc6-4* temperature-sensitive lethality. This suppression was independent of the well-known silencing roles for the SIR proteins at the *HM* loci, at telomeres, or at the rDNA locus. A deletion of *SIR2* uniquely rescued both the DNA synthesis defect of the *cdc6-4* mutant and its severe plasmid instability phenotype for many origins. A *SIR2* deletion suppressed additional initiation mutants affecting pre-RC assembly but not mutants that act subsequently. These findings suggest that Sir2p negatively regulates the initiation of DNA replication through a novel mechanism and reveal another connection between proteins that initiate DNA synthesis and those that establish silent heterochromatin in budding yeast.**

[*Keywords*: DNA replication; *SIR2*; deacetylase]

Supplemental material is available at http://www.genesdev.org.

Received November 26, 2003; revised version accepted March 3, 2004.

DNA replication occurs during the S phase of each cell cycle and initiates at discrete sites called origins of replication (Newlon and Theis 1993). Although DNA synthesis is restricted to the S phase, the ability to initiate replication is determined by molecular events at each origin beginning during anaphase of the previous cell cycle and continuing into early G1 phase. During this period, the assembly of prereplicative complexes (pre-RCs) directly at origins establishes replication competence. Pre-RCs contain the proteins necessary but not sufficient to initiate DNA replication (for review, see Diffley 1996; Bell and Dutta 2002). Subsequent events during G1 lead to origin unwinding and the recruitment of polymerases that initiate bidirectional DNA synthesis. Importantly, the ability to reinitiate replication from any one origin is not possible once cells have entered S phase because pre-RC assembly is prevented during the S, G2, and M phases. The inhibition of pre-RC assembly occurs through a variety of mechanisms controlling the availability and nuclear localization of key pre-RC components (Labib et al. 1999; Nguyen et al. 2001). This temporal determination of replication is necessary to prevent reinitiation of DNA replication within a single cell cycle that, if allowed to occur, would cause increases in ploidy and promote genomic instability.

In *Saccharomyces cerevisiae*, the origin recognition complex (ORC) determines the sites that initiate DNA replication by binding to a bipartite consensus sequence within the origin (Bell and Stillman 1992; Rao and Stillman 1995). ORC then serves as a "landing pad" for the assembly of the multiple initiation proteins at origins prior to DNA synthesis (Stillman 1996). The ORC–origin interaction occurs in a nucleosome-free region for the *ARS1* origin, and both ORC binding and the initiation of replication are inhibited by nucleosomes occupying this binding site (Simpson 1990; Lipford and Bell 2001). Positioning nucleosomes distal to the origin also inhibits initiation but not ORC binding, additionally suggesting that protein–nucleosomal contacts or chromatin structure is important for pre-RC assembly (Lipford and Bell 2001).

The first step in pre-RC assembly occurs when the Cdc6 protein binds to ORC and promotes MCM (minichromosome maintenance) helicase loading (Liang et al. 1995; Cocker et al. 1996; Aparicio et al. 1997; Tanaka et al. 1997). Cdc6p is a member of the large AAA$^+$ family of ATP-binding proteins (Neuwald et al. 1999; Davey et al.

[1]**Corresponding author.**
**E-MAIL michael.weinreich@vai.org; FAX (616) 234-5307.**
Article and publication are at http://www.genesdev.org/cgi/doi/10.1101/gad.1173204.

2002). This family includes proteins that use ATP binding or hydrolysis to promote conformational changes in nucleic acids or protein substrates. An X-ray crystallographic structure of an archaebacterial Cdc6-like protein bound to ADP (Liu et al. 2000) has revealed that it shares striking structural similarity with other members of this family, including the bacterial initiator protein DnaA (Erzberger et al. 2002), the pentameric γ–δ clamp loader (an analog of eukaryotic RFC; Jeruzalmi et al. 2001), and the human NSF-d2 hexamer that is involved in intracellular protein trafficking (Lenzen et al. 1998; Yu et al. 1998). Molecular genetic evidence indicates that ATP binding and/or hydrolysis is required for Cdc6p activity because mutation of conserved residues within the ATP-binding motifs of Cdc6p homologs either impairs or eliminates cellular growth and the ability to load the MCM helicase (Perkins and Diffley 1998; DeRyckere et al. 1999; Weinreich et al. 1999; Frolova et al. 2002). We had shown previously that substitutions of a conserved lysine residue in the Cdc6p Walker A box, which is predicted to contact β–γ phosphates of ATP, compromised or eliminated *CDC6* function (Weinreich et al. 1999). Although Cdc6p may act as a direct loader of the MCM helicase, there are no mechanistic data demonstrating how Cdc6p performs its essential function during initiation.

In addition to its essential role in DNA replication, ORC is involved in the formation of heterochromatin (Bell et al. 1993; Foss et al. 1993). ORC is required for heterochromatin assembly at the two silent mating-type *HM* loci in budding yeast, it influences heterochromatin structure at telomeres (Fox et al. 1997), and is present at heterochromatic regions in *Drosophila* (Pak et al. 1997). ORC has been shown to interact with both the *Drosophila* and *Xenopus* heterochromatin protein 1 (HP1). Budding yeast Orc1p recruits Sir1p to the *HM* loci (Triolo and Sternglanz 1996; Fox et al. 1997), which is required to help establish the silent chromatin state. However, the Orc1p domain that recruits Sir1p to the silencers is not required for ORC's role in DNA replication (Bell et al. 1995).

There are three additional silent information regulator genes (*SIR2–4*; Rine and Herskowitz 1987) that have well-established roles for formation of an alternative chromatin structure at the heterochromatic *HM* loci (Rusche et al. 2003) and at telomeres (Gottschling et al. 1990). *SIR2* is the founding member of a conserved family of $NAD^+$-dependent histone and protein deacetylases (Imai et al. 2000; Landry et al. 2000; Smith et al. 2000). *SIR2* is required for transcriptional silencing at the *HM* loci and telomeres as well as suppression of recombination between the 100–200 directly repeated copies of the ribosomal DNA (rDNA) genes (Gottlieb and Esposito 1989). This latter function of Sir2p promotes longevity in yeast (Guarente 2000; Sinclair 2002). Sir2p deacetylates specific acetyl-lysine residues within the N-terminal tails of histones H3 and H4 in *S. cerevisiae*, and this catalytic activity is required for its ability to function as a silencing protein. Mammalian *SIR2* homologs have been shown to deacetylate nonhistone proteins including p53 (Luo et al. 2001; Vaziri et al. 2001), tubulin (North et al. 2003), and also the $TAF_I68$ subunit of RNA polymerase I (Muth et al. 2001), raising the possibility that histones may not be the only targets of the budding yeast Sir2p. The closest murine *SIR2* homolog, SIRT1, is essential for normal development, and few homozygous mice survive to birth (Cheng et al. 2003). *SIR3* and *SIR4* encode chromatin-binding proteins unique to budding yeast. Sir3p and Sir4p interact with hypoacetylated histone H3 and H4 N-terminal tails (Hecht et al. 1995; Carmen et al. 2002) and are essential for heterochromatin formation at the silent-mating-type loci and at telomeres. Sir4p is thought to initiate the formation of heterochromatin through its ability to interact with multiple proteins, including the DNA-binding protein Rap1p (Luo et al. 2002), which is present at telomeres and silencers; histones H3 and H4 (Hecht et al. 1995); and Sir1p, Sir2p, and Sir3p (Gasser and Cockell 2001).

To further understand the process of pre-RC assembly, we isolated chromosomal suppressors of a *cdc6* temperature-sensitive allele that altered a conserved lysine residue (K114A) in the ATP-binding pocket. Some of these suppressors mapped to (and inactivated) the heterochromatin genes *SIR2*, *SIR3*, and *SIR4*. Our findings established that inactivation of the *SIR2–4* genes suppress an initiation mutant, although likely by different genetic pathways, and that this suppression is independent of the known roles for the SIRs in the cell. Because a deletion of *SIR2* (but not of *SIR3* or *SIR4*) suppressed additional pre-RC mutants and reversed many of the replication defects of *cdc6-4*, we suggest that Sir2p is acting to inhibit pre-RC assembly through its enzymatic activity as a protein deacetylase.

## Results

### *Deletion of SIR2, SIR3, or SIR4 suppresses a replication initiation mutant*

A strain containing the *cdc6-4* mutation, which changes the conserved lysine at position 114 in the Walker A motif to an alanine, was plated at 37°C to select spontaneous suppressors of its temperature-sensitive lethality. We isolated multiple independent clones that could grow at 37°C but observed that many of the suppressor strains were sterile because they could not mate with a strain of the opposite mating type. There are four *SIR* genes in yeast required for full transcriptional repression at the silent-mating-type cassettes, *HMR* and *HML*, and mutations within these chromatin-modifying genes lead to defects in mating (Rine and Herskowitz 1987). Therefore, we tested whether wild-type plasmid copies of the *SIR* genes complemented the sterility of the suppressors. The mating defects of two strains were complemented by *SIR2*, four were complemented by *SIR3*, and one was complemented by *SIR4*. None of the mating-defective strains was complemented by *SIR1*. We subsequently confirmed that in each case the suppressor of temperature sensitivity was linked to the same *SIR* gene that complemented the mating deficiency (see Materials and Methods), suggesting that a loss of *SIR2–4* function was

likely also responsible for the suppression of the *cdc6-4* mutant.

We tested whether null mutations in the *SIR* genes would suppress the *cdc6-4* temperature-sensitive defect. ORC also has a role in silencing by binding to the *HM* silencers and recruiting Sir1p through an Orc1p N-terminal domain that is dispensable for its role in DNA replication (Bell et al. 1995; Triolo and Sternglanz 1996; Gardner et al. 1999). Deleting *SIR2*, *SIR3*, or *SIR4* (but not *SIR1* or amino acids 2–235 of Orc1p) suppressed the temperature sensitivity of *cdc6-4*. We examined the degree of suppression by spotting serial 10-fold dilutions of each double mutant strain both at the permissive temperature of 25°C and also at the nonpermissive temperature of 37°C to solid media (Fig. 1A). Deleting *SIR2* almost fully restored wild-type growth at 37°C. Deletions of *SIR3* and *SIR4* were less efficient suppressors than a *SIR2* deletion, by 10-fold and 100-fold, respectively. However, deletion of *SIR1* or of the *ORC1* N terminus had little or no ability to suppress *cdc6-4*. The deletions of *SIR2*, *SIR3*, or *SIR4* were required to suppress the *cdc6-4* temperature sensitivity because transformation of the corresponding wild-type *SIR* gene into the *sir*Δ *cdc6-4* double-mutant strains reversed both the $TS^+$ phenotype and the sterility (data not shown). Because deletion of *SIR2* (but not of *SIR3* or *SIR4*) rescued the plasmid stability and S-phase defects of the *cdc6-4* mutant (see below), the *SIR3* and *SIR4* deletions are likely partially rescuing the growth of *cdc6-4* by a different mechanism than the loss of *SIR2*, which is the focus of this report.

### *The loss of mating-type or telomeric silencing does not indirectly suppress the* cdc6-4 *mutant*

Because *SIR2* is required for transcriptional silencing at the *HM* loci and telomeres, we first tested whether a deletion of *SIR2* could be indirectly affecting replication in the *cdc6-4* mutant through its roles in these processes. However, a priori, it seemed very unlikely that a loss of *HM* or telomeric silencing alone suppressed the *cdc6-4* mutant because the disruption of *SIR2*, *SIR3*, or *SIR4* completely abolished both TPE and *HM* silencing, but these same deletions had a 100-fold differential effect on the growth of *cdc6-4* at 37°C (Fig. 1A).

Disruption of *SIR2*, *SIR3*, or *SIR4* in a haploid strain causes expression of the genes at both the *HMR**a*** and *HML*α silent mating-type loci with accompanying transcriptional changes at the mating-type-responsive genes (Rusche et al. 2003). We, therefore, compared the *cdc6-4* temperature sensitivity in cells expressing **a**, α, or both **a** and α information. The *MAT**a***, *MAT*α, and the *MAT**a***/*MAT*α *cdc6-4* diploid were all temperature sensitive (Fig. 1B). We also constructed a *MAT*α *cdc6-4 HMR**a**-ss** haploid strain that was completely defective for silencing at *HMR**a*** because of a *cis* mutation of the Rap1p-binding site in the synthetic silencer (McNally and Rine 1991). The **a** and α gene expression caused by a loss of silencing at *HMR**a*** in the haploid also did not suppress the *cdc6-4* temperature sensitivity (Fig. 1B). These data indicate that expression of **a**, α, or both **a** and α mating-type genes does not suppress the temperature sensitivity of the *cdc6-4* mutant. Neither did a duplication (tandem integration) of *cdc6-4* rescue the temperature-sensitive phenotype (Fig. 1B), even though this strain increased *cdc6-4* mRNA expression 7.8-fold (data not shown). Therefore, increasing the number of *cdc6-4* copies either in the diploid or with a double integration also does not suppress its temperature sensitivity.

We next examined whether a loss of telomeric silencing could suppress the *cdc6-4* mutant because the Sir2–4 proteins are required for the transcriptional silencing of genes near the telomeres, termed the telomere position effect (TPE; Gottschling et al. 1990; Aparicio et al. 1991). The specialized telomeric chromatin structure requiring

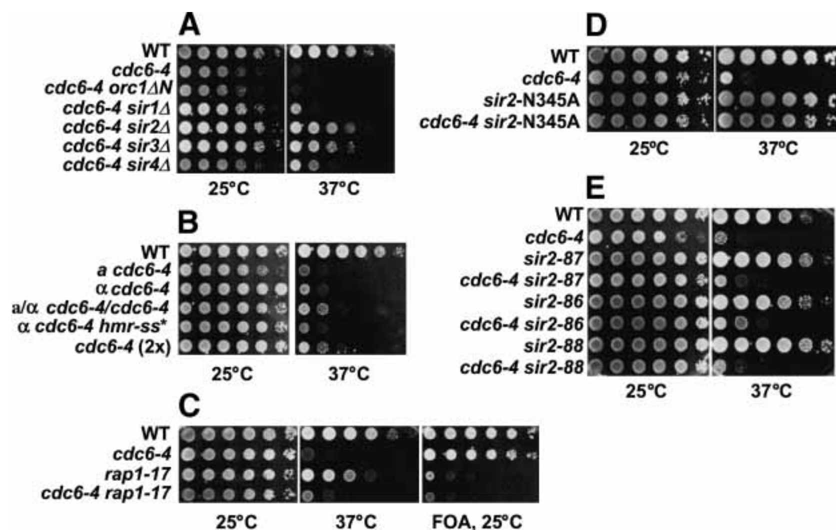


**Figure 1.** Tenfold serial dilutions of strains were spotted onto plates and incubated at 25°C (3 d) and 37°C (2 d). (*A*) Deletion of *SIR2-4* suppresses the temperature-sensitivity of *cdc6-4*. M138 (W303-1A), M386 (*cdc6-4*), M636 (*cdc6-4 orc1*$\Delta N_{6-235}$), M638 (*cdc6-4 sir1*Δ), M922 (*cdc6-4 sir2*Δ), M971 (*cdc6-4 sir3*Δ), and M974 (*cdc6-4 sir4*Δ). (*B*) Simultaneous *MAT**a*** and *MAT*α expression does not suppress *cdc6-4*. M138 (WT), M386 (*MAT**a** cdc6-4*), M599 (*MAT*α *cdc6–4*), M1101 (*MAT**a***/*MAT*α *cdc6-4/cdc6-4*), M1102 (*MAT*α *cdc6-4 hmr**a**-*ss*), and M576 (*MAT*α *cdc6–4[2×]*). (*C*) Disruption of telomeric silencing does not suppress *cdc6-4*. M1020 (WT, VIIL::*URA3*-tel), M1021 (*cdc6-4* VIIL::*URA3*-tel), AJL369-4d (*rap1-17* VIIL::*URA3*-tel), M1010 (*cdc6-4 rap1-17* VIIL::*URA3*-tel). (*D*) Loss of Sir2p deacetylase activity suppresses *cdc6-4*. M138 (WT), M386 (*cdc6-4*), M795 (*sir2*-N345A), and M1100 (*cdc6-4 sir2*-N345A). (*E*) Loss of Sir2p rDNA localization does not suppress *cdc6-4*. M138 (WT), M386 (*cdc6-4*), M1117 (*sir2-87*), M1118 (*cdc6-4 sir2-87*), M1155 (*sir2-86*), M1164 (*cdc6-4 sir2-86*), M1156 (*sir2-88*), and M1166 (*cdc6-4 sir2-88*).

the SIR proteins also contributes to the late replication timing of origins located within the subtelomeric domain (Stevenson and Gottschling 1999; Cosgrove et al. 2002), and it was possible that a loss of TPE might be affecting more origins than expected. Rap1p is required for formation of the telomeric and subtelomeric chromatin structure in part through recruiting Sir3p and Sir4p to the telomeres (Lustig et al. 1990; Kyrion et al. 1993; Cockell et al. 1995; Strahl-Bolsinger et al. 1997; Moretti and Shore 2001). Therefore, we crossed *cdc6-4* to the TS+ *rap1-17* allele that is known to abolish telomeric silencing and the Rap1p–Sir4p interaction. The *cdc6-4 rap1-17* double mutant lost TPE as evidenced by the derepression of the *URA3* reporter near telomere VII-L (Fig. 1C). However, all of the double mutants we recovered were still temperature sensitive, indicating that the loss of telomeric silencing or of the specialized telomeric chromatin structure per se is not capable of suppressing the *cdc6-4* initiation mutant.

### *The loss of Sir2p deacetylase activity but not its rDNA localization suppresses the growth of the cdc6-4 initiation mutant*

*SIR2* encodes an NAD+-dependent histone deacetylase that targets acetyl lysines at the histone H3 and H4 N termini. An N345A mutation in the catalytic domain of Sir2p abolishes its deacetylase activity together with its ability to transcriptionally silence its targets (Imai et al. 2000), although the N345A and additional Sir2p catalytic mutant proteins still bind in a spatially restricted pattern at the *HMR-E* silencer and the rDNA locus (Hoppe et al. 2002; Rusche et al. 2002). We isolated *cdc6-4 sir2*-N345A double mutants and observed that this catalytically inactive *sir2* mutant suppressed the *cdc6-4* temperature sensitivity similarly to a deletion of *SIR2* (Fig. 1D). Therefore, a loss of Sir2p enzymatic activity suppressed the *cdc6-4* mutant.

Sir2p has a unique role within the nucleolus independent of the other SIR proteins. Sir2p inhibits recombination between the 100–200 copies of the directly repeated rDNA gene cassettes (Gottlieb and Esposito 1989) and also silences Pol II transcribed genes placed directly adjacent to or within the rDNA locus (Bryk et al. 1997; Smith and Boeke 1997; Buck et al. 2002). Sir2p, Net1p, and Cdc14p are subunits of the nucleolar RENT complex (regulator of nucleolar silencing and telophase exit), and Sir2p localization to the nucleolus requires Net1p (Shou et al. 1999; Straight et al. 1999; Visintin et al. 1999). It was therefore possible that a loss of the nucleolar function of Sir2p suppressed the *cdc6-4* mutant in an indirect manner. We examined whether separation-of-function *sir2* mutations that cause a loss of Sir2p localization to the nucleolus (but still allow silencing at the *HM* loci and at telomeres) suppressed the *cdc6-4* mutant. A *sir2-87* mutant that deletes the last 15 amino acids of Sir2p is specifically defective for rDNA silencing (Cuperus et al. 2000). The Sir2-87 protein does not localize to the nucleolus because of an impaired interaction with Net1p. We verified that the *sir2-87* mutant was defective for rDNA silencing and crossed it to the *cdc6-4* mutant. However, because the double-mutant *sir2-87 cdc6-4* strains were still temperature sensitive (Fig. 1E), a loss of rDNA localization in this mutant did not suppress the *cdc6-4* mutant. Two additional rDNA silencing alleles (*sir2-86* and *sir2-88*), that we confirmed were defective for rDNA silencing (Cuperus et al. 2000), also did not suppress the temperature sensitivity of the *cdc6-4* mutant (Fig. 1E). Taken together, these data indicate that a loss of Sir2p function at the rDNA locus is not responsible for the *cdc6-4* suppression seen in the *sir2*-null mutant.

Cdc6p is an unstable protein that has a half-life of ~5 min (Drury et al. 2000). This instability varies during the cell cycle and is mediated by ubiquitin-mediated proteolysis of Cdc6p. Although *SIR2* is not known to regulate ubiquitin-mediated protein stability, we tested whether deletion of *SIR2* had an effect on Cdc6p protein levels. We compared total cell extracts of wild-type and *sir2*Δ strains expressing 3HA-Cdc6p as well as 3HA-Cdc6-4p at the permissive temperature. Because the levels of wild-type and Cdc6-4p are the same in a wild-type strain

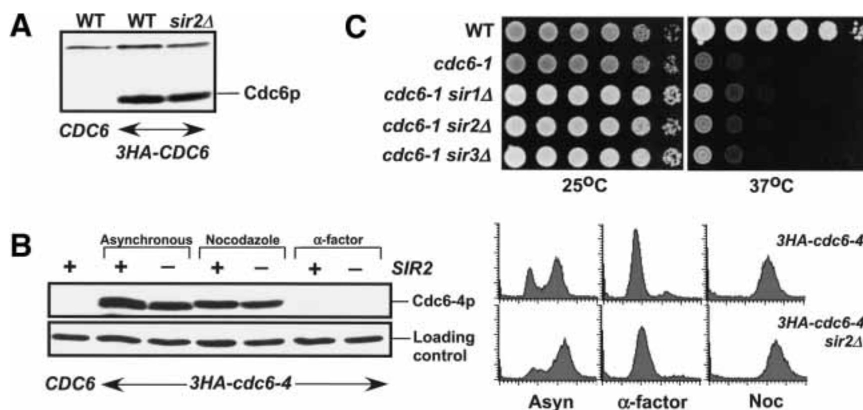


**Figure 2.** Deletion of *SIR2* does not alter the abundance of the wild-type or Cdc6-4 proteins. (*A*) 12CA5 Western blot for Cdc6p from asynchronous total cell extracts of W303-1A (untagged), M276 (*3HA-CDC6*), and M1065 (*3HA-CDC6 sir2*Δ). (*B*) 12CA5 Western blot of Cdc6-4p and FACS samples from asynchronous cells and cells completely arrested in G2/M phase using 15 µg/mL nocodazole or in G1 phase using 10 µg/mL α-factor; W303-1A (untagged), M1257 (*3HA-cdc6-4*), M1274 (*3HA-cdc6-4 sir2*Δ), M1299 (*3HA-cdc6-4 sir2*Δ *hml*Δ). (*C*) Deletion of *SIR1*, *SIR2*, or *SIR3* does not suppress the *cdc6-1* temperature sensitivity. W303-1A (WT), M379 (*cdc6-1*), M1103 (*cdc6-1 sir1*Δ), M1104 (*cdc6-1 sir2*Δ), M1105 (*cdc6-1 sir3*Δ).

and in the strains deleted for *SIR2* (Fig. 2A,B), a deletion of *SIR2* is not causing a significant stabilization of Cdc6p. We also compared Cdc6-4p levels in $SIR2^{WT}$ and *sir2*Δ strains using G2/M and G1 synchronized cells (Cdc6p is highly unstable in α-factor-arrested cells) and again saw no significant differences in Cdc6-4p abundance (Fig. 2B). In addition, deletion of *SIR1*, *SIR2*, or *SIR3* does not suppress the *cdc6-1* temperature sensitivity (Fig. 2C), indicating that the loss of the *SIR* genes is not bypassing the requirement for *CDC6*.

### *Deletion of SIR2 rescues the DNA replication defect of cdc6-4*

If deletion of *SIR2* is suppressing the initiation defect of the *cdc6-4* mutant, the double-mutant strain should progress through S phase when released from a G1 block at the restrictive temperature. The *cdc6-4* mutant progresses through S phase very slowly even at the permissive temperature and is defective in loading the MCM proteins to chromatin (Weinreich et al. 1999). The wild-type, *cdc6-4*, and *cdc6-4 sir2*Δ strains were arrested in G1 phase, shifted to the restrictive temperature at the G 1-arrest point, and then released into the cell cycle at the restrictive temperature for *cdc6-4* and examined for DNA content by flow cytometry (Fig. 3). The wild-type strain entered S phase within 30 min of the G1 release and had largely completed S phase by 60 min as evidenced by the subsequent reappearance of cells with 1C DNA content. The *cdc6-4* strain, however, remained arrested at the G1 stage with largely a 1C DNA content and did not progress through S phase. In contrast to the *cdc6-4* mutant, the *sir2*Δ *cdc6-4* double mutant exhibited a nearly wild-type S phase because the cells begin S phase between 30 and 40 min. These cells do not re-enter G1 phase with the same timing as the wild-type cells, perhaps indicating that DNA replication is not completed as accurately as in wild type or that additional functions of Cdc6p during G2/M are not effectively bypassed by a deletion of *SIR2*. Asynchronous cultures of *cdc6-4 sir2*Δ grown at 25°C also exhibit a substantial portion of cells in the G2/M phase, indicative of a cell cycle delay (data not shown). Importantly, the deletion of *SIR2* allowed S phase to occur in the *cdc6-4* mutant in a manner reflecting its nearly wild-type growth at 37°C, indicating that the loss of *SIR2* is suppressing the initiation defect of *cdc6-4*.

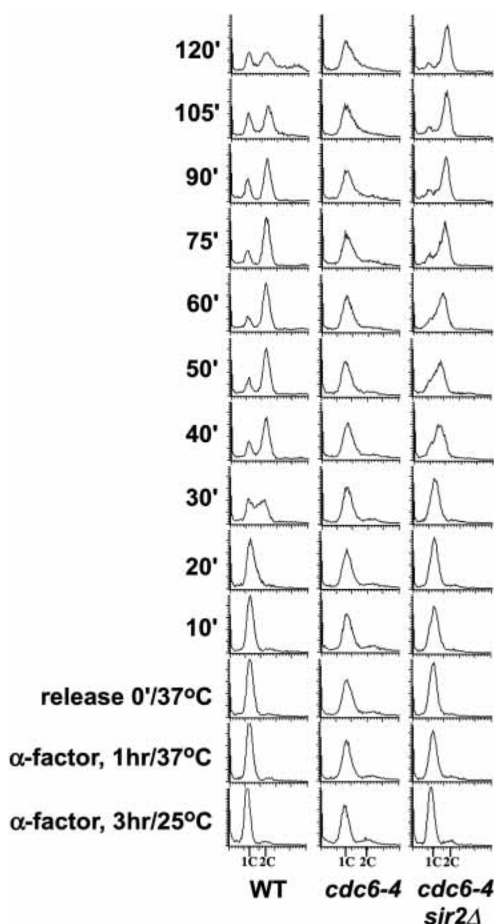


**Figure 3.** Deletion of *SIR2* promotes entry into S phase in the *cdc6-4* mutant. M138 (WT), M386 (*cdc6-4*), and M940 (*cdc6-4 sir2*Δ *hml*Δ) were arrested with α-factor for 3 h at 25°C, shifted for 1 h to 37°C, and then released from the arrest into fresh YPD medium at 37°C. Cell cycle progression was monitored by flow cytometry as described (Weinreich et al. 1999).

### *Loss of SIR2 rescues additional initiation mutants, but not mutants that act after pre-RC assembly*

Given that the loss of *SIR2* suppressed the *cdc6-4* initiation mutant, we tested whether temperature-sensitive mutations in other initiation mutants could be suppressed by deletion of *SIR2*. Deletion of *SIR2* almost completely suppressed the temperature-sensitive growth of the *orc5-1* and *mcm2-1* pre-RC mutants at 35°C, but not at 37°C (Fig. 4A). In contrast, deletion of *SIR2* did not suppress the temperature-sensitive phenotypes of the *cdc7-1* (protein kinase) or the *cdc17-1* (DNA polymerase α) mutants that act in steps subsequent to pre-RC assembly at temperatures ranging from 37°C to 30°C (Fig. 4A). Because the *ORC* and *MCM* genes are required for pre-RC assembly together with *CDC6*, this suggested that *SIR2* negatively regulates initiation at the level of pre-RC assembly but not at stages subsequent to this step.

We also examined the specificity of the *sir2* suppression by testing whether additional histone deacetylase mutants could suppress the *cdc6-4* temperature sensitivity. Based on sequence comparisons among organisms, *RPD3*, *HDA1*, and *SIR2* form three distinct classes of histone deacetylases; class I, II, and III, respectively (Marks et al. 2001; Kurdistani and Grunstein 2003). *RPD3* encodes a global histone deacetylase that negatively regulates the expression of many genes and, importantly, a deletion of *RPD3* has been shown to advance the replication time of both early and late origins in a manner correlated with the loss of histone acetylation

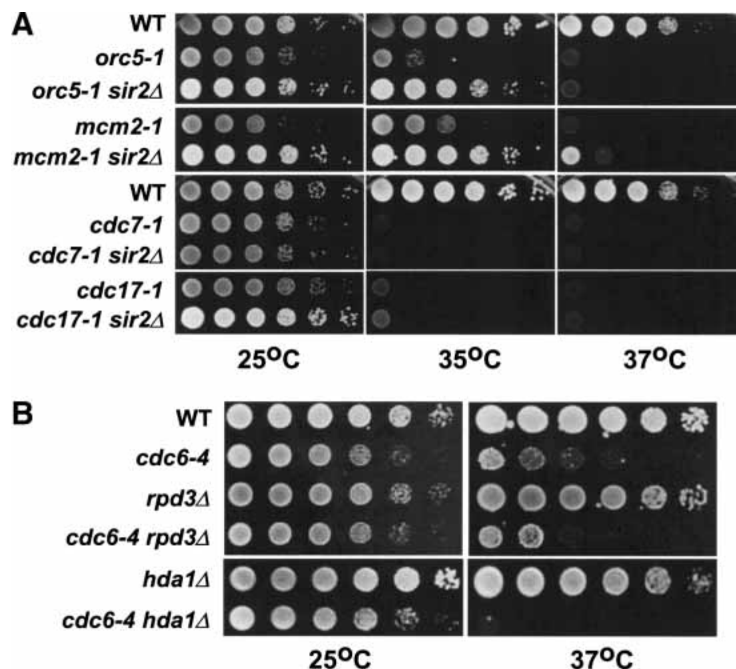


**Figure 4.** (*A*) Deletion of *SIR2* can partially rescue additional pre-RC mutants. M138 (WT), M198 (*orc5-1*), M1096 (*orc5-1 sir2*Δ), M359 (*mcm2-1*), M1097 (*mcm2-1 sir2*Δ), M444 (*cdc7-1*), M1098 (*cdc7-1 sir2*Δ), M354 (*cdc17-1*), and M1099 (*cdc17-1 sir2*Δ). (*B*) Deletion of the histone deacetylases *RPD3* or *HDA1* will not suppress *cdc6-4*. M138 (WT), M386 (*cdc6-4*), WJY140 (*rpd3*Δ), M1080 (*cdc6-4 rpd3*Δ), M1161 (*hda1*Δ), and M1174 (*cdc6-4 hda1*Δ).

near origins (Vogelauer et al. 2002). Rpd3p deacetylates all four histones in vitro, but interestingly, it does not deacetylate H4 K16, an important Sir2p target (Rusche et al. 2003). The Hda1p histone deacetylase also negatively regulates transcription of many genes throughout the genome, but it preferentially acts in a subtelomeric region termed the HAST domain (Robyr et al. 2002) and is thought to deacetylate only histones H3 and H2A. We constructed double mutants of *rpd3*Δ or *hda1*Δ with *cdc6-4* and found that deletions of these histone deacetylases would not suppress the temperature sensitivity of the *cdc6-4* mutant (Fig. 4B). This indicates that loss of a *SIR2* specific deacetylase function is required to suppress *cdc6-4*.

### *Deletion of* SIR2 *rescues the plasmid instability phenotype of* cdc6-4

As a third test of the effect of *SIR2* on replication initiation, we compared the plasmid loss rates of wild-type, *cdc6-4*, and *cdc6-4 sir2*Δ strains. Initiation mutants exhibit a high plasmid loss rate because they fail to initiate DNA replication from the plasmid origin in every cell cycle, and this is also true for *cdc6* mutants (Hogan and Koshland 1992). The high plasmid loss rate of an initiation mutant can be rescued by increasing the frequency at which the plasmid origin fires or by adding additional origins to the plasmid, which increases the probability that any one origin will fire. If deleting *SIR2* is causing an increase in initiation frequencies at specific origins, then the *sir2*Δ should also reverse the plasmid instability phenotype of the *cdc6-4* mutant. We therefore measured plasmid loss rates in wild-type, *sir2*Δ, *cdc6-4*, and the *cdc6-4 sir2*Δ strains at the permissive temperature for six different origin sequences. Wild-type strains typically lose plasmids at rates between 3% and 6% per generation under nonselective conditions, and this was true for all of the origins we tested (Fig. 5). In the wild-type background, deletion of *SIR2* slightly improved the plasmid loss rates for the *ARS1* and *ARSH4* plasmids but substantially improved loss rates for the remaining origins. The *cdc6-4* mutant had a highly elevated plasmid loss rate that varied from 15% to 25% per generation for these six origins. Significantly, deletion of *SIR2* rescued the high plasmid loss rate of *cdc6-4* but only for some of the origins we tested, suggesting that *SIR2* does not negatively regulate all origins with the same efficiency. A *sir2*Δ completely reversed the instability phenotype for the *ARS305* and *ARS501* plasmids and substantially reversed the loss rate of the *ARS315* plasmid. However, a *sir2*Δ had a partial effect on the *ARS1* plasmid and no effect on the loss rates of the *ARSH4* and *HML*-E (*ARS301*) plasmids. Because *ARS1*, *ARS301*, *ARS305*, and *ARS315* were present within an otherwise identical plasmid context, the *SIR2*-dependent variation in plasmid stability in the *cdc6-4* strain was caused by the origin sequences present on the plasmids.

### *Deletion of* SIR2 *promotes MCM origin binding in the* cdc6-4 *mutant*

Cdc6p is required to load MCM proteins at origins of replication (Cocker et al. 1996; Aparicio et al. 1997; Tanaka et al. 1997). We therefore used chromatin immunoprecipitation (ChIP) to address whether MCM protein could be loaded in the *cdc6-4* and *cdc6-4 sir2*Δ mutants at several of the origins that were differentially affected by deletion of *SIR2* in the plasmid instability assay. We used PCR primers to amplify short sequences that contained the *ARS1*, *ARS315*, and *ARS501* origins following

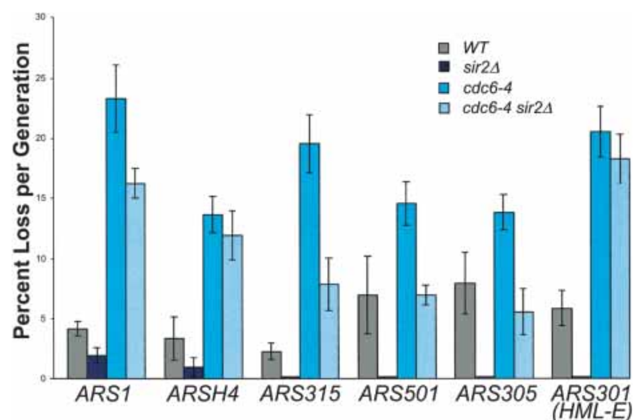


**Figure 5.** Deletion of *SIR2* rescues the plasmid instability defect conferred by *cdc6-4* at certain origins and improves plasmid stability in wild-type cells. Values were determined as in Dani and Zakian (1983) and are reported are percentage plasmid loss rate per generation in nonselective medium at 25°C. They represent the average of at least four measurements. The *ARS501* plasmid is pR151 (Ferguson et al. 1991), *ARSH4* is pRS416, and the remaining plasmids have ~300–500-bp origin fragments replacing the *ARS1* origin on pARS1-WT (Marahrens and Stillman 1992).

ChIP. Adjacent non-origin sequences within several kilobases of the *ARS* elements were amplified as negative controls (Fig. 6A). Wild-type, *cdc6-4*, and *cdc6-4 sir2*Δ cells were arrested at the permissive temperature in G2/M and then either released at the permissive temperature or raised to the nonpermissive temperature of 37°C prior to release into G1. During progression into G1, MCM proteins are loaded at origins in a *CDC6*-dependent manner (outlined in Fig. 6B). We used a monoclonal antibody against Mcm2p to identify MCM protein loading. In all cases, no Mcm2p was associated with the origins in G2/M-arrested cells (Fig. 6C,D). In the wild type, Mcm2p specifically associated with origin-containing fragments for these three origins at 25°C and 37°C during release to the G1 arrest. For *cdc6-4*, we observed a similar, weak recovery for all of the origins at 25°C, but the *cdc6-4* mutant was completely defective for loading Mcm2p at 37°C. Deletion of *SIR2* in the *cdc6-4* background had a differential effect for the origins we tested. We saw a partial recovery of *ARS1* Mcm2p loading at 25°C but very little at 37°C. For the *ARS315* and *ARS501* origins, deletion of *SIR2* allowed more efficient Mcm2p loading at both the permissive and nonpermissive temperatures. These data correlate well with the plasmid instability assays (Fig. 5) and also the flow cytometry data (Fig. 3), showing that deleting *SIR2* promoted S phase in the *cdc6-4* mutant.

## Discussion

We have uncovered a negative regulatory role for Sir2p in DNA replication using a *cdc6-4* mutant defective for initiation. Because a deletion of *SIR2* promoted a nearly wild-type S phase in the *cdc6-4* mutant background, a loss of Sir2p function is restoring the ability to replicate DNA (and therefore initiate DNA replication) on a genome-wide level. A *SIR2* deletion also partially rescued the temperature-sensitive growth of two other pre-RC mutants, *orc5-1* and *mcm2-1*, but not of replication mutants that act after pre-RC assembly. This suggests that Sir2p is affecting some early step in pre-RC formation and also that the loss of *SIR2* is not enhancing DNA replication in a generalized or nonspecific manner.

*SIR2* is required for silent heterochromatin formation in yeast; however, the loss of *SIR2* is not indirectly suppressing the initiation defect of *cdc6-4* through its known functions in the genome, either by transcriptional silencing at the *HM* loci, at telomere proximal genes, or by some indirect mechanism affected by increased recombination or a loss of silencing within the rDNA repeats. Although we initially isolated the *sir2* suppressor using the *cdc6-4* allele integrated at the *LEU2* locus, a deletion of *SIR2* also suppresses the temperature-sensitive phenotype of *cdc6-4* when it is present at its normal chromosomal location (D.L. Pappas Jr. and M. Weinreich, unpubl.). Importantly, we have shown that both Cdc6p and Cdc6-4p abundance is the same in a wild-type and in a *SIR2* deletion strain. Thus, a *SIR2* deletion suppresses *cdc6-4* in a context-independent fashion that is also not explained by an increase in *cdc6-4* expression. Taken together, these data suggest that *SIR2* has additional targets in the cell distinct from the silent heterochromatic loci.

Both the plasmid stability and ChIP assays indicated that loss of *SIR2* did not affect all origins equally. A *SIR2* deletion substantially reversed the *cdc6-4* plasmid instability phenotype for the *ARS305*, *ARS315*, and *ARS501* origin plasmids but only partially rescued *ARS1* plasmid instability and had no effect for the *ARSH4* and *ARS301* plasmids. In a wild-type strain, the *SIR2* deletion caused similar effects with the exception of the *ARS301* origin plasmid, which was now very efficiently maintained. The fact that the high plasmid loss rate of some origins was rescued by deletion of *SIR2* but others were unaffected or minimally affected, suggests that Sir2p is having specific effects at particular origins or that some origins are less sensitive to Sir2p inhibition is this assay. The different effects on plasmid stability seen by deletion of *SIR2* could also reflect different chromatin structures surrounding the origins, differences in origin sequence, or efficiency of origin usage. The observation that a *sir2*Δ in an otherwise wild-type background brings some plasmid retention rates to nearly 100% suggests that *SIR2* inhibits origin usage in wild-type cells.

A *SIR2* deletion had a differential effect on MCM loading at origins in the *cdc6-4* mutant. To examine pre-RC formation, we tested Mcm2p binding to three efficient origins (by ChIP) in the wild-type, *cdc6-4*, and *cdc6-4 sir2*Δ mutant strains. These experiments showed that the *cdc6-4* mutant was defective for Mcm2p origin loading at the permissive temperature and inactive for Mcm2p loading at the nonpermissive temperature at all three origins, as suggested previously (Weinreich et al. 1999). Deletion of *SIR2* promoted Mcm2p binding to the

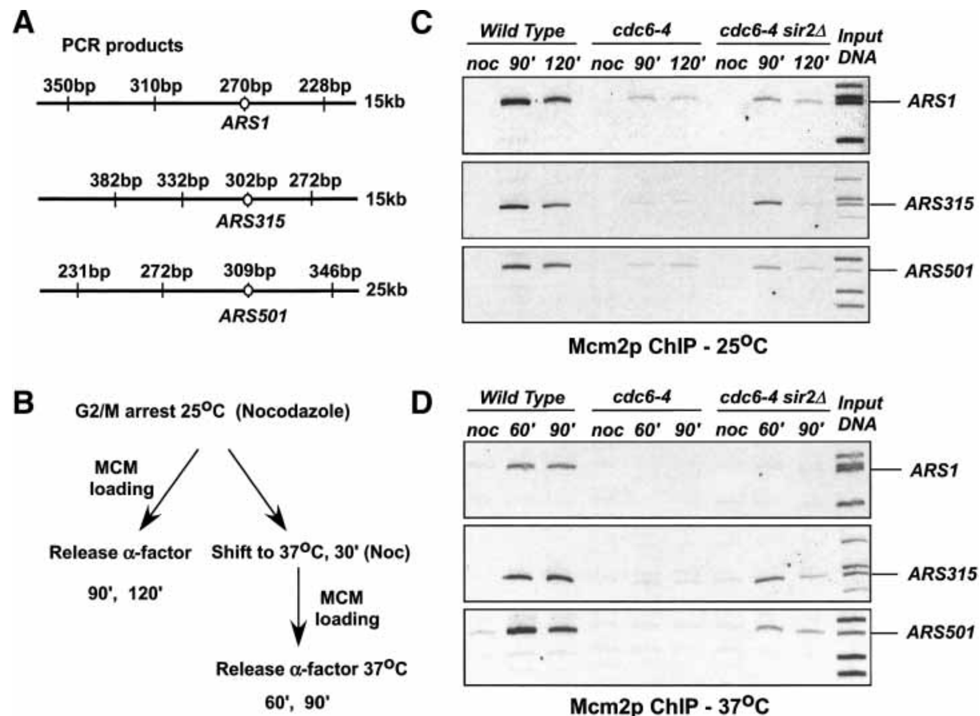


**Figure 6.** Deletion of *SIR2* restores Mcm2p loading at origins. ChIP assays were performed as described in Materials and Methods using cells grown in YPD medium to mid-log phase, arrested in G2/M with 15 µg/mL nocodazole, and then released into YPD medium containing 5 µg/mL α-factor for 60, 90, or 120 min, so that 95% of cells had a 1C DNA content as determined by flow cytometry. W303-1A (WT), M386 (*cdc6-4*), and M940 (*cdc6-4 sir2Δ hmlΔ*). A representative input DNA PCR sample is shown for each origin examined.

*ARS315* and *ARS501* chromosomal origins at 25°C and 37°C; however, a *sir2Δ* had only a minimal effect at the *ARS1* origin at the permissive temperature and did not promote Mcm2p binding to *ARS1* at the nonpermissive temperature. These results correlated well with the plasmid instability data and further suggested that the loss of *SIR2* differentially affects pre-RC formation at these origins. Because *ARS1*, *ARS315*, and *ARS501* are efficient chromosomal origins that fire at different times during S phase (Raghuraman et al. 2001), it appears that the rescue of origin activity is not strictly correlated with their time of activation but more properly with pre-RC assembly.

Consistent with our finding that Sir2p is a negative regulator of chromosomal DNA replication, a recent report found that Sir2p negatively regulated initiation events within the rDNA locus using the technique of molecular combing (Pasero et al. 2002). Each 9.1-kb rDNA repeat contains an origin of replication, although only ~20%–25% of these origins are used during the cell cycle. A *SIR2* deletion resulted in a twofold increase in origin initiation within the rDNA locus on Chromosome XII. Another systematic ChIP study across the rDNA repeat has shown that Sir2p is enriched at two positions: at the NTS1 (non-transcribed 1) spacer and also at the NTS2 near the Pol I 35S gene promoter and extending within the 35S gene (Huang and Moazed 2003). These experiments suggest that the replication fork barrier protein Fob1 and RNA polymerase I target Sir2p to the NTS1 and NTS2, respectively. Thus, the current understanding of Sir2p localization in the cell suggests that potentially three strategies are used to bring Sir2p to its targets: Sir4p recruits Sir2p to the HM loci and telomeres; Fob1p and Pol I recruit Sir2p (i.e., RENT) to the rDNA locus. It is possible that Sir2p has additional chromosomal targets in the cell that have not been seen because of their transient nature or that Sir2p can interact with an initiation protein. At the rDNA locus, because Sir2p deacetylates nucleosomes throughout the entire region, a loss of Sir2p would likely also affect the nucleosomal acetylation state near the origin, and this could contribute to the increased initiation frequencies observed.

Another precedent indicating that Sir2p affects chromosome maintenance comes from the finding that increased expression of Sir2p from the regulated *GAL1* promoter caused lethality in budding yeast, and a transient increase in Sir2p levels (that did not result in lethality) substantially increased chromosome loss rates (Holmes et al. 1997). Although the precise mechanism of the chromosome loss was not known, increased minichromosome loss also occurred for plasmids that did not contain telomeric sequences, suggesting that this effect was independent of Sir2p binding near telomeres. Both the lethality and the increased chromosome loss rates upon Sir2p induction could certainly be explained by the negative regulation of DNA replication that we have uncovered, because suppression of initiation frequencies

throughout the genome would lead to chromosome loss and cell death.

It is interesting to note that temperature-sensitive mutations within the *ORC* genes were first isolated as silencing-defective mutants using the *HMR**a*** synthetic silencer (Foss et al. 1993), and we have found that deletion of *SIR*2 restores growth to *cdc6-4* and *orc5-1* mutants. It is well established that ORC is required for silencing at the *HM* loci. However, ORC promotes silencing through a replication-independent mechanism whereby Orc1p recruits Sir1p to the silencer via the ORC-binding sites within the silencer DNA elements (Triolo and Sternglanz 1996; Fox et al. 1997). Although there is currently no evidence for this, it is not inconceivable that nonsilencer origins could also recruit one or more SIR proteins, because Sir1p interacts with ORC and the Sir2–4 proteins are present on the chromatin throughout an ~3–4-kb region surrounding the silencer that naturally includes the ORC-binding sites (Rusche et al. 2002; Zhang et al. 2002). Because a deletion of *SIR1* or the Orc1p interaction domain with Sir1p will not suppress the *cdc6-4* initiation mutant, it is very unlikely that the effect of Sir2p on replication is mediated through a known heterochromatic role for ORC. How then might Sir2p negatively affect DNA replication?

Sir2p could directly inhibit origin usage by deacetylating origin-proximal nucleosomes, which either hinders recruitment of a key initiation factor or promotes binding of an inhibitory factor. Sir2p is unique among the three classes of deacetylases in that it requires $NAD^+$ as a cofactor for its enzymatic activity (Moazed 2001), and it has a distinct substrate specificity from both the Rpd3p and Hda1p deacetylases. Because deleting neither *RPD3* nor *HDA1* restored growth to the *cdc6-4* mutant, this suggests that either Sir2p-specific histone modifications are influencing the initiation of DNA replication or that an unknown function of Sir2p not shared by Rpd3p or Hda1p is negatively affecting initiation frequencies. A recent report has shown that histone acetylation near origins promotes earlier firing for both early and late origins. A deletion of the *RPD3* deacetylase was shown to advance replication timing for many individual origins and was correlated with earlier binding of Cdc45p to origins (Vogelauer et al. 2002). Cdc45p associates with origins after pre-RC formation in a manner correlated with their time of activation and thus is a temporal marker for the initiation of DNA synthesis (Aparicio et al. 1999). The authors also targeted the histone acetyltransferase Gcn5p to the internal late origin *ARS1412*, which caused its earlier activation and increased histone acetylation near *ARS1412*. These data established that histone acetylation promotes replication initiation and that one consequence of histone acetylation is the earlier recruitment of Cdc45p to origins. Thus, if *SIR2* is directly targeted to some origins in the cell, localized histone deacetylation could inhibit initiation.

Although Sir2p is a histone deacetylase, it could also deacetylate a nonhistone protein such as a pre-RC component or regulator of initiation and thereby inhibit DNA replication. As mentioned earlier, mammalian *SIR2* orthologs have been shown to deacetylate nonhistone proteins such as p53 (Luo et al. 2001; Vaziri et al. 2001) and tubulin (North et al. 2003). Although there is no evidence that Sir2p normally silences genes apart from the silent-mating-type cassettes, the rDNA locus, or near telomeres, it is also possible that Sir2p regulates the transcription of an unknown gene that is limiting for initiation. Whatever the exact mechanism, Sir2p inhibits a subset of origins, and therefore some particular aspect of origin sequence or structure not shared among all origins makes them sensitive to *SIR2* inhibition. Because initiation from all origins is not restored in the *cdc6-4 sir2Δ* mutant, the G2/M delay seen in this strain could be caused by slowed or incomplete DNA replication.

*SIR2* is the only *SIR* gene that is conserved in metazoans (Brachmann et al. 1995), and human cells alone contain seven *SIR2* orthologs, SIRT1–SIRT7 (Frye 2000). Because Sir2p is required for heterochromatin formation and also negatively regulates DNA replication in the budding yeast, we speculate that a conservation of this function in metazoans could afford one mechanism to link replication domains with heritable transcriptional states during development or in response to cell-autonomous signals.

## Materials and methods

### *Construction of yeast strains, growth media, and genetic methods*

The yeast strains used in this work are listed in Supplementary Table 1. Genetic manipulations were performed according to standard techniques. YPD denotes rich medium, and FOA denotes synthetic complete medium containing 1 mg/mL 5-fluoro-orotic acid.

*SIR2* was PCR-amplified from wild-type genomic DNA with forward oligonucleotide *SIR2*-Sal and reverse oligonucleotide *SIR2*-Xba (Supplementary Table 2). The PCR reaction contained 10 ng of genomic DNA, 200 µM dNTP, 50 pmoles of each oligonucleotide, and 2.5 units of *Pfu*Turbo (Stratagene) in a 50-µL reaction volume. The PCR product was digested with SalI and XbaI and cloned into the respective sites in pRS416 to generate pDP56. pDP56 served as a template for *SIR2* mutagenesis. Mutagenesis of *SIR2* was performed using the corresponding oligonucleotides in Supplementary Table 2 with the QuikChange method (Stratagene) according to manufacturer's specifications. In each case, the entire *SIR2* gene was sequenced to verify the presence of only a single mutation.

*sir2* mutations were integrated at the *SIR2* locus in W303-1A as outlined below. For *sir2*-N345A and *sir2-87*, a 1.4-kb BsrGI/XbaI fragment was cloned into the Acc65I/XbaI sites of YIplac211 (Gietz and Sugino 1988). For *sir2-88*, a 1.4-kb BsrGI/XbaI fragment was cloned into the Acc65I/XbaI sites of YIplac204 (Gietz and Sugino 1988). For *sir2-86*, a 1.6-kb SphI/StuI fragment was cloned into the SphI/SmaI sites of YIplac204. The resulting plasmids, pDP135 (*sir2*-N345A), pDP139 (*sir2-87*), pDP309 (*sir2-88*), and pDP307 (*sir2-86*), respectively, were linearized with BglII to direct integration. All integrations were confirmed by PCR of genomic DNA. PCR reactions were performed with 5 ng of genomic DNA, 15 pmoles of oligonucleotides *SIR2*-Sal (*SIR2*-specific) and M13-reverse sequencing primer (vector-specific), and 1.5 units of *Taq* DNA polymerase

(Invitrogen) in a 25-µL reaction volume. In the case of *sir2-86*, reaction conditions were the same except that 15 pmoles of *SIR2*-Xba was used as the *SIR2*-specific oligonucleotide. PCR products were visualized by agarose gel electrophoresis followed by ethidium bromide staining (0.5 µg/mL). PCR products were also subjected to automated DNA sequencing using an ABI 3700 Genetic Analyzer (Applied Biosystems). Individual sequencing reactions contained 50 ng of PCR product and 3.2 pmoles of a *SIR2*-specific sequencing oligonucleotide (*SIR2*-SP1, SP2, SP3 or SP4; Supplementary Table 2).

To construct M1161, *hda1::kanMX* was PCR-amplified from the Research Genetics strain 5347 using primers *HDA1*-1F and *HDA1*-1R (Supplementary Table 2) and integrated into W303-1A in a single step. The correct insertion was verified by PCR using the original primers and a second set of primers (*HDA1*-2F and *HDA1*-2R; Supplementary Table 2) flanking these and the *HDA1* ORF.

### *Isolation and characterization of extragenic suppressors of* cdc6-4

Strain M386 (W303-1A *cdc6-4*) was grown at 25°C in YPD until stationary phase. Approximately $2 \times 10^6$ cells each were plated onto multiple YPD plates containing 1% formamide and incubated for 3 d at 37°C. This concentration of fomamide did not affect the growth of the wild-type strain at 37°C but did tighten up the ts phenotype of the *cdc6-4* strain. Multiple independent spontaneous $Ts^+$ revertants (*rgc* mutants [restores growth of *cdc6-4*]) were isolated that had a secondary nonmating phenotype. Plasmids containing *SIR1*, *SIR2*, *SIR3*, or *SIR4* (from C. Fox, UW-Madison) were introduced into suppressor strains and then assayed for mating proficiency. To confirm that suppression of *cdc6-4* was conferred by a single gene mutation, strains transformed with their respective complementing *SIR* plasmid were crossed to M599 (*MATα cdc6-4*). Diploids were selected, cured of the wild-type *SIR* plasmid, and sporulated. In all cases, the tetrad analysis showed a 2:2 ratio of $Ts^+$:$Ts^-$ demonstrating the suppression of *cdc6-4* in each strain was caused by a single gene mutation. The sterile phenotype cosegregated with suppression of *cdc6-4*, indicating that it is a pleiotropic phenotype associated with the suppressor mutation. *SIR2*, *SIR3*, and *SIR4* were tested to determine if they were, indeed, allelic to the original mutations. A *TRP1* nutritional marker was integrated adjacent to the *SIR2*, *SIR3*, and *SIR4* loci individually in strain M599 (*MATα cdc6-4*). The three resulting integrant strains were crossed to *rgc* strains transformed with the respective complementing *SIR* plasmid. Diploids were selected and sporulated as above. In all cases, $Ts^+$:$Ts^-$ segregated 2:2 and the $Trp^+$ phenotype (wild-type *SIR*) segregated opposite the suppressor. These data indicate that the *rgc* mutants are bona fide suppressors of *cdc6-4* and are allelic to *SIR2*, *SIR3*, and *SIR4*.

To quantitate growth of strains, cells were 10-fold serially diluted and spotted onto YPD at 25°C and 37°C or YPD +1% formamide at 37°C for the *cdc6-4*-containing strains shown in Figures 1 and 4. Formamide diminished the appearance of sporadic *cdc6-4* revertants, which made scoring difficult. However, the same quantitative suppression could be seen for the *cdc6-4 sir* mutants in the absence of formamide.

### *FACS analysis*

Strains M138 (WT), M386 (*cdc6-4*), and M940 (*cdc6-4 sir2Δ hmlΔ*) were grown in YPD medium at 25°C to $OD_{600}$ = 0.25 and synchronized in G1 by the addition of 10 µg/mL α-factor for 3 h. Cultures were shifted for 1 h to 37°C in the presence of α-factor and then released into fresh medium in the absence of α-factor at 37°C. Aliquots of cells were harvested at the indicated times, processed for flow cytometry as described (Weinreich et al. 1999), and analyzed using a Becton-Dickenson FACScalibur machine.

### *Cloning of* ARS *elements from Chromosome III*

*ARS* elements for Chromosome III were defined in Poloumienko et al. (2001). *ARS305* and *ARS315* were PCR-amplified from genomic DNA with EcoRI and HindIII linkers (Supplementary Table 2), whereas *ARS301* was PCR-amplified from genomic DNA with SacI and HindIII. All were then cloned in place of the 192-bp *ARS1*-containing fragment from pARS1-WT (Marahrens and Stillman 1992). The wild-type DNA sequence was verified for each *ARS* element, and each recombinant plasmid was shown to contain a functional origin by the high-frequency transformation assay. Chromosomal coordinates contained on the plasmids are for *ARS301* (11,045–11,532), *ARS305* (39,382–39,724), and *ARS315* (224,804–225,318).

### *Plasmid instability assays*

Plasmids pARS1-WT, pRS416 (*ARSH4*), pRF21 (*ARS315*), pR151 (*ARS501*), pRF4 (*ARS305*), and pRF12 (*ARS301*) were introduced into strains M138 (WT), CFY366 (*sir2Δ*), M386 (*cdc6-4*), and M922 (*cdc6-4 sir2Δ*). Plasmid instability was performed as previously described (Dani and Zakian 1983). Results are reported as the average of four to six measurements with accompanying standard errors.

### *ChIP*

ChIP was performed as described (Strahl-Bolsinger et al. 1997) with the following modifications: the lysis buffer contained 300 mM NaCl and the immunoprecipitation (IP) was performed using an Mcm2p monoclonal antibody (kindly provided by B. Stillman) cross-linked to protein A Sepharose beads. 1/25-th of the IP DNA and 1/500-th of the input DNA were subjected to 30 cycles of multiplex PCR for *ARS1* and 28 cycles for *ARS315* and *ARS501* using the indicated primers (Supplementary Table 2). PCR products were separated on 5% polyacrylamide gels.

## Acknowledgments

We thank Catherine Fox, Art Lustig, Dan Gottschling, David Shore, Carol Newlon, Jeff Smith, Michael Grunstein, Steve Bell, and Bruce Stillman for generously providing yeast strains and reagents. We also thank Catherine Fox for encouragement and critical reading of the manuscript, and Marleah Russo and Kelli VanDussen for expert technical assistance.

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## References

Aparicio, O.M., Billington, B.L., and Gottschling, D.E. 1991. Modifiers of position effect are shared between telomeric and silent mating-type loci in *S. cerevisiae*. *Cell* **66:** 1279–1287.

Aparicio, O.M., Weinstein, D.M., and Bell, S.P. 1997. Components and dynamics of DNA replication complexes in *S. cer-*

*evisiae*: Redistribution of MCM proteins and Cdc45p during S phase. *Cell* **91:** 59–69.

Aparicio, O.M., Stout, A.M., and Bell, S.P. 1999. Differential assembly of Cdc45p and DNA polymerases at early and late origins of DNA replication. *Proc. Natl. Acad. Sci.* **96:** 9130–9135.

Bell, S.P. and Dutta, A. 2002. DNA replication in eukaryotic cells. *Annu. Rev. Biochem.* **71:** 333–374.

Bell, S.P. and Stillman, B. 1992. ATP-dependent recognition of eukaryotic origins of DNA replication by a multiprotein complex. *Nature* **357:** 128–134.

Bell, S.P., Kobayashi, R., and Stillman, B. 1993. Yeast origin recognition complex functions in transcription silencing and DNA replication. *Science* **262:** 1844–1849.

Bell, S.P., Mitchell, J., Leber, J., Kobayashi, R., and Stillman, B. 1995. The multidomain structure of Orc1p reveals similarity to regulators of DNA replication and transcriptional silencing. *Cell* **83:** 563–568.

Brachmann, C.B., Sherman, J.M., Devine, S.E., Cameron, E.E., Pillus, L., and Boeke, J.D. 1995. The SIR2 gene family, conserved from bacteria to humans, functions in silencing, cell cycle progression, and chromosome stability. *Genes & Dev.* **9:** 2888–2902.

Bryk, M., Banerjee, M., Murphy, M., Knudsen, K.E., Garfinkel, D.J., and Curcio, M.J. 1997. Transcriptional silencing of Ty1 elements in the RDN1 locus of yeast. *Genes & Dev.* **11:** 255–269.

Buck, S.W., Sandmeier, J.J., and Smith, J.S. 2002. RNA polymerase I propagates unidirectional spreading of rDNA silent chromatin. *Cell* **111:** 1003–1014.

Carmen, A.A., Milne, L., and Grunstein, M. 2002. Acetylation of the yeast histone H4 N terminus regulates its binding to heterochromatin protein SIR3. *J. Biol. Chem.* **277:** 4778–4781.

Cheng, H.L., Mostoslavsky, R., Saito, S., Manis, J.P., Gu, Y., Patel, P., Bronson, R., Appella, E., Alt, F.W., and Chua, K.F. 2003. Developmental defects and p53 hyperacetylation in Sir2 homolog (SIRT1)-deficient mice. *Proc. Natl. Acad. Sci.* **100:** 10794–10799.

Cockell, M., Palladino, F., Laroche, T., Kyrion, G., Liu, C., Lustig, A.J., and Gasser, S.M. 1995. The carboxy termini of Sir4 and Rap1 affect Sir3 localization: Evidence for a multicomponent complex required for yeast telomeric silencing. *J. Cell Biol.* **129:** 909–924.

Cocker, J.H., Piatti, S., Santocanale, C., Nasmyth, K., and Diffley, J.F. 1996. An essential role for the Cdc6 protein in forming the pre-replicative complexes of budding yeast. *Nature* **379:** 180–182.

Cosgrove, A.J., Nieduszynski, C.A., and Donaldson, A.D. 2002. Ku complex controls the replication time of DNA in telomere regions. *Genes & Dev.* **16:** 2485–2490.

Cuperus, G., Shafaatian, R., and Shore, D. 2000. Locus specificity determinants in the multifunctional yeast silencing protein Sir2. *EMBO J.* **19:** 2641–2651.

Dani, G.M. and Zakian, V.A. 1983. Mitotic and meiotic stability of linear plasmids in yeast. *Proc. Natl. Acad. Sci.* **80:** 3406–3410.

Davey, M.J., Jeruzalmi, D., Kuriyan, J., and O'Donnell, M. 2002. Motors and switches: AAA$^+$ machines within the replisome. *Nat. Rev. Mol. Cell. Biol.* **3:** 826–835.

DeRyckere, D., Smith, C.L., and Martin, G.S. 1999. The role of nucleotide binding and hydrolysis in the function of the fission yeast cdc18$^+$ gene product. *Genetics* **151:** 1445–1457.

Diffley, J.F.X. 1996. Once and only once upon a time: Specifying and regulating origins of DNA replication in eukaryotic cells. *Genes & Dev.* **10:** 2819–2830.

Drury, L.S., Perkins, G., and Diffley, J.F. 2000. The cyclin-dependent kinase Cdc28p regulates distinct modes of Cdc6p proteolysis during the budding yeast cell cycle. *Curr. Biol.* **10:** 231–240.

Erzberger, J.P., Pirruccello, M.M., and Berger, J.M. 2002. The structure of bacterial DnaA: Implications for general mechanisms underlying DNA replication initiation. *EMBO J.* **21:** 4763–4773.

Ferguson, B.M., Brewer, B.J., Reynolds, A.E., and Fangman, W.L. 1991. A yeast origin of replication is activated late in S phase. *Cell* **65:** 507–515.

Foss, M., McNally, F.J., Laurenson, P., and Rine, J. 1993. Origin recognition complex (ORC) in transcriptional silencing and DNA replication in *S. cerevisiae*. *Science* **262:** 1838–1844.

Fox, C.A., Ehrenhofer-Murray, A.E., Loo, S., and Rine, J. 1997. The origin recognition complex, SIR1, and the S phase requirement for silencing. *Science* **276:** 1547–1551.

Frolova, N.S., Schek, N., Tikhmyanova, N., and Coleman, T.R. 2002. *Xenopus* Cdc6 performs separate functions in initiating DNA replication. *Mol. Biol. Cell* **13:** 1298–1312.

Frye, R.A. 2000. Phylogenetic classification of prokaryotic and eukaryotic Sir2-like proteins. *Biochem. Biophys. Res. Commun.* **273:** 793–798.

Gardner, K.A., Rine, J., and Fox, C.A. 1999. A region of the Sir1 protein dedicated to recognition of a silencer and required for interaction with the Orc1 protein in *Saccharomyces cerevisiae*. *Genetics* **151:** 31–44.

Gasser, S.M. and Cockell, M.M. 2001. The molecular biology of the SIR proteins. *Gene* **279:** 1–16.

Gietz, R.D. and Sugino, A. 1988. New yeast–*Escherichia coli* shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. *Gene* **74:** 527–534.

Gottlieb, S. and Esposito, R.E. 1989. A new role for a yeast transcriptional silencer gene, SIR2, in regulation of recombination in ribosomal DNA. *Cell* **56:** 771–776.

Gottschling, D.E., Aparicio, O.M., Billington, B.L., and Zakian, V.A. 1990. Position effect at *S. cerevisiae* telomeres: Reversible repression of Pol II transcription. *Cell* **63:** 751–762.

Guarente, L. 2000. Sir2 links chromatin silencing, metabolism, and aging. *Genes & Dev.* **14:** 1021–1026.

Hecht, A., Laroche, T., Strahl-Bolsinger, S., Gasser, S.M., and Grunstein, M. 1995. Histone H3 and H4 N-termini interact with SIR3 and SIR4 proteins: A molecular model for the formation of heterochromatin in yeast. *Cell* **80:** 583–592.

Hogan, E. and Koshland, D. 1992. Addition of extra origins of replication to a minichromosome suppresses its mitotic loss in cdc6 and cdc14 mutants of *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci.* **89:** 3098–3102.

Holmes, S.G., Rose, A.B., Steuerle, K., Saez, E., Sayegh, S., Lee, Y.M., and Broach, J.R. 1997. Hyperactivation of the silencing proteins, Sir2p and Sir3p, causes chromosome loss. *Genetics* **145:** 605–614.

Hoppe, G.J., Tanny, J.C., Rudner, A.D., Gerber, S.A., Danaie, S., Gygi, S.P., and Moazed, D. 2002. Steps in assembly of silent chromatin in yeast: Sir3-independent binding of a Sir2/Sir4 complex to silencers and role for Sir2-dependent deacetylation. *Mol. Cell. Biol.* **22:** 4167–4180.

Huang, J. and Moazed, D. 2003. Association of the RENT complex with nontranscribed and coding regions of rDNA and a regional requirement for the replication fork block protein Fob1 in rDNA silencing. *Genes & Dev.* **17:** 2162–2176.

Imai, S., Armstrong, C.M., Kaeberlein, M., and Guarente, L. 2000. Transcriptional silencing and longevity protein Sir2 is

an NAD-dependent histone deacetylase. *Nature* **403:** 795–800.

Jeruzalmi, D., O'Donnell, M., and Kuriyan, J. 2001. Crystal structure of the processivity clamp loader gamma (γ) complex of *E. coli* DNA polymerase III. *Cell* **106:** 429–441.

Kurdistani, S.K. and Grunstein, M. 2003. Histone acetylation and deacetylation in yeast. *Nat. Rev. Mol. Cell. Biol.* **4:** 276–284.

Kyrion, G., Liu, K., Liu, C., and Lustig, A.J. 1993. RAP1 and telomere structure regulate telomere position effects in *Saccharomyces cerevisiae*. *Genes & Dev.* **7:** 1146–1159.

Labib, K., Diffley, J.F., and Kearsey, S.E. 1999. G1-phase and B-type cyclins exclude the DNA-replication factor Mcm4 from the nucleus. *Nat. Cell Biol.* **1:** 415–422.

Landry, J., Sutton, A., Tafrov, S.T., Heller, R.C., Stebbins, J., Pillus, L., and Sternglanz, R. 2000. The silencing protein SIR2 and its homologs are NAD-dependent protein deacetylases. *Proc. Natl. Acad. Sci.* **97:** 5807–5811.

Lenzen, C.U., Steinmann, D., Whiteheart, S.W., and Weis, W.I. 1998. Crystal structure of the hexamerization domain of N-ethylmaleimide-sensitive fusion protein. *Cell* **94:** 525–536.

Liang, C., Weinreich, M., and Stillman, B. 1995. ORC and Cdc6p interact and determine the frequency of initiation of DNA replication in the genome. *Cell* **81:** 667–676.

Lipford, J.R. and Bell, S.P. 2001. Nucleosomes positioned by ORC facilitate the initiation of DNA replication. *Mol. Cell* **7:** 21–30.

Liu, J., Smith, C.L., DeRyckere, D., DeAngelis, K., Martin, G.S., and Berger, J.M. 2000. Structure and function of Cdc6/Cdc18: Implications for origin recognition and checkpoint control. *Mol. Cell* **6:** 637–648.

Luo, J., Nikolaev, A.Y., Imai, S., Chen, D., Su, F., Shiloh, A., Guarente, L., and Gu, W. 2001. Negative control of p53 by Sir2α promotes cell survival under stress. *Cell* **107:** 137–148.

Luo, K., Vega-Palas, M.A., and Grunstein, M. 2002. Rap1–Sir4 binding independent of other Sir, yKu, or histone interactions initiates the assembly of telomeric heterochromatin in yeast. *Genes & Dev.* **16:** 1528–1539.

Lustig, A.J., Kurtz, S., and Shore, D. 1990. Involvement of the silencer and UAS binding protein RAP1 in regulation of telomere length. *Science* **250:** 549–553.

Marahrens, Y. and Stillman, B. 1992. A yeast chromosomal origin of DNA replication defined by multiple functional elements. *Science* **255:** 817–823.

Marks, P., Rifkind, R.A., Richon, V.M., Breslow, R., Miller, T., and Kelly, W.K. 2001. Histone deacetylases and cancer: Causes and therapies. *Nat. Rev. Cancer* **1:** 194–202.

McNally, F.J. and Rine, J. 1991. A synthetic silencer mediates SIR-dependent functions in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **11:** 5648–5659.

Moazed, D. 2001. Enzymatic activities of Sir2 and chromatin silencing. *Curr. Opin. Cell Biol.* **13:** 232–238.

Moretti, P. and Shore, D. 2001. Multiple interactions in Sir protein recruitment by Rap1p at silencers and telomeres in yeast. *Mol. Cell. Biol.* **21:** 8082–8094.

Muth, V., Nadaud, S., Grummt, I., and Voit, R. 2001. Acetylation of TAF(I)68, a subunit of TIF-IB/SL1, activates RNA polymerase I transcription. *EMBO J.* **20:** 1353–1362.

Neuwald, A.F., Aravind, L., Spouge, J.L., and Koonin, E.V. 1999. AAA$^+$: A class of chaperone-like ATPases associated with the assembly, operation, and disassembly of protein complexes. *Genome Res.* **9:** 27–43.

Newlon, C.S. and Theis, J.F. 1993. The structure and function of yeast ARS elements. *Curr. Opin. Genet. Dev.* **3:** 752–758.

Nguyen, V.Q., Co, C., and Li, J.J. 2001. Cyclin-dependent kinases prevent DNA re-replication through multiple mechanisms. *Nature* **411:** 1068–1073.

North, B.J., Marshall, B.L., Borra, M.T., Denu, J.M., and Verdin, E. 2003. The human Sir2 ortholog, SIRT2, is an NAD$^+$-dependent tubulin deacetylase. *Mol. Cell* **11:** 437–444.

Pak, D.T., Pflumm, M., Chesnokov, I., Huang, D.W., Kellum, R., Marr, J., Romanowski, P., and Botchan, M.R. 1997. Association of the origin recognition complex with heterochromatin and HP1 in higher eukaryotes. *Cell* **91:** 311–323.

Pasero, P., Bensimon, A., and Schwob, E. 2002. Single-molecule analysis reveals clustering and epigenetic regulation of replication origins at the yeast rDNA locus. *Genes & Dev.* **16:** 2479–2484.

Perkins, G. and Diffley, J.F. 1998. Nucleotide-dependent prereplicative complex assembly by Cdc6p, a homolog of eukaryotic and prokaryotic clamp-loaders. *Mol. Cell* **2:** 23–32.

Poloumienko, A., Dershowitz, A., De, J., and Newlon, C.S. 2001. Completion of replication map of *Saccharomyces cerevisiae* chromosome III. *Mol. Biol. Cell* **12:** 3317–3327.

Raghuraman, M.K., Winzeler, E.A., Collingwood, D., Hunt, S., Wodicka, L., Conway, A., Lockhart, D.J., Davis, R.W., Brewer, B.J., and Fangman, W.L. 2001. Replication dynamics of the yeast genome *Science* **294:** 115–121.

Rao, H. and Stillman, B. 1995. The origin recognition complex interacts with a bipartite DNA binding site within yeast replicators. *Proc. Natl. Acad. Sci.* **92:** 2224–2228.

Rine, J. and Herskowitz, I. 1987. Four genes responsible for a position effect on expression from HML and HMR in *Saccharomyces cerevisiae*. *Genetics* **116:** 9–22.

Robyr, D., Suka, Y., Xenarios, I., Kurdistani, S.K., Wang, A., Suka, N., and Grunstein, M. 2002. Microarray deacetylation maps determine genome-wide functions for yeast histone deacetylases. *Cell* **109:** 437–446.

Rusche, L.N., Kirchmaier, A.L., and Rine, J. 2002. Ordered nucleation and spreading of silenced chromatin in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* **13:** 2207–2222.

———. 2003. The establishment, inheritance, and function of silenced chromatin in *Saccharomyces cerevisiae*. *Annu. Rev. Biochem.* **72:** 481–516.

Shou, W., Seol, J.H., Shevchenko, A., Baskerville, C., Moazed, D., Chen, Z.W., Jang, J., Charbonneau, H., and Deshaies, R.J. 1999. Exit from mitosis is triggered by Tem1-dependent release of the protein phosphatase Cdc14 from nucleolar RENT complex. *Cell* **97:** 233–244.

Simpson, R.T. 1990. Nucleosome positioning can affect the function of a *cis*-acting DNA element in vivo. *Nature* **343:** 387–389.

Sinclair, D.A. 2002. Paradigms and pitfalls of yeast longevity research. *Mech. Ageing Dev.* **123:** 857–867.

Smith, J.S. and Boeke, J.D. 1997. An unusual form of transcriptional silencing in yeast ribosomal DNA. *Genes & Dev.* **11:** 241–254.

Smith, J.S., Brachmann, C.B., Celic, I., Kenna, M.A., Muhammad, S., Starai, V.J., Avalos, J.L., Escalante-Semerena, J.C., Grubmeyer, C., Wolberger, C., et al. 2000. A phylogenetically conserved NAD$^+$-dependent protein deacetylase activity in the Sir2 protein family. *Proc. Natl. Acad. Sci.* **97:** 6658–6663.

Stevenson, J.B. and Gottschling, D.E. 1999. Telomeric chromatin modulates replication timing near chromosome ends. *Genes & Dev.* **13:** 146–151.

Stillman, B. 1996. Cell cycle control of DNA replication. *Science* **274:** 1659–1663.

Strahl-Bolsinger, S., Hecht, A., Luo, K., and Grunstein, M. 1997. SIR2 and SIR4 interactions differ in core and extended telo-

meric heterochromatin in yeast. *Genes & Dev.* **11:** 83–93.

Straight, A.F., Shou, W., Dowd, G.J., Turck, C.W., Deshaies, R.J., Johnson, A.D., and Moazed, D. 1999. Net1, a Sir2-associated nucleolar protein required for rDNA silencing and nucleolar integrity. *Cell* **97:** 245–256.

Tanaka, T., Knapp, D., and Nasmyth, K. 1997. Loading of an Mcm protein onto DNA replication origins is regulated by Cdc6p and CDKs. *Cell* **90:** 649–660.

Triolo, T. and Sternglanz, R. 1996. Role of interactions between the origin recognition complex and SIR1 in transcriptional silencing. *Nature* **381:** 251–253.

Vaziri, H., Dessain, S.K., Ng Eaton, E., Imai, S.I., Frye, R.A., Pandita, T.K., Guarente, L., and Weinberg, R.A. 2001. hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase. *Cell* **107:** 149–159.

Visintin, R., Hwang, E.S., and Amon, A. 1999. Cfi1 prevents premature exit from mitosis by anchoring Cdc14 phosphatase in the nucleolus. *Nature* **398:** 818–823.

Vogelauer, M., Rubbi, L., Lucas, I., Brewer, B.J., and Grunstein, M. 2002. Histone acetylation regulates the time of replication origin firing. *Mol. Cell* **10:** 1223–1233.

Weinreich, M., Liang, C., and Stillman, B. 1999. The Cdc6p nucleotide-binding motif is required for loading Mcm proteins onto chromatin. *Proc. Natl. Acad. Sci.* **96:** 441–446.

Yu, R.C., Hanson, P.I., Jahn, R., and Brunger, A.T. 1998. Structure of the ATP-dependent oligomerization domain of N-ethylmaleimide sensitive factor complexed with ATP. *Nat. Struct. Biol.* **5:** 803–811.

Zhang, Z., Hayashi, M.K., Merkel, O., Stillman, B., and Xu, R.M. 2002. Structure and function of the BAH-containing domain of Orc1p in epigenetic silencing. *EMBO J.* **21:** 4600–4611.