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])

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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 Difilley 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. 2000].
been shown to deacetylate nonhistone proteins including histones H3 and H4 in specific acetyl-lysine residues within the N-terminal tails of histones H3 and H4 (Guarente 2000; Sinclair 2002). Sir2p deacetylates ribosomal DNA (rDNA) genes (Gottlieb and Esposito 1989). This latter function of Sir2p promotes longevity in yeast (Cheng et al. 2003). Sir3p and Sir4p encode chromatin-binding proteins unique to budding yeast. Sir3p and Sir4p interact with hypoacetylated histones 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 acdc6 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 theSir2–4genes 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 ofacdc6–4, 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 theacdc6–4mutation, 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 suppressors 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 bySir2, 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 Sternlitz 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 sirΔ 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 HMRα and HMLα 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 MATα, MATα, and the MATα/ MATα cdc6-4 diploids were all temperature sensitive [Fig. 1B]. We also constructed a MATα cdc6-4 HMRα-ss haploid strain that was completely defective for silencing at HMRα 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 HMRα 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, 636 [cdc6-4 orc1ΔNα3]], M638 [cdc6-4 sir1Δ], M922 [cdc6-4 sirΔ2], M971 [cdc6-4 sir3Δ], and M974 [cdc6-4 sir4Δ]. [B] Simultaneous MATα and MATα expression does not suppress cdc6-4. M138 [WT], M386 [MATα cdc6-4], M599 [MATα cdc6-4], M1101 [MATα cdc6-4 hmrα-ss1], and M576 [MATα cdc6-4[2×]]. (C) Disruption of telomeric silencing does not suppress cdc6-4. M1020 [WT, VII:URA3-te], M1021 [cdc6-4 VIII:URA3-te], AJL396-4d [rap1-17 VII:URA3-te], M1010 [cdc6-4 rap1-17 VII:URA3-te], M1105 [cdc6-4 sir2-86], and M1117 [cdc6-4 sir2-87]. (D) Loss of Sir2p deacetylase activity suppresses cdc6-4. M138 [WT], M386 [cdc6-4], M795 [ sir2-86], and M1166 [cdc6-4 sir2-88].

[cdc6-4 sir2-N345A]. | Loss of Sir2p teDNA 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].

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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 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; Kyron 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 Cdc4p 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 sir2Δ 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 sir2Δ). (B) 12CA5 Western blot of Cdc6-4p and FACT 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 γ-factor, W303-1A (untagged), M1257 (3HA-cdc6-4), M1274 (3HA-cdc6-4 sir2Δ), M1299 (3HA-cdc6-4 sir2A hmlΔ). (C) Deletion of SIR1, SIR2, or SIR3 does not suppress the cdc6-1 temperature sensitivity. W303-1A (WT), M379 [cdc6-1], M1103 [cdc6-1 sir1Δ], M1104 [cdc6-1 sir2Δ], M1105 [cdc6-1 sir3Δ].
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 SIR2WT and sir2Δ 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 sir2Δ 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 sir2Δ 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 sir2Δ 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.

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.
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 rpd3Δ or hda1Δ 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 Sir2p 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 sir2Δ strains. Initiation mutants exhibited 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 sir2Δ should also reverse the plasmid instability phenotype of the cdc6-4 mutant. We therefore measured plasmid loss rates in wild-type, sir2Δ, cdc6-4, and the cdc6-4 sir2Δ 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 ARS4 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 sir2Δ completely reversed the instability phenotype for the ARS305 and ARS501 plasmids and substantially reversed the loss rate of the ARS315 plasmid. However, a sir2Δ 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 sir2Δ 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
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 origin plasmid instability and had no effect for the ARSH4 and ARS501 plasmids. In a wild-type strain, the SIR2 deletion caused similar effects with the exception of the ARS305 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 in 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 sir2Δ 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 sir2Δ 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
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 HMRα synthetic silencer [Foss et al. 1993], and we have found that deletion of SIR2 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 Ori1p recruits Sir1p to the silencer via the ORC-binding sites within the silencer DNA elements [Triolo and Stengelanz 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 Ori1p 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 Gen5p 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 Supporting 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 (Supporting Table 2). The PCR reaction contained 10 ng of genomic DNA, 200 µM dNTP, 50 pmol 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 sir2-343A 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/Stul fragment was cloned into the SphI/SmaI sites of YIpLac204. The resulting plasmids, pDP135 [sir2-343A], 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 pmol 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 pmol 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 pmol of a SIR2-specific sequencing oligonucleotide (SIR2-SP1, SP2, SP3 or SP4; Supplementary Table 2).

To construct M1161, hda1-1 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 x 10^6 cells each were plated onto multiple YPD plates containing 1% formamide and incubated for 3 d at 37°C. This concentration of formamide 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 nonmatting 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 plasmids were crossed to M599 [MATa cdc6-4-0]. 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 [MATa cdc6-4-0]. The three resulting integrant strains were crossed to rgg 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 rgg 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. 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 Polomierko 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, pBS416 [ARSH4], pRF12 [ARSL1], pRS151 [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 Mrc2p 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 ARS301 using the indicated primers (Supplementary Table 2). PCR products were separated on 5% polyacrylamide gels.

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