Chromatin assembly factor I contributes to the maintenance, but not the re-establishment, of silencing at the yeast silent mating loci

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CAC1/RLF2 encodes the largest subunit of chromatin assembly factor I (CAF-I), a complex that assembles newly synthesized histones onto recently replicated DNA in vitro. In vivo, *cac1/rlf2* mutants are defective in telomeric silencing and mislocalize Rap1p, a telomere-binding protein. Here, we report that in cells lacking CAF-I the silent mating loci are derepressed partially. *MATa cac1* cells exhibit an unusual response to α -factor: They arrest and form mating projections (shmoo) initially, but are unable to sustain the arrest state, giving rise to clusters of shmooing cells. *cac1 MATa HMLa HMRa* strains do not form these shmoo clusters, indicating that derepression of *HML α* causes the shmoo cluster phenotype in *cac1* cells. When *SIR3* is reintroduced into *sir1 sir3* cells, *HML* remains derepressed indicating that *SIR1* is required for the re-establishment of silencing at *HML*. In contrast, when *SIR3* is reintroduced into *cac1 sir3* cells, silencing is restored to *HML*, indicating that CAF-I is not required for the re-establishment of silencing. Loss of the other CAF-I subunits (*Cac2p* and *Cac3p/Msi1p*) also results in the shmoo cluster phenotype, implying that loss of CAF-I activity gives rise to this unstable repression of *HML*. Strains carrying certain mutations in the amino terminus of histone H4 and strains with limiting amounts of Sir2p or Sir3p also form shmoo clusters, implying that the shmoo cluster phenotype is indicative of defects in maintenance of the structural integrity of silent chromatin. *MATa cac⁻ sir1* double mutants have a synergistic mating defect, suggesting that the two silencing mechanisms, establishment and maintenance, function cooperatively. We propose a model to explain the distinctions between the establishment and the maintenance of silent chromatin.

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In differentiated cells, two identical genomic sequences can sometimes be found in two distinct states of expression. For example, in female mammals, one of the two X chromosomes is inactivated, whereas the other remains fully active (Latham 1996). Similarly, chromosomal imprinting ensures that a specific locus, when inherited from one parent, is completely inactive, whereas the same locus inherited from the other parent is completely active (Ferguson-Smith 1996). The imprinted state of the locus is inherited through many mitotic divisions and is generally reset only during meiosis. Inappropriate genomic imprinting can cause serious developmental defects, and several human genetic disorders are caused by mutations affecting imprinted genes (Hall 1990; Lalande 1996). Although the molecular mechanisms by which X inactivation and genomic imprinting are initiated and maintained are not well understood, the inactive X chro-

mosome is in a highly condensed heterochromatic state and a similar chromatin state may occur at silenced, imprinted loci (John and Surani 1996).

One of the best studied examples of silencing occurs at the *HM* loci in the budding yeast *Saccharomyces cerevisiae*. *S. cerevisiae* has three mating type loci. Mating type genes expressed from the *MAT* locus normally determine the yeast mating type, either **a** or α , in haploid cells. Haploid cells normally respond to the mating pheromone of the opposite mating type by arresting in late *G*₁ and forming mating projections (shmoo). In addition, wild-type strains have functional but transcriptionally repressed mating information at the *HM* loci, *HML* and *HMR*. If the *HM* loci become derepressed in haploid cells, both **a** and α mating information is expressed and the cells do not arrest growth or form mating projections in response to mating pheromones. Thus, by monitoring the pheromone response of haploid cells one can infer the expression state of the *HM* loci.

The silent state of the *HM* loci is attributable to a

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specialized form of chromatin that is the yeast version of metazoan heterochromatin (Grunstein 1995; Braunstein et al. 1996). Genes within the *HM* loci are inaccessible to DNA modification enzymes, RNA polymerases II and III, and excision repair enzymes (for review, see Fox and Rine 1996). The acetylation state of histones H3 and H4 in the nucleosomes of silent chromatin is different from that of bulk chromatin or of the active *MAT* locus; at the *HM* loci, histone H4 is hypoacetylated except on lysine-12 (Braunstein et al. 1996). This is similar to the acetylation pattern conferred on newly synthesized histone H4 by the cytoplasmic histone acetyltransferase Hat1p (Kleff et al. 1995; Parthun et al. 1996). A number of mutations in acetylated lysines in the amino termini of histones H3 and H4 weaken silencing at the *HM* loci or at telomeres (for review, see Grunstein 1995). Thus, histone acetylation may play an important role in the inheritance of chromatin expression states.

The Sir complex proteins (composed of Sir2p, Sir3p, and Sir4p and not including Sir1p) are structural components of yeast heterochromatin that associate with histones (Hecht et al. 1996; Strahl-Bolsinger et al. 1997). Loss of any one of these Sir complex proteins abrogates silencing completely (Rine and Herskowitz 1987). Sir3p and Sir4p interact with one another genetically (Ivy et al. 1986; Marshall et al. 1987) and in two-hybrid screens (Moretti et al. 1994), and all three Sir complex proteins can be isolated in complexes with each other (Moazed et al. 1997; Strahl-Bolsinger et al. 1997). Histones H3 and H4 coprecipitate with Sir3p (Hecht et al. 1996), and mutations in the amino termini of either H3 or H4 that affect silencing *in vivo* also affect the interaction of H3 and H4 with the Sir complex *in vitro* (Hecht et al. 1995).

The concentration of Sir complex proteins is critical for silencing. Changes in the stoichiometry of Sir complex proteins alters silencing (Ivy et al. 1986; Marshall et al. 1987; Sussel et al. 1993). The Sir complex proteins localize to a number of perinuclear foci that are often associated with silent telomeric DNA (Gotta et al. 1996). These foci are thought to reflect subnuclear domains of high Sir complex concentration in which silent chromatin is localized (Gotta et al. 1996, 1997). The *HM* loci and telomeres compete for Sir proteins, and the proximity of the *HM* loci to telomeres contributes to *HM* silencing (Buck and Shore 1995; Maillet et al. 1996).

The DNA sequences at the *HM* loci differ from the sequences at the *MAT* locus in that each *HM* locus is flanked by two silencers, E and I. Each E or I silencer contains an autonomously replicating (ARS) consensus sequence that is bound by the origin recognition complex (ORC) (Bell et al. 1993). In addition, each silencer contains a binding site for the ARS-binding factor 1 (Abf1p) or a binding site for the repressor/activator protein 1 (Rap1p). The E and I silencers, as well as the individual binding sites and the factors that bind them directly, have redundant functions. In most situations, one silencer is sufficient to silence an *HM* locus and any two of the three individual sites within a silencer are sufficient for *HM* silencing (Brand et al. 1987; Mahoney and Broach 1989; McNally and Rine 1991). Specific muta-

tions in the sites (or in the factors that bind them) reduce the redundancy of *HMR* silencing and can reveal the roles of silencing factors such as Rap1p (Sussel and Shore 1991), ORC (Bell et al. 1993; Micklem et al. 1993; Loo et al. 1995a), and Abf1p (Loo et al. 1995b; Fox et al. 1997).

The study of situations in which silencing is weakened, but not abrogated, has provided important insights into the mechanisms by which silencing occurs. *sir1* mutants exhibit epigenetic silencing of *HML*. In a subset of the *sir1* cells, *HML* is fully repressed and the repressed state is inherited in most of their progeny; in the remaining *sir1* cells, *HML* is fully derepressed and the derepressed state is inherited (Pillus and Rine 1989). Sir1p interacts physically with both Orc1p and with Sir4p (Triolo and Sternglanz 1996). Sir1p, when tethered to the *HML* locus in the absence of a silencer, is sufficient to direct silencing (Chien et al. 1993). Deletion of the ORC-binding site also causes defects in the establishment of silencing, which lead to derepression of the *HM* loci in a subset of the mutant cells (Mahoney et al. 1991; Sussel et al. 1993). Thus, Sir1p contributes to the establishment of silencing in wild-type cells by interacting with ORC and recruiting structural components of silent chromatin, such as Sir4p, to the silent loci.

Pillus and Rine (1989) proposed that there are two steps in *HM* silencing: (1) maintenance of the current state of the silent chromatin, and (2) re-establishment of the repressed state when *HML* becomes derepressed. Although deletion of *SIR1* and mutation of single sites within the *HM* loci cause defects in the re-establishment of silencing, they do not affect the ability to inherit the repressed chromatin state (Pillus and Rine 1989; Mahoney et al. 1991). Derepression of *HMR* (by inactivation of a temperature-sensitive Sir3 protein) can be restored only after passage through S phase (Miller and Nasmyth 1984), indicating that the re-establishment of silencing requires passage through S phase. Conversely, Holmes and Broach (1996) demonstrated that if the *cis*-silencer is excised from the chromosome, the repressed state of the chromatin can be maintained during α -factor arrest, but cannot be inherited efficiently. Taken together, these studies indicate that the establishment, maintenance, and inheritance of silencing all contribute to the formation of fully silenced *HM* loci.

Mammalian chromatin assembly factor I (CAF-I) was identified by its ability to assemble histones into nucleosomes in a DNA replication-dependent manner *in vitro* (Stillman 1986). CAF-I assembles preferentially histones H3 and H4 with the acetylation pattern of newly synthesized cytoplasmic histones (Smith and Stillman 1991; Kaufman et al. 1995; Verreault et al. 1996). *S. cerevisiae* CAF-I is encoded by *CAC1*, *CAC2*, and *CAC3* (Kaufman et al. 1997). *CAC1*, the largest subunit of CAF-I, is identical to *RLF2*, a gene that we identified in a screen for mutants defective in telomere-related functions (Enomoto et al. 1994, 1997), and *CAC3* is identical to *MSI1*, a gene identified in high-copy suppressor screens (Ruggieri et al. 1989; Hubbard et al. 1992). All three *cac* mutant strains display similar phenotypes; cells grow well but are defective in telomeric silencing, the segregation

of TEL + CEN plasmids, and Rap1p localization (Enomoto et al. 1997; Kaufman et al. 1997). Similar phenotypes have been observed in strains carrying mutations in either *SIR2*, *SIR3*, or *SIR4*, in strains carrying mutant alleles of histones H3 and H4, and in strains carrying *rap1^s* mutations (Enomoto et al. 1994). Because many of these genes are involved in *HM* silencing, as well as telomeric silencing, we examined the role of CAF-I in *HM* silencing.

In this paper we show that CAF-I contributes to the maintenance, but not the re-establishment, of silencing at the *HM* loci. In *cac⁻* mutants, we observed a transient loss of α -factor response, at the individual cell level. Cells form mating projections and divide slowly on α -factor, forming clusters of shmooing cells. The formation of shmoo clusters requires α -mating information at *HML*, indicating that this α -factor response reflects a defect in the maintenance of *HML* silencing. We have investigated the relationship between the maintenance and the re-establishment of silencing at *HML* by analyzing the roles of CAF-I, histones, Sir complex proteins, and Sir1p using α -factor confrontation assays.

Results

cac1 mutations affect *HML* silencing

Many of the factors that contribute to telomere position effect also contribute to silencing at the two *HM* loci *HML* and *HMR*. Although *cac1/rif2* mutants are defective in the repression of telomere adjacent genes, quantitative mating assays did not detect a mating defect in *cac⁻* strains (Enomoto et al. 1997; Kaufman et al. 1997). We tested *HML* repression in *cac1* cells using an α -factor response assay that is more sensitive to derepression of *HML α* than are quantitative mating assays. Exponentially growing *MATa* cells were resuspended in liquid medium containing α -factor and the proportion of dividing cells (cells with one or more growing buds) and the proportion of arrested cells (unbudded cells with or without mating projections) was determined. Three hours after the addition of α -factor, 1% of the wild-type cells were dividing, whereas 10–20% of the cells in *cac1* strains (*cac1- Δ 1* and *cac1-1*) were dividing. This difference between wild-type and *cac1* cells suggested that either *HML* is slightly derepressed in all *cac1* cells or that *HML* is derepressed in a population of the *cac1* cells. For comparison, in an isogenic *MATa sir1* strain, 32% of the cells were dividing in α -factor.

CAC1, together with the *ORC*-binding site, contributes to silencing at *HMR*

To measure the role of *CAC1* at *HMR*, we used an *HMR::TRP1* construct in which the *a1* and *a2* genes are replaced by the *TRP1* gene (Hardy et al. 1992). Assays that measure expression of *HMR::TRP1* are more sensitive to low levels of *HMR* derepression than are mating assays. We constructed a series of isogenic strains carrying the *cac1- Δ 1* allele and either *HMR::TRP1* (including the intact silencer) or derivatives missing binding sites

for either *ORC*, *Abf1p*, or *Rap1p*. We compared the ability of these strains to grow on medium lacking tryptophan with the growth of isogenic *CAC1* strains (Fig. 1). Consistent with published results (e.g., Sussel and Shore 1991), only the *hmr::TRP1* strain missing the *Rap1p* site grew on medium lacking tryptophan (Fig. 1). In the *cac1* series of strains, *Trp⁺* colonies also appeared in the strain lacking the *ORC* site (Fig. 1), suggesting that *CAC1* contributes to silencing at *HMR*. In addition, this result suggests that *CAC1* and the *ORC*-binding site in *HMR E* are necessary together for *HMR* silencing.

Interestingly, on medium lacking tryptophan, colonies of *cac1 hmr::TRP1* strains lacking the *ORC* site are smaller than colonies formed by either *sir4 HMR::TRP1* (data not shown) or *sir1 HMR::TRP1* mutants (Fig. 1). *sir4* strains are derepressed completely at the *HM* loci, whereas *sir1* strains include two populations of cells, those that are repressed and those that are derepressed (Pillus and Rine 1989). Like *sir1* mutants, strains carrying the *rap1-12* mutation (which also causes derepression of *hmr::TRP1* in strains lacking the *ORC* site) give rise to a population of cells that grows without tryptophan and each of these forms a colony that is larger than the *cac1* colonies (Fig. 1). Because on complete medium colonies of *cac1 hmr::TRP1* strains lacking the *ORC* site are similar in size to colonies of isogenic wild-type, *sir1*,

Figure 1. CAF-I contributes to the repression of *HMR*. Cells with the indicated genotype at the *CAC1*, *CAC3*, *RAP1*, or *SIR1* locus and deleted for the listed sites within *HMR E::TRP1* were plated in 10-fold serial dilutions onto medium lacking tryptophan (left) or complete medium (right). Colonies were photographed after 2 days at 30°C. (Top) Strains used: WT WT, YJB959; WT orf YJB955; WT abf1, YJB1143; WT rap1, YJB1104; *cac1* WT, YJB1960; *cac1* orf YJB958; *cac1* abf1, YJB1139; *cac1* rap1, YJB1101; *rap1-12* orf, YJB1638; and *sir1* WT, YJB2006. (Bottom) Strains used in a separate experiment photographed after 3 days at 30°C were: *cac1* orf, YJB958; *cac3* orf, YJB2011; *cac1 cac3* orf, YJB2009; and *rap1-12* orf, YJB1638.

or *rap1-12* strains (Fig. 1), the “minicolony” phenotype observed for *cac1* cells on medium lacking tryptophan is related specifically to expression of the *TRP1* allele in the *hmr* locus. Because we do not observe a population of large Trp⁺ colonies in these strains, this observation also implies that, in contrast to *sir1* mutants, either none of the *cac1* cells are derepressed completely when *hmr::TRP1* is missing the ORC site, or derepressed cells and their descendants do not remain derepressed as long as *sir1* mutant cells.

cac1 cells exhibit an unusual budding shmoo response to α -factor

MATa cells expressing only **a** mating information form mating projections, termed shmoo, and arrest in the G₁ stage of the cell cycle in response to α -factor. However, α -factor does not affect the growth and division of cells expressing both **a** and α mating type genes. To understand the α -factor response of *cac1* strains, we assayed the response of individual *MATa cac1* cells to prolonged α -factor treatment on solid medium. After 18 hr at 23°C, 94% of the wild-type cells arrested as shmoo in response to the α -factor treatment (Fig. 2A). In *sir1* strains, two populations of cells were observed; ~60% of the cells arrested as shmoo, and ~40% of the cells divided actively and formed colonies of round yeast cells (Fig. 2A). The *cac1* strains exhibited an entirely different response to α -factor (Fig. 2). After 18 hr, the vast majority of the *cac1* cells (85%) had formed clusters of cells with multiple shmoo-like projections extending in different directions (Fig. 2A). We have observed similar clusters of shmooing cells in *cac1* strains in a number of genetic backgrounds (data not shown). In all cases, the vast majority of *cac1* cells formed these unusual shmoo clusters on α -factor at a time that wild-type cells were arrested as individual shmoo cells. Individual mating projections contained a nucleus (as determined by DAPI staining) and eventually could be separated by micromanipulation (data not shown), indicating that the mating projections are buds that give rise to individual cells. Eventually (~12 hr later than *cac1* cells) wild-type cells formed similar clusters of shmoo cells. After longer periods of time, small colonies of shmooing *cac1* cells are evident. The presence of shmoo at the colony edges indicated that the dividing cells in the colony were not resistant to α -factor. Furthermore, it demonstrated that the α -factor in the medium was still active.

To analyze the dynamics of shmoo cluster formation in *cac1* cells, we observed cells after different times on α -factor (Fig. 2B) and followed individual cells by time-lapse microscopy (Fig. 2B, bottom row). Virtually all *MATa cac1* cells responded with an initial period of cell cycle arrest. Within the first 3–5 hr on α -factor at 23°C they formed mating projections at a time when α -factor-resistant *sir1* cells were dividing. However, after 8–9 hr of arrest, virtually all of the *cac1* cells formed a second shmoo-like projection. This second projection continued to grow and a third projection, often projecting perpendicular to the surface of the medium, appeared on most

cells by 12 hr. At 16 hr, a fourth projection appeared on many of the *cac1* cells, whereas a few of the arrested wild-type shmoo began to form a second projection. α -Factor-resistant *sir1* cells divided approximately once every 2 hr. In contrast, in *cac1* cells, new mating projections appeared approximately once every 4 hr, and eventually, each mating projection gave rise to an individual cell. This suggests that *cac1* cells arrested in response to α -factor, but eventually resume and complete a cell cycle. We term the groups of cells with multiple mating projections shmoo clusters and the individual cells that arise from each mating projection budding shmoo.

The budding shmoo phenotype occurs in virtually all *cac1* cells, indicating that, unlike *sir1* cells, *cac1* cells do not exist in two distinct epigenetic states. Rather, our observations suggest that *cac1* cells are all in a similar state that responds to α -factor initially, but cannot sustain the α -factor response over time.

cac1 α -factor-resistant colonies are attributable to expression of α genes from HML

When *MATa* cells encounter α -factor, they must commit to a new developmental program by repressing pathways that lead to continued cell division and by activating pathways required for cell cycle arrest and polarized growth toward the α -factor source. Later on, the process of recovery or adaptation to α -factor stimulation is induced. The most parsimonious explanation for the appearance of shmoo clusters in *MATa cac1* strains exposed to α -factor and for the formation of small colonies of *cac1 hmr::TRP1* strains lacking the ORC site on medium lacking tryptophan, is that silencing of the *HML* loci is weakened in *cac1* strains. If this is the case, *cac1* cells lacking $\alpha 1$ or $\alpha 2$ genes should remain arrested on α -factor. However, an alternative possibility is that the loss of CAF-I activity alters directly the transcriptional efficiency of genes that either control cell division or that affect the adaptation of cells to α -factor. If this is the case, *cac1* cells lacking $\alpha 1$ and $\alpha 2$ genes should form shmoo clusters in response to α -factor. To distinguish between these two alternatives, we analyzed the α -factor response of strain YJB2057 (*MATa cac1 HMRa HMLa*), in which **a** mating information was substituted for α mating information at *HML*. YJB2057 did not form a second mating projection; only individual shmoo cells were observed (Fig. 2A). Like wild-type strains, these cells remained arrested for >18 hr. This result indicates that *HMLa* information is required for the budding shmoo phenotype in *MATa cac1* strains and implies that the unusual α -factor response of *MATa cac1* strains is attributable to weakened repression of *HML* in cells lacking *Cac1p*. Because the *cac1* cells continue to shmoo and arrest, our results suggest that in all *cac1* cells, *HML* oscillates between the repressed state and the derepressed state.

cac1 mutations enhance the mating defect of *sir1* strains

Pillus and Rine (1989) demonstrated that in *sir1* strains,

Figure 2. α -Factor response of *cac* strains on solid media. Yeast cells were spread onto α -factor-YPD plates and maintained at 23°C. Cells were analyzed at indicated times after exposure to α -factor. (A) Analysis of yeast cell populations after 18 hr on α -factor. More than 100 cells per strain were analyzed. (Shmoo) Individual cells that formed mating projections and remained arrested; (shmoo cluster) individual cells that formed multiple mating projections and eventually divided at least once; (colony) cells that formed colonies of round cells and did not appear to respond to α -factor. (Left) Strains used: WT, YJB276; *cac1*, YJB469; *cac1 HMLa*, YJB2057; *sir1*, YJB335; and *sir1 cac1*, YJB744. (Right) Strains used: WT, YJB195; *cac1*, YJB1838; *cac2*, YJB1803; *cac3*, YJB1581; and *cac2 cac3*, YJB1865. (Left) χ^2 tests indicated that the difference between WT and *cac1 HMLa* strains was not significant, whereas differences between all other pairwise combinations were significant. (Right) All pairwise combinations were significantly different except that the *cac1* and the *cac2 cac3* strains were not significantly different. (B) Analysis of cells over time. (Top four rows) Populations of cells; (bottom row) the same individual cells photographed at indicated times after exposure to α -factor.

silencing of a derepressed *HML* locus can be reestablished at a rate of 4×10^{-3} changes in state per cell generation. In contrast, if the *HML* locus is repressed, its repressed state can be maintained in >90% of the cells in the absence of Sir1p. We analyzed the response of *MATa cac1 sir1* cells to α -factor to determine whether the residual *HML* repression in *cac1* single mutants is dependent on Sir1p functions. In other words, when *cac1* mutants become derepressed after incubation on α -factor, is

the re-establishment of α -factor responsiveness in the next cell cycle dependent on Sir1p function? We reasoned that if residual *HML* silencing in *cac1* mutants as independent of Sir1p re-establishment, then the proportion of α -factor-resistant cells in the double mutant population should remain similar to the number of α -factor-resistant cells in the *sir1* single mutant population. On the other hand, if *HML* repression is relieved transiently in a *cac1* mutant, and if Sir1p was required

for re-establishment of the repressed state of *HML*, then we would expect an increase in the proportion of α -factor-resistant colonies in the *cac1 sir1* double mutants. Consistent with the latter expectation, virtually all of the *MATa cac1 sir1* cells were resistant to α -factor (Fig. 2A). The proportion of α -factor-resistant cells in the double mutant strains was significantly greater than the proportion of α -factor-resistant cells in the *sir1* single mutant strain in two different strain backgrounds (data not shown). In addition, quantitative mating assays confirmed that *MATa cac1 sir1* strains have reduced mating efficiency (Fig. 4, below). It is paradoxical that almost 100% of *cac1 sir1* cells form "colonies" rather than shmoo clusters on α -factor, yet the mating efficiency of *cac1 sir1* strains is reduced only 10- to 100-fold (depending on the strain background). We think this difference is due to some of the *cac1 sir1* colonies having elongated cells, which we presume to be mating competent. Thus, we conclude that derepression caused by the *cac1* mutation requires Sir1p to reestablish repression in cells where *HML* becomes derepressed.

CAF-I is not required for the re-establishment of silencing

The re-establishment of silencing at a derepressed *HM* locus occurs readily in wild-type cells but is a very rare event in *sir1* cells (Pillus and Rine 1989). We compared the role of *cac1* and *sir1* in the re-establishment of silencing by monitoring the state of *HML* in *sir3* strains in which Sir3p expression was restored by transformation with a centromere plasmid-carrying *SIR3* (pSIR3). In all cases, *sir3* cells not carrying pSIR3 did not mate (Fig. 3) and did not respond to α -factor (data not shown). These strains were then transformed with pSIR3 to provide a single copy of the *SIR3* gene expressed from its own promoter. In the otherwise wild-type *sir3* pSIR3 strain, mating competence was readily restored and, when exposed to α -factor, arrested shmoo cells appeared. As expected, as *SIR1* is an important contributor to the re-establishment of *HML* silencing, the opposite result was seen in the isogenic *sir1* strain (Fig. 3); *sir1 sir3* pSIR3 cells did not mate (Fig. 3) and, when exposed to α -factor, did not give rise to arrested shmoo cells. Cotransformation with both pSIR3 and a centromere plasmid-carrying *SIR1* (pSIR1) in this strain restored mating competence and α -factor responsiveness, indicating that it was the lack of

Sir1p that limited the ability of this strain to restore *HML* to the repressed state. In contrast, transformation of the *cac1 sir3* cells with pSIR3 led to the appearance of mating competent cells and these cells arrested as shmoo clusters when exposed to α -factor, indicating that *HML* was restored to the silent state. These results clearly demonstrate that *cac1* is not required for the re-establishment of silencing when *HML* has been derepressed.

The HM silencing defect in cac1 strains is attributable to the loss of CAF-I function

CAC1 encodes the large subunit of CAF-I, a trimeric complex that includes Cac1p/Rlf2p, Cac2p, and Cac3p/Msi1p (Kaufman et al. 1997). To determine whether the *HM* silencing defect in *cac1* mutants was attributable to the absence of CAF-I function, we constructed strains carrying mutations in one, two, or all three genes encoding CAF-I subunits and performed quantitative mating assays on both *MATa* and *MAT α* mutant strains. Like *cac1* mutants, the single, double, and triple *cac* mutants of both mating types were able to mate with wild-type efficiency in quantitative mating assays (Fig. 4). However, all of the single and double *cac* mutants have measurable *MATa* mating defects in combination with *sir1* (Fig. 4). Single and double *cac* mutants in combination with *sir1* behaved like the *sir1 cac1* strain; the *MATa* strains mated with reduced efficiency, whereas the *MAT α* strain mating was not significantly different from the wild-type, *sir1*, or *cac* single mutant strains (Fig. 4).

Like *MATa cac1* strains, the *MATa cac2* and *MATa cac3* strains produced shmoo clusters by 18 hr at 23°C (see Fig. 2A). Interestingly, *cac2* and *cac3* mutations caused less severe silencing defects (61% and 42% shmoo clusters, respectively) than did *cac1* mutants (97% shmoo clusters), which encodes the largest CAF-I subunit (see Fig. 2A). This may occur because *CAC2* and *CAC3* both encode small proteins that include WD40 repeats (Verreault et al. 1996; Kaufman et al. 1997), which may be partially redundant with one another. Consistent with this idea, the silencing defect of *cac2 cac3* double mutants (89% shmoo clusters) is not significantly different from that of *cac1* mutants (see Fig. 2A).

To determine whether loss of any one of the CAF-I components also causes derepression of *hmr::TRP1* strains lacking the ORC site, we analyzed the Trp phenotype of strains carrying either *cac3* alone or as a double

Figure 3. *SIR1* is required, and *CAC1* is not required, for the re-establishment of *HML* silencing. Plasmids pSIR3 (pSE334 or pJR273) and pSIR1 (pJR910) were introduced (indicated by +) into strains carrying *sir3* and the other indicated mutations. Two days after transformation, transformants were allowed to mate for 18 hr with a *Mat α* tester strain (TD1). Diploids were then selected by replica plating onto SDC medium lacking adenine and histidine. Strains used were *sir3 sir1*, YJB2471; *sir3 cac1*, YJB2109; *sir3*, YJB2544; WT, YJB195.

Figure 4. Mutation of CAF-I subunits causes subtle *MATa* mating defects. At least four individual quantitative mating assays were performed for each strain. The median value of the assays is shown. All values are normalized to the isogenic wild type. Solid bars indicate that results were statistically different from wild type at the $P < 0.05$ level. Strains (*MATa*, *MATα*): WT, YJB195, YJB209; *cac1*, YJB1838, YJB1578; *cac2*, YJB1803, YJB1599; *cac3*, YJB1581, YJB1836; *cac1 cac2*, YJB1804, YJB1802; *cac2 cac3*, YJB1865, YJB1864; *cac1 cac3*, YJB1862, YJB1863; *sir1*, YJB1940, YJB1941; *sir1 cac1*, YJB1962, YJB1961; *sir1 cac2*, YJB2000, YJB2034; *sir1 cac3*, YJB1945, YJB1946; *sir1 cac1 cac2*, YJB2044; *sir1 cac2 cac3*, YJB2048; *sir1 cac1 cac3*, YJB2007, YJB1993.

mutant with *cac1-Δ1* (see Fig. 1). The *cac3* ORC site mutant grew slower and formed smaller colonies than the *cac1* ORC site mutant, although the number of Trp⁺ colonies was similar in both strains (see Fig. 1). This effect of *cac3* on *hmrΔA::TRP1* silencing is reminiscent of the mini-colony phenotype of *cac3* strains relative to *cac1* and *cac2* strains in telomeric silencing assays (Kaufman et al. 1997). The *cac1 cac3* mutant was derepressed to the same degree as the *cac1* single mutant, suggesting that *cac3* mutants may retain some CAF-I function that is lost in *cac1* mutants. Taken together, our results indicate that loss of CAF-I function, rather than the loss of Cac1p alone, causes derepression of both *HML* (in *sir1* strains) and *HMR* (when the ORC site is missing).

Can defects in histones give rise to *shmoo* clusters?

CAF-I is unlikely to be a structural component of silent chromatin, because CAF-I localizes to replication foci in mammalian cells (Krude 1995) and overexpressed epitope-tagged Cac1p localizes to nuclear foci that do not colocalize with Rap1p (Enomoto et al. 1997), a structural

component of the silencers. In vitro studies identified CAF-I as an activity that preferentially assembles specifically acetylated histones H3 and H4 into nucleosomes on recently replicated DNA. Yet genes encoding CAF-I subunits are not essential for yeast cell viability, indicating that alternative chromatin assembly mechanisms must function in *cac* mutants (Kaufman et al. 1997). If weakened silencing at the *HM* loci in *cac1* mutants is attributable to subtle alterations in the nucleosomes assembled at these loci, we hypothesized that mutations in CAF-I and certain mutations in histones H3 and H4 should give rise to similar phenotypes. To test this hypothesis, we analyzed the α -factor response of strains carrying mutations in either histone H3 or histone H4.

A number of mutations in lysine 16 of histone H4 virtually eliminate *HM* silencing (e.g., histones H4 K16A and H4 K16Q), whereas H4 K16R causes a small, but measurable reduction in mating of *MATa* strains (Johnson et al. 1990; Megee et al. 1990; Park and Szostak 1990). Other mutations in the amino termini of histones H3 and H4, such as deletion of the entire H3 amino

terminus or mutation of H4 lysines 5, 8, or 12 to arginine, have little, if any, effect on silencing, when monitored by quantitative mating assays (Johnson et al. 1990; Megee et al. 1990; Park and Szostak 1990; Morgan et al. 1991; Thompson et al. 1994). As expected, we found that strains carrying the histone H4 K16A and H4 K16Q mutations did not arrest at all in response to α -factor (data not shown), and therefore, were not informative with regard to the mechanism by which *HM* silencing was defective. Also consistent with published data, the H4 K16R strain included a population of cells that did not respond to α -factor. However, like *cac1* strains, strains carrying mutations in the amino terminus of histone H4 (K5R, K5R K8R, K5R K12R, and K16R; Megee et al. 1990), or strains carrying deletions in the histone H3 amino terminus (Δ 2-20 and Δ 2-29; Morgan et al. 1991) that are competent to mate gave rise to shmoo clusters in response to α -factor (Fig. 5). The fact that changes within the amino terminus of histones H3 or H4 are sufficient to give rise to the shmoo cluster phenotype implies that this phenotype reflects subtle defects in the structural integrity of silenced chromatin. These results also suggest that silencing defects in the histone mutant strains (which carry a wild-type allele of *CAC1*), are similar to the silencing defects in strains lacking CAF-I (which carry only wild-type histone alleles).

Is the budding shmoo phenotype sensitive to Sir complex protein concentration?

The stoichiometry of Sir complex proteins is critical for silencing (Ivy et al. 1986; Marshall et al. 1987), and the concentration of Sir complex proteins is likely limiting,

Figure 5. α -Factor response of strains with mutations in the amino termini of histones H3 or H4. Yeast cells were treated as described in Fig. 2A. χ^2 tests indicated that HHT HHF and HHT hhtK16R were significantly different from each other and from the other histone mutants. Strains used: HHT HHF, YJB2166; hhtΔ2-20 HHF, YJB2167; hhtΔ2-29 HHF, YJB2168; HHT hhtK5R, YJB2169; HHT hhtK5R K8R, YJB2170; HHT hhtK5R K12R, YJB2171; and HHT hhtK16R, YJB2172.

as under a number of conditions the telomeres and *HM* loci compete for Sir complex proteins (Buck and Shore 1995). Our working hypothesis is that silencing is maintained by the efficient assembly (by CAF-I) of nucleosomes and the strong association of these CAF-I-assembled nucleosomes with Sir complex proteins, which render the underlying DNA inaccessible to enzymes. This hypothesis predicts that the efficiency of silencing should be dependent on the concentration of Sir complex proteins. We tested whether providing an additional copy of each *SIR* gene could improve the repression of the *HML* locus in *cac1* mutants. *MATa cac1* strain YJB469 was transformed with a plasmid carrying either *SIR1*, *SIR2*, *SIR3*, or *SIR4* or with the vector (YCplac33) alone, and the response to α -factor was monitored by time-lapse microscopy. All *MATa cac1* cells carrying only the vector plasmid formed shmoo clusters within 18 hr of exposure to α -factor; no individual shmoo cells were observed. Similarly, all *MATa cac1* cells expressing an extra copy of *SIR1* formed shmoo clusters on α -factor, indicating that Sir1p was not limiting in *MATa cac1* cells. In contrast, the presence of an extra copy of either *SIR2*, *SIR3*, or *SIR4* improved the silencing and α -factor response of *MATa cac1* cells; 20–30% of the cells arrested to form individual shmoos.

The ability of additional Sir2p, Sir3p, or Sir4p to restore α -factor arrest to *cac1* cells is consistent with the idea that *cac1* mutants have subtle defects in the maintenance of the heterochromatin at *HML*, perhaps attributable to a limiting amount of Sir complex proteins in the complex. If this is the case, reduced concentrations of Sir complex proteins in otherwise wild-type cells should give rise to phenotypes similar to those seen in strains lacking CAF-I activity. To determine whether limiting Sir complex protein concentration can give rise to shmoo clusters, we used *sir* mutant strains that expressed the wild-type *SIR* gene from the *GAL10* promoter. Cells were pregrown on raffinose, which permitted sufficient expression of the *SIR* genes to repress *HML*. The strains were then released into glucose medium (to repress the *SIR* gene expression) and plated either immediately or after 2, 4, 6, or 8 hr onto α -factor plates containing glucose. The response to α -factor was then monitored by time-lapse microscopy. In cultures in which either *SIR2* expression or *SIR3* expression was repressed (by growth on glucose for 8 hr before α -factor exposure), shmoo clusters appeared (Fig. 6). Furthermore, the proportion of cells giving rise to shmoo clusters (and α -factor-resistant colonies) increased with increasing time of glucose repression of either *SIR2* or *SIR3* before α -factor exposure (data not shown). Thus, limiting the amount of Sir2p or Sir3p in otherwise wild-type cells is sufficient to weaken silencing and generate the budding shmoo phenotype.

Discussion

Complete *HM* silencing requires CAF-I

Mutations in CAF-I subunits cause derepression of the *HM* loci. Sensitive assays that detect derepression of the

Figure 6. Limiting amounts of Sir2p or Sir3p weaken the maintenance of silencing at *HML*. Strains limiting for Sir2p (YJB285 [pAR14]) or Sir3p (YJB397 [pAR16]) were generated by pregrowth on raffinose, transfer to glucose for 8 hr, and then plating on α -factor lacking leucine and containing glucose. Elongated shmoo clusters that arose in these cultures are shown.

HM loci, such as α -factor arrest or *HMR::TRP1* expression, reveal silencing defects in *cac* mutant strains at both *HML* and *HMR*. The unusual budding shmoo phenotype observed in *MATa cac1* cells grown on α -factor plates provides a new tool for analyzing the mechanisms of silencing. Derepression of the *HML α* locus causes the budding shmoo phenotype seen in *cac1* cells, because we do not see shmoo clusters in a *MATa cac1 HMLa* strain.

Sir1p and CAF-I contribute to different aspects of mating silencing

Mutation of either *SIR1* or *CAC1* causes subtle mating defects. However, there are important differences between *sir1* and *cac1* mutant strains. *cac1* and *sir1* mutants have different relationships with the sites in the *HMRE* silencer. The silencing defect of *sir1* cells is enhanced when the Abf1 site is deleted from *HMRE* (Chien et al. 1993). In contrast, the silencing defect of *cac1* cells is enhanced only in strains lacking the ORC site at *HMRE*. Most important, *sir1* cells exist in one of two distinct epigenetic states that can be detected by their responses to α -factor: (1) the derepressed state that forms colonies, and (2) the repressed state that arrests as shmoo. In contrast, *cac1* cells are not found in two distinct states; they all appear to form shmoo clusters with similar kinetics. Moreover, *sir1* and *cac1* cells respond very differently when *SIR3* is restored to *sir3* cells (Fig. 3). Clearly, *SIR1* is required for the re-establishment of the silent state at *HML*, whereas *CAC1* is not required for the re-establishment process.

We propose that the budding shmoo phenotype reflects a transient defect in the maintenance, rather than the re-establishment of *HML* silencing. The following observations lead us to this proposal. First, the budding shmoo phenotype does not occur in *cac1* mutants that lack α information at *HML*, indicating that it is derepression of *HML α* that gives rise to the phenotype. Second, in *hmr::TRP1* strains lacking the ORC site, *cac1* colonies are smaller than *sir1* or *sir4* colonies on medium lacking tryptophan. Similarly, clusters of *MATa cac1* cells dividing on α -factor are much smaller than *MATa sir4* or *MATa sir1* colonies. The smaller size of *cac1* colonies is consistent with the idea that, in *cac1* cells, both *HML*

and *HMR* are partially, rather than completely, derepressed. Third, the shmoo–bud–shmoo cycle observed in *cac1* cells suggests that *HML* derepression occurs because the *HML* locus in each *cac1* cell oscillates between the repressed state (because cells initially respond to α -factor) and the derepressed state (because cells form new mating projections/buds at a time when wild-type cells have not recovered from α -factor arrest). Fourth, this oscillation between the two states of *HML*, and the loss of this oscillation in most *MATa cac1 sir1* cells, implies that Sir1p facilitates the re-establishment of repression at the *HML* locus in *cac1* cells. Finally, alterations in either the quantity or quality of the major components of yeast heterochromatin (Sir complex proteins and histones, respectively) give rise to the budding shmoo phenotypes, suggesting that the shmoo cluster response to α -factor reflects subtle defects in the structural integrity of the heterochromatin itself. Thus, we propose that Cac1p (and CAF-I) are required for the structural integrity, or maintenance, of yeast silent chromatin.

A model for silencing at HM loci

Previously, we proposed that CAF-I is required for telomeric silencing because it promotes efficient nucleosome assembly and permits rapid association of Sir complex proteins with telomeres and telomere-adjacent regions (Enomoto et al. 1997). The study of *HM* silencing in *cac1* mutants allows us to extend this model to the maintenance of silencing at the *HM* loci as well. We propose that nucleosomes assembled from appropriately acetylated histones form a solid “foundation” on which a strong “wall” of silent chromatin is built (Fig. 7). The Sir complex proteins are essential “bricks” in this silencing wall. We propose that DNA and nucleosomes protected by a stable Sir protein wall remain completely inaccessible to enzymes including histone acetylases and histone deacetylases. In addition, the wall of Sir complex proteins may become unstable if the foundation of nucleosomes is weakened by the presence of histones with inappropriately acetylated amino termini (Fig. 7).

Role of CAF-I in the assembly of silent chromatin

We propose that CAF-I contributes to the association of Sir complex proteins with the heterochromatin domain in two ways. First, CAF-I ensures that replication-coupled nucleosome assembly occurs soon after the replication fork has passed through the heterochromatin. Second, CAF-I ensures that the nucleosomes are assembled from the appropriately acetylated histones, forming a solid foundation for Sir complex propagation.

Timing of nucleosome assembly We posit that CAF-I-mediated nucleosome assembly facilitates the rapid association of Sir complex proteins whose local concentration is likely to be high immediately after replication. Because Rap1p localization and presumably Sir complex localization as well are perturbed in *cac* mutants (Enomoto et al. 1997), it appears that the concentration of

Figure 7. Model for the mechanism by which CAF-I contributes to formation of stable heterochromatin. Heterochromatin is represented as a “wall” of Sir complex proteins (Sir2p, Sir3p, and Sir4p) built on a foundation of nucleosomes (circles) composed of appropriately acetylated histones. After replication, existing nucleosomes (white circles) are randomly distributed between daughter strands of DNA. (Top) CAF-I assembles newly synthesized nucleosomes (gray circles) into chromatin. Existing Sir complex proteins (white rectangles), as well as newly synthesized Sir complex proteins (gray rectangles), associate with the nucleosomes to form a wall of proteins that restrict accessibility to the DNA. If Sir complex proteins are limiting, the wall is thinner or weaker. (Middle) If derepression of the locus occurs, nucleosomes with the “active” acetylation patterns generated during the previous cell cycle (white circles with X) form an unstable foundation that does not associate as tightly with the Sir complex proteins. In the absence of CAF-I, these nucleosomes are recycled onto daughter strands and a fragile wall of Sir complex proteins (recruited by Sir1p) is subject to “leaking” or eventual derepression. If extra Sir complex proteins are provided, the wall can become thicker and thus, more stable. (Bottom) In *cac sir1* double mutants, an unstable foundation (attributable to the lack of CAF-I) and limited recruitment of Sir complex proteins (attributable to the lack of Sir1p) leads to more derepression of the locus.

heterochromatin proteins at telomeres (and presumably at the *HM* loci) is reduced in cells lacking CAF-I, and that these proteins are distributed more randomly throughout the nucleus. If chromatin assembly is delayed, the local Sir complex concentration near silencers may decrease as a result of diffusion of the proteins over the time required for assembly of nucleosomes. This would lead to the formation of a silencer wall with fewer bricks (Fig. 7). Because the efficiency of silencing is a function of competition between transcription activators and the silent chromatin components such as Sir3p (Renauld et al. 1993), a wall composed of fewer Sir proteins at *HML* would be less effective at restricting the accessibility of the transcriptional machinery to the α -genes at *HML*.

Assembly of nucleosomes from appropriately acetylated histones How is the repressed chromatin state inherited and how does CAF-I contribute to that inheritance? Sogo et al. (1986) demonstrated that preexisting nucleosomes segregate randomly after DNA replication. Thus, the replicated chromatin is composed of histones present in the previous cell cycle as well as newly synthesized histones. Because the acetylation pattern of histone H4 in silent chromatin (Braunstein et al. 1996) resembles the acetylation pattern conferred on histone H4 by the cytoplasmic histone acetyltransferase Hat1p (Kleff et al. 1995; Parthun et al. 1996), we propose that the acetylation pattern of histones within silent chromatin remains unaltered and old nucleosomes from the silent domain can be recycled within the silent domain after replication. The incorporation of these recycled nucleosomes (that resemble nucleosomes assembled from newly syn-

thesized histones) into silent chromatin would facilitate the inheritance of the silent chromatin state (Fig. 7).

Active chromatin is characterized by a histone acetylation pattern that is different from newly synthesized or silent chromatin. Mammalian CAF-I preferentially assembles newly synthesized, cytoplasmic histones (Verreault et al. 1996). Assuming that yeast CAF-I has a similar activity, we propose that the role of CAF-I is to ensure that only appropriately acetylated histones are assembled into silent chromatin (Fig. 7). CAF-I may exclude histones with the “active” acetylation pattern from being recycled into silent chromatin, which is especially relevant when an *HM* locus has become derepressed in the previous cell cycle.

Shmoo clusters appear when structural components of heterochromatin are altered

Our experiments with histone H3 and histone H4 mutants support the idea that defects in the nucleosome foundation lead to defects in the maintenance of silencing. Mutations that alter the histone amino termini give rise to budding shmoo cells, suggesting that a defect in the ability of histones H3 or H4 to be acetylated is sufficient to cause a problem with the maintenance of silencing.

Our model suggests that CAF-I ensures that local Sir2p, Sir3p, and Sir4p concentrations are elevated enough to permit assembly of a strong silencer and that in the absence of CAF-I, the local concentration of Sir complex proteins decreases, because of simple diffusion of the proteins over time (Fig. 7). Our observation that an additional copy of either Sir2p, Sir3p, or Sir4p improves

Table 1. Yeast strains used in this study

Strain names	Genotype	Source
S150B-2		
YJB276	<i>MATα; leu2-3,112; ura3-52; trp1-289; his3Δ; ade2Δ, [cir⁺]</i>	Berman lab
YJB277	<i>MATα; leu2-3,112; ura3-52; trp1-289; his3Δ; ade2Δ [cir⁺]</i>	Berman lab
YJB485	276 <i>cac1-1</i> (<i>rfl2-1</i> , Enomoto et al. 1997)	Berman lab
YJB335	276 <i>sir1::LEU2</i>	Berman lab
YJB744	276 <i>HIS⁺ cac1-1 sir1::LEU2</i>	this study
YJB1289	277 <i>lys2; VR-ADE2-TEL</i>	this study
YJB469	276 <i>cac1Δ1</i> (<i>=rfl2-Δ1::LEU2</i> ; Enomoto et al. 1997)	Berman lab
YJB2057	276 <i>HMLα cac1-Δ1</i>	this study
YJB285	276 <i>sir2::HIS3</i>	Berman lab
YJB397	276 <i>sir3::TRP1</i>	Berman lab
W303		
YJB195	<i>MATα ura3-1, ade2-1, his3-11, leu 2-3,112, can1-100, trp1-1</i>	Berman lab
YJB209	<i>MATα ura3-1, ade2-1, his3-11, leu 2-3,112, can1-100, trp1-1</i>	Berman lab
YJB959	195 <i>HMR::TRP1</i>	D. Shore
YJB955	195 <i>hmrΔA::TRP1</i> (missing ORC site)	D. Shore
YJB1143	195 <i>hmrΔB::TRP1</i> (missing Abf1 site)	D. Shore
YJB1104	195 <i>hmrΔE::TRP1</i> (missing Rap1 site)	D. Shore
YJB1960	195 <i>cac1-Δ1 HMR::TRP1</i>	this study
YJB958	195 <i>cac1-Δ1 hmrΔA::TRP1</i>	this study
YJB1139	195 <i>cac1-Δ1 hmrΔB::TRP1</i>	this study
YJB1101	195 <i>cac1-Δ1 hmrΔE::TRP1</i>	this study
YJB1638	195 <i>rap1-12 hmrΔA::TRP1</i>	D. Shore
YJB2006	195 <i>sir1::HIS3 HMR::TRP1</i>	this study
YJB2011	195 <i>msil::hisG-URA3-hisG hmrΔA::TRP1</i>	this study
YJB2009	195 <i>cac1-Δ1 msil::hisG-URA3-hisG hmrΔA::TRP1</i>	this study
YJB1838	195 <i>cac1-Δ1</i>	this study
YJB1578	209 <i>cac1-Δ1</i>	this study
YJB1803	195 <i>cac2::TRP1</i>	this study
YJB1599 (=pkY086)	209 <i>cac2::TRP1</i>	P. Kaufman
YJB1581	195 <i>msil::hisG</i>	P. Kaufman
YJB1836	209 <i>msil::his G-URA3-hisG</i>	this study
YJB1804	195 <i>cac1-Δ1 cac2::TRP1</i>	this study
YJB1802	209 <i>cac1-Δ1 cac2::TRP1</i>	this study
YJB1865	195 <i>cac2::TRP1 msil::hisG-URA3-hisG</i>	this study
YJB1864	209 <i>cac2::TRP1 msil::hisG-URA3-hisG</i>	this study
YJB1862	195 <i>msil::hisG-URA3-hisG cac1-Δ1</i>	this study
YJB1863	209 <i>msil::hisG-URA3-hisG cac1-Δ1</i>	this study
YJB1919	195 <i>cac1-Δ1 cac2::TRP1 msil::his G-URA3-hisG</i>	this study
YJB1918	209 <i>cac1-Δ1 cac2::TRP1 msil::hisG-URA3-hisG</i>	this study
YJB1940	195 <i>sir1::HIS3</i>	this study
YJB1941	209 <i>sir1::HIS3</i>	this study
YJB1962	195 <i>sir1::HIS3 cac1-Δ1</i>	this study
YJB1961	209 <i>sir1::HIS3 cac1-Δ1</i>	this study
YJB2000	195 <i>sir1::HIS3 Δcac2::TRP1</i>	this study
YJB2034	209 <i>sir1::HIS3 Δcac2::TRP1</i>	this study
YJB1945	195 <i>sir1::HIS3 Δmsil::hisG-URA3-hisG</i>	this study
YJB1946	209 <i>sir1::HIS3 Δmsil::hisG-URA3-hisG</i>	this study
YJB2044	195 <i>sir1::HIS3 Δcac1-Δ1 cac2::TRP1</i>	this study
YJB2048	195 <i>sir1::HIS3 Δcac2::TRP1 msil::hisG-URA3-hisG</i>	this study
YJB2007	195 <i>sir1::HIS3 Δmsil::hisG-URA3-hisG cac1-Δ1</i>	this study
YJB1993	209 <i>sir1::HIS3 Δmsil::hisG-URA3-hisG cac1-Δ1</i>	this study
YJB2109	195 <i>cac1-Δ1 sir3::TRP1</i>	this study
YJB2471	195 <i>sir1::HIS3 sir3::TRP1</i>	this study
YJB2544	195 <i>sir3::TRP1</i>	this study
MX4-22A (S288C)		
YJB2166(=MSY552)	<i>MATα ura3-52 lys2Δ201 leu2-3,112 Δ(HHT1 HHF1) Δ(HHT2 HHF2)</i> pMS337[CEN ARS LEU2 HHT1 HHF1]	M.M. Smith
YJB2167 (MSY343)	2166 <i>hht1-1</i> (Δ 2-20)	M.M. Smith
YJB2168 (MSY344)	2166 <i>hht1-2</i> (Δ 2-29)	M.M. Smith
YJB2169 (MSY541)	2166 <i>hhf1-21</i> (KR5)	M.M. Smith
YJB2170 (MSY613)	2166 <i>hhf1-14</i> (KR5KR8)	M.M. Smith
YJB2171 (MSY641)	2166 <i>hhf1-15</i> (KR5KR12)	M.M. Smith
YJB2172 (MSY742)	2166 <i>hhf1-13</i> (KR16)	M.M. Smith
Miscellaneous		
A364A	<i>MATα ade1 ura1 gal1 ade2 tyr1 his7 lys2</i>	L. Hartwell
B364B	<i>MATα ade1 ura1 gal1 ade2 tyr1 his7 lys2</i>	L. Hartwell
TD1	<i>MATα his4-38 ura3-52 trp1-289</i>	Berman lab

silencing is consistent with the idea that in strains lacking CAF-I activity, the concentration of Sir complex proteins at the silencers is suboptimal for silencing. Also consistent with this idea, decreasing the concentration of Sir2p or Sir3p in *CAC1* cells results in the appearance of budding shmoos, indicating that, in otherwise wild-type cells, suboptimal Sir complex protein concentrations are sufficient to give rise to silencing defects like those seen in *cac1* strains.

Role of CAF-I in the *Sir1* independent maintenance of silencing

Our studies indicate that CAF-I and Sir1p act synergistically to silence *HML* in *MATa* cells. In *SIR1* cells, if *HML* becomes derepressed re-establishment of silencing occurs, presumably because Sir1p can attract more Sir complex proteins and can nucleate the formation of a silencer when the local concentration of Sir complex proteins decreases below the threshold for Sir1p independent maintenance. In most *sir1Δ* cells, repressed *HM* loci remain silent (Pillus and Rine 1989). Because this is not true in *sir1Δ cac1Δ* cells, we presume that CAF-I-assembled nucleosomes contribute to the ability of recycled and new Sir complex proteins to associate with the heterochromatin and to form a silencer wall of sufficient "thickness" (Fig. 7). In *sir1Δ* cells, derepressed *HM* loci remain active, presumably because once expression of an *HM* locus occurs, histone acetylation patterns are altered by the transcriptional machinery, and the local concentration of Sir complex proteins decreases (Maillet et al. 1996). In this case, recycled histones would not have the appropriate acetylation pattern (Fig. 7) and the local concentration of Sir complex proteins would not be sufficient to attract newly synthesized Sir complex proteins to the region. We assume that *sir1Δ cac1Δ* cells become derepressed more frequently (because of the lack of CAF-I) and, once derepressed, silencing cannot be reestablished (because of the lack of Sir1p).

Materials and methods

Plasmids and strains

Plasmids pJR910, pJR69, pJR273, and pJR368, carrying *SIR1*, *SIR2*, *SIR3*, and *SIR4*, respectively, were provided by Jasper Rine, University of California, Berkeley. Yeast strains used in this study are listed in Table 1. Strains were constructed by standard crosses within isogenic genetic backgrounds. *SIR1* was disrupted in W303 using pJR533 (Kimmerly and Rine 1987). To construct strain YJB2057, pJH132 (carrying GAL-HO) was induced to switch *HMLα* to *HMLa* in a *sir4* strain (Klar et al. 1981), allowing it to mate as a *MATα* cell. A *Leu*⁺ segregant from this cross, which was unable to switch mating type in the presence of GAL-HO expression, was selected. The strain was then cured of pJH132. pSIR3 (pSE334) includes the complete *SIR3* gene in YCPlac111 (Gietz and Sugino 1988).

Quantitative mating assay

To assay mating of specific strains, 10⁵ cells were mixed with 10⁶ tester cells for 4.5 hr at 30°C on solid complete synthetic

medium (Rose et al. 1990). Mating mixtures were excised from the solid medium, resuspended in sterile water, and serial dilutions of the mixtures were plated on appropriate solid media to select for diploids or for haploid parents. Four assays were performed for each strain. The mating competence of the mutants (proportion of diploids to total cells) was expressed as a proportion of the mating competence of wild-type cells, which were always included in the same sets of experiments. A rank sum test (Snedecor and Cochran 1980) was performed on the ratio of diploid/total for each experiment in pairwise combination with all other strains tested in the same experiment.

α-Factor response

Liquid assays were performed by incubating the relevant strains in YPAD (Rose et al. 1990) containing 500 ng/ml of α-factor (Sigma, St. Louis, MO) for 3 hr. Time lapse assays were performed on the appropriate solid YPAD medium. Five microliters of α-factor (200 μg/ml) was placed on a 5 mm diameter region of the plate. The appropriate *MATa* strains were streaked across this region. Just after streaking (time = 0), areas containing well-separated cells were identified and marked by puncturing the agar surface nearby with a dissecting needle. The location and cell shape were detected and recorded with an Olympus BX-40 Photomicroscope III, equipped with a CoolCAM liquid-cooled three-chip color CCD camera (Cool Camera Co.) and captured using Image Pro Plus version 1.3 software (Media Cybernetics). Cells were incubated at 23°C and then scored at different times after streaking. The χ^2 test of goodness to fit (Snedecor and Cochran 1980) was performed by taking the distribution of wild type into three classes as the null model and testing each strain against this model. Similarly, pairs of mutant strains were tested against one another. Samples were considered significantly different at the $p < 0.01$ level.

Limiting Sir protein experiments

sir2 (YJB285) and *sir3* (YJB397) strains carrying either *GAL-SIR2* (pAR14) or *GAL-SIR3* (pAR16) (Holmes et al. 1997), respectively, were pregrown on raffinose overnight, which allowed sufficient expression of the GAL-SIR constructs to maintain *HM* silencing. Cultures were diluted into SDC-Leu medium containing 2% glucose for 2, 4, 6, or 8 hr, to repress Sir complex protein expression, before assaying α-factor response on solid medium containing glucose as described above.

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Chromatin assembly factor I contributes to the maintenance, but not the re-establishment, of silencing at the yeast silent mating loci

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