Molecular Cell, Vol. 6, 769–780, October, 2000, Copyright ©2000 by Cell Press

A Histone Variant, Htz1p, and a Sir1p-like Protein, Esc2p, Mediate Silencing at *HMR*

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Summary

Silencing at *HMR* requires silencers, and one of the roles of the silencer is to recruit Sir proteins. This work focuses on the function of Sir1p once it is recruited to the silencer. We have generated mutants of Sir1p that are recruited to the silencer but are unable to silence, and we have utilized these mutants to identify four proteins, Sir3p, Sir4p, Esc2p, and Htz1p, that when overexpressed, restored silencing. The isolation of Sir3p and Sir4p validated this screen. Molecular analysis suggested that Esc2p contributed to silencing in a manner similar to Sir1p and probably helped recruit or stabilize the other Sir proteins, while Htz1p present at *HMR* assembled a specialized chromatin structure necessary for silencing.

Introduction

A key characteristic of cellular differentiation is the ability of cells to specifically activate certain genes and stably repress other genes. The mating type of haploid cells of the yeast *Saccharomyces cerevisiae* is determined by just such a mechanism. Mating type is determined by which allele is present at the *MAT* locus. **a** cells possess *MAT***a** genes, while α cells possess *MAT*α genes. In addition to the allele at the *MAT* locus, haploid cells contain copies of both the **a** and α genes at the *HMR***a** and *HML*α loci, respectively. However, these loci are transcriptionally silenced by a mechanism that involves the packaging of these genes into a chromatin structure that is inaccessible to the transcription machinery (Loo and Rine, 1994). Silencing requires regulatory sites called silencers and numerous proteins, some of which bind the silencer directly.

Two silencers called E and I flank the repressed *MAT***a** and *MAT*α genes at the *HMR* and *HML* loci. The *HMR*-E silencer is essential for silencing, whereas the *HMR*-I silencer is important for silencing (reviewed in Kamakaka, 1997). The silencers contain binding sites for Rap1p and Abf1p and autonomous regulatory sequence (ARS) elements that bind the origin recognition complex (ORC). A synthetic *HMR*-E silencer comprised of just single copies of these three binding sites arranged in the same orientation and spacing as the wild-type silencer is sufficient for efficient silencing (McNally and Rine, 1991).

In addition to the proteins that bind the silencers, the core histones and the Sir proteins Sir1p, Sir2p, Sir3p, and Sir4p are also required for silencing. It is believed that the positively charged amino-terminal tails of the histones interact with Sir3p and Sir4p to form a repressed domain (Grunstein, 1998), while Sir2p has been recently shown to possess multiple enzymatic activities (reviewed in Guarente, 2000). Sir1p, on the other hand, interacts with ORC at the silencer (Triolo and Sternglanz, 1996; Gardner et al., 1999). One of the roles of the silencer-bound proteins (ORC and Rap1p) is to recruit Sir1p, since a Gal4-Sir1p chimera anchored to the *HMR* silencer can bypass the functions of ORC and Rap1p (Chien et al., 1993).

Both the silencer-bound proteins (Sussel et al., 1993) and Sir1p (Pillus and Rine, 1989) are required for the establishment of the repressed state and are believed to efficiently recruit the remaining Sir proteins to the silenced chromatin domain. The silencers are also required for the inheritance of the repressed state during DNA replication and cell division, thereby providing the genomic memory that promotes the reformation of the silent state in the daughter cells following DNA replication (Holmes and Broach, 1996; Cheng and Gartenberg, 2000).

Establishment of the silenced state only occurs following passage through the S-phase of the cell cycle (Miller and Nasmyth, 1984; Fox et al., 1997). While the S-phase event necessary for the establishment of the silent state is not known, efficient establishment requires the silencer and Sir1p (reviewed in Kamakaka, 1997). To improve our understanding of the mechanism of assembly of the silenced chromatin, we have generated a series of point mutants in *SIR1* that were efficiently recruited to the silencer but were still unable to silence. These mutants were employed to identify proteins that when overproduced, could overcome the establishment defect and function downstream of the recruitment of Sir1p to the silencer. Two of the proteins were identified as Sir3p and Sir4p, thus validating the model that the role of the silencer-associated Sir1p is to recruit the other Sir proteins. We also isolated two genes (*ESC2* and *HTZ1*) that were required for efficient silencing. We show that while Esc2p behaved in a manner similar to Sir1p, Htz1p, a histone H2A variant, was present at silenced loci. These results strengthen the argument that silencing is mediated by the formation of a specialized chromatin structure.

Results

In this study, we were interested in understanding the events that occurred following the recruitment of Sir1p to the silencer. We generated mutant alleles of *SIR1* that were efficiently recruited to the silencer but were still deficient in the establishment of silencing. We reasoned that these mutants were defective in their ability to recruit or stabilize other factors necessary for silencing, and therefore, overexpression of these factors would overcome the silencing defect in the *sir1* mutants and in the process help identify factors necessary for silencing.

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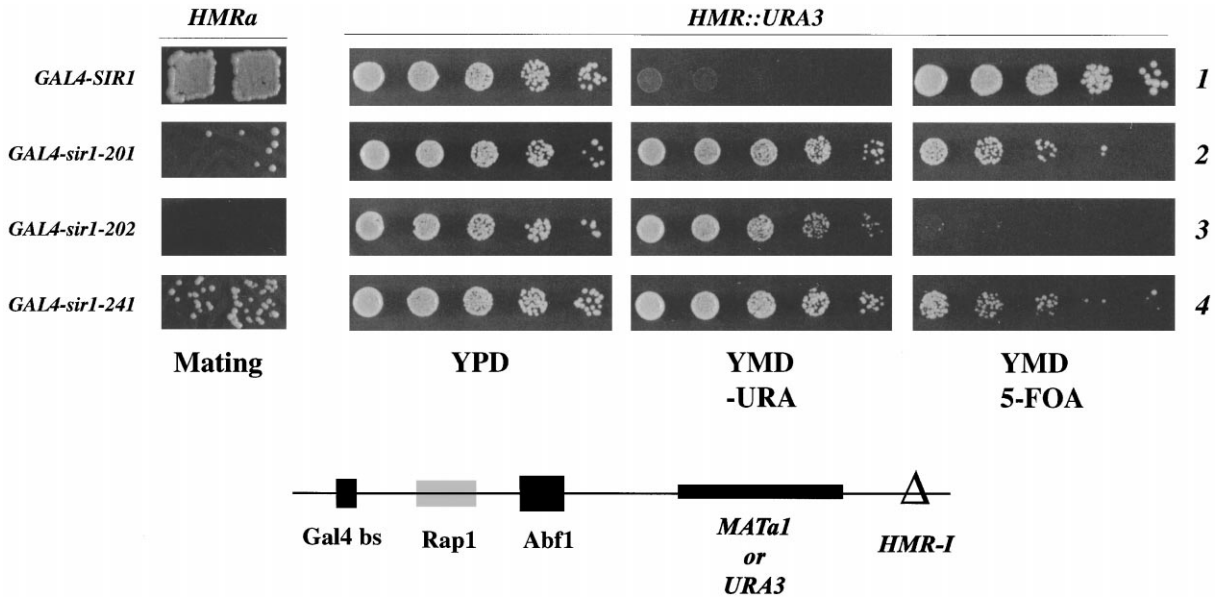

Figure 1. *HMRa* Silencing in *GAL4-sir1* Mutants

Transcriptional repression of *MAT*a1 was examined by patch-mating assays in *HMR*ss(Gal4-Rap-Abf)ΔI strains (column labeled *HMRa*) carrying either wild type (BUY144, row1) or *GAL4-sir1* mutants *GAL4-sir1-201* (Q381P, V390A) (BUY150, row 2), *GAL4-sir1-202* (M472K, L608P) (BUY180, row 3) or *GAL4-sir1-241* (Y447C, F494S) (BUY171, row 4).

Transcriptional repression of *URA3* at *HMR* was examined by monitoring the growth of *HMR*ss(Gal4-Rap-Abf)::*URA3* strains (columns labeled *HMR::URA3*) carrying either wild type (BUY345, row 1) or *GAL4-sir1* mutants *GAL4-sir1*-201 (BUY 383, row 2), *GAL4-sir1*-202 (BUY344, row 3), or *GAL4-sir1*-241 (BUY363, row 4). Cells were grown in YPD medium and serial dilutions were spotted onto YPD plates, YMD plates lacking uracil, and onto fully supplemented YMD plates with 0.5 mg/ml 5-FOA.

Generation of *sir1* Mutants Defective in Silencing

Previous studies showed that Sir1p can establish silencing when it is targeted to the silencer via a DNA binding domain such as Gal4p (Chien et al., 1993). We used this observation as the basis of a screen for mutants of Gal4-Sir1p that were unable to establish silencing, even though Sir1p was recruited to the silencer via its Gal4 DNA binding domain. To perform this study, we designed a strain where the *MAT*a1 genes were under the control of a silencer containing binding sites for Gal4p, Rap1p, and Abf1p. The *MAT*a1 gene is silenced in the presence of a Gal4-Sir1p fusion protein. Silencing was monitored using a mating assay. In wild-type α cells, the *MAT*a1 gene at *HMR* is repressed, and the α cell is capable of mating with an **a** cell, giving rise to diploid colonies. However, if the *MAT*a1 gene is derepressed, the α cell is unable to mate and fails to form diploid colonies.

The *GAL4-SIR1* fusion gene was mutagenized in the *SIR1* coding sequence using standard PCR-based mutagenesis techniques (Muhlrad et al., 1992). Yeast strains (*MAT*α *sir1* Δ *HMR*ss(Gal4-Rap-Abf)ΔI) were cotransformed with a gapped *GAL4-SIR1* plasmid and the mutagenized portion of the *SIR1* gene. The resulting gap-repaired transformants were replica plated to the appropriate mating type lawns and assayed for their mating phenotypes. The nonmating phenotypes of three representative *sir1* mutant alleles that were chosen for further analysis are shown in Figure 1 (compare row 1 to rows 2, 3, and 4).

We also examined silencing of a *URA3* gene at *HMR* by monitoring the growth of cells on medium lacking uracil or containing 5-FOA. As seen for *MATa1*, the *URA3* gene at *HMR* was derepressed in the *sir1* mutants (see Figure 1).

Strains harboring these mutations were subjected to immnuoblot analysis using antibodies against the DNA binding domain of Gal4 to identify missense mutants and eliminate any truncation mutants. This analysis also revealed that the mutants were expressed at levels similar to the wild-type Gal4-Sir1 protein (data not shown). The mutations were mapped by DNA sequence analysis.

Isolation of Multicopy Suppressors of *sir1* Mutants

The isolation of missense alleles of Sir1p suggests a weakening of its interactions with other proteins that are required for silencing. We next sought to identify proteins that, when overexpressed, lead to restoration of silencing in the presence of *sir1* mutants. Overproducing such proteins in a *sir1* mutant should compensate for the weakened interaction (Rine, 1991).

These experiments were performed using two mutant alleles of Gal4-Sir1p (*GAL4-sir1–201* or *GAL4-sir1–202*), and two multicopy plasmid libraries were screened for proteins that restored silencing in these mutant backgrounds. Several genes, when overexpressed, restored silencing in these *sir1* mutants, and DNA sequence analysis of the clones identified Sir3p (silent information regulator 3), Sir4p (silent information regulator 4), Ssf2p (suppressor of *STE4*), Ste12p (sterile 12), Esc2p (establishment of silent chromatin 2), and Htz1p (histone H2az 1). Sir4p is known to physically interact with Sir1p (Chien et al., 1991) and its isolation in this screen validated our approach.

All the suppressors were next tested for their ability to silence *HMR*ss(Gal4-Rap-Abf)::*URA3* (data not shown). This analysis showed that *STE12* and *SSF2* were unable to silence *HMR*ss(Gal4-Rap-Abf)::*URA3* and were most likely not involved in silencing but instead affected the mating pathway and were not analyzed further.

A closer analysis of the reestablishment of silencing by the remaining four genes revealed differences in their proficiency to restore silencing (Figure 2A). Overexpression of only Sir3p and Sir4p in a *sir1-202* mutant restored robust silencing. In a *sir1-201* allele, Sir3p, Sir4p, and Esc2p reestablished silencing, while Htz1p was a poor suppressor. In a *sir1-241* allele, all four genes restored silencing. Interestingly, in a *sir1* null mutant strain, only Sir3p and Sir4p restored efficient silencing.

Effects of the Suppressors on Mutations in the Silencers

The *HMR*-E silencer is essential for the establishment and inheritance of silencing (Holmes and Broach, 1996). It is possible, therefore, that the reestablishment of silencing by the dosage suppressors in a *sir1* mutant was being mediated in part through interactions with the silencer-bound proteins. To address this, we determined whether the dosage suppressors could restore silencing in strains where the silencer-bound proteins themselves were absent. In a synthetic silencer lacking the Abf1 binding site, silencing was only weakly compromised (Figure 2B, panel i, row 1), and all four dosage suppressors (rows 2–5) restored silencing in the absence of the Abf1p.

An *HMR* silencer lacking the ARS element such that ORC was unable to bind to the silencer was completely derepressed (Figure 2B, panel ii, row 1). Under these circumstances, Sir3p and Sir4p (panel ii, rows 2 and 3) were able to restore silencing when overexpressed, albeit less efficiently than in a silencer lacking Abf1 binding sites. Esc2p, on the other hand, only weakly restored silencing in the absence of an ARS element at the silencer (panel ii, row 4).

Silencers lacking a Rap1p binding site were also fully derepressed (Figure 2B, panel iii, row 1), and surprisingly, under these circumstances only Sir3p was able to partially restore silencing when overexpressed (compare row 2 to rows 3, 4, and 5). This analysis suggests that these suppressors act at least in part through interactions with the silencer-bound factors.

An alternative possibility for Esc2p- and Htz1p-mediated suppression was that these proteins indirectly suppressed the *sir1* mutants by increasing the levels of the Sir proteins. To test this, we performed protein immunoblots with antibodies against Sir3p and Sir4p and observed no change in the levels of these proteins when either Esc2p or Htz1p was overexpressed (Figure 2C, compare vector control against each suppressor).

Esc2p and the Establishment of Silencing

The above data suggest that Esc2p function required the presence of ORC, Rap1p, and Sir1p. It has previously been shown that tethering Sir1p to the silencer can bypass Rap1p and ORC function at the silencer (Chien et al., 1993). We therefore asked whether Esc2p was able to restore silencing if it was tethered to the silencer and whether this Esc2p-mediated silencing required Sir1p. This experiment was performed in the presence or absence of Sir1p, using a Gal4-Esc2p fusion protein and analyzing Gal4-Esc2p-mediated silencing of an *HMR*ss (Gal4-Rap-Abf) silencer. As shown in Figure 3A, tethering Gal4-Esc2p to the silencer allowed the efficient formation of silenced chromatin in an *HMR*ss(Gal4-Rap-Abf) background (compare vector control with Gal4-Esc2p). Furthermore, Gal4-Esc2p was able to restore silencing to the same extent in the absence of Sir1p as in its presence (compare *SIR1* with *sir1*Δ). This suggests that targeting Esc2p to the silencer could substitute for Sir1p. Silencing mediated by Gal4-Esc2p did require the other Sir proteins (data not shown). Furthermore, Gal4-Esc2p could also restore silencing to a synthetic silencer in which the Gal4p binding sites replaced the Rap1 binding site (data not shown). These data collectively demonstrate that tethered Esc2p could bypass the requirement for Sir1p, ORC, or Rap1p binding sites.

The demonstration that Gal4-Esc2p could bypass Sir1p indicated that Esc2p functioned in a manner similar to Sir1p or downstream of Sir1p in the establishment/stabilization of silencing. Previous data have shown that the *HMR* domain is completely derepressed in strains lacking *sir1*, and *sir3* and reestablishment of silencing requires both Sir1p and Sir3p (Enomoto and Berman, 1998). We therefore analyzed the role of Esc2p in the reestablishment of silencing and asked whether Esc2p could substitute for Sir1p at a completely derepressed *HMR* locus. For this experiment, we generated a strain lacking Sir1p, Sir3p, and Esc2p in which the *HMR*ss(Ars-Rap-Abf)ΔI locus was fully derepressed (Figure 3B, upper panel). Reintroduction of Sir3p (on an ARS/CEN plasmid) or Esc2p (on a 2μ plasmid) alone or together did not restore silencing, consistent with our earlier data in Figure 2A demonstrating Esc2p's inability to suppress a *sir1* null strain.

Silencing mediated by *HMR*-E is enhanced by the presence of the *HMR*-I silencer (Donze et al., 1999; Rivier et al., 1999). The previous experiments were all performed in strains lacking the *HMR*-I silencer to avoid complications in interpretation. We wanted to determine whether *HMR*-I would contribute toward Esc2p-mediated reestablishment of silencing in the absence of *SIR1*. This analysis was therefore performed in a strain containing a synthetic *HMR*-E silencer together with *HMR*-I (*HMR*ss(Ars-Rap-Abf)+I). Reintroduction of Sir3p or Esc2p alone did not restore silencing, but expressing both Sir3p and Esc2p established silencing even in the absence of Sir1p (Figure 3B, lower panel), demonstrating that Esc2p, when overexpressed, could substitute for Sir1p in the reestablishment of silencing in the presence of *HMR*-I.

Loss of *HTZ1* and *ESC2* Lead to Derepression of Silencing

Having identified two novel gene products that affect silencing when overproduced, we asked whether silencing was affected in cells lacking these proteins. The loss of either gene had a very subtle growth defect. To determine whether the loss of either Htz1p or Esc2p affected silencing, we used a sensitive assay that monitors the expression of the *ADE2* gene positioned at *HMR*

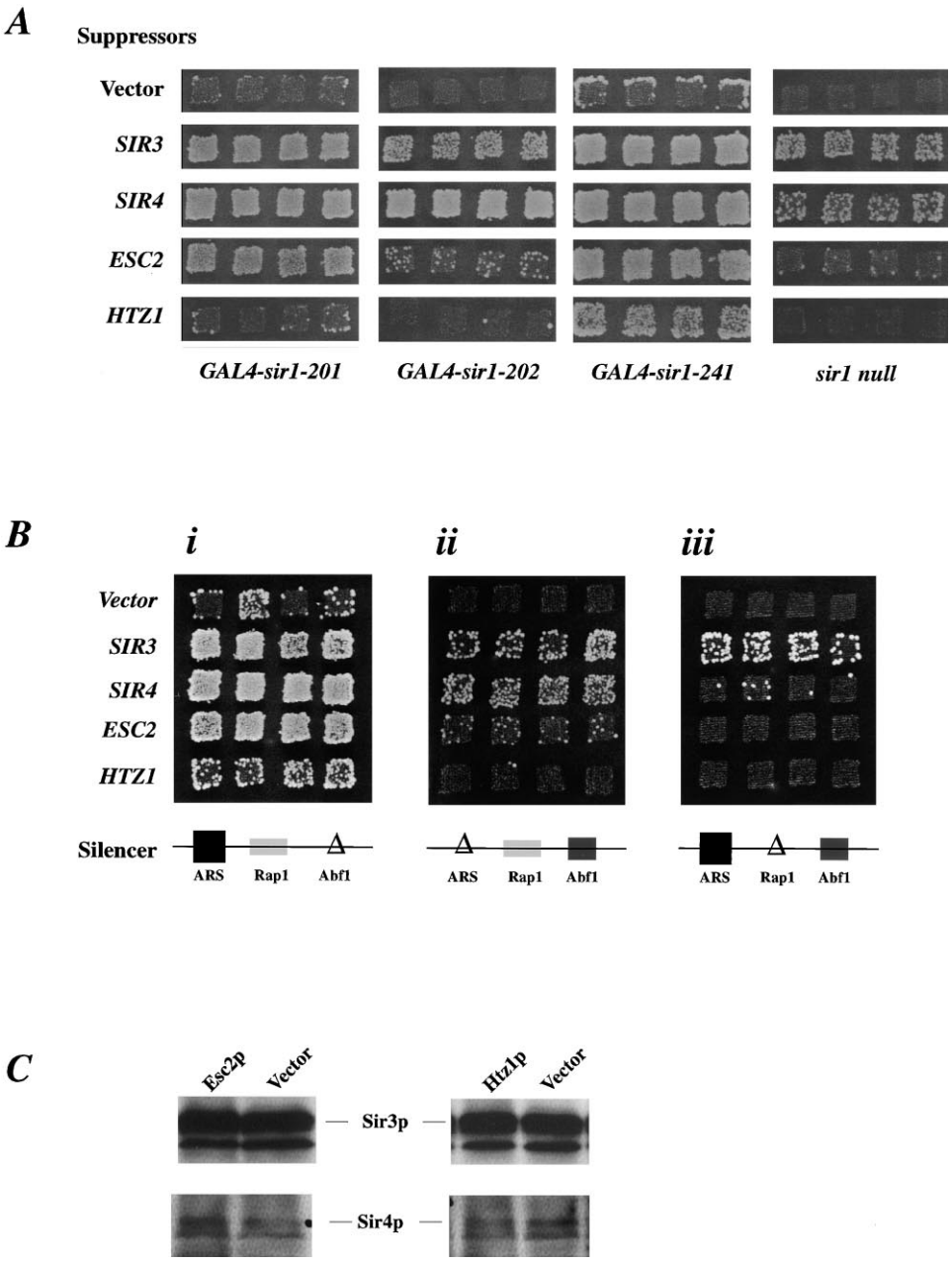

Figure 2. Isolation and Characterization of Dosage Suppressors of *GAL4-sir1* Mutants

(A) Dosage suppressors of silencing defects in *GAL4-sir1* mutants. Patch mating assays were employed to assess the ability of 2 micron plasmid-borne *SIR3* (pRO329), *SIR4* (pOS46), *ESC2*(pOS48), and *HTZ1* (pOS65) to restore silencing of *MAT*a1 in an *HMR*ss(Gal4-Rap-Abf)ΔI strain carrying various mutant alleles of *GAL4-sir1*, *GAL4-sir1-201* (BUY150), *GAL4-sir1-202* (BUY180), *GAL4-sir1-241* (BUY171), or *sir1*Δ (BUY121).

(B) Effect of dosage suppressors on silencer mutations. Patch-mating assays were employed to assess the ability of 2 micron plasmid-borne *SIR3* (pRO329), *SIR4* (pOS46), *ESC2* (pOS48), and *HTZ1* (pOS65) to restore silencing of *MAT*a1 in *HMR*ssΔI silencers lacking either (i) the Abf1p binding site (Ars-Rap-abfΔ, JRY4889), (ii) the Ars element (arsΔ-Rap-Abf, JRY4474), or (iii) the Rap1p binding site (Ars-rapΔ-Abf, JRY3936).

(C) Sir3p or Sir4p levels are not affected by overexpression of either *ESC2* or *HTZ1*. Crude lysates of yeast cells carrying either a 2 micron–based vector control or 2 micron plasmid-borne ESC2 (pOS46, left panel) or HTZ1 (pOS65, right panel) were subjected to immunoblot analysis using polyclonal antibodies against Sir3p (rabbit) and Sir4p (donkey). Arrows are used to indicate the Sir3p or Sir4p species.

(Sussel et al., 1993). In wild-type cells, repression of *ADE2* at *HMR* results in the accumulation of a red pigment, giving rise to red colonies. A complete loss of silencing of *ADE2* at *HMR* results in white colonies, while a partial derepression yields pink or sectored colonies. Loss of either *HTZ1* or *ESC2* gave rise to pink colonies, suggesting that the loss of these proteins led to a partial derepression of the silenced state (Figure 4A). Unfortu-

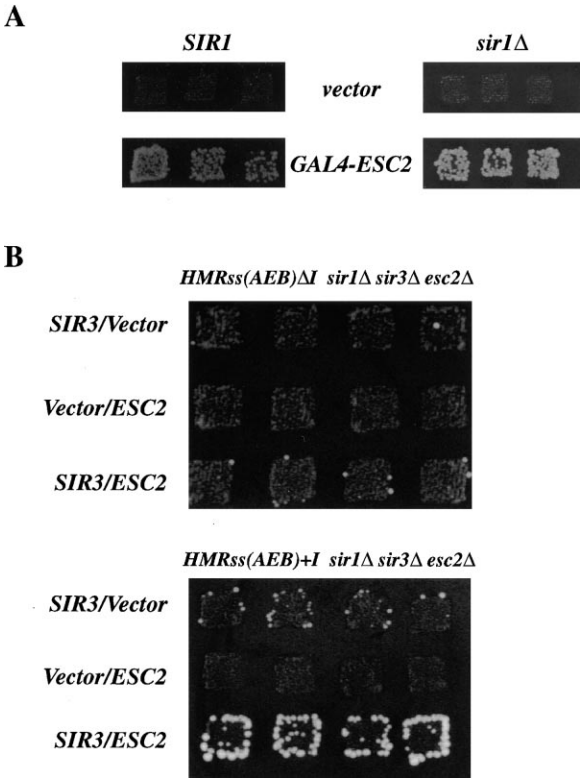

Figure 3. Analysis of Esc2p-Mediated Silencing

(A) Gal4-Esc2p-mediated silencing. *HMR*ss(Gal4-Rap-Abf)ΔI strains either carrying wild-type Sir1p (JRY4806) or lacking Sir1p (BUY123) were transformed either with a control vector or a chimera of the Gal4p DNA binding domain fused to the NH2 terminus of Esc2p. Silencing of *MAT*a1 was assayed in representative colonies from each transformation by patch mating.

(B) Esc2p could substitute for Sir1p in the reestablishment of silencing. Strains lacking Sir1p, Sir3p, and Esc2p were transformed with each of the following plasmid pairs: *SIR3* on a *TRP1*-containing vector (pRO144) and a *URA3*-containing vector control, *ESC2* on a *URA3*-containing vector (pOS007) and a *TRP1*-containing vector control, or *SIR3* on a *TRP1*-containing vector and *ESC2* on a *URA3*-containing vector. The triple-delete strains carried either *HMR*ss(Ars-Rap-Abf)ΔI (BUY593, top panel) or *HMR*ss(Ars-Rap-Abf)+I (BUY595, lower panel). Patch-mating assays were used to monitor transcriptional repression of *MAT*a1.

nately, semiquantitative analysis did not show any significant derepression, suggesting that this loss of *HMR::ADE2* silencing was less than 5-fold.

We also tested the effects of these mutations on silencing at telomeres (Figure 4B) by monitoring expression of *URA3* present at telomere VIIL. Strains containing the marked telomere and either *esc2*Δ*::HIS3* or *htz1*Δ*::*kanMX were monitored for growth on media lacking uracil or containing 5-FOA. While the loss of Htz1p led to a profound loss of telomeric silencing as monitored by growth of cells on 5-FOA, the absence of Esc2p caused only a partial derepression of *URA3* at the telomere (Figure 4B).

Since the rDNA locus is also silenced in a Sir2-dependent manner (Smith and Boeke, 1997), we also monitored expression of a *mURA3* gene at the *RDN1* locus. Strains lacking Esc2p did not show any loss of silencing, while *htz1*Δ strains showed a slight increase in silencing at the rDNA (data not shown).

Localization of Esc2p and Htz1p

Since *ESC2* and *HTZ1* were uncharacterized genes in *S. cerevisiae*, we were interested in determining the localization of these proteins in the cell. The subcellular localization of Esc2p was determined with a protein chimera containing GFP fused in frame with *ESC2*. Fluorescent microscopy indicated that the GFP-Esc2p fusion protein was exclusively nuclear and remained nuclear even during mitosis (Figure 5A). However, within the limits of optical microscopy we did not observe any subnuclear localization of this protein.

The localization of Htz1p was determined with a protein chimera containing an HA epitope fused in frame with *HTZ1*. Yeast strains containing the N-terminal-tagged Htz1p were fixed and immunostained with antibodies against the HA epitope (Figure 5A). The immunofluorescence data indicate that Htz1p was present throughout the nucleus with increased localization to subnuclear structures that appeared to be the nucleolus. Staining of yeast cells with antibodies against the nucleolar protein Net1p (Shou et al., 1999) also stained similar structures (data not shown).

To gain further insight into the distribution of HA-tagged Htz1p, we investigated the association of this histone variant with particular DNA sequences *HMR* and *RDN1*. To map the distribution of this protein, we performed a chromatin immunoprecipitation analysis of formaldehyde cross-linked yeast cells using antibodies directed against the HA epitope (Figure 5B). The coimmunoprecipitated DNA was analyzed by PCR amplification using specific primers against *HMR* and *RDN1*. Control immunoprecipitations using antibodies against the Myc epitope or a strain lacking the HA epitope demonstrated the specificity of the immunoprecipitation. The results are shown in Figure 5B and demonstrated that Htz1p appeared to be present at the *HMR* locus as well as at either end of the *RDN1* locus (centromere-proximal *RDN1* repeat as well as the centromere-distal *RDN1* repeat). There appears to be a subtle but consistent reduction in Htz1p on a fragment encompassing the *HMR*-E silencer (data not shown), which may reflect the absence of nucleosomes at the silencer (Ravindra et al., 1999).

HTZ1 but Not H2A Suppressed the *sir1* Mutant Defect

The histones H3 and H4 play a central role in the formation of silenced chromatin through direct physical interactions with Sir3p and Sir4p (Hecht et al., 1995). However, no role has been ascribed to the other two histones (H2A and H2B) in silencing. The isolation of *HTZ1*, a nonallelic variant of histone H2A, as a dosage suppressor of *sir1* mutants was unexpected and led us to reexamine the role of the other two core histones.

We first addressed the question of whether overexpression of the predominant H2A (*HTA1*) would also suppress the silencing defect of a *sir1* mutant. To perform this analysis, we used GAL4-*sir1–241* in an *HMR*ss(Gal4-Rap-Abf)::*URA3* background and monitored expression of the *URA3* gene following overexpression of Htz1p or H2A. In the presence of just the

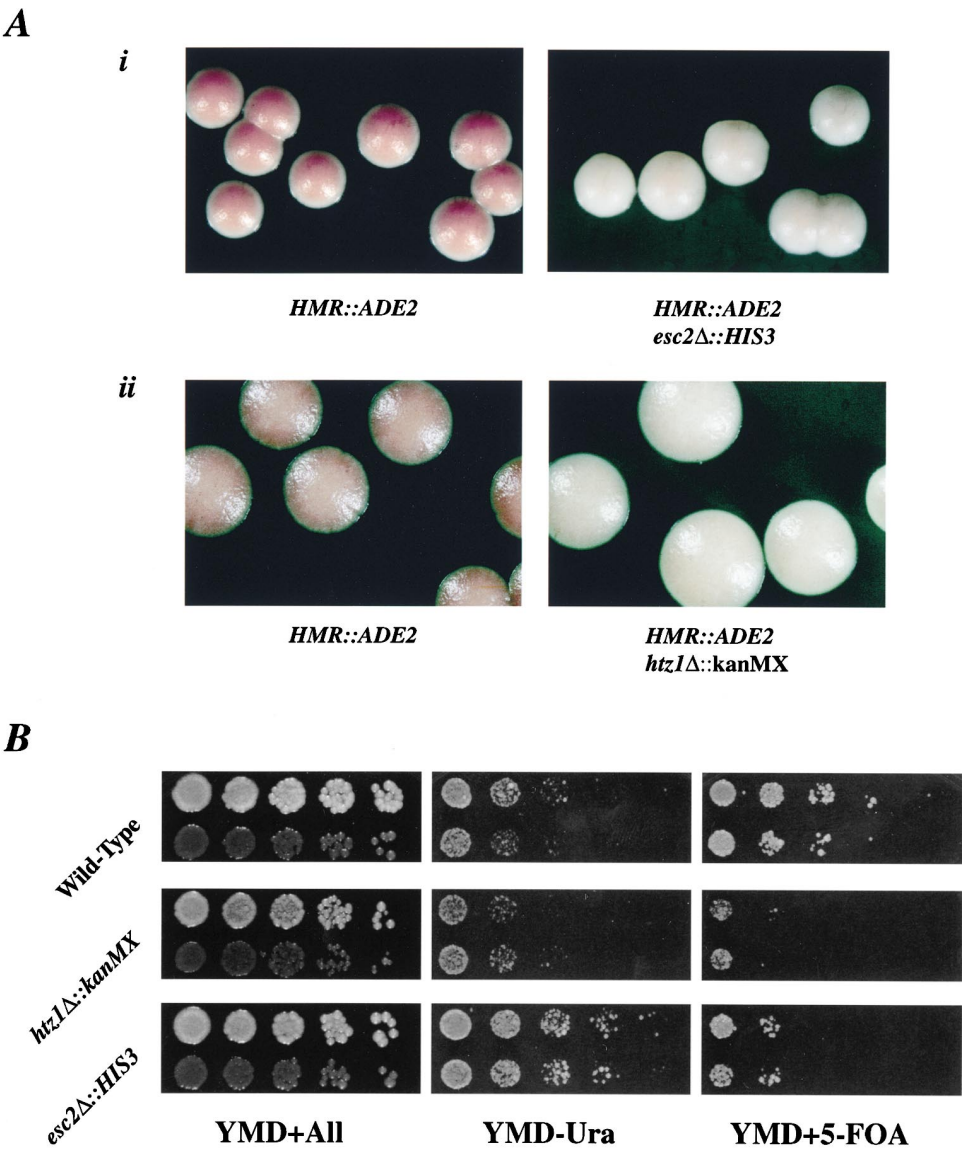

Figure 4. Loss of Esc2p and Htz1p Derepress the Silenced Loci

(A) Loss of Esc2p or Htz1p led to de-repression of *HMR::ADE2* silencing. (i) Isogenic strains bearing either wild-type (BUY488) or an *esc2* null allele (BUY495) were grown to log phase, and serial dilutions were plated on YPD medium to obtain well-dispersed, discrete colonies. Plates were incubated at 4°C for 8–10 days before they were photographed. (ii) Isogenic strains bearing either wild-type (BUY540) or an *htz1* null allele (BUY532) were grown to log phase, and serial dilutions were plated on YPD medium to obtain well-dispersed, discrete colonies. Plates were incubated at 4°C for 8–10 days before they were photographed

(B) Loss of Esc2p or Htz1p lead to derepression of telomeric silencing. Isogenic strains bearing *URA*3 on the left arm of telomere VII and either *esc2*Δ::*HIS3* (BUY703, BUY704), *htz1* Δ::*kanMX* (BUY696, BUY699), or wild-type copies of *ESC2* and *HTZ1* (BUY668, BUY695) were grown to log phase, and 5-fold serial dilutions were spotted on YPD, YMD lacking uracil, and fully supplemented YMD with 0.1% 5-FOA.

mutant, Sir1p silencing of the *URA3* gene was lost and cells were unable to grow on 5-FOA plates. While Htz1p was able to restore silencing to some extent, H2A was unable to silence *URA3* at *HMR* (Figure 6A). Since H2A forms a stable heterodimer in nucleosomes with H2B (*HTB1*), we also asked whether the simultaneous overexpression of both Htz1p and H2B would further increase silencing. However, as shown in Figure 6B, the extent of Htz1p-mediated silencing remained the same regardless of whether H2B was overexpressed or not. The simultaneous expression of both H2A and H2B also did not restore silencing in *sir1* mutants (data not shown). These results argue for a specific role for Htz1p in silencing at *HMR* rather than a generic role for the H2A/H2B dimer.

Htz1p Function Was Essential in a Histone H4 N-Terminal Tail Mutant

Several mutations in the N-terminal tails of histone H4 have been shown to affect silencing at both *HML* and *HMR* (Grunstein et al., 1995), leading to partial derepression at these loci. Since the absence of Htz1p also led

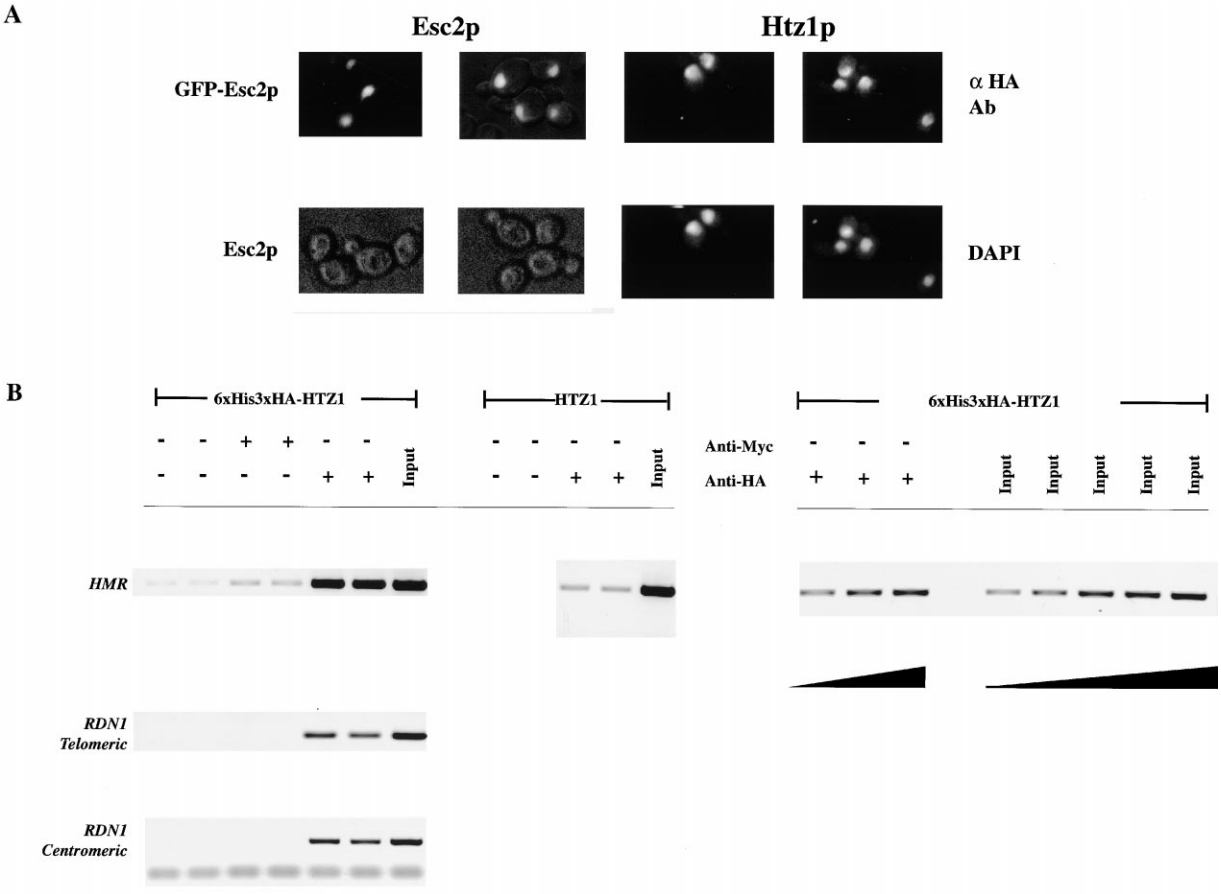

Figure 5. Cellular Localization of Esc2p and Htz1p

(A) Subcellular localization of GFP-Esc2p and HA-Htz1p. A plasmid-borne chimera of GFP(S65T) fused to the N terminus of Esc2p (GFP-Esc2p) or an untagged control (Esc2p) was transformed into a *MATa/MAT*a diploid strain (BYB80). Representative transformants were grown to early log phase and were visualized by fluorescence microscopy under a FITC filter (left panel). The 12CA5 monoclonal antibody was used in indirect immunofluorescence studies to localize HA-Htz1p (α-HA Ab) in *a MATa/MAT*α diploid strain (BUY645). Cells were costained with DAPI to visualize the nuclei. Images were captured by a CCD camera and processed using IPLab software.

(B) Chromatin Immunoprecipitation of HA-Htz1p. Chromatin isolated from formaldehyde-fixed strains BUY629 (with 3× HA-Htz1p) and BUY68 (with Htz1p) was subjected to immunoprecipitation with either mock antibody, 9E10 (α-Myc), or HA.11 (α-HA) monoclonal antibodies. PCR-based amplification reactions of the input and coimmunoprecipitating DNA were performed using primers specific to the *HMR* locus (top left panel), the telomere-proximal *RDN1* locus (middle panel), and the centromere-proximal *RDN1* locus (bottom panel). The top right panel represents *HMR*-specific PCR amplification products from 2-fold serially diluted input DNA (0.01–0.16 μl) and coimmunoprecipitated DNA templates (0.5–2.0 μl).

to a partial derepression of *HMR*, we rationalized that cells lacking Htz1p and either the H3 or H4 N-terminal tail (H4 NΔ4-28 and H3 NΔ 4-30) might exacerbate the silencing defect of each individual mutant. To investigate this, we attempted to generate strains carrying mutations in both of these proteins. Initially, we constructed a *htz1Δ hhf1-hht1Δ hhf2-hht2Δ* where wild-type histones H3 and H4 were provided by copies of these genes on a *URA3*-bearing plasmid. We next introduced a wild-type H3 and a mutant version of histone H4 (H4 NΔ4-28) on a *TRP1*-bearing plasmid. Attempts to replace the wild-type H4 genes with the N-terminal mutant using plasmid shuffle (Kayne et al., 1988) by growth on 5-FOA (which would result in the loss of the *URA3*-bearing plasmid containing the wild-type histone H4) did not yield any Ura-FOA^R viable cells (Figure 7A). These data indicate that cells lacking histone H4 N-terminal tails were unable to survive in the absence of Htz1p. Similar experiments were also performed with histone H3 tail delete mutants. While these mutants were able to survive, they grew very slowly.

Previous studies have demonstrated an absolute requirement of an N-terminal histone tail on either H3 or H4 for viability. Furthermore, strains lacking acetylatable lysines at residues 5, 8, and 12 are also inviable in the absence of an H3 tail, and this lethality is most likely due to defects in chromatin assembly. We were therefore interested in determining whether the lethality seen in H4(NΔ4-28) *htz1Δ* strains was also due to incomplete chromatin assembly. Unlike the histone H4 (K5, 8, 12) point mutant substitutions in a histone H3(N4-30Δ) strain, *htz1Δ* mutants were viable in combination with substitutions in residues K5, 8, 12R/Q of histone H4 (Figure 7B). The *htz1Δ* strains were also able to survive with a histone H4 K16R/Q substitution. These results suggest that the inviability associated with *htz1Δ* and

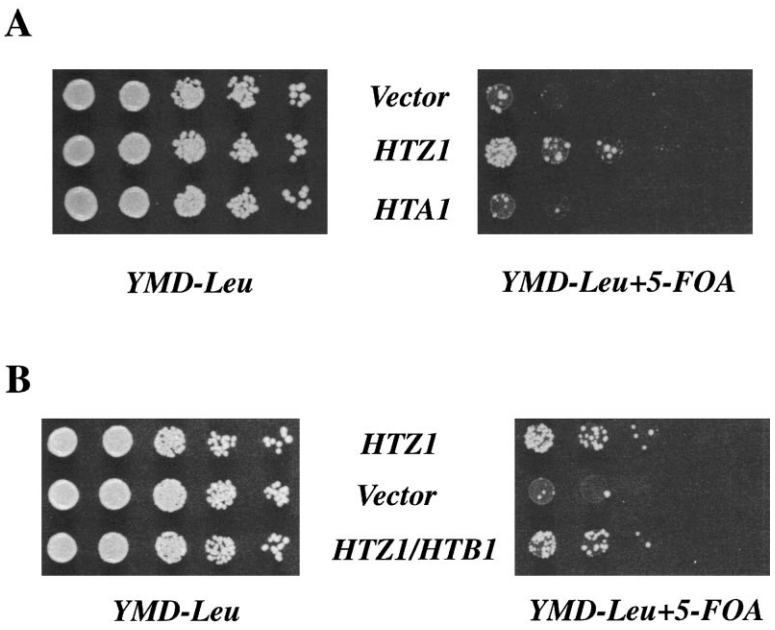

Figure 6. Analysis of Htz1p-Mediated Silencing

(A) *HTZ1* but not *HTA1* (H2A) restored silencing in a *GAL4-sir1* mutant. *GAL4-sir1-241* (BUY363) mutant cells bearing *HMR*ss(GEB)::*URA3* were transformed with either a control plasmid or a 2 micron–based plasmid carrying either *HTZ1* (pOS65) or *HTA1* (H2A, pOS67). Transcriptional repression of *URA3* at *HMR* was monitored by growth of representative transformants on YMD medium containing 5-FOA. Cells were grown in YMD medium lacking leucine (to maintain plasmid selection), and serial dilutions were spotted onto YMD plates lacking leucine and onto YMD plates lacking leucine but with 0.5 mg/ml 5-FOA.

(B) Simultaneous expression of *HTZ1* and *HTB1* (H2B) did not improve silencing over that mediated by *HTZ1* alone. *GAL4-sir1-241* (BUY363) mutant cells bearing *HMR*ss(Gal4-Rap-Abf)::*URA3* were transformed with either a control plasmid or with a 2 micron–based plasmid carrying either *HTZ1* alone (pOS65) or both *HTZ1* and H2B (*HTB1* pOS70). Repression of *URA3* at *HMR* was monitored by growth of representative transformants on YMD medium containing 5-FOA. Cells were grown in YMD medium lacking leucine (to maintain plasmid selection), and serial dilutions were spotted onto YMD plates lacking leucine and onto YMD plates lacking leucine but with 0.5 mg/ml 5-FOA.

an H4 tail deletion is probably not a consequence of defects in chromatin assembly.

CAF-1 is required for the proper deposition of histones H3 and H4 during replication-coupled chromatin assembly (Smith and Stillman, 1989), and mutations in CAF-1 subunits (*cac1*) lead to "misassembled" nucleosomes. One of the consequences of misassembled nucleosomes is a loss of silencing (reviewed in Adams and Kamakaka, 1999). Since Htz1p is an integral component of nucleosomes, it was unclear whether the *cac1*-dependent misassembly of nucleosomes coupled with the absence of Htz1p would further cripple the Sir–histone interactions. Results from this double mutant analysis revealed that the loss of both *CAC1* and *HTZ1* in the same cell did not cause lethality nor did it lead to increased derepression of *HMR*::*ADE2* (data not shown).

Discussion

A popular view of silencing is that the silenced domain is maintained in a repressed state through interactions between the proteins bound to the silencer and the proteins associated with nucleosomes. Establishment of silencing, defined as the switch that leads a derepressed *HMR* locus to become repressed, can only be established following passage through the S-phase of the cell cycle (Miller and Nasmyth, 1984; Fox et al., 1997). While the S-phase event that is required for the establishment of the silent state is not known, efficient establishment of silencing requires the silencer and Sir1p and is presumed to be a consequence of replication-associated chromatin assembly.

Isolation of Sir1p Mutants Defective in the Establishment of Silencing

It is believed that the role of the silencer-associated components (specifically Rap1p and ORC) is to efficiently recruit the Sir proteins, including Sir1p (Chien et al., 1993; Sussel et al., 1993; Fox et al., 1997).

In this study, we undertook a molecular genetic approach to further understand Sir1p function. We have generated mutations in Sir1p that are efficiently recruited to the silencer but were still unable to silence the locus (*sir1*^{esc} alleles). The effects of these mutations were not due to reduced levels of these proteins, since immunoblot analysis indicated that the fusion protein was present at levels comparable to wild-type Sir1p. In addition, characterization of these alleles suggest multiple roles for Sir1p in silencing, since differential interactions were observed with other proteins such as Sir4p and the acetyltransferase Sas2p (data not shown).

Our *sir1*^{esc} mutants were distinct from *sir1* mutants that have been shown to be defective in silencer recognition (*sir1*^{srd}) (Gardner et al., 1999; Hollenhorst et al., 2000). In addition to the three alleles described, we have isolated additional *sir1*^{esc} alleles, and none are defective in the silencer recognition domain defined by the *sir1*^{srd} alleles.

Having isolated mutants in Sir1p, we reasoned that these point mutations weakened interactions with other proteins involved in silencing, and therefore, overproducing such proteins in the *sir1* mutants should compensate for the weakened interaction. We isolated six suppressors, of which Ste12p and Ssf2p suppressed the mating defect but not the silencing defect and were therefore not pursued. It is, however, interesting to note that although Ssf2p is a suppressor of Ste4, it is a nucleolar protein (Kim and Hirsch, 1998).

The Role of the Silencer-Bound Proteins in Recruiting Sir3p and Sir4p

Of the other four proteins, the isolation of Sir3p and Sir4p was consistent with previous observations that these proteins interact with Sir1p and the silencer and confirmed the validity of this screen.

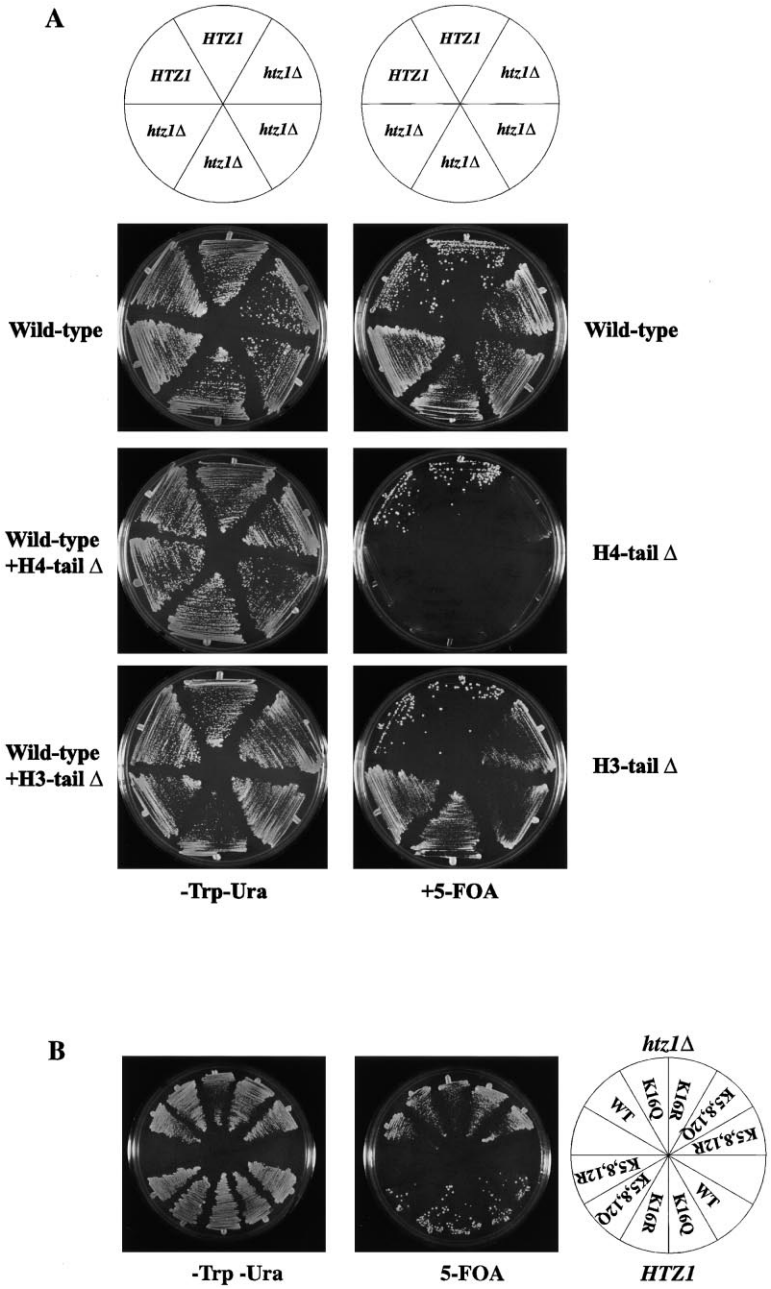

Figure 7. Characterization of the Genetic Interactions between Htz1p and the Core Histones H3 and H4

(A) HTZ*1* function was essential in strains lacking the histone H4 N-terminal tail but was dispensable in strains lacking the histone H3 N-terminal tail. Wild-type copies of H3 and H4 were provided on a centromere-based *URA3* plasmid (pRO149) in strains where both copies of the histones H3 and H4 genes had been deleted either alone (*HTZ1 hhf1-hht1*Δ *hhf2-hht2*Δ ROY1280 and ROY 1281) or in conjunction with *HTZ1* (*htz1*Δ *hhf1-hht1*Δ *hhf2-hht2*Δ ROY1433, ROY1434, ROY1439, ROY1440). These strains were transformed with a centromere-borne *TRP1* plasmid (pRM200) carrying both wild-type histones H3 and H4 or wild-type histone H3 and a histone H4 mutant lacking the N-terminal tail (H4N4-28Δ, pGF29) or a wild-type histone H4 and histone H3 lacking the N-terminal tail (H3NΔ4-30, pRM430). Representative transformants were streaked on YMD medium lacking tryptophan (to maintain pRM200, pGF29, or pRM430) but containing 1 mg/ml 5-FOA to expel the *URA3*-based wild-type H4 allele and thereby generate either an H4 (NΔ4-28) or an H3 (ND) allele in an *HTZ1* null background.

(B) N-terminal tail point mutants in histone H4 did not require *HTZ1* function for viability. ROY1280 (*HTZ1 hhf1-hht1*Δ *hhf2-hht2*Δ) and ROY1434 (*htz1*Δ *hhf1-hht1*Δ *hhf2-hht2*Δ) carrying pRO149 were transformed with centromere-borne *TRP1* plasmids carrying wild-type histone H3 and either wild-type (pOS107), K16Q (pOS108), K16R (pOS109), K5, 8, 12Q (pOS110) or K5, 8, 12Q (pOS111) H4 point mutant alleles. Representative transformants were streaked on YMD medium lacking tryptophan but containing 1 mg/ml 5-FOA to expel the *URA3*-based wild-type H4 allele and thereby generate H4 point mutant alleles in a *htz1*Δ::kanMX background.

Our overexpression studies with Sir3p suggest that this protein could be recruited or stabilized by either Rap1p, ORC, or Sir1p, since overexpression of Sir3p could bypass any one of these three silencer-bound proteins. These results are consistent with previous observations that Sir3p interacts with Rap1p (Moretti et al., 1994), and that increased levels of Sir1p suppress specific *sir3* mutant alleles (Stone et al., 1991). Together, these results reveal a redundancy in the recruitment/stabilization of Sir3p and suggest that Sir3p could be recruited or stabilized by either Rap1p or ORC, but robust recruitment probably requires both proteins.

Our results with Sir4p suggest that the requirements to recruit or stabilize this protein are different from those for Sir3p. Overexpressing Sir4p could overcome defects in Sir1p and ORC but not defects in Rap1p. These results were consistent with previous data demonstrating that overexpression of Sir4p restores silencing in a strain with a silencer lacking an ARS element (Sussel et al., 1993), and that Sir1p interacts with Sir4p in a two-hybrid assay (Triolo and Sternglanz, 1996). The observation that overexpression of Sir4p did not restore silencing in a strain where the Rap1p binding site at the silencer was absent suggests that the recruitment of Sir4p absolutely requires Rap1p. This result is also consistent with previous observations demonstrating that Rap1p interacts with Sir4p (Moretti et al., 1994). Since Rap1p has been shown to physically interact with Sir3p and Sir4p (Moretti et al., 1994; Liu and Lustig, 1996), our results would argue that in the absence of Rap1p, wild-type levels of Sir3p are not efficiently recruited to the *HMR* locus (via ORC), and increased levels of Sir4p cannot compensate

for this defect. It is interesting to note that neither of these two proteins required Abf1p at the silencer, and the precise role of Abf1p in silencing remains unclear.

Esc2p and Silencing

Esc2p is a novel protein that was identified by its ability to restore silencing in strains lacking Sir1p function. An analysis of the predicted amino acid sequence revealed that this protein was enriched in charged amino acids (primarily acidic and serine residues) with three putative nuclear localization signals in the amino terminus and a coiled-coil region from amino acid residues 300–359. The extreme carboxyl terminus (residues 384–456) showed a significant homology (28% identity and 60% similarity) to a SUMO-2 motif (small ubiquitin-like modifier). The SUMO polypeptides are covalently conjugated to other proteins (Johnson and Hochstrasser, 1998), but the role of the SUMO modification is not clear. The SUMO domain of Esc2p appears to be required for silencing (data not shown), suggesting that this region of the protein may interact with other silencing proteins. Future experiments should shed light on the proteins that interact with Esc2p.

Overproduction of Esc2p suppressed all the *sir1* point mutants but was unable to restore silencing in the absence of Sir1p. However, Sir1p function could be bypassed by overproducing Esc2p but only if the silencers contained multiple binding sites for ORC. Consistent with this observation was the demonstration that Gal4-Esc2p could also robustly silence in the absence of Sir1p, suggesting that once Esc2p is recruited to a silencer, it is able to establish silencing. On the other hand, Abf1p binding at the silencer was not a prerequisite for Esc2p-mediated silencing.

One possible mechanism by which Esc2p could affect silencing is in the efficient recruitment or stabilization of other Sir proteins at the silenced chromatin domain. Consistent with this role, loss of Esc2p leads to a partial derepression of the *HMR::ADE2* locus as well as a *URA3* reporter gene placed at a telomere. A second possibility is that Esc2p sequesters the silenced domain into the perinuclear region, thereby favoring the establishment of silencing. Based on our microscopic observations of GFP-Esc2p, we currently do not favor this model. The recent demonstration that Sir2p is associated with an enzymatic activity raises the possibility that Esc2p helps increase the activity of the Sir proteins that have been recruited to the silenced domain.

HTZ1 Is Required for Efficient Silencing

The isolation of *HTZ1* in our screen was intriguing and exciting. The H2Az variants of histone H2A have been identified in all eukaryotes (Jackson et al., 1996). These genes are either essential for development (Clarkson et al., 1999) or affect growth rates and chromosome segregation (Carr et al., 1994).

The demonstration in this study that overexpression of Htz1p restored silencing in *sir1*-impaired cells, while deletion of the *HTZ1* gene partially reduced silencing suggests that this histone H2A variant is normally present at the silenced loci. Chromatin immunoprecipitation experiments directly demonstrated that Htz1p was present at the *HMR* locus as well as at the rDNA locus, and this latter observation was also confirmed by immunofluorescence data. These data are consistent with previous observations demonstrating the presence of Htz1p homologs in the nucleoli of metazoan cells (Allis et al., 1982) as well as in centromeric heterochromatin (Russanova et al., 1989; Leach et al., 2000).

It is significant that overexpression of predominant H2A was not able to restore silencing in *sir1* mutants and is consistent with previous observations that mutations in the predominant H2A genes do not affect silencing at *HMR* (see Lenfant et al., 1996 and references therein). However, it is not clear whether all of the H2A molecules at *HMR* were replaced by Htz1p, or whether some nucleosomes possessed H2A and others Htz1p. Based on the fact that Htz1p overexpression increases silencing, we believe that normally there is a mixed population of nucleosomes at *HMR*, with some containing Htz1p and others containing H2A. We hypothesize that increasing the dose of Htz1p increases the number of nucleosomes containing Htz1p, and this favors silencing of *HMR*.

Interestingly, deletion of Htz1p led to loss of silencing at *HMR* and telomeres but not rDNA. Although these results are surprising in light of the fact that Htz1p was concentrated in the nucleolus, it is possible that the Sir proteins are redistributed from the telomeres to the rDNA in an *htz1*Δ strain, resulting in continued silencing at the rDNA. Alternatively, Htz1p may exert a positive effect on rDNA transcription, since it has been reported to have a role in transcription activation in *Tetrahymena* (Stargell et al., 1993). The likelihood that Htz1p may have roles in both activation and repression is not altogether surprising, since the N-terminal tails of histone H4 also have functions in transcription activation (Durrin et al., 1991) and repression (Kayne et al., 1988) through specific interactions with activators and repressors.

The unexpected finding that double mutants lacking the H4 N-terminal tail and Htz1p were inviable argue that as in larger eukaryotes, the yeast histone variant performs other important functions. The lethality of these double mutants could be due to incomplete chromatin assembly, since H4 (N4-28Δ) H3 (N4-30Δ) double mutants have previously been shown to be inviable due to defects in chromatin assembly. However, the observation that Htz1p function was dispensable in strains carrying point mutant alleles of histone H4 (K5, 8, 12) (sites required for assembly) strongly argues against chromatin misassembly as a major cause for the inviability of *htz1*Δ H4 (N4-28Δ) mutants.

We propose that Esc2p contributed to silencing by functioning in the recruitment/stabilization of Sir proteins via interactions with the silencer, while the presence of Htz1p in nucleosomes at *HMR* leads to a more stable binding of the Sir complex to the nucleosomes.

Experimental Procedures

Silencing Assays

Patch-mating assays and silencing of *URA3* at *HMRss*(Gal4-Rap-Abf) were essentially as described (Kamakaka and Rine, 1998).

Construction of *HMR*ss(Gal4-Rap-Abf)::*URA3*

The ARS element in pJR1237 was replaced with 4$\times$ Gal4 binding sites, and this *HMRss*(Gal4-Rap-Abf) silencer was integrated into

the *HMR* locus on chromosome III at *Saccharomyces* Genome Database (SGD) coordinates 290816–291786. A 1.1 kb HindIII fragment containing *URA3* was targeted between the *HMRss*(Gal4-Rap-Abf) and *HMR-I* silencers at SGD coordinates 291,786 and 293,136.

Generation of *GAL4-SIR1* Mutant Alleles

GAL4-SIR1 mutants were generated by PCR mutagenesis using the following set of primers: 5′-CTA TCT CCA ACC TGG ATT ATT CAG ACA TAA AAA AAC AGC AGT TTA CTG AAG CAG AGG TTG TAA AAA GAA A-3′ (chromosome XI, SGD coordinates 641062–641132) and 5′-CTC GGA ATT AAC CCT CAC TAA AGG GAA CAA AAG CTG GGT ACC GGG CCC CCC CTC GAG GTC GAC GGT ATC G-3′ (pRS316) to amplify the *SIR1* gene. We relied on the inherent infidelity of Taq polymerase to generate mutations in *SIR1* under standard amplification conditions. PCR products were cotransformed into JRY4806 with the *GAL4-SIR1*::pRS316 plasmid gapped with BglII and MluI and plated on medium lacking uracil. Approximately 10,000 potential *SIR1* mutants were screened by mating assays, and of these, 43 were further screened for missense mutations by immunoblot analysis.

Multicopy Suppressor Screen

Two yeast genomic libraries in Yep352 and Yep13 were used to transform either BUY180 (*GAL4-sir1-202*) or BUY383 (*GAL4-sir1-201*) by the lithium acetate method. Approximately 20,000 Ura^+ transformants from the Yep352 library were screened for their ability to restore mating to BUY180 by replica plating the transformants on a MATa mating tester. Approximately 20,000 Leu^+ transformants from the Yep13 library were plated on synthetic medium containing 0.1% 5-FOA to select for clones that could restore repression of *HMRss*(Gal4-Rap-Abf)::*URA3* in BUY383.

Generation of *esc2Δ::HIS3* and *htz1Δ*::KanMX Alleles

The entire coding sequence of *ESC2* (chromosome IV, SGD coordinates 1199217–1200587) was replaced with *HIS3* (amplified from pRS403) flanked by ~500 bp of the *ESC2* promoter region and ~500 bp of the 3′UTR region of *ESC2*.

The *htz1Δ*::KanMX strain was generated by eight consecutive backcrosses between JRY2334 or JRY4013 and an *htz1Δ*::KanMX strain carrying a start-to-stop codon deletion of *HTZ1* procured from the *Saccharomyces* Genome Deletion Project.

Chromatin Immunoprecipitation

Formaldehyde fixation of strains BUY619 and BYB68 and subsequent chromatin preparation and immunoprecipitation reactions were essentially as described (Kamakaka and Thomas, 1990; Meluh et al., 1998). PCR-based amplification of the coimmunoprecipitating DNA was performed using primers specific only to the *HMR* locus (SGD coordinates: chromosome III, nt 292939–292962 [Watson strand]; chromosome III, nt 293464–293488 [Crick strand]), the telomere-proximal *RDN1* locus (SGD coordinates: chromosome XII, nt 468730–468761 [Watson strand]; chromosome XII, nt 469348–469378 [Crick strand]), and the centromere-proximal *RDN1* locus (SGD coordinates: chromosome XII, nt 451386–451418 [Watson strand]; chromosome XII, nt 451912–451942 [Crick strand]).

Immunoblotting and Indirect Immunofluorescence

The preparation of yeast lysates and subsequent immunoblot analysis and indirect immunofluorescence studies were performed as described (Pringle et al., 1991).

Plasmids

The following yeast vectors were used to generate the plasmids used in this study: pRS314, pRS425 (Sikorski and Hieter, 1989), Yep351, Yep352 (Rine, 1991), and pAS1 (Durfee et al., 1993), YCp22 (Gietz and Sugino, 1988). Plasmids pRO144 and pRO329 were derived from pRS314 and pRS425 (Sikorski and Hieter, 1989), respectively, and they carry the *SIR3* gene. Plasmid pOS46 carrying *SIR4*::Yep351 was derived from Yep351 (Rine, 1991). Plasmids pOS48 and pOS007 carrying *ESC2* were derivatives of Yep351 and Yep352, respectively.

The following plasmids were used in this study, and the authors will provide details of these on request: pRO144, *SIR3*::pRS314; pRO149, HTA1-HTB1 HHT1-HHF1 (URA3 2μ); pRO329, *SIR3*::pRS425; pGF29, *HHT2-HHF2*(N4-28Δ) (*TRP1* Ars/Cen); pRM200, *HHT2-HHF2* (*TRP1* Ars/Cen); pRM430, *HHT2*(N4-30Δ)-*HHF2* (*TRP1* Ars/Cen); pOS46, *SIR4*::Yep351; pOS48, *ESC2*::Yep351; pOS007, *ESC2*::Yep352; pOS59, *GAL4-ESC2*::pAS1; pOS77, GFP(S65T) at Age1 site in *ESC2*::pRS424; pOS65, *HTZ1*::pRS425; pOS67, *HTA1*::pRS425; pOS68, *esc2Δ::HIS3*::Bluescript II KS^+; pOS70, *HTZ1*+*HTB1*::pRS425; pOS107, *HHT2-HHF2*::Ycp22; pOS108, *HHT2-HHF2* K16Q::Ycp22; pOS109, *HHT2-HHF2* K16R::Ycp22; pOS110, *HHT2-HHF2* K5, 8, 12Q::Ycp22; and pOS111, *HHT2-HHF2* K5, 8, 12R::Ycp22.

The detailed genotypes of the strains used in this study are available as supplemental data (see table at www.molecule.org/cgi/content/full/6/4/769/DC1).

Acknowledgments

The authors would like to thank J. Rine, M. Grunstein, D. Shore, F. Winston, and P. Kaufman for providing strains and plasmids and J. Rine, A. Dillin, A. Wolffe, P. Wade, A. Ehrenhofer-Murray, D. J. Clark, and members of the Kamakaka lab for helpful comments. This work was supported by a NIH grant to R. T. K.

Received March 23, 2000; revised July 24, 2000.

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Note Added in Proof

Santisteban et al. present independent evidence for a role of Htz1p in gene regulation: Santisteban, M., Kalishnikova, T., and Smith, M. (2000). Histone 112AZ regulates transcription and is partially redundant with nucleosome remodeling complexes. Cell, in press.