Copyright © 2002 by the Genetics Society of America

A Role for the *Saccharomyces cerevisiae* RENT Complex Protein Net1 in *HMR* Silencing

Daniela Kasulke, Stefanie Seitz and Ann E. Ehrenhofer-Murray[1]

Max-Planck-Institute for Molecular Genetics, 14195 Berlin, Germany

Manuscript received December 13, 2001
Accepted for publication May 7, 2002

ABSTRACT

Silencing in the yeast *Saccharomyces cerevisiae* is known in three classes of loci: in the silent mating-type loci *HML* and *HMR*, in subtelomeric regions, and in the highly repetitive rDNA locus, which resides in the nucleolus. rDNA silencing differs markedly from the other two classes of silencing in that it requires a DNA-associated protein complex termed RENT. The Net1 protein, a central component of RENT, is required for nucleolar integrity and the control of exit from mitosis. Another RENT component is the NAD^+-dependent histone deacetylase Sir2, which is the only silencing factor known to be shared among the three classes of silencing. Here, we investigated the role of Net1 in *HMR* silencing. The mutation *net1-1*, as well as *NET1* expression from a 2μ-plasmid, restored repression at silencing-defective *HMR* loci. Both effects were strictly dependent on the Sir proteins. We found overexpressed Net1 protein to be directly associated with the *HMR*-E silencer, suggesting that Net1 could interact with silencer binding proteins and recruit other silencing factors to the silencer. In agreement with this, Net1 provided ORC-dependent, Sir1-independent silencing when artificially tethered to the silencer. In contrast, our data suggested that *net1-1* acted indirectly in *HMR* silencing by releasing Sir2 from the nucleolus, thus shifting the internal competition for Sir2 from the silenced loci toward *HMR*.

SILENCING is a form of transcriptional repression that converts regions of eukaryotic chromosomes into an inaccessible chromatin state, which in higher eukaryotes is referred to as heterochromatin. Silenced chromatin is generally refractory to transcription and recombination, replicates late in the S phase of the cell cycle, and is usually located in discrete subnuclear domains (Loo and Rine 1995; Lustig 1998). On a molecular level, silenced chromatin consists of specialized heterochromatin proteins and of nucleosomes carrying deacetylated histones. In the budding yeast *Saccharomyces cerevisiae*, three classes of loci are known to be silenced: the silent mating-type loci *HML* and *HMR*, the telomeres, and the rDNA locus.

The rDNA cluster is the most abundant repetitive sequence in the yeast genome and consists of 100–200 copies of a 9-kb rDNA gene unit. However, only about half of these repeats are active at any given time, whereas the other half is transcriptionally repressed (Warner 1989). Likewise, marker genes inserted in the rDNA locus become metastably repressed (Smith and Boeke 1997). rDNA silencing is promoted by a protein complex termed RENT (for *re*gulator of *n*ucleolar silencing and *t*elophase; Shou *et al.* 1999), which contains the proteins Net1, Sir2, Cdc14, and at least one more, yet uncharacterized component. Net1, a 128.5-kD protein with similarity to the topoisomerase interacting factor Tof2, plays a central role in RENT: Net1 is required for nucleolar integrity and for the localization of both Cdc14 and Sir2 to the rDNA (Straight *et al.* 1999; Visintin *et al.* 1999). Furthermore, a mutation in *NET1* (*net1-1*) was identified in a genetic screen for mutants that bypassed the anaphase arrest of *cdc15*Δ cells, showing that Net1 is required for the control of exit from mitosis (Shou *et al.* 1999). The current model posits that the protein phosphatase Cdc14 is tethered to the rDNA via its interaction with Net1 during G1, S, and early M phase. Net1 thereby acts as a competitive inhibitor of Cdc14, thus preventing it from executing its enzymatic activity (Traverso *et al.* 2001). Through a Tem1-dependent signal, Cdc14 is released from the nucleolus during anaphase and thus becomes able to dephosphorylate its targets Sic1, Cdh1, and Swi5 in the nucleus (Visintin *et al.* 1998; Zachariae *et al.* 1998). Significantly, the two functions of Net1 in cell cycle regulation and in sustaining the general nucleolar integrity are independent of each other (Shou *et al.* 2001). In addition, Net1 also recruits RNA polymerase I (PolI) to the rDNA and thereby stimulates the synthesis of rRNA (Shou *et al.* 2001). The roles of the Net1 protein emphasize its functional complexity and suggest a widespread ability to participate in different cellular processes. However, as Net1 is not likely to bind DNA directly, the mechanism by which the rDNA silencing complex RENT is tethered to the nucleolar DNA still remains to be resolved.

Like Cdc14 and PolI, the silent information regulator

[1] *Corresponding author:* Max-Planck-Institute for Molecular Genetics, Otto-Warburg-Laboratories, Ihnestrasse 73, D-14195 Berlin, Germany. E-mail: ehrenhof@molgen.mpg.de

protein Sir2 is tethered to the rDNA via Net1 (Straight *et al.* 1999). Sir2 is a NAD^+-dependent histone deacetylase specific for lysines 9 and 14 of histone H3 and lysine 16 of H4, and it requires this deacetylase activity for its silencing function (Imai *et al.* 2000; Landry *et al.* 2000). Sir2 is the only Sir protein required for rDNA silencing, suggesting a distinct mechanism of silencing at this locus. The first indication for a role of Sir2 in the rDNA came from the observation that Sir2 represses rDNA recombination (Gottlieb and Esposito 1989). Recombination between the repetitive rDNA units produces extrachromosomal rDNA circles, which are a cause of cellular senescence in yeast (Sinclair and Guarente 1997). Hence, Sir2 is an anti-aging factor that may provide a link between caloric intake and aging through its NAD^+ dependence (Lin *et al.* 2000).

Apart from its participation in RENT, nuclear Sir2 is also found at the *HM* loci, where it forms a complex with the silencing proteins Sir3 and Sir4 (Moazed *et al.* 1997). The complex is recruited to the target region by an interaction of Sir3/4 with the DNA-binding factor Rap1 (Moretti *et al.* 1994) and with the deacetylated tails of histone H3 and H4 (Hecht *et al.* 1995), presumably enabling a spreading of the complex along chromatin. Sir1 also takes part in forming the repressed chromatin, but becomes essential only for silencing in compromised silencer mutants. Sir1 is anchored at the silencer by its interaction with the origin recognition complex (ORC; Triolo and Sternglanz 1996; Gardner *et al.* 1999). Binding sites for ORC, Rap1, and for a third DNA-binding protein, Abf1, are found in the *HM* silencers. These silencers flank the mating-type genes at *HMR* and result in their repression, whereas the identical genes at *MAT* are expressed and determine the cell type. The silencer binding sites will here be referred to as ORC, RAP, and ABF. The factors binding these sites are a prerequisite for the formation of repressed chromatin. A comparable spreading of the Sir complex takes place at the telomeres, where the interaction with telomeric Rap1 enables the anchoring of the complex (Moretti *et al.* 1994) and where a subsequent arrangement of a telomeric loop structure may drive the formation of a repressed state (Pryde and Louis 1999). The Sir2, Sir3, and Sir4 proteins are limiting within the cell, leading to an internal competition between the telomeres and the *HM* loci (Buck and Shore 1995) as well as between the telomeres and the rDNA (Smith *et al.* 1998) for these proteins.

In this study, we investigated the role of the RENT factor Net1 in silencing the *HMR* locus. We found that a mutation in *NET1* (*net1-1*) as well as *NET1* overexpression restored repression at silencing-defective *HMR* alleles. When overexpressed, Net1 was physically associated with the *HMR*-E silencer, suggesting that in this scenario, Net1 had a direct role in silencing. Net1 was also capable of providing silencing when artificially tethered to the *HMR*-E silencer. Interestingly, this repression was dependent upon ORC, but independent of Sir1. Furthermore, we characterized the effect of the *net1-1* mutation on silencing. Our data suggested that *net1-1* acted indirectly by relieving the competition between the rDNA locus and *HMR* for the silencing factor Sir2.

MATERIALS AND METHODS

Plasmid constructions: The *NET1* gene was cloned into a 2μ-plasmid by PCR amplifying a 3925-bp fragment from genomic DNA and ligating it into the *Sma*I-site of pRS 426 (Sikorski and Hieter 1989), resulting in pAE 567. To obtain a 2μ-plasmid with a *LEU2* marker, the insert was released from pAE 567 with *Xho*I/*Spe*I and was inserted into *Xho*I/*Spe*I-treated pRS 425 (Sikorski and Hieter 1989), resulting in pAE 548. To construct the Gal4 DNA-binding domain fusion to Net1, the C terminus of *NET1* (amino acids 566–1189) was amplified by PCR from genomic DNA with flanking *Bam*HI and *Sal*I sites and introduced into *Bam*HI/*Sal*I-treated pAE 107 behind the Gal4 DNA-binding domain, yielding pAE 610. pAE 107 is identical to pJR1639 (Fox *et al.* 1997) and is a *CEN/URA3* vector in which *GAL4* (amino acids 1–147) is cloned between the GPD promoter and the PGK terminator. To fuse *NET1* with an epitope tag (pAE 622), we made use of 6×c-myc containing pRS 426 (pAE 569). In a first step, the promoter of *NET1* was inserted upstream of the tag sequence as a PCR fragment with flanking *Kpn*I and *Xho*I sites. In a second step, the *NET1* open reading frame and terminator region were inserted downstream of the tag as a *Spe*I/*Not*I PCR fragment. The complete insert of pAE 622 was cloned as a *Kpn*I/*Not*I fragment into the *CEN*-based plasmid pRS 316 to yield pAE 711. All fusions were verified by DNA sequence analysis.

Yeast strains and methods: The relevant genotypes of the yeast strains used in this study are listed in Table 1. Strains were constructed by standard techniques of crossing, subsequent sporulation, and tetrad analysis. Genomic tagging by 3×HA was performed as described (Knop *et al.* 1999). Growth and manipulation of yeast were carried out according to standard procedures (Sherman 1991). Patch-mating assays and quantitative mating assays were performed as described (Ehrenhofer-Murray *et al.* 1997), using AEY 264 (*MAT***a** *his4*) as the **a** mating tester strain. All quantitative mating efficiencies are the average of at least two independent determinations and were normalized to the wild-type strain AEY 1.

Chromatin crosslinking and immunoprecipitation: Formaldehyde fixation as well as subsequent chromatin preparation and immunoprecipitation reactions were performed essentially as described (Hecht *et al.* 1996). Fixation was performed with 2% formaldehyde for 1 hr and quenched with 250 mm glycine. The crude lysate was precleared with protein A sepharose beads (Pharmacia, Piscataway, NJ) for 30 min and the resulting supernatant was subsequently incubated with α-myc antibody (Invitrogen, San Diego) overnight at 4°. Immune complexes were isolated by incubating the extracts for 1 hr with protein A sepharose beads. Elution was performed at room temperature for 15 min, using 1% SDS/0.1 m $NaHCO_3$. To analyze the presence of specific DNA loci, 1/15 of the purified material was amplified by PCR for 28 cycles (*HMR*: *HMR*-1 5′-gctgatgcatgccaaacaaaaccc-3′ and *HMR*-2 5′-ccctctcct cagacactactaag-3′; NTS: NTS-up 5′-tcgcatgaagtacctcccaactac-3′ and NTS-down 5′-tccgcttccgcttccgcagtaaaa-3′; 25S: 25S-up 5′-aggacgtcatagagggtgagaatc-3′ and 25S-down 5′-ttgacttacgtcg cagtcctcagt-3′; actin: act-up 5′-cggtagaccaagacaccaagg-3′ and act-down 5′-gtcagtcaaatctctaccggcc-3′). PCR products were

TABLE 1

***S. cerevisiae* strains used in this study**

Strain[a]	Genotype	Reference[b]
AEY 1558*	*MAT***a** *leu2 trp1 ura3-52 prc1-407 pep4-3 prb1-1122*	E. W. Jones
AEY 1778*	*MAT*α *his3*Δ*200 leu2*Δ*1 ura1-167 RDN1::Ty1-mURA3*	Smith *et al.* (1998)
AEY 2548*	AEY 1558 *RAP1-3HA-KlTRP1*	
AEY 2551*	AEY 1558 *SIR2-3HA-KlTRP1*	
AEY 1	*MAT*α *ade2-101 his3-11,15 trp1-1 leu2-3,112*	Thomas and Rothstein (1989)
AEY 69	AEY 1 *HMR* Δ*RAP*Δ*ABF*	Brand *et al.* (1987)
AEY 70	AEY 1 *HMR* Δ*ACS*Δ*ABF*	Brand *et al.* (1987)
AEY 72	*MAT***a** Δ*Ahmr::TRP1 rap1-13::LEU2*	Sussel and Shore (1991)
AEY 98	AEY 1 *HMR* Δ*ACS*Δ*RAP*	Brand *et al.* (1987)
AEY 346	*MAT***a** *sir1*Δ *sas2*Δ	
AEY 403	AEY 1 *HMR***a**-*e***	Ehrenhofer-Murray *et al.* (1997)
AEY 454	AEY 1 *HMR-SS*Δ*I***a** (ACS-Gal4-ABF)	Ehrenhofer-Murray *et al.* (1997)
AEY 500	AEY 1 *HMR-SS*Δ*I***a** (5×Gal4-ACS-ABF) *gal4*Δ*::HIS*	
AEY 567	*MAT***a** Δ*Ahmr::TRP1 rap1-12::LEU2*	Sussel and Shore (1991)
AEY 576	MAT**a** *orc5-1*	
AEY 726	AEY 1 *HMR-SS abf1*$^-$Δ*I*	Ehrenhofer-Murray *et al.* (1997)
AEY 760	*MAT*α Δ*Ahmr::TRP1 rap1-12::LEU2*	
AEY 848	*MAT***a** *HMR-SS*Δ*I orc2-1*	
AEY 1017	AEY 1 VIIL-TEL::*URA3*	
AEY 1793	AEY 70 *net1-1*	
AEY 1882	AEY 70 *net1-1 sir1*Δ*::LEU2*	
AEY 1885	AEY 726 *net1-1*	
AEY 1888	AEY 403 *net1-1*	
AEY 1889	AEY 69 *net1-1*	
AEY 1891	AEY 98 *net1-1*	
AEY 1898	AEY 70 *net1-1 sir3*Δ*::HIS3 lys2*Δ	
AEY 1899	AEY 70 *sir1*Δ*::LEU2*	
AEY 1909	AEY 726 *sir1*Δ*::LEU2*	
AEY 1912	AEY 726 *net1-1 sir1*Δ*::LEU2 lys2*Δ	
AEY 1916	AEY 726 *sir2*Δ*::TRP1 ADE2*	
AEY 1917	AEY 726 *net1-1 sir2*Δ*::TRP1*	
AEY 1925	AEY 70 *sir2*Δ*::TRP1*	
AEY 1927	AEY 70 *net1-1 sir2*Δ*::TRP1 ADE2 lys2*Δ	
AEY 1934	AEY 454 *sir1*Δ*::LEU2*	
AEY 1937	AEY 70 *sir4*Δ*::LEU2 lys2*Δ	
AEY 1939	AEY 726 *net1-1 sir3*Δ*::HIS3*	
AEY 1944	AEY 726 *sir4*Δ*::LEU2*	
AEY 1945	AEY 726 *net1-1 sir4*Δ*::LEU2*	
AEY 1968	AEY 454 *sir2*Δ*::TRP1 lys2*Δ	
AEY 2132	AEY 454 *orc2-1*	
AEY 2143	*MAT*α VIIL-TEL::*URA3::ADE2 orc2-1*	
AEY 2741	AEY 1 *URA3*	
AEY 2742	AEY 1 *RDN1::Ty1:mURA3*	
AEY 2743	AEY 567 *RDN1::Ty1:mURA3*	
AEY 2744	AEY 72 *RDN1::Ty1:mURA3*	
AEY 2745	AEY 576 *RDN1::Ty1:mURA3*	
AEY 2746	AEY 848 *RDN1::Ty1:mURA3*	

[a] All strains except those marked with an asterisk (*) were derivatives of W303.

[b] All strains except those indicated were from the laboratory collection or were constructed during the course of this study.

separated on 1% agarose gels and visualized with ethidium bromide.

Co-immunoprecipitations: The preparation of extracts and co-immunoprecipitations (co-IP) were performed as described (Shou *et al.* 1999) except that extracts were incubated with α-myc or with α-hemagglutinin (HA) overnight at 4°, followed by a 1-hr incubation with protein A sepharose beads.

Cell extract preparation and Western blotting: Whole cell extracts were prepared by glass bead lysis (Hampton and Rine 1994), and proteins were separated on 8% SDS-polyacrylamide gels and immunoblotted with the following antibodies: Sir2, Sir3, and Sir4 (Santa Cruz Biotechnology; Santa Cruz, CA); α-myc (Invitrogen); and α-HA (Covance).

DNA preparation and Southern blotting: Genomic DNA

was prepared as described (Hoffman and Winston 1987), digested with *Hae*II, and separated on a 0.8% agarose gel, followed by DNA blot hybridization and detection of DNA using a *URA3* probe.

Immunostaining: Fixation, spheroblasting, and spreading of yeast nuclei were performed as described (Trelles-Sticken *et al.* 1999).

RESULTS

The *net1-1* mutation improved silencing at a defective *HMR* allele: The protein complex RENT is required for rDNA silencing, and mutations in *NET1* cause derepression of marker genes inserted at the rDNA locus. Furthermore, the deletion of *NET1* causes a slight increase in telomeric silencing (Straight *et al.* 1999). In this study, we sought to investigate in detail if Net1 also exerted an influence on *HM* silencing. Derepression of the *HMR* locus results in the simultaneous expression of **a** and α information in *MAT*α cells, which abolishes the mating ability of the cells. Thus, a loss of *HMR* silencing in *MAT*α strains can be measured as a loss of mating ability.

In a first attempt, we determined the influence of a mutation in *NET1*, *net1-1* (Shou *et al.* 1999), on silencing of the *HMR* locus. We chose to use the *net1-1* allele instead of a *NET1* deletion, because the poor growth of *net1*Δ cells would have complicated the analysis (Visintin *et al.* 1999). *net1-1* cells display a temperature-sensitive phenotype and have an abnormal, elongated shape. The capacity of Net1 to interact with Cdc14 is abolished in *net1-1* cells as shown by co-immunoprecipitation experiments (Shou *et al.* 1999). Introducing the mutation *net1-1* did not derepress the wild-type *HMR* and *HML* loci, as *MAT***a** or *MAT*α *net1-1* strains showed no mating defect in a patch-mating assay (data not shown). However, in silencing, genes can act as both positive or negative regulators. Thus, to have an experimental setup with the possibility of observing both increases or decreases in silencing, we made use of a version of the synthetic *HMR*-E silencer in which the Abf1-binding site is mutated and where *HMR*-I is deleted (*HMR-SS abf1*$^-$ Δ*I*; McNally and Rine 1991). The synthetic silencer is a minimal version of *HMR*-E that lacks most of the functional redundancies present at natural *HMR*-E. The introduction of the additional mutation in the Abf1-binding site causes a moderate 50-fold derepression.

A strain that was *MAT*α *HMR-SS abf1*$^-$ Δ*I* and carried *net1-1* was constructed, and its mating ability was compared to that of an isogenic *NET1* strain (Figure 1A). The *NET1* strain displayed a reduced α-mating ability due to the expression of **a**1 information from the mutant *HMR-SS abf1*$^-$ Δ*I* allele. However, introducing *net1-1* improved the mating ability, suggesting that silencing at the defective *HMR* locus was restored.

***NET1* overexpression enhanced silencing at defective *HMR* alleles:** The observation that *net1-1* improved mating suggested that Net1 had a negative effect on *HMR* silencing. We next determined the effect of *NET1* overexpression on silencing of the defective *HMR-SS abf1*$^-$ Δ*I* locus. For this purpose, a 2μ-plasmid containing *NET1* under the control of its own promoter was introduced into a *MAT*α strain carrying *HMR-SS abf1*$^-$ Δ*I*, and the mating ability of the strain was determined. Surprisingly, expression of *NET1* from this plasmid improved the mating ability of the strain by ~130-fold compared to the strain transformed with an empty vector (Figure 1B), suggesting that the overexpression of *NET1* increased silencing at *HMR*. To determine whether a lower amount of Net1 might already lead to an increase in silencing capacity, we introduced *NET1* on a low-copy *CEN*-based plasmid into the *MAT*α *HMR-SS abf1*$^-$ Δ*I* strain and measured the mating ability. Indeed, the slightly overexpressed *NET1* could improve the mating ability, albeit to a lesser extent than the 2μ-overexpression (Figure 1B). These observations suggested that upon increase of the Net1 protein amount, *HMR* silencing in this yeast strain could be restored, depending upon the amount of additional Net1. The extent of *NET1* expression was determined by measuring the amount of a 6×myc-epitope-tagged version of *NET1* when present on the high-copy 2μ-plasmid or on the low-copy *CEN*-based vector. The epitope-tagged Net1 was functional in that it complemented the temperature sensitivity of the *net1-1* mutation and suppressed *HMR* silencing defects when overexpressed (data not shown). 2μ-expressed 6×myc-*NET1* was expressed at least sixfold higher than that from the *CEN*-based vector as determined by Western blotting analysis (Figure 1D).

The fact that *NET1* overexpression improved repression at an *HMR* allele mutated in the Abf1-binding site of *HMR*-E (*HMR-SS abf1*$^-$ Δ*I*) suggested that Abf1 was not required for Net1-mediated silencing. We next determined the *cis* requirements for this silencing. Mutations in the ORC- and Rap1-binding sites of the synthetic *HMR*-E silencer cause complete derepression and therefore are not suitable for this analysis. In contrast, the deletion of a combination of binding sites in the natural *HMR*-E silencer in the presence of *HMR*-I causes a moderate derepression (Brand *et al.* 1987), comparable to *HMR-SS abf1*$^-$ Δ*I*, and thus was used here. High-copy expression of *NET1* improved the mating ability of a *MAT*α strain carrying deletions in the ORC- and Abf1-binding sites of the wild-type *HMR*-E silencer (*HMR* Δ*ACS*Δ*ABF*) by ~10-fold, whereas *HMR* Δ*ACS*Δ*RAP* was only slightly affected and *HMR* Δ*RAP*Δ*ABF* was not affected by *NET1* overexpression (Figure 1B). These results suggested that overexpressed *NET1* required an intact Rap1-binding site at *HMR*-E to improve silencing. Moreover, *rap1-12* and *rap1-13* mutants, carrying a substitution of the *HMR***a** information by the *TRP1* gene, also did not show an enhanced silencing of the *TRP1* reporter upon *NET1* overexpression, as measured by

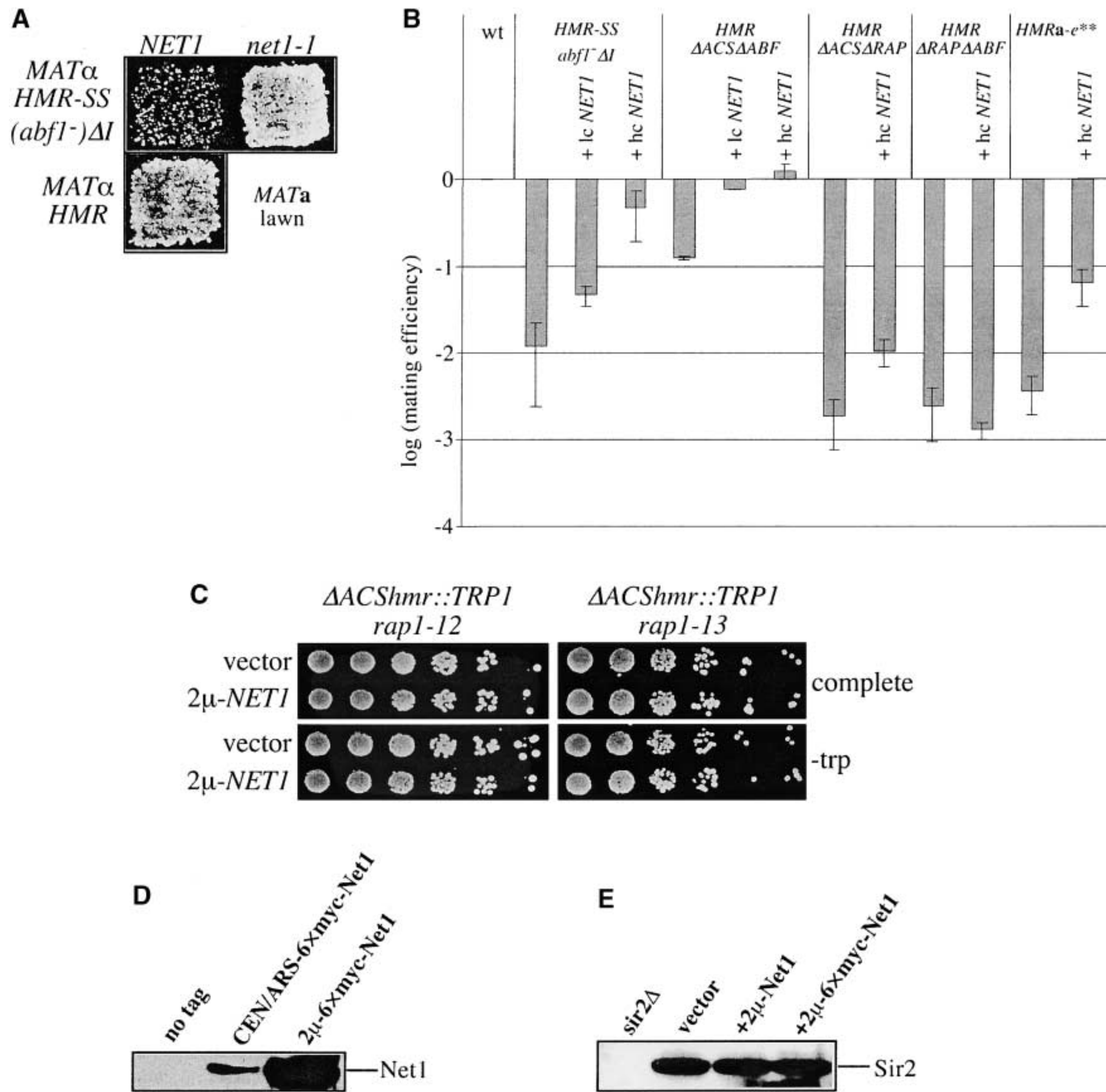

Figure 1.—The *net1-1* mutation and the overexpression of *NET1* from a 2μ-plasmid increased the silencing capacity of mutated *HMR* alleles without affecting the Sir2 protein level. (A) The mating ability of *MAT*α *HMR-SS abf1*⁻ Δ*I* cells that were either *NET1* (AEY 726) or *net1-1* (AEY 1885) was tested in a patch-mating assay. (B) Expression of *NET1* from a 2μ-plasmid increased the silencing ability of a *MAT*α *HMR-SS abf1*⁻ Δ*I* (AEY 726), of a *MAT*α *HMR*Δ*ACS*Δ*ABF* (AEY 70), and of a *MAT*α *HMR***a**-*e*** (AEY 403) strain, as measured by quantitative mating analysis. lc, low copy; hc, high copy. (C) Expression of *NET1* from a 2μ-plasmid did not increase the silencing ability of a *MAT*α Δ*ACS hmr::TRP1* strain with mutations in the *RAP1* gene (AEY 760, AEY 72) as measured by growth on medium lacking tryptophane. (D) Expression of *NET1* from a 2μ-plasmid was approximately sixfold higher than that from a *CEN*-based vector, as tested by Western blotting analysis of 6×myc-tagged *NET1* (pAE 711, pAE 622) in AEY 1558. (E) Sir2 protein levels were unaffected by additional *NET1* expression. Protein extracts of AEY 726, transformed with pRS 426, pAE 711, or pAE 622, were analyzed by Western blotting.

growth on medium lacking tryptophane (Figure 1C), suggesting that a functional Rap1 protein was also required. However, silencing at an *HMR* allele with subtle point mutations in the Rap1- and Abf1-binding sites (*HMR***a**-*e***; Kimmerly *et al.* 1988) was also affected by *NET1* overexpression. Perhaps the point mutation in the Rap1-binding site in this context has a less severe effect than the deletion of the Rap1 site and thus could be suppressed more easily.

Silencing by *NET1* overexpression depended on the Sir proteins: In principle, the suppression of mating defects by high-copy *NET1* could occur by mechanisms other than those that improve classical Sir-mediated silencing. To determine whether the restoration of mating was caused by a restoration of this type of silencing, we tested whether the presumptive silencing by *NET1* overexpression depended upon the known structural components of silenced chromatin, the Sir proteins. *MAT*α strains were constructed that carried a defective *HMR* allele (*HMR-SS abf1*⁻ Δ*I* or *HMR* Δ*ACS*Δ*ABF*) and a deletion of *SIR1*, *SIR2*, *SIR3*, or *SIR4*, and the mating ability of these strains in the presence or absence of

overexpressed *NET1* was determined. All strains were complete non-maters (data not shown), showing that *HMR* was derepressed in these strains. This was similarly the case in the *sir2*Δ, *sir3*Δ, and *sir4*Δ strains carrying the wild-type *HMR* allele and for a *HMR-SS* Δ*I sir1*Δ strain (data not shown). Thus, the effect of *NET1* overexpression required the *SIR* genes, suggesting that it established *bona fide* silencing at the mutant *HMR* alleles and that it functioned there via the Sir proteins.

***NET1* overexpression did not influence expression of the Sir proteins:** How does Net1 increase silencing at *HMR*? One possibility is that Net1 would do so by increasing the cellular levels of silencing proteins, since higher expression of Sir proteins has been shown to lead to improved silencing (Xu *et al.* 1999b). To test whether *NET1* overexpression affected Sir protein levels, we measured the amount of Sir2, Sir3, and Sir4 in a *MAT*α *HMR-SS abf1*$^{-}$ Δ*I* strain that carried *NET1* or 6×myc-tagged *NET1* on a 2μ-plasmid. As shown in Figure 1E, the amount of Sir2 as well as of Sir3 and Sir4 (data not shown) did not change upon additional expression of *NET1*. This showed that Net1 did not function in silencing by changing Sir protein levels and suggested a more direct role for Net1 at *HMR*.

Overexpressed Net1 was physically associated with the *HMR*-E silencer in chromatin: One hypothesis for Net1's role in *HMR* silencing is that Net1, when overexpressed, interacts with the silencer binding proteins and helps to attract other silencing factors to establish silenced chromatin, perhaps through its ability to interact with Sir2. If Net1 is associated with the *HMR*-E silencer in chromatin, then *HMR*-E DNA should be enriched in a protein-DNA fraction prepared by immunoprecipitation of Net1. Yeast cells harboring the *HMR-SS abf1*$^{-}$ Δ*I* or the *HMR-SS rap1*$^{-}$ Δ*I* allele and overexpressing 6×myc-*NET1* were crosslinked with formaldehyde to generate covalent linkages between closely associated proteins and between proteins and DNA. Chromatin was isolated and sonicated to an average size of 0.5–1.0 kb, and 6×myc-Net1 was immunoprecipitated from this mixture by using an α-myc antibody. Net1-associated DNA was subsequently analyzed by PCR using specific primers. The overexpressed, myc-tagged Net1 protein could be found associated with rDNA loci (NTS, 25S), as has been shown previously (Straight *et al.* 1999; data not shown). Similarly, *HMR*-E was enriched in the immunoprecipitates of the *HMR-SS abf1*$^{-}$ Δ*I* strain but not of the *HMR-SS rap1*$^{-}$ Δ*I* strain, and the enrichment required both 6×myc-*NET1* and the α-myc antibody (Figure 2A and data not shown). Conversely, *ACT1* sequences were not enriched in the immunoprecipitates, arguing against unspecific binding of Net1 to chromatin. We further tested the possibility that overexpressed Net1 was bound on non-rDNA chromatin by performing immunostaining on chromatin of spread nuclei with 6×myc-Net1. Even at high levels of *NET1* expression, Net1 was located specifically on rDNA, but not on other

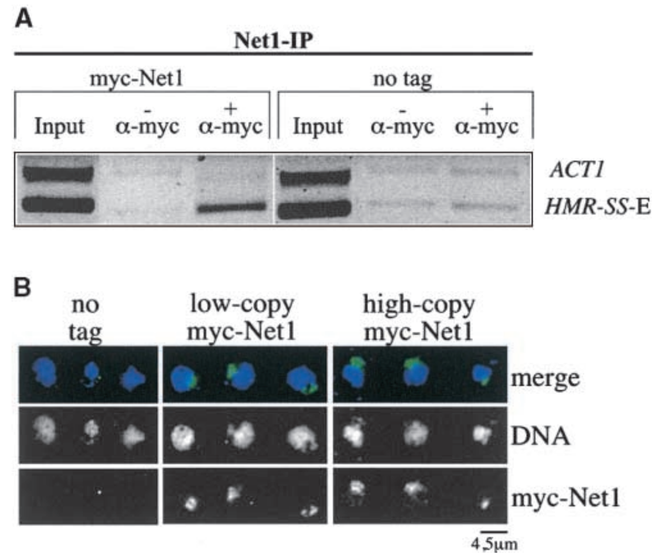

Figure 2.—Overexpressed Net1 protein was specifically associated with the *HMR*-E silencer. (A) Chromatin immunoprecipitates from formaldehyde crosslinked cells (*MAT*α *HMR-SS abf1*$^{-}$ Δ*I*, AEY 726) were analyzed by PCR using primers specific to the *HMR*-E silencer and to the *ACT1* gene simultaneously. Identical immunoprecipitations were carried out without addition of antibody (−α-myc) and with cells lacking the overexpressed, tagged *NET1* (no tag). The inverse image of ethidium-bromide-stained gels with representative amplifications is shown. (B) Net1-immunostaining experiments showed the nucleolar Net1 staining pattern that was independent of the extent of *NET1* expression. The images show spread nuclei of fixed cells of AEY 726, transformed with pAE 426, pAE 711, or pAE 622.

chromatin (Figure 2B). *HMR* staining was not detected in this assay, presumably because the Net1 concentration at this locus was too low for detection. These results demonstrated that overexpressed *NET1* was physically present at *HMR-SS abf1*$^{-}$ Δ*I*, but did not unspecifically coat chromatin, and thus likely had a direct role in *HMR* silencing, which depended upon the architecture of the *HMR* allele.

Tethered Gal4-Net1 promoted ORC-dependent, Sir1-independent repression of *HMR*: Since overexpressed Net1 was physically associated with the *HMR*-E silencer, we reasoned that it might have the capacity to recruit silencing proteins and to nucleate a repressive chromatin structure. To test this possibility, we asked whether Net1 could provide silencing when artificially tethered to the silencer in a so-called targeted silencing assay (Chien *et al.* 1993). In this assay, one of the protein binding sites in the *HMR* silencer is replaced by a varying number of Gal4-binding sites, which on their own have no silencing capacity. Silencing is then achieved by expressing fusions of silencing proteins with the Gal4 DNA-binding domain (amino acids 1–147, referred to as Gal4). The prototype silencing protein used in this assay is Gal4-Sir1 (Chien *et al.* 1993).

To test tethered silencing by Net1, a Gal4-Net1 hybrid

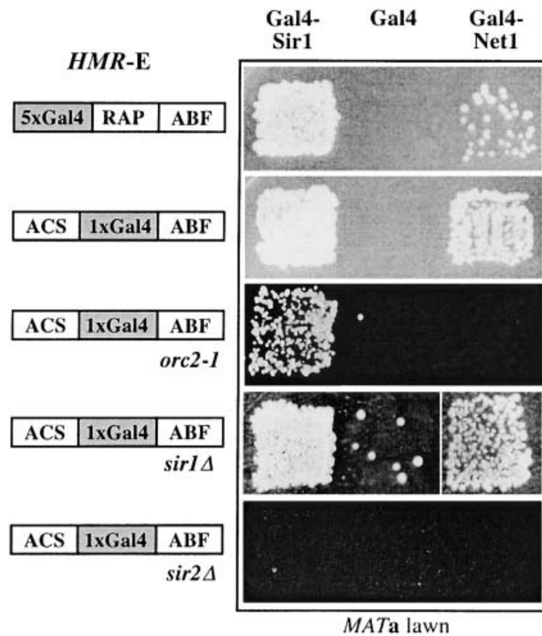

FIGURE 3.—Net1 induced silencing when tethered to the Rap1-binding site of the *HMR*-E silencer. Repression at *HMR* was measured by patch-mating assays in *MAT*α *HMR*-5×Gal4-RAP-ABF Δ*I* (AEY 500) and *MAT*α *HMR*-ACS-Gal4-ABF Δ*I* (AEY 454) cells. Gal4-Sir1 served as a positive control for targeted silencing. The dependence of Gal4-Net1-mediated silencing on Sir1, Sir2, and ORC was investigated in strains carrying a *SIR1* or *SIR2* deletion (AEY 1934 and AEY 1968, respectively) or the *orc2-1* mutation (AEY 2132).

was constructed by fusing the *NET1* C terminus (amino acids 566–1189) with the Gal4-binding domain. We chose this part of Net1 because it shows an interaction with Sir2 in the yeast two-hybrid assay (Cuperus *et al.* 2000). *HMR* silencing by Gal4-Net1 was tested in *MAT*α strains in which either the ORC- or the Rap1-binding site was replaced by a single Gal4-binding site (*HMR*-Gal4-RAP-ABF Δ*I* and *HMR*-ACS-Gal4-ABF Δ*I*, respectively). As expected, in the absence of a fusion protein, these strains were non-maters due to derepression of *HMR*, but became efficient maters upon introduction of Gal4-Sir1 (Figure 3). When Gal4-Net1 was expressed in these strains, efficient mating was observed in the *HMR*-ACS-Gal4-ABF strain, showing that tethered Net1 could provide silencing. However, no silencing was found when Net1 was tethered to the ORC-binding site (data not shown). Since the number of Gal4-binding sites for some silencing proteins has previously been shown to be limiting (Fox *et al.* 1997), we also tested Gal4-Net1-mediated silencing when three or five Gal4-binding sites replaced the ACS. Only with five binding sites did we observe weak tethered silencing by Gal4-Net1 (Figure 3). These results showed that tethered Net1 had the capability of establishing repressive chromatin at *HMR* and suggested that Net1 required the presence of specific silencer binding proteins to mediate silencing.

The observation that Gal4-Net1 silencing was efficient only when the single Gal4-binding site replaced the Rap1-binding site suggested that tethered silencing mediated by Net1 required a functional ORC-binding site. We therefore tested whether Gal4-Net1-mediated silencing also required one of the ORC proteins, Orc2, by introducing *orc2-1* into the *MAT*α *HMR*-ACS-Gal4-ABF strain. Silencing by Gal4-Net1 was abolished in this strain (Figure 3), showing that ORC was required for tethered Net1 silencing. ORC is thought to act in silencing by recruiting Sir1 to the silencer via an interaction between Sir1 and Orc1 (Triolo and Sternglanz 1996). If this recruitment were the major task of ORC in silencing, then we would also expect the Net1-driven silencing to be dependent on Sir1. We therefore tested the tethered Gal4-Net1 silencing in a *sir1*Δ strain. Interestingly, tethered Net1 was still able to establish silencing in the absence of Sir1 (Figure 3), suggesting that Sir1 function in silencing became dispensable in this scenario.

If the silencing property of tethered Net1 was based on the interaction between Net1 and Sir2 (Cuperus *et al.* 2000), then Gal4-Net1-mediated silencing was expected to depend upon Sir2. Indeed, silencing by tethered Net1 could not be detected in the absence of Sir2 (Figure 3), suggesting that tethered Net1 established a repressive chromatin structure recruiting Sir2, and potentially other silencing proteins, to the silencer.

***NET1* overexpression did not affect rDNA silencing and telomeric silencing:** Since increased cellular levels of Net1 were capable of improving *HMR* silencing, we next asked whether silencing at the other known silenced loci was influenced by *NET1* overexpression. For this purpose, silencing of the *URA3* reporter gene was monitored when inserted in the rDNA locus (Smith and Boeke 1997) or at an artificial telomere (Gottschling *et al.* 1990) by testing growth on medium lacking uracil or on medium containing 5-fluoroorotic acid (FOA), which inhibits growth of *URA3*-expressing cells due to the synthesis of the toxic compound 5-fluorouracil. However, *NET1* overexpression had no effect on telomeric *URA3* silencing, even when telomeric silencing was compromised by an *orc2-1* mutation (Figure 4A). Also, expression of *URA3* in the rDNA locus was unaffected by high-copy *NET1* (Figure 4B). We also sought to investigate the effect of *NET1* on *HML* silencing. Since no suitable *HML* silencer mutants are available, we tested whether *NET1* overexpression could suppress the intermediate silencing defect at *HML* in a *sir1*Δ*sas2*Δ strain (Ehrenhofer-Murray *et al.* 1997). However, *NET1* overexpression was unable to suppress, probably because *NET1*-mediated silencing required Sir1 (Figure 4C).

Net1-based *HMR* silencing did not involve a stable interaction with Rap1: The above data led us to further investigate the functional importance of Rap1 in Net1-based silencing mechanisms. Rap1- and ORC-binding sites also exist inside the repeated rDNA locus, where

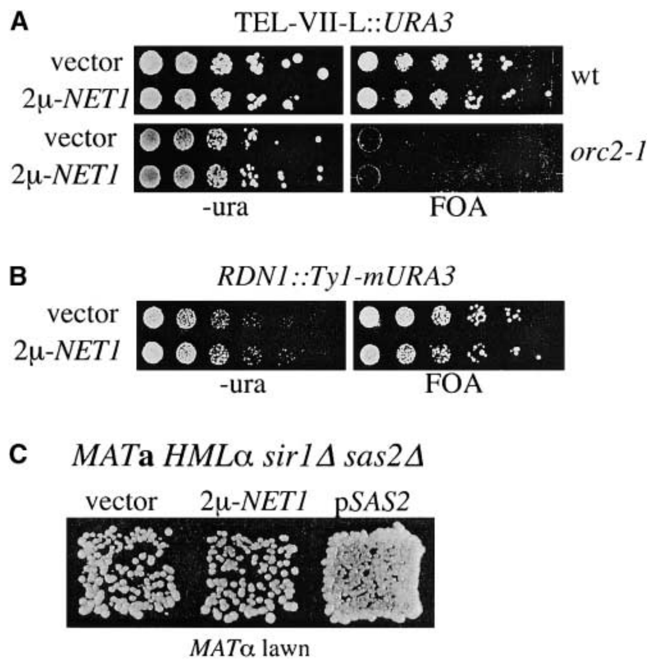

Figure 4.—*NET1* overexpression did not affect telomeric, rDNA, or *HML* silencing. (A) Telomeric silencing was determined by measuring the expression of *URA3*, when inserted in the telomeric region of chromosome VII (AEY 1017), by testing growth on medium lacking uracil and on FOA-containing medium. Experiments were also performed with a telomeric silencing-deficient *orc2-1* strain (AEY 2143). (B) rDNA silencing was determined by measuring the expression of the *URA3* gene inserted in the nontranscribed region of an rDNA unit (AEY 1778). (C) *HML* silencing was measured in a silencing-deficient *MAT***a** *sir1*Δ *sas2*Δ strain (AEY 346), using a patch-mating assay. Introduction of a *SAS2*-containing plasmid but not a *NET1* overexpressing plasmid restored its mating deficiency.

Net1 is present in the RENT complex. As it is not yet clear how RENT is tethered to the DNA, we sought to elucidate whether Rap1 or ORC was important for rDNA silencing.

For this purpose, we integrated *Ty1-mURA3* into the 25S region of the rDNA locus (*RDN1*) in strains that either were wild type or carried mutations in *RAP1* (*rap1-12*, *rap1-13*) or ORC (*orc2-1*, *orc5-1*). To estimate the number of tandem integrations, the band intensity of *URA3* in *RDN1::Ty1-mURA3* was compared to that of the native *URA3* locus in a Southern blot. Transformants were chosen that carried one or two integrated copies of the *URA3* gene. The level of *URA3* expression was measured by growth on medium lacking uracil as well as on FOA-containing medium. While mutations in the *RAP1* gene had only a minor influence on the cells' ability to grow in the presence of FOA, *orc* mutants were FOA sensitive, indicating a higher level of *URA3* expression and, thus, less *Ty1-mURA3* silencing in these strains (Figure 5A). However, neither *orc* nor *rap* mutations repressed *URA3* at its native locus, since such strains were completely FOA sensitive (Figure 5A and data not shown). Taken together, these results suggested that ORC, but not Rap1, was required for rDNA silencing.

We further tested whether Net1 interacted physically with Rap1 by co-IP. For this purpose, the 2μ-plasmid carrying the 6×myc-tagged version of *NET1* was introduced into a yeast strain that expressed an HA-tagged Rap1 protein (Rap1-3×HA) from its native promoter. As a positive control, a co-IP was also performed between 6×myc-tagged Net1 and Sir2-3×HA, since Sir2 and Net1 have previously been shown to interact (Ghidelli *et al.* 2001; Shou *et al.* 2001). Whereas Sir2 co-immunoprecipitated with Net1, we could not detect coprecipitated Rap1 (Figure 5B), suggesting that Rap1 and Net1 were not or were only weakly associated with each other.

Increased dosage of Sir2 recapitulated the *net1-1* silencing phenotype: The experiments above supported the view that the Net1 protein, when overexpressed, interacted with silencer binding proteins at the *HMR*-E silencer and recruited the Sir proteins to form repressed chromatin. Thus, one might propose that, under normal expression, Net1 would be present at *HMR* and that the mutation of *NET1* would therefore decrease silencing. However, contrary to this view, we had observed that *net1-1* improved the mating of a *MAT*α *HMR-SS abf1*$^-$ Δ*I* strain (see above), thus arguing that the influence of *net1-1* in silencing was distinct from that of *NET1* overexpression. We therefore sought to determine by what mechanism *net1-1* was restoring silencing.

In a first set of experiments, we further characterized the *net1-1* effect on defective silencer variants. *net1-1* was able to improve the mating ability of strains carrying all the tested *HMR* alleles (Figure 6), confirming the hypothesis that the *net1-1* mutation and the *NET1* overexpression nucleated silencing in two distinct ways. The increase in the mating capacity most likely reflected an increase in silencing at *HMR*, because the *net1-1* strains became complete non-maters upon a deletion of *SIR1*, *SIR2*, or *SIR3* (data not shown).

Net1 interacts with Sir2 in the RENT protein complex at the rDNA locus and is required for Sir2's association with the rDNA (Straight *et al.* 1999). We therefore reasoned that in the *net1-1* strain, the interaction between Net1 and Sir2 might be disturbed such that Sir2 failed to localize to the nucleolus. Perhaps Sir2 would then be released into the nucleus, thus increasing the level of Sir2 available for *HMR* silencing. If the effect of *net1-1* on *HMR* was indirect through the release of nucleolar Sir2, one prediction would be that the third class of transcriptional repression, telomeric silencing, would also improve in the absence of Net1. In agreement with this, a slightly increased level of telomeric silencing has been detected in *net1*Δ cells (Straight *et al.* 1999). Also supporting this theory, *SIR2* overexpression has previously been shown to suppress the silencing defect of the *HMR***a**-*e*** allele (Xu *et al.* 1999a). Therefore, we tested the effect of increased Sir2 dosage on

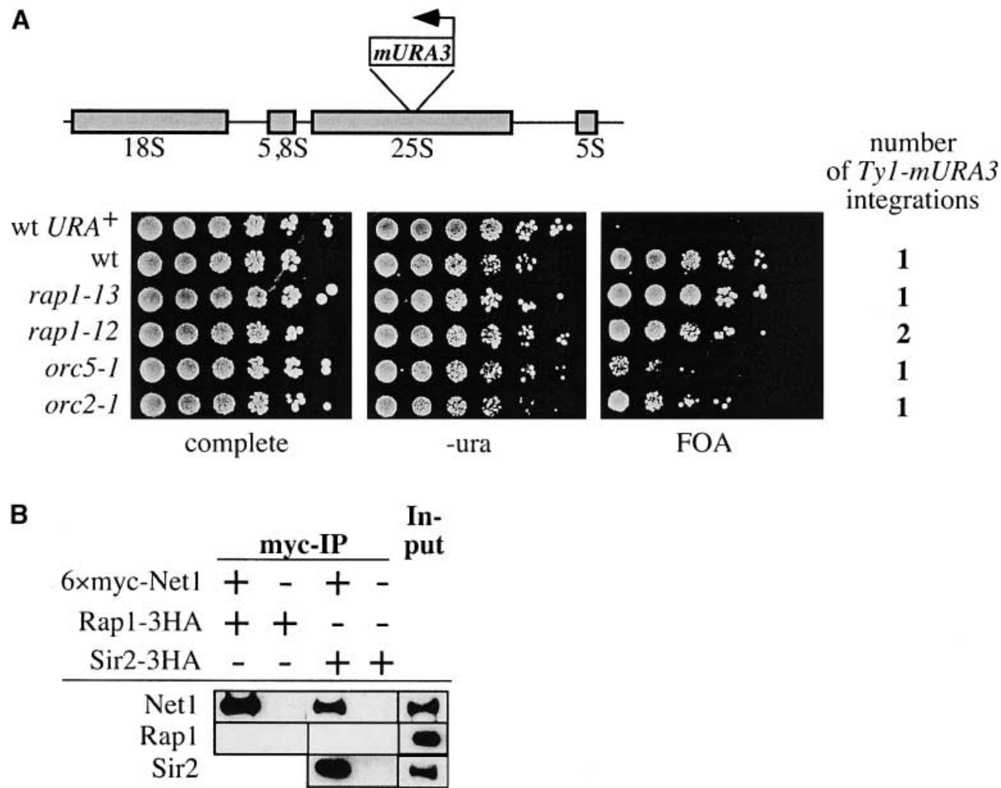

Figure 5.—*orc* mutations reduced rDNA silencing. (A) *RDN1::Ty1-mURA3* expression was tested by plating serial dilutions of the respective strains on complete medium, on medium lacking uracil, or on FOA-containing medium. The number of *Ty1-mURA3* integrations in each strain was measured by Southern blotting analysis. The strains used were AEY 2741 (wt *URA3*$^+$), AEY 2742 (wt), AEY 2744 (*rap1-13*), AEY 2743 (*rap1-12*), AEY 2745 (*orc5-1*), and AEY 2746 (*orc2-1*). (B) Net1 and Rap1 did not interact in co-immunoprecipitation experiments. The interaction of Sir2 and Net1 served as a positive control. The respective strains were obtained by transforming AEY 2548 (Rap-3×HA) and AEY 2551 (Sir2-3×HA) with a 6×myc-Net1-containing plasmid (pAE 622).

the other defective *HMR* alleles. All *HMR* alleles tested were suppressed by high-copy Sir2 to a similar extent as by the *net1-1* mutation (Figure 6), indicating that the enhanced availability of nuclear Sir2 in *net1-1* cells might be responsible for the improved *HMR* silencing.

Sir2 was distributed throughout the nucleus in *net1-1* cells: Our hypothesis posits that *net1-1* liberates Sir2 from the nucleolus and increases the amount of Sir2 in the whole nucleus. The Sir2 association with rDNA has previously been shown to be lost upon deletion of *NET1* (Straight *et al.* 1999), but it remained unclear what happened to Sir2 in *net1-1* mutant cells. To follow the fate of Sir2 in *net1-1* cells, we introduced a green fluorescent protein (GFP)-tagged Sir2 (Cuperus *et al.* 2000) into a wild-type *NET1* and into a mutant *net1-1* strain and observed its cellular distribution by fluorescence microscopy. In a wild-type strain, as well as in a *NET1* overexpressing strain, nucleolar Sir2 was present as a half-moon-shaped structure at the edge of the nucleus. In contrast, Sir2-GFP showed a strikingly different staining pattern in *net1-1* cells, in that the nucleolar half-moon structure was completely absent (Figure 7). Instead, the whole nucleus appeared weakly stained by Sir2-GFP. We next measured the amount of Sir2-GFP protein in wild-type and *net1-1* strains by Western blotting analysis. Both strains displayed equal levels of Sir2-GFP (data not shown), showing that *net1-1* did not decrease the cellular level of Sir2. Therefore, the nuclear staining pattern of Sir2-GFP demonstrated that Sir2 became distributed throughout the nucleus in *net1-1* strains, which supported the notion that *net1-1* acted indirectly in *HMR* silencing by liberating Sir2 from the nucleolus. Thus, these experiments suggested that the rDNA locus and *HMR* competed for limiting amounts of Sir2.

DISCUSSION

The Net1 protein is a central component of the nucleolar protein complex RENT that promotes rDNA silencing and nucleolar integrity and regulates the exit from mitosis in yeast. In this study, we have identified a role for Net1 in repression of the silent mating-type locus *HMR* of *S. cerevisiae*. Significantly, both high-copy expression and mutation of *NET1* suppressed mutations in the *HMR*-E silencer. Both effects were dependent upon the function of the Sir proteins, suggesting that *bona fide* silencing was established under these conditions. Thus, *NET1* joins genes such as the cell cycle genes *CDC7* (Axelrod and Rine 1991), *CDC45*, and *POL30* (Ehrenhofer-Murray *et al.* 1999) as well as the *SAS* genes *SAS2*, *SAS3*, *SAS4*, and *SAS5* as genes that, when mutated, improve silencing (Reifsnyder *et al.* 1996; Ehrenhofer-Murray *et al.* 1997; Xu *et al.* 1999b).

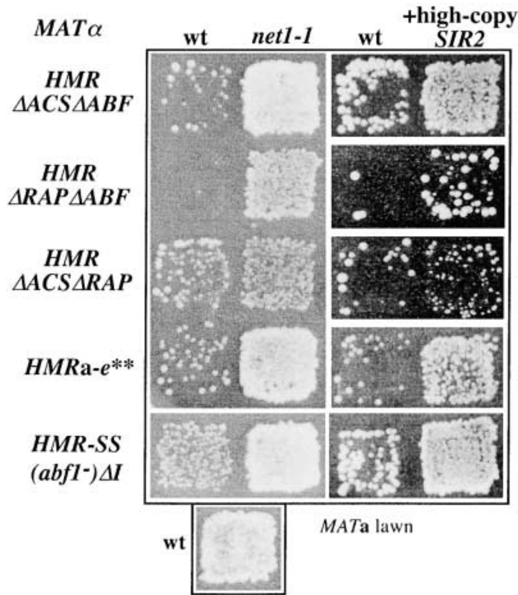

Figure 6.—Increased *HMR* silencing in *net1-1* cells was recapitulated by high-copy expression of Sir2. The effect of *net1-1* and *SIR2* overexpression was tested in *MAT*α strains carrying various mutations in the *HMR*-E silencer. Strains AEY 70, 69, 98, 403, and 726 (from top to bottom) were *NET1*, and strains AEY 1793, 1889, 1891, 1888, and 1885 were *net1-1*. *SIR2* overexpression was tested in the *NET1* series of strains by introducing *SIR2* on the d*LEU2* plasmid pJDB 207 (Buchman *et al.* 1988).

However, the mechanisms of how these genes act in silencing are likely to be quite distinct. We invoke two models for the effect of Net1 on *HM* silencing: (1) a direct role in silencing for overexpressed Net1, since it was physically associated with *HMR*-E sequences, and (2) an indirect role for *net1-1*, namely, by increasing the amount of Sir2 available for *HMR* silencing by releasing Sir2 from the nucleolus.

There are several possibilities for how elevated levels of Net1 could improve silencing. Overexpressed Net1 could have indirect effects on silencing, for instance, by disrupting the RENT complex in a dominant-negative fashion and releasing Sir2 or other components from the nucleolus or by influencing cell cycle progression, the deceleration of which has been proposed to increase the likelihood of establishing repressed chromatin (Laman *et al.* 1995). However, we do not favor these interpretations, because Sir2 was still nucleolar in cells overexpressing *NET1* (Figure 7). Additionally, these cells did not show any detectable growth defect or differences in doubling time, arguing against an effect on cell cycle progression. Importantly, we found that Net1 was physically associated with *HMR*-E DNA, suggesting a direct role for Net1 in *HMR* silencing. Since Net1 has no recognizable DNA-binding capacity (Straight *et al.* 1999), this interaction is likely to be mediated by silencer binding proteins. The observation that Net1-mediated *HMR* silencing required a functional Rap1-binding site at *HMR*-E and that *NET1* overexpression could not overcome the silencing defect of a *rap1-12* or a *rap1-13* strain suggests a direct or indirect recruitment of Net1 to *HMR*-E through Rap1. However, we were unable to detect an interaction between Rap1 and Net1 by co-immunoprecipitation, which supports the argument that a potential association, if present, may be unstable or bridged by other proteins. Alternatively, Net1 may localize to *HMR* via its interaction with Sir2, which in turn may require an intact Rap1-binding site. Notably, silencing at an artificial telomere, which depends upon tandem Rap1-binding sites, was not strengthened by *NET1* overexpression (Figure 4A), indicating that other *cis*-acting elements at *HMR*-E were required to mediate the *NET1* silencing effect. In agreement with this, rDNA silencing was not weakened by mutations in Rap1 (*rap1-12*, *rap1-13*). However, the possibility remains that these mutations alter only rDNA-independent Rap1 functions. Interestingly, in tethered silencing experiments, Net1 was able to promote silencing only when Gal4-Net1 substituted for Rap1, further suggesting that the position of Net1 within the silencer was important for its silencing effect. Notably, Net1 established Sir1-independent silencing in the artificial tethering experiments, whereas silencing upon *NET1* overexpression required Sir1. Perhaps Sir1 is required to recruit Net1 to the silencer and hence is dispensable in the tethered silencing experiments. Alternatively, tethered Net1

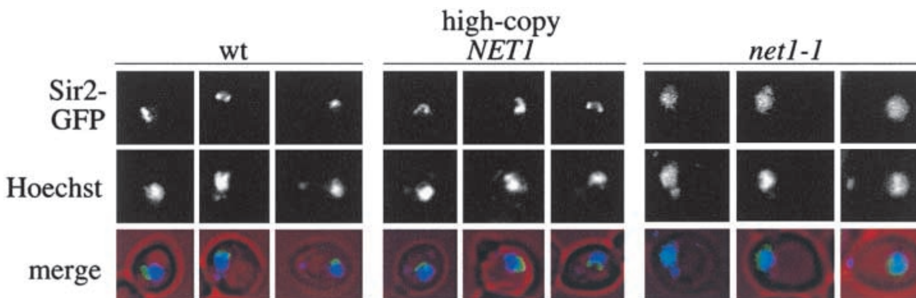

Figure 7.—Delocalization of Sir2 from the nucleolus in a *net1-1* mutant. Sir2-GFP was visualized in living cells by fluorescence microscopy under a FITC filter. Hoechst was used to stain the cellular DNA. Sir2-GFP appeared as a distinct nucleolar shape at the nuclear periphery in wild type (wt) and *NET1* overexpressing cells, but was spread over the whole nucleus in *net1-1* cells. The strains used were *HMR-SS abf1*⁻ Δ*I NET1* (AEY 726) and *HMR-SS abf1*⁻ Δ*I net1-1* (AEY 1885), transformed with GLC462 (Cuperus *et al.* 2000), and, for overexpression of *NET1*, with pAE 622.

might be associated with the silencer in a more stable fashion than overexpressed Net1. Tethered Net1 may therefore recruit the Sir2/3/4 complex more efficiently, thus circumventing the need for Sir1. Interestingly, the Net1-tethered silencing was abolished in an *orc2-1* mutant, showing its dependence upon ORC. Also, rDNA silencing, which is independent of Sir1, was compromised in an *orc2-1* strain. Thus, ORC displayed functions in silencing that went beyond the recruitment of Sir1. In light of this, it is interesting that the association of Sir1 at *HMR* requires not just ORC, but also Sir2 (Gardner and Fox 2001), suggesting that the roles of both ORC and Sir1 are more complex than the simple Sir1 recruitment by ORC. With respect to rDNA silencing, our data suggest the possibility that ORC is involved in RENT binding to rDNA.

Interestingly, overexpressed *NET1* yielded silencing at the *HMR* Δ*ACS*Δ*ABF* allele, suggesting that this silencing was ORC independent due to the deletion of the ACS site. This seems at odds with the observation that tethered Net1 silencing required ORC. Thus, a possible connection between ORC and Net1 may depend upon the way Net1 is recruited to the silenced region. Alternatively, silencing at the *HMR* Δ*ACS* allele may still require ORC, because several origins close to *HMR-E* become active upon mutation of the silencer origin (Palacios DeBeer and Fox 1999).

How does Net1 promote silencing once it is recruited to the silencer? Since Net1 interacts with Sir2 in the nucleolus, it may also primarily attract Sir2 (and potentially other unidentified RENT components) to the *HMR*-E silencer. Sir2 may then deacetylate histones in the *HMR* chromatin domain, which would lead to a more efficient recruitment of Sir3 and Sir4 through their interaction with unacetylated histone tails (Hecht *et al.* 1995). The Net1-driven anchoring of the Sir2/3/4 complex to DNA may be related to the attraction of this complex to the nucleolar rDNA that takes place in aging mother cells and in *sir4-42* cells (Kennedy *et al.* 1997). In this respect, it is interesting to note that cells coexpressing **a** and α information display accelerated aging (Kaeberlein *et al.* 1999). Thus, the prevention of *HMR* expression by excess Net1 may be interpreted as an additional mechanism to promote longevity in yeast.

An alternative possibility as to how silencer-bound Net1 promotes silencing is that Net1 may locally stabilize the Sir2 protein or the whole Sir complex at *HMR*, similar to Cdc14, whose degradation is prevented through its nucleolar association with Net1 (Shou *et al.* 1999). This may lead to increased local concentrations of Sir2, which would then improve silencing at *HMR*. Net1 may also act at *HMR* by directly modifying the deacetylase activity of Sir2, much as it regulates the phosphatase activity of Cdc14 (Traverso *et al.* 2001), for instance, by activating Sir2 through physical contact or by deactivating a yet unidentified inhibitor of Sir2.

Formally, the silencing improvement in a *net1-1* mutant invokes a negative effect of Net1 on *HMR* silencing, since the lack of Net1 function leads to enhanced *HMR* silencing. For instance, Net1 might act in silencing by inhibiting a silencing factor, or *net1-1* might indirectly improve silencing by changing cell cycle progression. However, we favor the model that the availability of Sir2 for *HMR* silencing is increased in the *net1-1* mutant, because we observed a Sir2 delocalization from the nucleolus in *net1-1* cells and because *SIR2* overexpression phenocopied the *net1-1* effect at *HMR* (Figures 6 and 7). Formally, we cannot exclude the possibility that the *net1-1* mutation releases other nucleolar factors that might participate in *HMR* silencing. In summary, our data suggest a competition for Sir2 (and potentially other silencing proteins) between the rDNA and *HMR*, similar to the competition that has previously been observed between rDNA and telomeric silencing (Smith *et al.* 1998) as well as between the telomeres and *HM* silencing (Buck and Shore 1995).

As in yeast, rDNA gene units are arranged in multiple tandem repeats in higher eukaryotes. For instance, rDNA arrays in Drosophila lie on the sex chromosomes, where they act as pairing sites between the X and Y chromosomes during male meiosis (Briscoe and Tomkiel 2000). Net1 homologs have been identified in Drosophila as well as in other eukaryotes (Costanzo *et al.* 2000). Thus, in analogy to Net1's role in yeast, these homologs may likewise be involved in nucleolar functions and in epigenetic gene regulation in these organisms. Furthermore, our experiments shed light on the dynamics between the different silenced regions in the genome of *S. cerevisiae*. Perhaps a similar intranuclear competition for limiting silencing factors also is in play in higher organisms.

We thank D. Shore, R. H. Deshaies, J. Boeke, and J. Rine for strains and plasmids and M. Grunstein for the ChIP protocol. We also thank A. Geissenhöner, A. Grünweller, and S. Meijsing for comments on the manuscript, A. Barduhn and K. Vogel for excellent technical assistance, H. Scherthan and E. Trelles-Sticken for support with immunostainings, and all members of our laboratory for many stimulating discussions.

LITERATURE CITED

Axelrod, A., and J. Rine, 1991 A role for *CDC7* in repression of transcription at the silent mating-type locus *HMR* in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **11:** 1080–1091.

Brand, A. H., G. Micklem and K. Nasmyth, 1987 A yeast silencer contains sequences that can promote autonomous plasmid replication and transcriptional activation. Cell **51:** 709–719.

Briscoe, A., and J. E. Tomkiel, 2000 Chromosomal position effects reveal different *cis*-acting requirements for rDNA transcription and sex chromosome pairing in *Drosophila melanogaster*. Genetics **155:** 1195–1211.

Buchman, A. R., W. J. Kimmerly, J. Rine and R. D. Kornberg, 1988 Two DNA-binding factors recognize specific sequences at silencers, upstream activating sequences, autonomously replicating sequences, and telomeres in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **8:** 210–225.

Buck, S. W., and D. Shore, 1995 Action of a RAP1 carboxy-terminal silencing domain reveals an underlying competition between *HMR* and telomeres in yeast. Genes Dev. **9:** 370–384.

Chien, C. T., S. Buck, R. Sternglanz and D. Shore, 1993 Targeting of *SIR1* protein establishes transcriptional silencing at *HM* loci and telomeres in yeast. Cell **75:** 531–541.

Costanzo, M. C., J. D. Hogan, M. E. Cusick, B. P. Davis, A. M. Fancher *et al.*, 2000 The yeast proteome database (YPD) and *Caenorhabditis elegans* proteome database (WormPD): comprehensive resources for the organization and comparison of model organism protein information. Nucleic Acids Res. **28:** 73–76.

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

Ehrenhofer-Murray, A. E., D. H. Rivier and J. Rine, 1997 The role of Sas2, an acetyltransferase homologue of *Saccharomyces cerevisiae*, in silencing and ORC function. Genetics **145:** 923–934.

Ehrenhofer-Murray, A. E., R. T. Kamakaka and J. Rine, 1999 A role for the replication proteins PCNA, RF-C, polymerase epsilon and Cdc45 in transcriptional silencing in *Saccharomyces cerevisiae*. Genetics **153:** 1171–1182.

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

Gardner, K. A., and C. A. Fox, 2001 The Sir1 protein's association with a silenced chromosome domain. Genes Dev. **15:** 147–157.

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

Ghidelli, S., D. Donze, N. Dhillon and R. T. Kamakaka, 2001 Sir2p exists in two nucleosome-binding complexes with distinct deacetylase activities. EMBO J. **20:** 4522–4535.

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

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

Hampton, R. Y., and J. Rine, 1994 Regulated degradation of HMG-CoA reductase, an integral membrane protein of the endoplasmic reticulum, in yeast. J. Cell Biol. **125:** 299–312.

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

Hecht, A., S. Strahl-Bolsinger and M. Grunstein, 1996 Spreading of transcriptional repressor SIR3 from telomeric heterochromatin. Nature **383:** 92–96.

Hoffman, C. S., and F. Winston, 1987 A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. Gene **57:** 267–272.

Imai, S., C. M. Armstrong, M. Kaeberlein and L. Guarente, 2000 Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. Nature **403:** 795–800.

Kaeberlein, M., M. McVey and L. Guarente, 1999 The SIR2/3/4 complex and SIR2 alone promote longevity in *Saccharomyces cerevisiae* by two different mechanisms. Genes Dev. **13:** 2570–2580.

Kennedy, B. K., M. Gotta, D. A. Sinclair, K. Mills, D. S. McNabb *et al.*, 1997 Redistribution of silencing proteins from telomeres to the nucleolus is associated with extension of life span in *S. cerevisiae*. Cell **89:** 381–391.

Kimmerly, W., A. Buchman, R. Kornberg and J. Rine, 1988 Roles of two DNA-binding factors in replication, segregation and transcriptional repression mediated by a yeast silencer. EMBO J. **7:** 2241–2253.

Knop, M., K. Siegers, G. Pereira, W. Zachariae, B. Winsor *et al.*, 1999 Epitope tagging of yeast genes using a PCR-based strategy: more tags and improved practical routines. Yeast **15:** 963–972.

Laman, H., D. Balderes and D. Shore, 1995 Disturbance of normal cell cycle progression enhances the establishment of transcriptional silencing in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **15:** 3608–3617.

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

Lin, S. J., P. A. Defossez and L. Guarente, 2000 Requirement of NAD and *SIR2* for life-span extension by calorie restriction in *Saccharomyces cerevisiae*. Science **289:** 2126–2128.

Loo, S., and J. Rine, 1995 Silencing and heritable domains of gene expression. Annu. Rev. Cell Dev. Biol. **11:** 519–548.

Lustig, A. J., 1998 Mechanisms of silencing in *Saccharomyces cerevisiae*. Curr. Opin. Genet. Dev. **8:** 233–239.

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

Moazed, D., A. Kistler, A. Axelrod, J. Rine and A. D. Johnson, 1997 Silent information regulator protein complexes in *Saccharomyces cerevisiae*: a SIR2/SIR4 complex and evidence for a regulatory domain in SIR4 that inhibits its interaction with SIR3. Proc. Natl. Acad. Sci. USA **94:** 2186–2191.

Moretti, P., K. Freeman, L. Coodly and D. Shore, 1994 Evidence that a complex of SIR proteins interacts with the silencer and telomere-binding protein RAP1. Genes Dev. **8:** 2257–2269.

Palacios DeBeer, M. A., and C. A. Fox, 1999 A role for a replicator dominance mechanism in silencing. EMBO J. **18:** 3808–3819.

Pryde, F. E., and E. J. Louis, 1999 Limitations of silencing at native yeast telomeres. EMBO J. **18:** 2538–2550.

Reifsnyder, C., J. Lowell, A. Clarke and L. Pillus, 1996 Yeast *SAS* silencing genes and human genes associated with AML and HIV-1 Tat interactions are homologous with acetyltransferases. Nat. Genet. **14:** 42–49.

Sherman, F., 1991 Getting started with yeast. Methods Enzymol. **194:** 3–21.

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

Shou, W., K. M. Sakamoto, J. Keener, K. W. Morimoto, E. E. Traverso *et al.*, 2001 Net1 stimulates RNA polymerase I transcription and regulates nucleolar structure independently of controlling mitotic exit. Mol. Cell **8:** 45–55.

Sikorski, R. S., and P. Hieter, 1989 A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics **122:** 19–27.

Sinclair, D. A., and L. Guarente, 1997 Extrachromosomal rDNA circles—a cause of aging in yeast. Cell **91:** 1033–1042.

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

Smith, J. S., C. B. Brachmann, L. Pillus and J. D. Boeke, 1998 Distribution of a limited Sir2 protein pool regulates the strength of yeast rDNA silencing and is modulated by Sir4p. Genetics **149:** 1205–1219.

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

Sussel, L., and D. Shore, 1991 Separation of transcriptional activation and silencing functions of the *RAP1*-encoded repressor/activator protein 1: isolation of viable mutants affecting both silencing and telomere length. Proc. Natl. Acad. Sci. USA **88:** 7749–7753.

Thomas, B. J., and R. Rothstein, 1989 Elevated recombination rates in transcriptionally active DNA. Cell **56:** 619–630.

Traverso, E. E., C. Baskerville, Y. Liu, W. Shou, P. James *et al.*, 2001 Characterization of the Net1 cell cycle-dependent regulator of the Cdc14 phosphatase from budding yeast. J. Biol. Chem. **276:** 21924–21931.

Trelles-Sticken, E., J. Loidl and H. Scherthan, 1999 Bouquet formation in budding yeast: Initiation of recombination is not required for meiotic telomere clustering. J. Cell Sci. **112:** 651–658.

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

Visintin, R., K. Craig, E. S. Hwang, S. Prinz, M. Tyers *et al.*, 1998 The phosphatase Cdc14 triggers mitotic exit by reversal of Cdk-dependent phosphorylation. Mol. Cell **2:** 709–718.

Visintin, R., E. S. Hwang and A. Amon, 1999 Cfi1 prevents prema-

ture exit from mitosis by anchoring Cdc14 phosphatase in the nucleolus. Nature **398:** 818–823.

Warner, J. R., 1989 Synthesis of ribosomes in *Saccharomyces cerevisiae*. Microbiol. Rev. **53:** 256–271.

Xu, E. Y., S. Kim, K. Replogle, J. Rine and D. H. Rivier, 1999a Identification of *SAS4* and *SAS5*, two genes that regulate silencing in *Saccharomyces cerevisiae*. Genetics **153:** 13–23.

Xu, E. Y., S. Kim and D. H. Rivier, 1999b *SAS4* and *SAS5* are locus-specific regulators of silencing in *Saccharomyces cerevisiae*. Genetics **153:** 25–33.

Zachariae, W., M. Schwab, K. Nasmyth and W. Seufert, 1998 Control of cyclin ubiquitination by CDK-regulated binding of Hct1 to the anaphase promoting complex. Science **282:** 1721–1724.

Communicating editor: J. Rine