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Net1, a Sir2-Associated Nucleolar Protein Required for rDNA Silencing and Nucleolar Integrity

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Summary

The Sir2 protein mediates gene silencing and repression of recombination at the rDNA repeats in budding yeast. Here we show that Sir2 executes these functions as a component of a nucleolar complex designated RENT (*r*egulator of *n*ucleolar silencing and *t*elophase exit). Net1, a core subunit of this complex, preferentially cross-links to the rDNA repeats, but not to silent DNA regions near telomeres or to active genes, and tethers the RENT complex to rDNA. Net1 is furthermore required for rDNA silencing and nucleolar integrity. During interphase, Net1 and Sir2 colocalize to a subdomain within the nucleolus, but at the end of mitosis a fraction of Sir2 leaves the nucleolus and disperses as foci throughout the nucleus, suggesting that the structure of rDNA silent chromatin changes during the cell cycle. Our findings suggest that a protein complex shown to regulate exit from mitosis is also involved in gene silencing.

Introduction

The process of gene silencing converts large regions of DNA within eukaryotic chromosomes into an inaccessible state, often called heterochromatin. Heterochromatic or silent DNA regions are refractory to transcription and recombination, replicate late in S phase of the cell cycle, and are usually located in discrete subnuclear domains near the nuclear periphery (Laurenson and Rine, 1992; Lustig, 1998). In the budding yeast, *Saccharomyces cerevisiae*, silencing has been observed at three distinct chromosome regions: the silent mating–type loci, telomeric DNA regions, and the tandemly repeated rDNA genes (Rine and Herskowitz, 1987; Gottschling et al., 1990; Bryk et al., 1997; Fritze et al., 1997; Smith and Boeke, 1997). The latter example presents an unexpected and unusual form of silencing because rDNA is very highly transcribed, accounting for approximately 60 percent of transcription in rapidly growing yeast cells (Woolford and Warner, 1991). However, only about half of the 100–200 copies of the genes encoding rRNA are active at any given time (Warner, 1989). The regulatory mechanisms that control the ratio of active to inactive rDNA repeats are poorly understood, but silencing is one of the factors that influences transcriptional activity at rDNA (Smith and Boeke, 1997). One known function of silencing is to repress recombination within the rDNA cluster (Gottlieb and Esposito, 1989). Recombination between the rDNA repeats can lead to excision of rDNA in the form of extrachromosomal circles, which have been identified as a cause of cellular senescence in yeast (Sinclair and Guarente, 1997).

At least four classes of proteins orchestrate silencing at the yeast silent mating–type loci and telomeres. The first class contains the DNA-binding proteins, Rap1, Abf1, and the origin recognition complex (ORC) (Shore and Nasmyth, 1987; Halfter et al., 1989; Rhode et al., 1989; Bell and Stillman, 1992). These proteins act primarily to recruit to DNA a second class of proteins, the dedicated silencing proteins, Sir2, Sir3, and Sir4 (Klar et al., 1979; Rine and Herskowitz, 1987; Aparicio et al., 1991; Moretti et al., 1994; Triolo and Sternglanz, 1996). In addition, the Sir1 protein is required for silencing at the silent mating–type loci but not telomeres (Pillus and Rine, 1989; Aparicio et al., 1991). Once recruited to the DNA, some of the Sir proteins spread along chromatin and create inactive domains that can extend for several kilobases from the initiation sites (Hecht et al., 1996; Strahl-Bolsinger et al., 1997). A third class of proteins comprises the histones. The N termini of histones H3 and H4 are required for silencing and are thought to be involved in both the initiation and spreading steps described above (Kayne et al., 1988; Thompson et al., 1994; Hecht et al., 1996). A fourth class of proteins, whose role in silencing is less clear, modulates the efficiency of silencing. This class includes histone acetylases and deacetylases, and enzymes of the ubiquitin pathway. For example, deletion of the *RPD3* histone deacetylase has been shown to cause an increase in the efficiency of telomeric silencing (De Rubertis et al., 1996; Rundlett et al., 1996). Deletion of *UBP3*, a deubiquitinating enzyme that binds to Sir4, results in more efficient silencing of reporter genes inserted near a telomere or at one of the silent mating–type loci (Moazed and Johnson, 1996) and promotes an expansion of silent chromatin domains (G. J. D., T. Gabriele, A. D. J., and D. M., unpublished). Finally, *UBC2/RAD6*, which encodes a ubiquitin-conjugating enzyme, is required for efficient silencing of telomere-proximal reporter genes and one of the silent mating–type loci (Huang et al., 1997; D. M., unpublished).

Much less is known about the mechanism and biological role of silencing at the rDNA repeats. Gottlieb and

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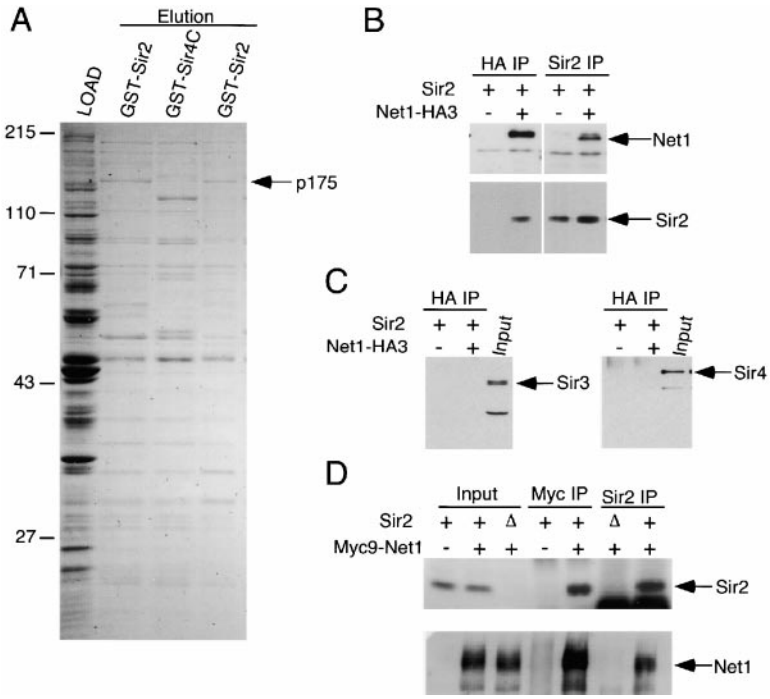

Figure 1. Purification of Net1 on a GST-Sir2 Affinity Column and Its Association with Sir2 in Yeast Extracts

(A) Coomassie-stained gel of the load and elution fractions of yeast proteins bound to GST-Sir2 and GST-Sir4-Cterm (GST-Sir4C) affinity columns. The p175 protein was excised from the Coomassie-stained gel (A) and identified by mass spectrometry. Approximate positions of prestained molecular weight markers are indicated on the left of the panel. (B–D) Western blots showing coimmunoprecipitation of Sir2 with Net1-HA3 and Myc9-Net1 from whole cell yeast extracts, and the absence of Sir3 or Sir4 in Net1-HA3 immunoprecipitates. + and − denote the presence or absence of the indicated protein. Δ denotes deletion of the *SIR2* gene.

Esposito (1989) originally discovered that *SIR2* is required for repressing mitotic and meiotic intrachromosomal recombination within the rDNA repeats. This observation provided the first clue that a silencing-related mechanism may operate in regulating rDNA chromatin structure. More recently it has been shown that expression of a number of pol II–transcribed genes is silenced when they are inserted within the rDNA repeats (Bryk et al., 1997; Fritze et al., 1997; Smith and Boeke, 1997), and that *SIR2* and *UBC2/RAD6* are required for this silencing. rDNA silencing does not require *SIR3* or *SIR4*, both of which are absolutely required for telomeric and mating-type silencing (Aparicio et al., 1991; Fritze et al., 1997; Smith and Boeke, 1997), and the role of histones in this process has not yet been fully evaluated. Thus, a distinct silencing mechanism operates at rDNA that uses some of the same components as silencing at the silent mating–type loci and telomeres.

The general requirement for Sir2 in all three examples of silencing described in *S. cerevisiae*, together with the universal conservation of *SIR2*-like genes (Brachmann et al., 1995), suggests that Sir2 plays a pivotal role in silencing that is likely to be conserved in other organisms. Sir2 forms a complex with the Sir3 and Sir4 proteins that mediates the conversion of telomeric and donor mating–type DNA regions into silent chromatin (Moazed et al., 1997; Strahl-Bolsinger et al., 1997). We have identified a new Sir2 protein complex that is distinct from the previously described Sir2 complexes containing the Sir3 and Sir4 proteins. Using protein affinity chromatography, we identified a 175 kDa protein, named Net1, that specifically bound to Sir2 but not to the Sir3 or Sir4 proteins. *NET1* was required for silencing of a *URA3* reporter gene inserted within the rDNA repeats, was specifically associated with rDNA, and was required for the association of Sir2 with rDNA. However, the association of Net1 with rDNA was independent of *SIR2*. In agreement with these results, immunolocalization experiments showed that the Net1 protein was localized to a fibrous subdomain of the nucleolus. Sir2, which has previously been localized to a nucleolar subdomain (Gotta et al., 1997), also localized to this fibrous structure but partially dissociated from it and dispersed throughout the nucleus in the anaphase period of the cell cycle.

Net1 appears to be a multifunctional protein that regulates several aspects of the organization and function of the nucleolus. For example, in addition to loss of rDNA silencing, the restricted nucleolar localization of Nop1, a major nucleolar protein, was partially lost in *net1*Δ cells, suggesting that Net1 also plays a role in maintaining nucleolar integrity. Furthermore, Net1 is also associated with the Cdc14 phosphatase and regulates exit from telophase (Shou et al., 1999 [this issue of *Cell*]). The complex containing Net1, Sir2, and Cdc14 has therefore been named RENT, for *r*egulator of *n*ucleolar silencing and *t*elophase exit (Shou et al., 1999 and this report). Like Net1 and Sir2, we found that Cdc14 was preferentially associated with rDNA.

Results

Identification of Net1 as a Sir2-Binding Protein

We used protein affinity chromatography to identify proteins that interact with Sir2. We previously reported that a nonoverlapping set of proteins bound to affinity columns composed of GST-Sir2 or GST-Sir4-C-terminal domain (Moazed and Johnson, 1996). A major protein of approximately 175 kDa was specifically retained on the GST-Sir2 affinity column in these experiments (Figure 1A). Mass spectrometry analysis identified this protein as the yeast YJL076w protein. *YJL076w* encodes a protein of 1189 amino acids with a predicted molecular weight of 128.5 kDa and has subsequently been renamed, *NET1*, for *NUS1/ESC5/TAB2*, to recognize its

independent identification as *NUS1* (Nucleolar Silencing protein; this study), *ESC5* (Establishes Silent Chromatin; R. Sternglanz, personal communication and cited in *Saccharomyces* Genome Database), and *TAB2* (Telophase Arrest Bypassed; Shou et al., 1999). For simplicity we propose to refer to *NET1* as Nucleolar silencing Establishing factor and Telophase regulator. Net1 shares significant sequence similarity with only one other protein in the sequence databases, the Topoisomerase-interacting Factor 2 (Tof2; Park and Sternglanz, 1999) of *S. cerevisiae*. Net1 and Tof2 are 22% identical (40% similar) over an 828–amino acid span. Most of the conserved residues are concentrated within the N-terminal 200 amino acids of each protein, which display 30% sequence identity (50% similarity).

To characterize further the interaction of Net1 with Sir2, we constructed yeast strains in which the endogenous copy of the *NET1* gene was modified to encode a protein with either three copies of the hemagglutinin (HA) epitope at its C terminus or nine copies of the Myc epitope at its N terminus. Unlike *net1*Δ cells, which grew very slowly (see below), the *NET1-HA3* and *Myc9-NET1* strains had no growth defect suggesting that the tagged proteins were functional. These strains were used to test the interaction of Net1 with Sir2 by coimmunoprecipitation from yeast whole cell extracts. Immunoprecipitation of Net1-HA3 using an anti-HA antibody (Figure 1B, top), or Myc9-Net1 using an anti-Myc antibody (Figure 1D, bottom), also immunoprecipitated Sir2 (Figures 1B, bottom and 1D, top). Similarly, immunoprecipitation of Sir2 using an anti-Sir2 antibody (Figures 1B and 1D), also immunoprecipitated Net1-HA3 (Figure 1B, top) and Myc9-Net1 (Figure 1D, bottom). However, while immunoprecipitation of Sir2 under these conditions efficiently coprecipitated Sir4 (Moazed et al., 1997, and data not shown), the Net1-HA3 immunoprecipitates contained no detectable Sir3 or Sir4 (Figure 1C). The coimmunoprecipitation of Sir2 and Myc9-Net1 or Net1-HA3 required both a functional *SIR2* gene (Figure 1D) and the presence of appropriately tagged Net1, verifying the specificity of the observed interactions (Figures 1B and 1D). These results provided an independent confirmation of the affinity column results and further indicated the existence of a distinct Sir2 complex that did not contain Sir3 or Sir4. Additional support for the existence of this complex comes from immunoaffinity purification of Net1 by Shou et al. (1999) who have identified Sir2 and Cdc14 as Net1-associated proteins.

NET1 Is Required for rDNA Silencing

To test the silencing phenotype of a *NET1* null mutation, we constructed a heterozygous diploid strain in which one of the two copies of the *NET1* gene was entirely replaced with *HIS3*. Haploid progeny resulting from the sporulation of this diploid strain produced two fast-growing colonies (Figure 2A) and two slow-growing colonies that were only visible after 5 days of growth (Figure 2B). In every case the *HIS3* marker cosegregated with slow growth, indicating that *NET1* was required for normal growth rates. *net1*Δ haploid colonies contained cells of abnormal size and morphology that often formed extended chains (Figure 2C).

Standard assays used for assessing silencing are based on growth of strains bearing reporter genes on selective or counterselective media (Gottschling et al., 1990; Smith and Boeke, 1997). In order to minimize the effect of the growth defect of *net1*Δ cells in interpretation of the results of such silencing assays, we constructed *net1*Δ strains that had reporter genes (wild-type *URA3* or a modified *URA3* gene referred to as *mURA3*) inserted within the rDNA repeats, near a telomere, or within the nonsilenced loci, *LEU2* or *ADH4* (Gottschling et al., 1990; Smith and Boeke, 1997). In this way, the Ura phenotype is solely a function of the location of the *URA3* gene in comparisons of strains of otherwise identical genotype and growth rates. As reported previously (Smith and Boeke, 1997), in a *NET1*$^+$ strain the *URA3* reporter gene inserted within the rDNA repeats was efficiently silenced, and the strain grew poorly on medium lacking Uracil (Ura$^-$) but grew well on medium containing 5-fluoro-orotic acid (5-FOA), which counterselects against Ura$^+$ strains (Figure 2D, row 2). An identical strain carrying a nonsilenced *URA3* reporter gene, inserted at *LEU2*, grew well on Ura$^-$ medium and did not form colonies on 5-FOA medium (Figure 2D, row 1). In contrast, *net1*Δ*::HIS3, mURA3::LEU2* and *net1*Δ*::HIS3, mURA3::rDNA* strains were indistinguishable from each other on Ura$^-$ media (Figure 2D, compare rows 5 and 6). Similar to what has been previously reported for *sir2*Δ strains, about 1% of the *net1*Δ*, mURA3::rDNA* cells formed colonies on 5-FOA plates (Smith and Boeke, 1997); all these colonies were Ura$^-$ (data not shown), indicating that they resulted from loss of the *URA3* marker due to hyperrecombination rather than residual silencing.

As a further control, we tested the rDNA silencing phenotype of an allele of *NET1* that has a less severe growth defect than *net1*Δ cells. This allele, *net1-1*, was isolated in a genetic screen for *cdc15*Δ bypass mutations (Shou et al., 1999). *net1-1* cells were also defective in rDNA silencing as indicated by their ability to grow on Ura$^-$ medium with equal proficiency whether the *URA3* reporter gene was located within the rDNA (Figure 2D, rows 10 and 11) or at a control nonsilenced locus (Figure 2D, row 9). As with the *net1*Δ cells, the *net1-1, mURA3::rDNA* strain produced many 5-FOA resistent colonies. Again, all of these colonies were Ura$^-$, indicating that they resulted from recombination and loss of the *URA3* reporter.

Silencing is sensitive to changes in the levels of structural components of silent chromatin. For example, increasing the dosage of the Sir3 protein results in more efficient telomeric silencing and deletion of one copy of the *SIR2* gene in a diploid strain results in less efficient rDNA silencing (Renauld et al., 1993; Smith et al., 1998). We took advantage of this phenomenon to test whether the requirement of *NET1* for rDNA silencing could be separated from its growth defect by assessing silencing in diploid strains that had one copy of the *NET1* gene deleted. Unlike *net1*Δ haploid cells, *net1*Δ*/NET1*$^+$ heterozygote diploids had no growth or cell separation defect (data not shown). However, compared to *NET1*$^+$*/NET1*$^+$*, mURA3::rDNA* diploids, *net1*Δ*/NET1*$^+$*, mURA3::rDNA* heterozygotes displayed a 500- to 1000-fold reduction in their ability to form colonies on 5-FOA medium and grew as well on Ura$^-$ medium as cells containing the *URA3* reporter at the nonsilenced *LEU2* locus (Figure 2E, compare rows 1–3). For comparison, as reported

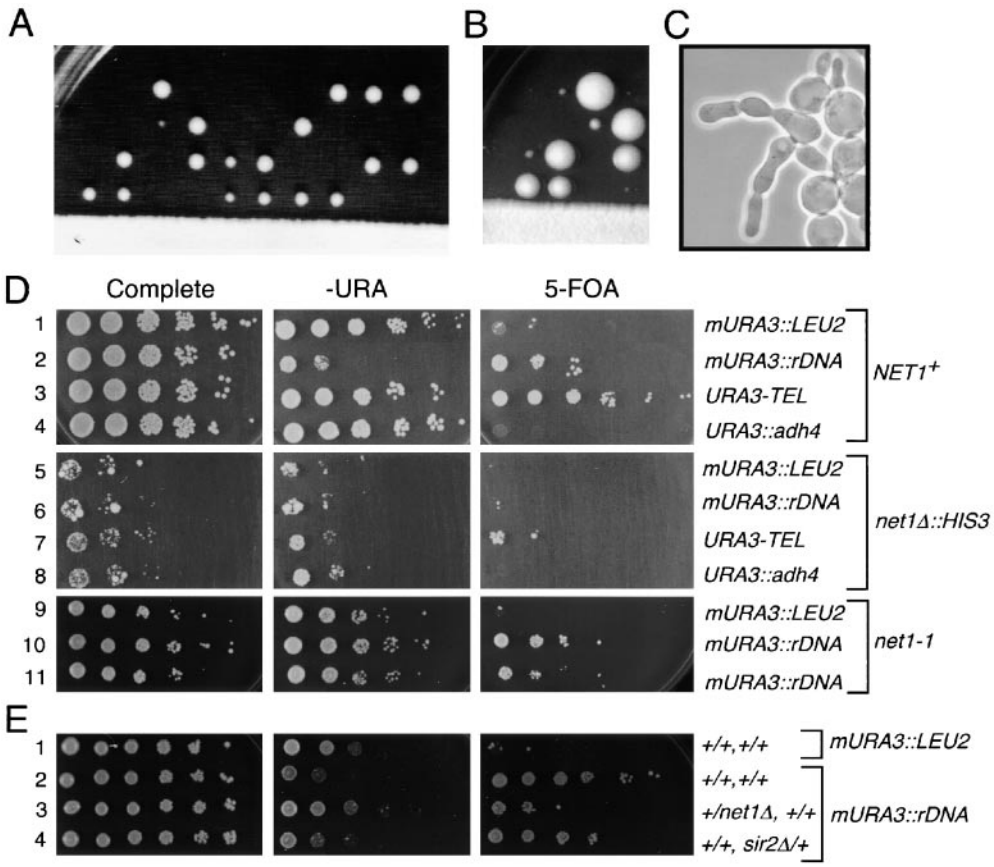

Figure 2. Deletion of *NET1* Causes a Growth Defect and Results in Loss of rDNA Silencing

For assessment of rDNA silencing, the *NET1* open reading frame was replaced in diploid strains that were either *ura3*$^-$ or contained a modified *URA3* gene (*mURA3*) within rDNA (*mURA3::*rDNA) or within the *LEU2* locus (*mURA3::LEU2*); to assess telomeric silencing, *NET1* was deleted in diploid strains that had a wild-type copy of the *URA3* gene integrated either near chromosome VIIL telomere (*URA3-TEL*), or at *ADH4* (*URA3::adh4*). Haploid progeny of these diploid strains were used to determine the rDNA and telomeric silencing phenotypes of *net1*Δ cells. Silencing was also assessed in diploids containing only one copy of *NET1*.

(A) Haploid progeny from sporulation of a diploid strain in which one copy of the *NET1* gene was replaced with *HIS3* after 2 days of growth at 30°C.

(B) Same as (A) but after 5 days of growth.

(C) Higher magnification image showing the growth of *net1*Δ cells as chains.

(D) Ten-fold serial dilutions of cultures of haploid strains showing loss of rDNA silencing in *net1*Δ*::HIS3* and *net1-1* cells.

(E) Reduced rDNA silencing of *mURA3* in *net1*Δ*::HIS3*/+ diploid cells.

previously, deletion of one copy of the *SIR2* gene resulted in a 10-fold decrease in growth on 5-FOA and also a 10-fold increase in growth on Ura$^-$ medium (Smith et al., 1998; and see Figure 2E). These results indicated that rDNA silencing was highly sensitive to a reduction in the dosage of *NET1* and established a role for *NET1* in rDNA silencing independent of its role in growth.

The effect of deletion of *NET1* on telomeric silencing was also tested. *net1*Δ cells containing *URA3* near a telomere grew slower on Ura$^-$ medium than did *net1*Δ cells containing *URA3* at *ADH4* (Figure 2D, rows 7 and 8, compare to rows 3 and 4 for *NET1*$^+$), suggesting that *net1*Δ cells have slightly increased levels of telomeric silencing. However, whereas about 75% of wild-type cells containing telomeric *URA3* were able to form colonies on 5-FOA medium (Figure 2D, row 3), only 1%–10% of *net1*Δ cells were able to grow on 5-FOA (Figure 2D, row 7). The poor plating efficiency of *net1*Δ*, URA3-TEL* cells on 5-FOA medium may be an indirect effect of their growth defect. For example, if the *net1*Δ cells are defective in cell separation or cytokinesis, as suggested by their growth as chains (Figure 2C), loss of silencing in one cell within a chain of cells may cause 5-FOA toxicity in all the cells within the chain. Deletion of one copy of either *NET1* or *SIR2* had no significant effect on silencing of a telomeric *URA3* gene in a diploid strain (data not shown).

Net1 Is Preferentially Associated with rDNA

The association of Net1 with Sir2 together with the silencing phenotype of *net1* mutant cells suggested that the Net1 protein may be associated with silent chromatin. Chromatin cross-linking and immunoprecipitation experiments have previously shown that the Sir2 protein is associated with rDNA, telomeric DNA, and the silent mating–type loci (Gotta et al., 1997; Strahl-Bolsinger et al., 1997). These loci comprise all the known silent chromatin domains in budding yeast. We used the chromatin

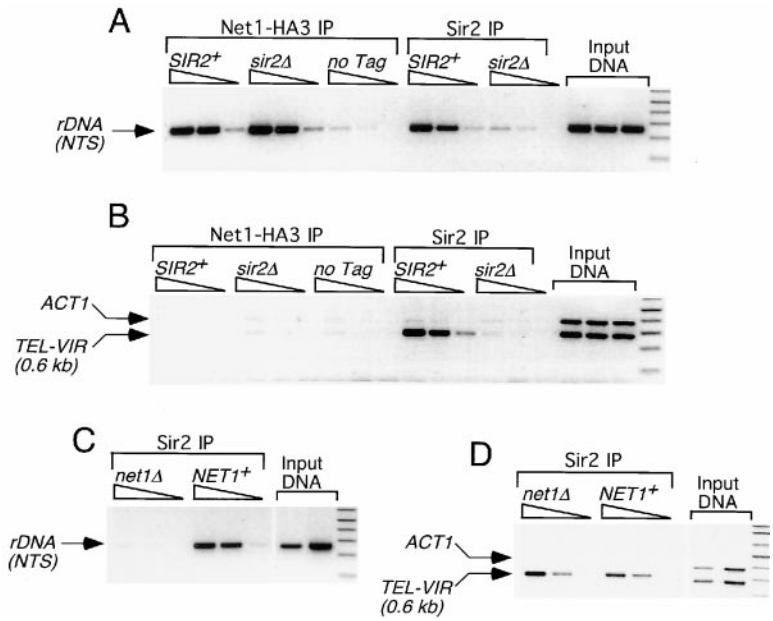

Figure 3. Net1 Preferentially Cross-Links to rDNA and Is Required for Cross-Linking of Sir2 to rDNA

(A) Immunoprecipitation of chromatin from formaldehyde cross-linked cells showing the association of Net1-HA3 with the nontranscribed spacer region of rDNA (NTS) in *SIR2*$^+$ and *sir2*Δ cells. As a control, immunoprecipitations were also carried out using an anti-Sir2 antibody. (B) Unlike Sir2, Net1-HA3 does not cross-link to telomeric DNA (*TEL-VIR*, 0.6 kb). Immunoprecipitation of cross-linked chromatin with an anti-Sir2 antibody from *net1*Δ and *NET1*$^+$ cells showing that cross-linking of Sir2 to rDNA (C) but not telomeric DNA (D) is Net1-dependent. PCR amplification was performed with 2.5-fold serial dilutions of immunoprecipitated DNA, corresponding to 1/50th, 1/125th, and 1/250th of immunoprecipitated DNA. Input DNA, represents PCR amplification of approximately 1/10,000th of crude chromatin used for each immunoprecipitation reaction.

cross-linking and immunoprecipitation assay to test whether Net1 is associated with some or all of these regions. We precipitated formaldehyde-cross-linked soluble chromatin using an HA-tagged Net1 protein (Net1-HA3). Control immunoprecipitations of chromatin were also carried out using an anti-Sir2 antibody and with strains that either lacked an HA-tagged Net1 or had a deletion of the *SIR2* gene. The results showed that Net1 is associated with rDNA (Figure 3A and data not shown). However, unlike Sir2, immunoprecipitation of Net1-HA3 did not coprecipitate telomeric DNA fragments near the right arm of chromosome VI (Figure 3B and data not shown). Neither Net1-HA3 nor Sir2 was associated with nonsilenced loci, including *ACT1*, *GAL1*, or the mildly repetitive *CUP1* gene (Figure 3B, Figures 4A and 4B, and data not shown). Precipitation of rDNA with anti-HA and anti-Sir2 antibodies depended on the presence of HA-tagged *NET1* and wild-type *SIR2*, respectively (Figure 3A). Interestingly, immunoprecipitation of rDNA fragments with Net1-HA3 occurred with the same efficiency regardless of whether chromatin was prepared from a *SIR2*$^+$ strain or a *sir2*Δ strain, indicating that Net1 associated with rDNA independently of Sir2 (Figure 3A). Net1 had a similar association pattern with DNA fragments within the *25S* rDNA transcribed region and did not associate with a DNA fragment located 1.4 kb from the right arm of chromosome VI; in addition, both Net1 and Sir2 cross-linked preferentially to a single copy *URA3* gene inserted at rDNA compared to the same *URA3* gene inserted at a nonsilenced locus (data not shown).

Net1 Tethers Sir2 to rDNA

Since Net1 associated with rDNA independently of Sir2, we asked whether Net1 was required to localize Sir2 to rDNA. To test this possibility, we compared the cross-linking of Sir2 to chromatin in *NET1*$^+$ and *net1*Δ cells. Deletion of *NET1* abolished the association of Sir2 with rDNA (Figure 3C) but had no effect on the association of Sir2 with a telomeric DNA fragment (Figure 3D). Similarly, cross-linking of Sir2 to rDNA was greatly reduced in *net1-1*, an allele of *NET1* that was also defective in rDNA

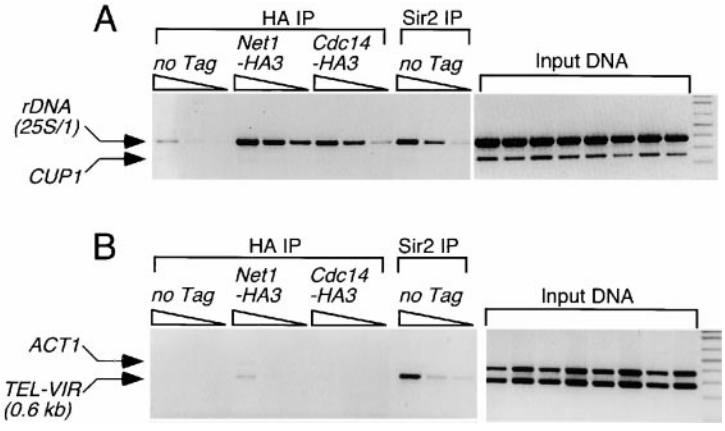

Figure 4. Preferential Association of Cdc14 with rDNA

(A) Immunoprecipitation of formaldehyde cross-linked chromatin showing the association of Cdc14-HA3 with the *25S* region of rDNA. Cdc14-HA3 cross-linked to rDNA to the same extent as Sir2 but less efficiently than Net1-HA3 (A). Like Net1-HA3, Cdc14-HA3 did not cross-link to DNA fragments from the *CUP1* locus, *ACT1*, or the right telomere of chromosome VI (*TEL-VIR*). PCR amplification was performed with three 2.5-fold serial dilutions of immunoprecipitated DNA. Two 2.5-fold serial dilutions of input DNA for each strains are shown. See Figure 3 legend for other details.

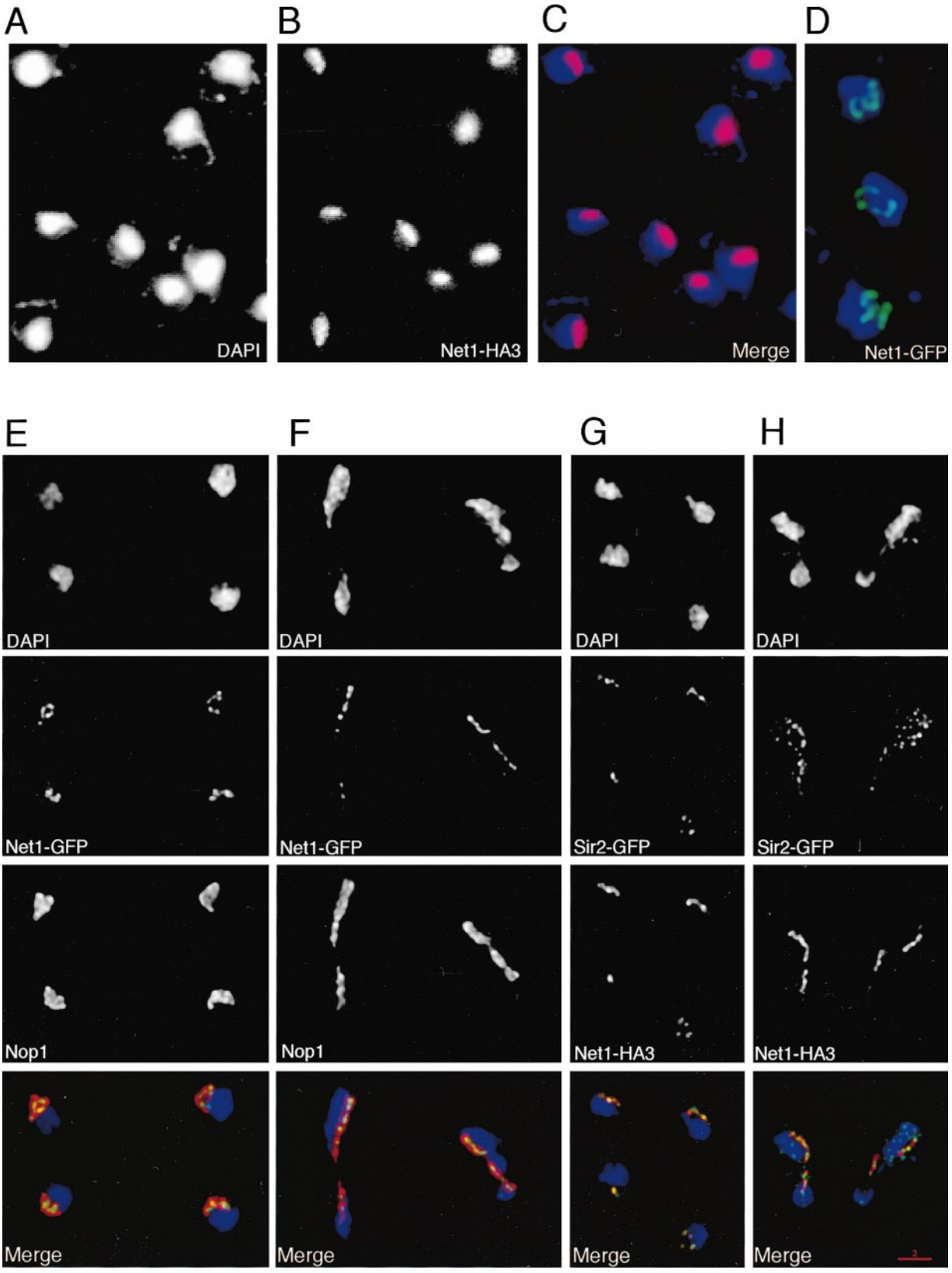

Figure 5. Localization of Net1 and Sir2 to a Fibrous Subdomain within the Nucleolus, and Release of Sir2 from the Nucleolus in Anaphase

(A–C) Indirect immunofluorescence showing the localization of Net1-HA3 to a nuclear subdomain resembling the nucleolus. (D) Three dimensional image reconstruction after deconvolution of yeast cells expressing a GFP-tagged Net1 protein (Net1-GFP). Net1 staining (green) appears as strings (top), a ring (middle), or rods (bottom) closely abutting the DAPI-stained DNA mass (blue).

silencing (data not shown, see Figure 2D). *NET1* therefore acts upstream of *SIR2* and provides the sole pathway for the localization of Sir2 to rDNA.

Cdc14 Is Preferentially Associated with rDNA

Net1 has been independently identified as a Cdc14-associated protein that regulates exit from mitosis (Shou et al., 1999). We used the chromatin cross-linking and immunoprecipitation assay to determine whether Cdc14, like Net1 and Sir2, is associated with rDNA. To perform these experiments, we used a strain in which the *CDC14* gene was modified to encode a protein with three HA epitopes at its C terminus (Shou et al., 1999). Immunoprecipitation of Cdc14-HA3 or Sir2 from formaldehyde-cross-linked cells using anti-HA and anti-Sir2 antibodies, respectively, coprecipitated rDNA with a similar efficiency but did not coprecipitate *CUP1* or *ACT1* DNA (Figures 4A and 4B). Similar results were obtained using primers that amplified fragments from a different region within the *25S* rDNA or from the rDNA nontranscribed spacer (data not shown). Similar to Net1-HA3 and unlike Sir2, Cdc14-HA3 did not cross-link to telomeric DNA (Figure 4B). As described above for Sir2, the cross-linking of Cdc14-HA3 to rDNA was abolished in *net1*Δ and *net1-1* cells (D. M., unpublished).

Net1 Is Localized to a Fibrous Subdomain in the Nucleolus

We examined the subcellular localization of the Net1 protein tagged at its C terminus with either 3 HA epitopes (Net1-HA3) or the green fluorescent protein (Net1-GFP). Immunofluorescence detection showed that both Net1-HA3 and Net1-GFP localized to a tightly restricted subdomain in the nucleus that overlaps the location of Nop1, a well-characterized nucleolar marker (Figures 5A–5H) (Aris and Blobel, 1988). Localization of both Net1-GFP and Net1-HA3 revealed previously undescribed structural details of the yeast nucleolus; in many images Net1 staining appeared as a series of dots often arranged in string-, ring-, or rod-like structures (Figure 5D–5H). Optical sectioning and image reconstruction showed that Net1 was localized to a fibrous structure that forms a subdomain within the Nop1-staining region (Figures 5E and 5F). This staining pattern is reminiscent of the yeast rDNA visualized by in situ hybridization using fluorescent probes (Guacci et al., 1994) and may correlate with the rDNA-containing fibrillar center previously described for nucleoli of multicellular eukaryotes.

Sir2 Is Released from the Nucleolus during Mitosis

The Sir2 protein has been previously shown to localize to a nucleolar subdomain as well as to telomeric foci located near the nuclear periphery (Gotta et al., 1997). We also tested the localization of Sir2 to the fibrous nucleolar subdomain containing Net1. This experiment was performed in a *sir4*Δ strain, because in *sir4*Δ cells, Sir2 localizes exclusively to the nucleolus (Gotta et al., 1997), making it easier to distinguish the details of its staining pattern. As expected from the biochemical interaction of Sir2 and Net1, we observed that Sir2-GFP colocalized predominately with Net1-HA3 in the fibrous nucleolar subdomain. Shou et al. (1999) have demonstrated that the Net1-dependent localization of Cdc14 in the nucleolus is lost at the end of mitosis. We therefore asked whether Sir2 was similarly released from the nucleolus. Surprisingly, most of the Sir2 signal was delocalized from the nucleolus and appeared as foci dispersed throughout the nucleus in anaphase (Figure 5H). In contrast, Net1-GFP remained associated with the nucleolar Nop1 staining domain in the same anaphase cells (Figures 5F and 5H).

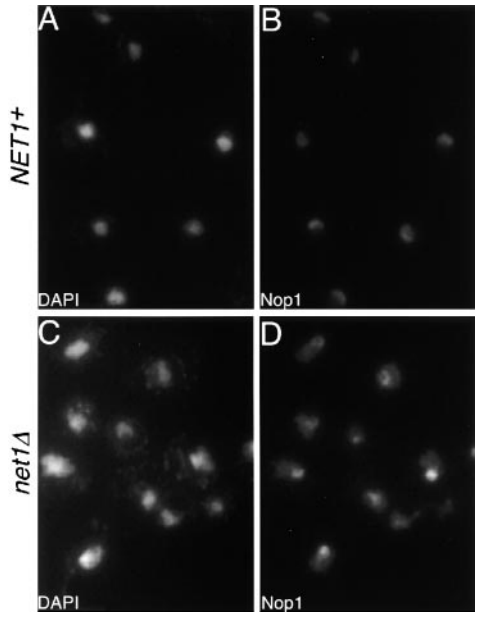

Figure 6. Delocalization of the Nucleolar Marker Nop1 in *net1*Δ Cells

(A) DAPI-stained *NET1*$^+$ cells, (B) Nop1 staining in *NET1*$^+$ cells, (C) DAPI-stained *net1*Δ cells, (D) Nop1 staining in *net1*Δ cells. Nop1 staining appears as a DAPI-excluded crescent area in *NET1*$^+$ cells (B), but spreads over the entire nucleus in *net1*Δ cells (D).

***NET1* Is Required for the Proper Nucleolar Localization of Nop1**

Net1 remains associated with rDNA in *sir2*Δ cells (Figure 3A) and in cells arrested at the *cdc14* block (D. M., unpublished results), and may therefore play a role in rDNA and nucleolar organization independent of its other functions. To test this idea, we determined whether *NET1* was required for maintaining nucleolar organization by comparing the staining pattern of Nop1 in *NET1*$^+$

(E and F) 3D image reconstruction of optical sections showing the colocalization of Net1-GFP with Nop1 in interphase and anaphase cells, respectively.

(G and H) *sir4*Δ cells showing the colocalization of Net1-HA3 with Sir2-GFP in interphase (G) but not anaphase cells (H). Color frames in (C), (D), and the bottom frame of panels (E) and (F), are merged images of DAPI (blue), FITC (green), and rhodamine (red) channels shown separately in black and white. In each case, Net1-HA3 and Nop1 were detected using anti-HA and anti-Nop1 primary antibodies and a rhodamine-conjugated secondary antibody; Net1-GFP and Sir2-GFP (E and F) were detected using an anti-GFP primary antibody and a FITC-conjugated secondary antibody. Scale bar, 2 μm.

and *net1*Δ cells. In wild-type cells, Nop1 staining appears as a crescent adjacent to the bulk of the DAPI-stained DNA mass (Aris and Blobel, 1988; see Figures 5E and 6B). In *net1*Δ cells, the restricted staining pattern of Nop1 was partially lost (Figures 6C and 6D). Instead of the tight crescent observed in wild-type cells, Nop1 staining was spread throughout the entire nucleus and a nucleolar-like subdomain was less evident. These results indicated that *NET1* was required for either the restricted subnuclear localization of the nucleolus or for preventing dissociation of nucleolar material from the nucleolus. Since the Nop1 protein is involved in rRNA processing and maturation and is therefore present at the site of rDNA transcription (Tollervey et al., 1993), Nop1 delocalization suggests that rDNA itself may be partially delocalized in *net1*Δ cells.

Discussion

Our findings suggest that Sir2 executes its rDNA silencing functions as a component of the nucleolar RENT complex. Net1, a core subunit of the RENT complex, appears to be a multifunctional protein that regulates several aspects of the structure and function of the nucleolus. In addition to its role in rDNA silencing, Net1 is required for the proper localization of the nucleolus itself (this study) and regulates the mitotic exit function of Cdc14 (Shou et al., 1999). Together, these studies identify the same protein complex with functions in rDNA silencing, nucleolar integrity, and the regulation of exit from mitosis, cellular processes heretofore thought to be unrelated. Below, we discuss the multiple functions of the RENT complex.

RENT and rDNA Silencing

Several lines of evidence argue that RENT is an rDNA-specific silencing complex. First, this complex contains Sir2 but lacks Sir3 or Sir4. These three Sir proteins are each required for silencing at the silent mating–type loci and telomeres, but only Sir2 is required for rDNA silencing (Aparicio et al., 1991; Bryk et al., 1997; Rine and Herskowitz, 1987; Smith and Boeke, 1997). Second, deletion of *NET1*, a specific subunit of this complex, results in an apparent complete loss of silencing of a *URA3* reporter gene inserted within the rDNA repeats in haploid cells; in diploid cells rDNA silencing is highly sensitive to a reduction in the dosage of *NET1*. In contrast, deletion of *NET1* only has a marginal effect on telomeric silencing, and this effect may well be an indirect consequence of the severe growth defect of *net1*Δ cells. Third, the Net1 protein is preferentially associated with rDNA repeats compared to other positions in the genome. Unlike the Sir2, Sir3, or Sir4 proteins, Net1 shows no significant association with telomeric DNA sequences. Finally, Net1 localizes exclusively to the nucleolus, as would be expected from a component of an rDNA-specific silencing complex. The evidence presented here and elsewhere (Moazed et al., 1997; Strahl-Bolsinger et al., 1997), indicates the existence of two types of Sir2-containing silencing complexes in yeast: a Sir2/Sir4 complex that together with the Sir3 protein acts in telomeric and mating-type silencing, and a Sir2/Net1 complex that acts in rDNA silencing (Figure 7). While both Net1 and Sir2 are required for efficient rDNA silencing, the role of Cdc14 in this process remains to be determined. Cdc14 is not required for the association of Sir2 with rDNA (D. M., unpublished). The possible role of Cdc14 in rDNA silencing would therefore have to follow the recruitment of Sir2 to rDNA. Alternatively, the function of Cdc14 in the RENT complex could be limited to its role in promoting exit from mitosis and regulating an unknown aspect of nucleolar structure that is important for nucleolar segregation during mitosis, but may not be required for rDNA silencing per se.

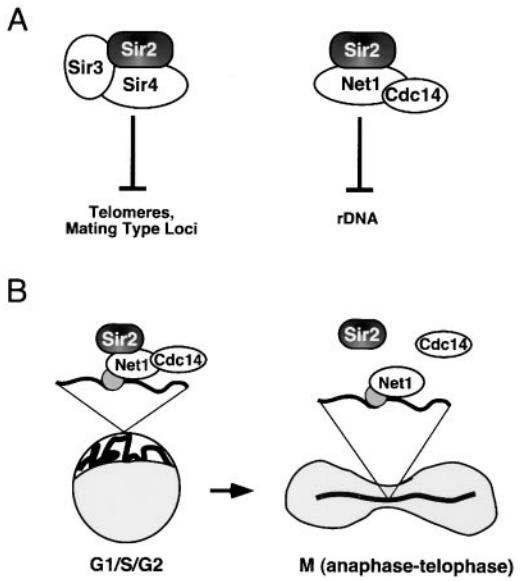

Figure 7. Schematic Summary of the Yeast Silencing Complexes and Dynamic Nature of the RENT Complex

(A) The Sir2, Sir3, and Sir4 proteins assemble into a complex that mediates the conversion of the mating-type loci and telomeric DNA regions into silent chromatin (Moazed et al., 1997; Strahl-Bolsinger et al., 1997). rDNA silencing is mediated by the RENT complex containing Sir2, Net1, and Cdc14 (this study; Shou et al., 1999).

(B) The rDNA-associated RENT complex localizes to a fibrous subdomain within the nucleolus (represented as a loopy black line within the nucleolar crescent). Net1 localizes to rDNA independently of the other known subunits of the RENT complex, through interactions that may require an unknown factor(s) (represented as gray circles) and anchors the remaining subunits of RENT to rDNA (see Discussion). Sir2 and Cdc14 are released from RENT at the end of mitosis; Cdc14 release promotes exit from mitosis (Shou et al., 1999), Sir2 release is likely to result in a change in the structure rDNA silent chromatin during late mitosis.

One function of Net1 involves the recruitment of the Sir2 protein to the rDNA repeats. In *net1*Δ cells the association of Sir2 with rDNA is abolished; however, Net1 localizes to the nucleolus and associates with rDNA in the absence of Sir2 (Figure 3; and D. M., unpublished). At the silent mating–type loci and telomeres, Sir2 appears to be recruited to DNA through the Sir4 protein, which has been shown to interact, directly or indirectly, with DNA-bound proteins Rap1 and ORC (Moretti et al., 1994; Triolo and Sternglanz, 1996). An analogous situation appears to exist at rDNA where Net1 localizes independently of Sir2 and is required for the localization of Sir2 to rDNA (Figure 7). How Net1 itself is localized to rDNA, however, is unknown. Net1 does not have a

recognizable DNA-binding domain and may localize to rDNA through interactions with other rDNA-bound factor(s), perhaps through interactions with a specific combination of transcriptional activators of rRNA genes. Alternatively, specific DNA binding could be accomplished through an unidentified component of the RENT complex. It also remains to be determined whether Net1 recruits other silencing proteins, for example, one or more of the yeast Sir2-like proteins (Hst1–4), to rDNA in addition to Sir2. Association of one of the yeast Sir2-like proteins, Hst1, with rDNA has been reported (Gotta et al., 1997).

The inactivation of pol II–transcribed genes that have been artificially inserted within the rDNA repeats provides clear experimental evidence of silencing within the rDNA repeats, but does not reveal the biological role of this phenomenon. The known biologically relevant functions of rDNA silencing include the repression of recombination within the repeats and delaying cellular senescence (Bryk et al., 1997; Gottlieb and Esposito, 1989; Sinclair and Guarente, 1997). The latter phenomenon involves the Sir2-dependent relocalization of the Sir3 and Sir4 proteins to the nucleolus in old cells (Gotta et al., 1997; Kennedy et al., 1997). However, silencing of rDNA repeats may also be involved in controlling the extent of transcriptional activity at rDNA. As mentioned in the introduction, less than half of all rDNA repeats are active at any given time, but deletion of *SIR2* causes only a modest increase in the ratio of active to inactive repeats (from 40% active to 50% active; Smith and Boeke, 1997). It is possible that the remaining inactive repeats in *sir2*Δ cells are indicative of rDNA silencing that is Sir2 independent, but that may still be Net1 dependent. According to this idea, the association of Net1 with rDNA independently of Sir2 may allow it to recruit silencing proteins that are at least partly redundant with Sir2 (e.g., Hst1–4, mentioned above). Consistent with this idea, *net1*Δ cells (unlike *sir2*Δ cells) have a severe growth defect. An intriguing possibility is that the loss of rDNA silencing in *net1*Δ cells reflects a more profound change in rDNA structure that may be the underlying cause of deleterious events, such as delocalization of the nucleolus and poor growth.

RENT and Mitotic Segregation of the Nucleolus

In addition to its role in rDNA silencing, RENT is a central player in a newly discovered mechanism that regulates exit from mitosis (Shou et al., 1999). The Cdc14 phosphatase is sequestered in the nucleolus in the RENT complex until telophase, at which time partial or complete disassembly of the complex releases Cdc14 from the nucleolus and allows it to promote exit from mitosis (Shou et al., 1999). The findings of Granot and Snyder (1991) suggest that Cdc14 has an additional role that involves the segregation of nucleolus during mitosis. In *cdc14*-arrested cells, a major nucleolar protein, Nop1, fails to segregate with the mass of DNA during mitosis (Granot and Snyder, 1991). Cdc14 may therefore be involved in a distinct mechanism that ensures the segregation of either a specialized chromosome domain (i.e., rDNA) or a specific nuclear compartment (i.e., the nucleolus). The presence of Cdc14 in the RENT complex (Shou et al., 1999) and its association with rDNA (this study) point to rDNA chromatin as a likely target for the nucleolar segregation function of Cdc14.

A Possible Change in rDNA Structure during Late Mitosis

The localization studies presented by Shou et al. (1999) and in this report suggest that RENT is a dynamic protein complex whose molecular composition changes during late mitosis. The Net1 subunit of RENT remains localized in the nucleolus throughout mitosis, but both Cdc14 and Sir2 dissociate from the nucleolus in the anaphase–telophase period of the cell cycle (Figure 7B). While the biological significance of Sir2 release from the nucleolus is unknown, it strongly suggests that the structure of rDNA changes in late mitosis. Such changes in rDNA structure or silencing during the cell cycle have not been reported, but have been suggested for telomeric silencing. Activation of a silenced telomere-proximal *URA3* reporter gene by the *URA3 trans*-activator, Ppr1, can only occur in the G2/M period of the cell cycle (Aparicio and Gottschling, 1994). This observation suggests that a change in the structure of telomeric silent chromatin takes place during the cell cycle. A restricted period during the cell cycle may therefore provide a window of opportunity for reprogramming of silent chromatin.

RENT and Subnuclear Localization of the Nucleolus

The nucleolus contains the rDNA repeats, the RNA polymerase I complex that transcribes rDNA, and the machinery that processes and assembles rRNA into ribosomes (Shaw and Jordan, 1995). In yeast, the nucleolus forms a tight crescent that occupies approximately one-third of the nuclear volume and is positioned adjacent to the nuclear periphery (Aris and Blobel, 1988). The molecular interactions that promote this distinct subnuclear localization have not yet been defined. The Net1 subunit of the RENT complex, in addition to its silencing function, may be required for the proper localization of the nucleolus within the nucleus. In *net1*Δ cells, a fraction of the nucleolar marker protein Nop1 spreads over the entire nucleus, and the intense Nop1 staining near the nuclear periphery is only weakly evident (Figure 6D). Since Nop1 is usually found at the site of rRNA transcription (Tollervey et al., 1993), Nop1 delocalization suggests that rDNA itself may be delocalized throughout the nucleus in *net1*Δ cells. Net1 may participate in interactions that tether the rDNA to the nuclear periphery, perhaps through interactions with the nuclear envelope. Alternatively, Net1 may promote the association of rDNA with the rest of the nucleolus, which may be localized independently of rDNA. Whether this delocalization of the nucleolus contributes to the rDNA silencing defect of *net1*Δ cells is unknown, but we note that telomere-associated silencing proteins, including Sir2, aggregate into foci near the nuclear periphery (Palladino et al., 1993), and this type of perinuclear localization has been shown to contribute to the ability of a weak silencer to initiate silencing (Andrulis et al., 1998).

Experimental Procedures

Strains and Plasmids

To tag Net1 with HA, a 584 bp Asp718-EagI DNA fragment corresponding to the C-terminal 195 amino acids of Net1 was PCR amplified and subcloned in frame into Yplac111d to produce pDM239. Yplac111d is a yeast integrating vector that contains the *LEU2* gene and three HA epitopes followed by a stop codon. pDM239 was linearized by digestion with AccI to target integration into *NET1* and

produce a *NET1-HA3* strain (DMY574). The entire GFP open reading frame (ORF) containing mutations S65→T and V163→A was PCR amplified from pAFS135 as an EagI-HindIII fragment (Straight et al., 1998). The HA tag in pDM239 was replaced with the EagI-HindIII GFP fragment to produce pDM266. pDM266 was linearized with BglII and transformed into yeast to produce *NET1-GFP* strains (DMY758). To tag Sir2 with GFP at its C terminus, a 405 bp EagI-HindIII DNA fragment corresponding to the C-terminal 135 amino acids of Sir2 was PCR amplified and subcloned in frame with GFP into the EagI-HindIII site of pDM266 to produce pDM268, which was linearized with StuI and transformed into yeast to produce *SIR2-GFP* strains (DMY752, DMY792, and DMY793). Correct integration was confirmed by PCR and all PCR-amplified DNA regions used for generating fusion proteins were sequenced to insure that they were free of PCR-introduced errors. The *MYC9-NET1* strain has been described (WY53; Shou et al., 1999).

Two *NET1* knockout plasmids were constructed by PCR amplification of a pair of DNA fragments flanking the *NET1* ORF and ligation of these fragments into pRS303, a yeast integrating vector containing the *HIS3* gene (Sikorski and Hieter, 1989). The first plasmid, pDM244, deletes the entire *NET1* ORF, in addition to about 350 bp of the *YJL075c* ORF, which is oriented in the opposite direction from *NET1* and overlaps 350 bp of the *NET1* ORF. The second plasmid, pDM262, deletes amino acids 1–1071 of *NET1* and does not delete any of the *YJL075c* ORF. pDM244 and pDM262 were digested with BamHI and transformed into the W303a/α diploid strain to replace *NET1* with *HIS3* (strains DMY583, and DMY725, respectively). Correct integrants were identified by PCR, sporulated, and their haploid progeny isolated by microdissection. Identical growth and silencing defects were observed for the resulting *NET1* deletions using either pDM244 or pDM262, indicating that *YJL075c* did not contribute to any of the phenotypes described here.

Protein Affinity Chromatography and Mass Spectrometry Analysis

Purification of proteins on GST-Sir2 affinity columns was as described previously (Moazed and Johnson, 1996). Elution fractions from GST-Sir2 and control columns were separated on an 8.5% SDS polyacrylamide gel and stained with Coomassie Brilliant Blue, R-250. A Coomassie-stained protein band of about 170 kDa that eluted specifically from the GST-Sir2 affinity column was excised from the gel and subjected to in-gel digestion with trypsin. Peptides were extracted from the gel and after clean up were infused into an ion-trap Mass Spectrometer (Finnigan Mat, San Jose, CA) using a nanospray capillary (Protana, Odense, Denmark). Several peptide ions were subjected to fragmentation and the resultant MS/MS data compared to the nonredundant protein database (NCBI, Bethesda, MD). One MS/MS spectrum derived from the doubly charged peptide ion 822.0 yielded a significant homology score using the SEQUEST program (University of Washington, Seattle, WA), with a hypothetical 128.5 kDa protein in the SCP160-SMC3 intergenic region of *S. cerevisiae* (# P47035, YJL076w).

Immunoprecipitation Reactions

Fifty-milliliter cultures of yeast strains DMY10 or DMY574 (Net1-HA3) were grown to mid-log phase (A660 of 0.5, approximately 4×10^8) in YEPD (1% yeast extract, 2% peptone, 2% glucose) medium. Cells were harvested by centrifugation, resuspended in 400 μl of lysis buffer (50 mM HEPES-KOH [pH 7.5], 500 mM NaCl, 10% glycerol, 0.5% NP-40, 1 mM EDTA, 2 mM Bezamidine-HCl, 1 mM PMSF, and 1 μg/ml each pepstatin, leupeptin, and bestatin. Cells were lysed in 1.5 ml plastic tubes by agitation with 1 ml of 0.5 mm glass beads (Sigma) for 30 min at 4°C, centrifuged at 14,000 rpm in a microfuge, and the supernatant was transferred to a new tube (Kaffman et al., 1994). For immunoprecipitation reactions, approximately 1 μg of either a monoclonal anti-HA antibody (HA11, Babco, Berkeley, CA) or a monoclonal anti-Sir2 antibody (Moazed et al., 1997) were added to 200–400 μl of extract ($\sim$10 mg/ml) and incubated at 4°C on a mixer for 2–3 hr. Twenty microliters of a 50% slurry of protein A–Sepharose (Pharmacia, washed 3 times with PBS) was added to the immunoprecipitation reaction and incubation and mixing were continued at 4°C for 1 hr. The immune complexes were collected by centrifugation at 2,000 rpm for 2 min in a microfuge, washed once with 1 ml lysis buffer and twice with 1 ml 50 mM HEPES-KOH (pH 7.5), 150 mM NaCl, 1 mM EDTA, resuspended in 15 μl of 2$\times$ SDS sample buffer, and heated to 85°C for 10 min. Six microliters of each sample was loaded on 8.5% SDS polyacylamide gels for Western analysis. Protein was blotted to PVDF membranes, and membranes were probed with mouse anti-HA, mouse or rabbit anti-Sir2, and rabbit anti-Sir3 and anti-Sir4. Horseradish peroxidase–coupled secondary antibodies and the ECL chemiluminescence reagents (Amersham) were used for Western detection. In these experiments, 80%–90% of Sir2 and Net1-HA3 were depleted from the extract using anti-Sir2 and anti-HA antibodies, respectively. Immunoprecipitation of Myc9-Net1 was carried out using the 9E10 anti-Myc monoclonal antibody as described in Shou et al. (1999).

Silencing Assays

rDNA and telomeric silencing were assayed as described (Gottschling et al., 1990; Smith and Boeke, 1997). Strains containing the *URA3* gene near the left telomere of chromosome VII, or at the *ADH4* locus, or a modified *URA3* gene (*mURA3*) located either within the rDNA repeats or at the *LEU2* locus were derived from diploid strains of desired genotype following sporulation and tetrad dissection. The *URA3* gene was placed near the left telomere of chromosome VII or at the *ADH4* gene by transformation of diploid cells with plasmids pVIIL,URA3-TEL and padh4::*URA3* digested with EcoRI and SalI, and BamHI and SalI, respectively (Gottschling et al., 1990). Correct integration near the telomere was determined by growth on both Ura$^-$ and 5-FOA media. The *mURA3* gene was placed either within the *LEU2* locus or within the rDNA repeats using plasmid pJSS60-2 (Smith and Boeke, 1997). pJSS60-2 contains the *LEU2* gene and a single rDNA repeat with the *mURA3* gene inserted in the 25S rRNA coding region. Yeast cells were transformed with pJSS60-2 digested with BstXI, which cuts the plasmid once within the *LEU2* gene to target integration of *mURA3* at *LEU2*, or with pJSS60-2 digested with MluI, which cuts the plasmid once within the rDNA region to target integration of *mURA3* at rDNA. Correct integration at rDNA resulted in transformants that could grow on 5-FOA-containing medium.

Chromatin Cross-Linking and Immunprecipitation

Chromatin cross-linking and immunoprecipitation reactions were performed as previously described (Strahl-Bolsinger et al., 1997). The following primers were used for PCR detection of immunoprecipitated DNA fragments: VIR-1, 5′-CAGGCAGTCCTTTCTATTTC-3′; VIR-2, 5′-GCTTGTTAACTCTCCGACAG-3′; VIR-3, 5′-AATGTCTTAT CAAGACCGAC-3′; VIR-4, 5′-TACAGTCCAGAAATCGCTCC-3′; ACT1-1, 5′-CCAATTGCTCGAGAGATTTC-3′; ACT1-2, 5′-CATGATA CCTTGGTGTCTTG-3′. VIR-1 and VIR-2, and VIR-3 and VIR-4, amplify 270 and 359 bp fragments that are 0.59 kb and 1.4 kb from the right arm end of chromosome VI. Primers for PCR amplification of *CUP1* and rDNA fragments corresponding to the rDNA nontranscribed region (NTS), and the 25S rDNA coding region (25S/1 and 25S/2) have been previously described (Gotta et al., 1997). PCR products were analyzed on 2.3% agarose gels containing 0.25 mg per liter ethidium bromide in both the gel and the TAE running buffer.

Microscopy and Image Analysis

Yeast cells were prepared for immunofluorescence following a standard protocol previously described (Pringle et al., 1991). Net1-HA3 was detected using a monoclonal anti-HA antibody (HA.11, Babco) at 1:1000 dilution. Net1-GFP and Sir2-GFP were detected using an affinity-purified rabbit polyclonal anti-GFP antibody at 1:5000 dilution or directly through GFP autofluorescence. Nop1 was detected using a mouse monoclonal antibody (kindly provided by John Aris) at 1:2000 dilution. Secondary antibodies, donkey fluorescein (FITC)-anti-rabbit and donkey rhodamine-anti-mouse (Jackson Laboratories), were used at 1:100 dilution. Images were obtained using a Zeiss Axioplan II fluorescent microscope equipped with a Hamamatsu digital camera (model #C4742–95) and Phase3 imaging system software (Media Cybernetics, Maryland). Three-dimensional image acquisition and reconstruction was performed on a DeltaVision platform (Applied Precision, Issaquah, Washington) using an Olympus IX70 microscope, a 100$\times$, 1.4NA lens, and a Photometrics Quantix camera. All images were acquired as sets of 150 nm axial sections

and processed by wide-field deconvolution microscopy (Agard et al., 1989). After deconvolution, three-dimensional image sets were projected onto two dimensions for the purpose of display.

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References

Agard, D.A., Hiraoka, Y., Shaw, P., and Sedat, J.W. (1989). Fluorescence microscopy in three dimensions. Methods Cell Biol. *30*, 353–377.

Andrulis, E.D., Neiman, A.M., Zappulla, D.C., and Sternglanz, R. (1998). Perinuclear localization of chromatin facilitates transcriptional silencing. Nature *394*, 592–595.

Aparicio, O.M., and Gottschling, D.E. (1994). Overcoming telomeric silencing: a trans-activator competes to establish gene expression in a cell cycle-dependent way. Genes Dev. *8*, 1133–1146.

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

Aris, J.P., and Blobel, G. (1988). Identification and characterization of a yeast nucleolar protein that is similar to a rat liver nucleolar protein. J. Cell Biol. *107*, 17–31.

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

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

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

De Rubertis, F., Kadosh, D., Henchoz, S., Pauli, D., Reuter, G., Struhl, K., and Spierer, P. (1996). The histone deacetylase RPD3 counteracts genomic silencing in Drosophila and yeast. Nature *384*, 589–591.

Fritze, C.E., Verschueren, K., Strich, R., and Easton Esposito, R. (1997). Direct evidence for SIR2 modulation of chromatin structure in yeast rDNA. EMBO J. *16*, 6495–6509.

Gotta, M., Strahl-Bolsinger, S., Renauld, H., Laroche, T., Kennedy, B.K., Grunstein, M., and Gasser, S.M. (1997). Localization of Sir2p: the nucleolus as a compartment for silent information regulators. EMBO J. *16*, 3243–3255.

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

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

Granot, D., and Snyder, M. (1991). Segregation of the nucleolus during mitosis in budding and fission yeast. Cell Motil. Cytoskeleton *20*, 47–54.

Guacci, V., Hogan, E., and Koshland, D. (1994). Chromosome condensation and sister chromatid pairing in budding yeast. J. Cell Biol. *125*, 517–530.

Halfter, H., Kavety, B., Vandekerckhove, J., Kiefer, F., and Gallwitz, D. (1989). Sequence, expression and mutational analysis of BAF1, a transcriptional activator and ARS1-binding protein of the yeast Saccharomyces cerevisiae. EMBO J. *8*, 4265–4272.

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

Huang, H., Kahana, A., Gottschling, D.E., Prakash, L., and Liebman, S.W. (1997). The ubiquitin-conjugating enzyme Rad6 (Ubc2) is required for silencing in Saccharomyces cerevisiae. Mol. Cell Biol. *17*, 6693–6699.

Kaffman, A., Herskowitz, I., Tjian, R., and O'Shea, E.K. (1994). Phosphorylation of the transcription factor PHO4 by a cyclin-CDK complex, PHO80-PHO85. Science *263*, 1153–1156.

Kayne, P.S., Kim, U.J., Han, M., Mullen, J.R., Yoshizaki, F., and Grunstein, M. (1988). Extremely conserved histone H4 N terminus is dispensable for growth but essential for repressing the silent mating loci in yeast. Cell *55*, 27–39.

Kennedy, B.K., Gotta, M., Sinclair, D.A., Mills, K., McNabb, D.S., Murthy, M., Pak, S.M., Laroche, T., Gasser, S.M., and Guarente, L. (1997). Redistribution of silencing proteins from telomeres to the nucleolus is associated with extension of life span in S. cerevisiae. Cell *89*, 381–391.

Klar, A.J.S., Fogel, S., and MacLeod, K. (1979). *MAR1*—a regulator of HMa and HMα loci in *Saccharomyces cerevisiae*. Genetics *93*, 37–50.

Laurenson, P., and Rine, J. (1992). Silencers, silencing, and heritable transcriptional states. Microbiol. Rev. *56*, 543–560.

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

Moazed, D., and Johnson, A.D. (1996). A deubiquitinating enzyme interacts with SIR4 and regulates silencing in S. cerevisiae. Cell *86*, 667–677.

Moazed, D., Kistler, A., Axelrod, A., Rine, J., and Johnson, A.D. (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., Freeman, K., Coodly, L., and Shore, D. (1994). Evidence that a complex of SIR proteins interacts with the silencer and telomere-binding protein RAP1. Genes Dev. *8*, 2257–2269.

Palladino, F., Laroche, T., Gilson, E., Axelrod, A., Pillus, L., and Gasser, S.M. (1993). SIR3 and SIR4 proteins are required for the positioning and integrity of yeast telomeres. Cell *75*, 543–555.

Park, H., and Sternglanz, R. (1999). Identification and characterization of the genes for two topoisomerase I–interacting proteins from Saccharomyces cerevisiae. Yeast *15*, 35–41.

Pillus, L., and Rine, J. (1989). Epigenetic inheritance of transcriptional states in S. cerevisiae. Cell *59*, 637–647.

Pringle, J.R., Adams, A.E., Drubin, D.G., and Haarer, B.K. (1991). Immunofluorescence methods of yeast. In Guide to Yeast Genetics and Molecular Biology, G. Guthrie and G.R. Fink, eds. (New York: Academic Press, Inc.), pp. 565–602.

Renauld, H., Aparicio, O.M., Zierath, P.D., Billington, B.L., Chhablani, S.K., and Gottschling, D.E. (1993). Silent domains are assembled continuously from the telomere and are defined by promoter distance and strength, and by SIR3 dosage. Genes Dev. *7*, 1133–1145.

Rhode, P.R., Sweder, K.S., Oegema, K.F., and Campbell, J.L. (1989). The gene encoding ARS-binding factor I is essential for the viability of yeast. Genes Dev. *3*, 1926–1939.

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

Rundlett, S.E., Carmen, A.A., Kobayashi, R., Bavykin, S., Turner, B.M., and Grunstein, M. (1996). HDA1 and RPD3 are members of distinct yeast histone deacetylase complexes that regulate silencing and transcription. Proc. Natl. Acad. Sci. USA *93*, 14503–14508.

Shaw, P.J., and Jordan, E.G. (1995). The nucleolus. Annu. Rev. Cell Dev. Biol. *11*, 93–121.

Shore, D., and Nasmyth, K. (1987). Purification and cloning of a DNA binding protein from yeast that binds to both silencer and activator elements. Cell *51*, 721–732.

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

Sikorski, R.S., and Hieter, P. (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 Guarente, L. (1997). Extrachromosomal rDNA circles—a cause of aging in yeast. Cell *91*, 1033–1042.

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

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

Strahl-Bolsinger, S., Hecht, A., Luo, K., and Grunstein, M. (1997). SIR2 and SIR4 interactions differ in core and extended telomeric heterochromatin in yeast. Genes Dev. *11*, 83–93.

Straight, A.F., Sedat, J.W., and Murray, A.W. (1998). Time-lapse microscopy reveals unique roles for kinesins during anaphase in budding yeast. J. Cell Biol. *143*, 687–694.

Thompson, J.S., Ling, X., and Grunstein, M. (1994). Histone H3 amino terminus is required for telomeric and silent mating locus repression in yeast. Nature *369*, 245–247.

Tollervey, D., Lehtonen, H., Jansen, R., Kern, H., and Hurt, E.C. (1993). Temperature-sensitive mutations demonstrate roles for yeast fibrillarin in pre-rRNA processing, pre-rRNA methylation, and ribosome assembly. Cell *72*, 443–457.

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

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

Woolford, J.L., and Warner, J.R. (1991). The ribosome and its synthesis. In The Molecular and Cellular Biology of the Yeast Saccharomyces, J.R. Broach, J.R. Pringle, and E.W. Jones, eds. (New York: Cold Spring Harbor Laboratory Press), pp. 587–626.